HALO® SC-IPS / HALO® RS

A Stem Cell Identity, Purity and Strength (Potency) Assay for Umbilical Cord Blood, Bone Marrow, and Peripheral Blood Cellular Therapy Processing

Technical Manual

(Version 8-19)

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

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

Preferred Cell Systems™

KM017.001

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *HALO® SC-IPS is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *HALO® SC-IPS 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

Strength or potency of a cellular therapy product can be defined as "the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties". According to the FDA Guidance for Industry on Potency Tests for Cellular and Gene Therapy Products, testing the strength or potency of a product is part of conformance and stability testing, which is associated with product quality and manufacturing controls to assure the identity, purity and strength (potency) of the product so that it meets defined specifications or acceptance criteria.

The purpose of treating patients by transplantation using cells from umbilical cord blood, bone marrow or mobilized peripheral blood, is to primarily cure malignancies of the hematopoietic and/or lymphopoietic system. The cells responsible for forming a new lympho-hematopoietic systems are the stem cells. According to regulatory agencies, these would be considered the "active ingredients" and, therefore, the identity, purity and strength or potency of the stem cell "active ingredients" should be quantitatively measured. This is the basis of the HALO® SC-IPS assay kit.

Since umbilical cord blood has been designated by the FDA as a "drug", the potency of all drugs must be determined from a reference standard for which the strength or potency is known. Unlike traditional drugs, cellular therapeutic products do not have "global" reference standards against which a sample can be compared to determine if the strength or potency is greater, similar or lower than the standard. Nevertheless, internal or in-house reference standards can be established to provide an indication as to whether the sample being tested exhibits an identity, purity and strength or potency that would predict or indicate that the cells being considered for use, will produce the intended result. That is, in this case, engraftment and reconstitution of the lympho-hematopoietic system. The assay kit developed by Preferred Cell Systems™ to help establish an in-house cellular reference standard for umbilical cord blood, bone marrow or mobilized peripheral blood is HALO® RS.

One of the important requirements of a strength or potency assay is that it must be validated. A validated assay provides trustworthy results. To validate an assay according to FDA Bioanlytical Method Validation Guidelines, the assay should be quantitative and include controls and standards that allow validation parameters to be established. Once these parameters have been established, the measurement assurance parameters that provide the ranges of results can be defined. These measurement assurance parameters also provide the necessary proficiency to demonstrate that the user is performing assay correctly. If results obtained by the user are within the measurement assurance parameters, the user can affirm that the results will be trustworthy. Both HALO® SC-IPS and HALO® RS are fully standardized and validated assays that include measurement assurance parameters (see **Section 15**).

The important property manifested by stem cell transplantation is cell proliferation. If the stem cells in umbilical cord blood, bone marrow or mobilized peripheral blood do not demonstrate sufficient proliferation, engraftment and reconstitution of the patient will not occur. Since proliferation occurs prior to differentiation and the latter occurs by default, the need for a differentiation assay, such as the colony-forming unit (CFU), which cannot be standardized or validated, is superfluous and unnecessary.

Both HALO® SC-IPS and HALO® RS are used to detect a minimum of two stem cell populations. These are: (a) the primitive hematopoietic stem cell population designated SC-GEMM 1 (Stem Cell - Granulocyte, Erythroid, Macrophage, Megakaryocyte) and (b) the more primitive lympho-hematopoietic stem cell population, designated SC-HPP (Stem Cell - High Proliferative Potential). Rather than using methylcellulose, both HALO® SC-IPS and HALO® RS incorporate Suspension Expansion Culture™ (SEC) Technology. The advantages of SEC™ are discussed later in this manual.

Both HALO® SC-IPS and HALO® RS also incorporate a fully standardized and validated ATP bioluminescence readout to measure stem cell proliferation potential and ability. These assays are therefore instrument-based and non-subjective. The bioluminescence signal measures intracellular ATP (iATP) concentrations using a luciferin/luciferase reaction. It is the most sensitive and accurate, non-radioactive readout available.

Stem cell proliferation, using HALO® SC-IPS and HALO® RS, are therefore based on the biochemical and metabolic ability of stem cells to produce iATP in response to being stimulated in culture by growth factor cocktails. When stem cells are induced into proliferation, the intracellular ATP (iATP) concentration increases proportionately. The iATP concentration and, therefore the proliferation status, correlates directly with the number of stem cells plated. To measure strength or potency, or rather to determine the potency ratio, a minimum 3-point cell dose response for each stem cell population from the sample and reference standard (RS) should be performed.

3. Use and Availability

HALO® RS is intended to be used to establish an in-house reference standard of cryopreserved umbilical cord blood, bone marrow or mobilized peripheral blood hematopoietic mononuclear cells (MNC) or purified stem cells.

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HALO® SC-IPS is intended to be used as an identity, purity and strength (potency) test for umbilical cord blood, bone marrow or mobilized peripheral blood hematopoietic mononuclear cells (MNC) or purified stem cells.

Both HALO® RS and HALO® SC-IPS are meant to be used with human:

- Umbilical cord blood
- Bone marrow
- Peripheral blood (mobilized)
- Purified stem cells from the above.

Suspension Expansion Culture™ (SEC™) Technology

Both HALO® RS and HALO® SC-IPS 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.
- Shorter cell incubation times; cell proliferation is measured on the exponential part of the growth curve.
- Coefficients of variation ≤15%.

Two stem cell populations are measured using HALO® RS and HALO® SC-IPS. These are:

- Primitive lympho-hematopoietic stem cell, SC-HPP 2
- Primitive hematopoietic stem cell, SC-GEMM 1

HALO® RS includes 1 vial of cryopreserved cells of the following tissues:

HALO® RS Assay Kit Availability

Catalog No.	Cryopreserved Tissue and Purity	Serum Formulation	No. of Plates
K2-PRS-1CB	Cord Blood / MNC	Low serum	1
K2SF-PRS-1CB	Cord Blood / MNC	Serum-free	1
K2-PRS-1CB34	Cord Blood / $CD34^+$	Low serum	1
K2SF-PRS-1CB34	Cord Blood / $CD34^+$	Serum-free	1
K2-PRS-1BM	Bone Marrow / MNC	Low serum	1
K2SF-PRS-1BM	Bone Marrow / MNC	Serum-free	1
K2-PRS-1BM34	Bone Marrow / $CD34^+$	Low serum	1
K2SF-PRS-1BM34	Bone Marrow / $CD34^+$	Serum-free	1
K2-PRS-1mPB	Mobilized Peripheral Blood / MNC	Low serum	1
K2SF-PRS-1mPB	Mobilized Peripheral Blood / MNC	Serum-free	1
K2-PRS-1mPB34	Mobilized Peripheral Blood / $CD34^+$	Low serum	1
K2SF-PRS-1mPB34	Mobilized Peripheral Blood / $CD34^+$	Serum-free	1

HALO® SC-IPS is meant to be used with cells from an established in-house reference standard (RS), which are compared to an unknown sample.

HALO® SC-IPS Assay Kit Availability

Catalog No.	Cryopreserved Tissue and Purity	Serum Formulation	No. of Plates
K2-PQR-1	Cord Blood, Bone Marrow or Mobilized Peripheral Blood	Low serum	1
K2SF-PQR-1	Cord Blood, Bone Marrow or Mobilized Peripheral Blood	Serum-free	1

4. Establishing a Reference Standard (RS) for Stem Cell Potency Testing using HALO® RS

To measure potency, a reference standard is required. This is because the measure of potency is the potency ratio. To estimate the potency ratio, the sample must be compared with that of a standard. For traditional drugs, establishing RSs for a compound is relatively easy since large quantities of the drug or compound are usually available. This is not the case for cells, and stem cells in particular. The establishment of a primary cell RS is not a standard procedure and there is no consensus on how this should be performed for cellular therapeutic products. The following is a suggestion for establishing internal primary, secondary and even tertiary reference standards for a specific tissue. One of the advantages of using a calibrated, standardized and validated assay such as HALO® RS or HALO® SC-IPS is that results can be directly compared over time. This means that one RS can be compared to another, both for intra- and inter-laboratory comparisons.

HALO® RS: The Strength or Potency Reference Standard Assay Kit
HALO® RS is an assay that helps establish an in-house RS. HALO® RS includes a cryopreserved RS of the same tissue and purification that is to be analyzed for stem cell potency. A batch of umbilical cord blood, bone marrow or mobilized peripheral blood RS should be red cell depleted and the mononuclear cell (MNC) fraction prepared by density gradient fractionation (DGF) as described in **Section 10, Step 1**. . Further purification (e.g. to $CD34^+$ cells) to should be performed using magnetic bead separation (e.g. Miltenyi Biotech). The concentration of the resulting MNC suspension is adjusted so that aliquots of 1-2 million cells are cryopreserved and stored in liquid nitrogen. Using an aliquot of the newly prepared, in-house cryopreserved RS cells, the stem cell potency and "quality" is determined against the cryopreserved RS included with the HALO® RS assay kit. This is performed using the general protocol described in **Section 10, Step 2-3.** This procedure establishes the Primary Reference Standard (1° RS).

Once a 1° RS has been established, a second batch of cells can be processed in the same way to produce a Secondary Reference Standard (2° RS). This 2° RS is tested against the 1° RS. The secondary RS should demonstrate similar or better potency parameters than the primary RS. The same procedure and testing is performed for a Tertiary Reference Standard (3° RS).

The last RS to be established and stored is the RS used to test against the unknown samples.

At regular intervals or when the number of aliquots remaining of the RS is low, a new RS should be established and compared to a previously established RS batch. In this way, several RS batches can be maintained at the same time.

The same procedure and testing is used for establishing reference standards of purified stem cells.

Please note HALO® RS is usually used only once. When one or more in-house reference standards have been established, all further reference standards can be tested using HALO® SC-IPS, unless a new tissue RS is required.

When is the Reference Standard Used?
A reference standard is used every time a stem cell product is to be released for use. Approximately 1-2 weeks prior to use, the potency of a sample of the product should be tested. As described later in this manual (**Section 14**), there are specific parameters that are required for release of the product to ensure engraftment potential. These include the potency ratio greater than 1 (>1) for both stem cell populations tested and an iATP concentration for both individual stem cell populations greater than 0.04**µ**M (> 0.04**µ**M). Other parameters should also be included in the release criteria, such as, total nucleated cell (TNC) or MNC count, viability and viable $CD34^+$ number as well as HLA compatibility, sterility etc.

When is Potency Measured?
Depending on the source of the cells, potency should be measured just prior to use in the patient. For cryopreserved samples, the potency and quality should be determined shortly after cryopreservation and again at a minimum of 7 days prior to use. Potency can not be measured after the product has been used in the patient since potency is a predictor of cell growth and dose.

PLEASE NOTE: Engraftment potential or the capacity of the cells to engraft is a different parameter to that of time to engraftment. Time to engraftment is the result of the engraftment process. It occurs after transplantation and is therefore

a retrospective parameter. Potency is a prospective parameter. Thus, time to engraftment cannot be used to measure potency.

5. The Concept of ATP Bioluminescence Assays

Both HALO® RS and HALO® SC-IPS are ATP bioluminescence assays. The fundamental concept underlying the assay is the measurement of the cell's chemical energy in the form of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When hematopoietic cells are stimulated to proliferate in culture with growth factors cocktails, 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 factorin the cocktail used to stimulate the cells.
- The plated cell concentration.

For HALO® RS and HALO® SC-IPS, two stem cell populations are cultured with their respective HALO® Master Mixes provided with the kit for a specific period of time (5-7 days). 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:

$$\text{ATP} + \text{Luciferin} + \text{O2} \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

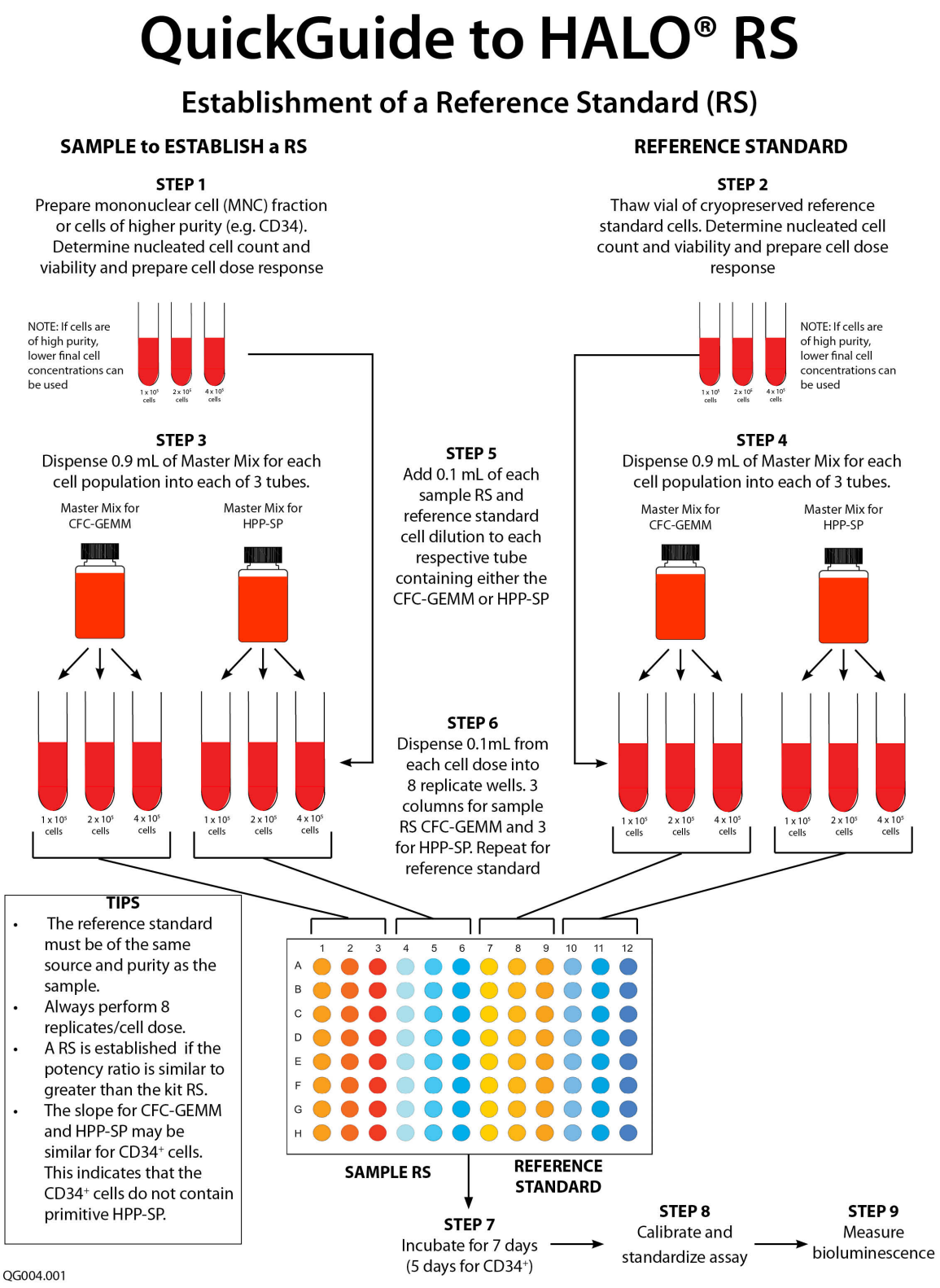
The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU). The assay can be calibrated and standardize, and controls and standards are included for this purpose. Performing an ATP standard curve and controls is very important because it has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

1. The controls calibrate the instrument and also ensure that the reagents are working correctly.
2. The ATP standard curve also ensures that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.
5. The results obtained from controls and standard curve should be compared with those provided in **Section 15**. 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 15**, the investigator can consider the results trustworthy.

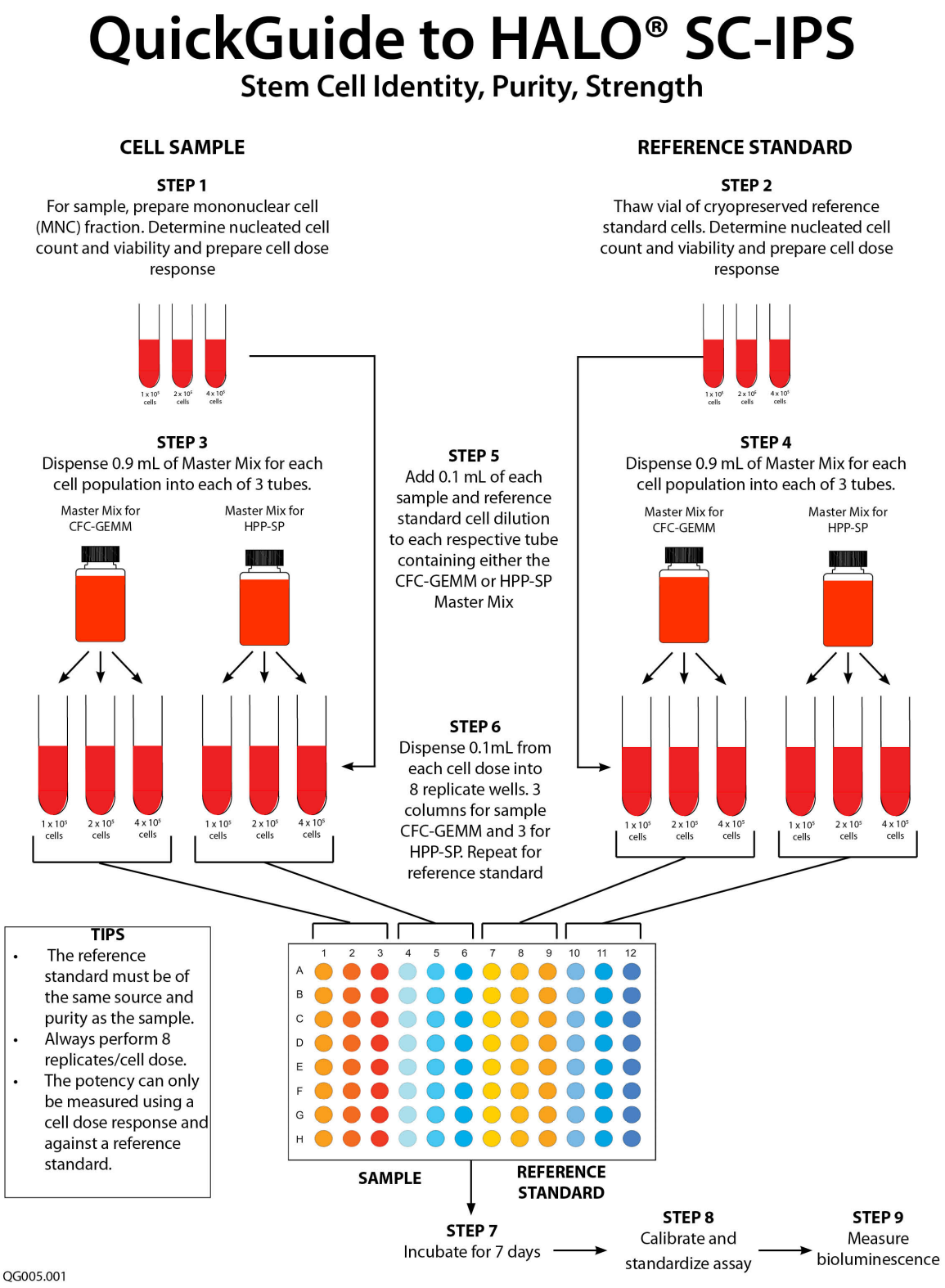
The ATP standard curve and controls need only be measured once on the day samples are to be processed. Do not use previous results from an ATP standard curve and controls performed on a different day. This will cause erroneous results.

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.

6. QuickGuide to HALO® RS (Figure 1)



7. QuickGuide to HALO® SC-IPS (Figure 2)



8. Kit Contents and Storage Conditions

HALO® RS and HALO® SC-IPS assay kits contain reagents that have been frozen and stored at -80°C prior to shipment. The kit is shipped with dry ice.

Components Included with HALO® RS Assay Kits

Item	Component	Storage
1	Cryopreserved vial of reference standard mononuclear cells (MNC) or $CD34^+$ cells.	Liquid N_2
2	HALO® Master Mix (serum-free or low serum) to detect primitive lympho-hematopoietic stem cells (SC-HPP 2).	-20°C until used
3	HALO® Master Mix (serum-free or low serum) to detect hematopoietic, multipotential stem cells (SC-GEMM 1).	-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, solid white 96-well plate for stem cell culture	Can be kept with other kit components
10	Non-sterile, solid white 96-well plate for ATP standard curve determination.	Can be kept with other kit components
	Technical manual can be downloaded from the HALO® SC-IPS page on the Perred Cell Systems website	

Components Included with HALO® SC-IPS Assay Kits

Item	Component	Storage
1	HALO® Master Mix (serum-free or low serum) to detect primitive lympho-hematopoietic stem cells (SC-HPP 2).	-20°C until used
2	HALO® Master Mix (serum-free or low serum) to detect hematopoietic, multipotential stem cells (SC-GEMM 1).	-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 plate for stem cell culture	Can be kept with other kit components
9	Non-sterile, solid white 96-well plate for ATP standard curve determination.	Can be kept with other kit components
	Technical manual can be downloaded from the HALO® SC-IPS page on the Perred 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.

9. 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% 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. HemoGro™ Hematopoietic Growth Medium for preparing cell suspensions and cell dilutions (Preferred Cell Systems™)
2. DNase (4000U/mL. Use for cell thawing).
3. Iscove's Modified Dulbecco's Medium (IMDM).
4. Density-gradient medium (e.g. Ficoll-Paque, Lymphoprep).
5. 7-AAD, propidium iodide or trypan blue for viability assay.
6. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

10. The HALO® RS and HALO® SC-IPS Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 11 BEFORE PERFORMING THE PROTOCOL.

- ***Perform all procedures under a laminar flow, bio-hazzard hood.***
- ***Wear protective clothing, including gloves for all operations.***
- ***Always use professionally calibrated, and preferably, electronic pipettes for all dispensing.***

Performing either HALO® RS or HALO® SC-IPS is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HALO® RS and HALO® SC-IPS cell culture master mix procedure, plating and incubation in 96-well plates.
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

A. Thawing of Cells Included with HALO® RS

1. Prepare 20mL of thaw medium (IMDM + 10% fetal bovine serum, FBS) warmed to 37ºC in a 50mL conical tube.
2. Prior to thawing the cells, thaw a vial of DNase (100µL at 4,000U/mL).
3. Add 100µL of thawed DNase to the 20mL of thaw medium in the 50mL conical tube and mix by inversion.
4. Carefully remove the vial of cells from liquid nitrogen and partially unsrew the cap.
5. Thaw the cells in a 37ºC water bath by gently swirling the vial for about 1 min or until a small ball of ice remains in the vial.
6. Remove the vial and spray with 70% alcohol.
7. Carefully remove the lid of the vial and transfer all of the contents down the side of the 50mL tube containing the thaw medium. Mix gently.
8. Remove about 1mL from the 50mL tube and use it to rinse the vial, returning the cell suspension back to the 50mL tube.
9. Close the 50mL and mix gently by inversion.
10. Centifuge the cells in the 50mL tube at 300 x g for 10min at room temperature.
11. After centrifugation, aspirate and discard the supernatant.
12. Add 1mL of IMDM to the tube and resuspend the cells.
13. Count the cells, preferably using an electronic cell counter, and perform a viability assessment. (See Step 1, Section C.).

B. Purity of Sample Cells

For establishing an in-house reference standard using HALO® RS or determining the strength or potency of an umbilical cord blood, bone marrow or mobilized peripheral blood sample using HALO® SC-IPS, the results will depend on the purity of the original cells used in the assay.

1. Although for umbilical cord blood and bone marrow, it is routine to prepare a red blood cell- / plasma-depleted preparation using automated instrumentation, the resulting total nucleated cell (TNC) fraction should neither be used to establish an in-house RS nor to measure the strength or potency of a sample. It has been conclusively demonstrated that a TNC fraction is unsatisfactory for measuring both the quality and potency usually produced by red blood cell reduction. This is due to:
   (a) Dilution of primitive stem cell due to large numbers of cell impurities, leading to
   (b) Underestimation of primitive stem cell populations.
   (c) Interference of high red blood cell concentrations (>25-30%) that contain high levels of ATP to cause a false positive result.
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.
3. Purified stem and progenitor cell populations prepared by cell sorting or magnetic bead separation.
4. Mobilized peripheral blood cells are prepared by apheresis. This produces a fractionation similar to a MNC fraction. No further fractionation is necessary unless the cells need to be further purified.

C. Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. Perform a dye exclusion viability preferably using 7-AAD and flow cytometry. A viability of 85% or greater should be obtained when using dye exclusion viability. It is recommended not to use a cell suspension with a viability of less than 85% since these cells will not be able to sustain proliferation ability. **PLEASE NOTE:** *Dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity. In addition, it is possible to observe a high dye exclusion viability, prior to culture, but a low or non-existent degree of proliferation detect using ATP bioluminescence, after culture. If this phenomenon is observed, it is due to the dye exclusion viability method giving a false positive result.* Use LIVEGlo™ (Preferred Cell Systems™) as a metabolic viability assay.
2. Determine the cell concentration using, preferably using an 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 the highest working cell concentration shown in Table 1 with HemoGro™ Medium.
   Note *the working cell concentration per ml is 100 x the final cell concentration per well.*

4. Using HemoGro™ Medium, prepare a 3-point serial cell dose response as shown in Table 1. The cell dose response for MNCs should be performed as follows:
   (i) Prepare and label 3 x sterile 5mL tubes for each of the 3 cell doses required.
   (ii) Prepare 0.6mL of the highest working concentration, i.e. 500,000 cells/mL (Tube 1).
   (iii) Add 0.3mL of HemoGro™ medium to the second tube (250,000 cells/mL, Tube 2).
   (iv) Add 0.2mL of HemoGro™ medium to the third tube (125,000 cells/mL, Tube 3).
   (v) Transfer 0.3mL of cells from Tube 1 to Tube 2 and mix.
   (vi) Transfer 0.2mL of cells from Tube 2 to Tube 3 and mix.

If using $CD34^+$ cells as the starting cell source, perform a reduced cell dose response shown in Table 1.

TABLE 1
Recommended Highest Working Cell Doses and Serial Dilutions for HALO® RS and HALO® SC-IPS

Species	Cell Type	Cell Preparation	Cell State	Highest Working Cell Concentration Required (100 x Final Cells/Well)	Cell Dose Response Prepare from Highest Working Cell Concentration
Human	Umbilical cord blood	MNC	Frozen	500,000 cells/mL	500,000, 250,000, 125,000
	Bone marrow	MNC	Frozen	500,000 cells/mL	500,000, 250,000, 125,000
	Mobilized peripheral blood	MNC	Frozen	500,000 cells/mL	500,000, 250,000, 125,000
	Bone marrow	$CD34^+$	Fresh	100,000 cells/mL	100,000, 50,000, 25,000
	Mobilized peripheral blood*	$CD34^+$	Frozen	100,000 cells/mL	100,000, 50,000, 25,000
	Umbilical cord blood	$CD34^+$	Frozen	100,000 cells/mL	100,000, 50,000, 25,000

STEP 2. HALO® RS and HALO® SC-IPS Cell Culture Procedure

- ***HALO® QC Master Mixes are complete and ready-.to-use.***
- ***Perform all procedures under a laminar flow, bio-hazzard hood.***
- ***Wear protective clothing, including gloves for all operations.***
- ***Always use professionally calibrated, and preferably, electronic pipettes for all dispensing.***

1. Remove the HALO® SC-HPP 2 and SC-GEMM 1 Master Mixes that came with the assay kit from the freezer and thaw at room temperature or in a beaker of cold water. Do not thaw in a 37°C water bath or incubator. Mix the contents when thawed by gentle inversion. Do NOT shake the bottles.
2. 3 x 5mL sterile tubes will be needed for the sample SC-HPP 2 cell dose response and 3 x 5mL sterile tubes will be needed for the SC-GEMM 1 cell dose response. The same number of tubes will be need for the reference standard
3. The tubes might be labeled as follows:
   Tube 1: Sample/1250 cells/SC-HPP 2
   Tube 2: Sample/2500 cells/SC-HPP 2
   Tube 3: Sample/5000 cells/SC-HPP 2
   Tube 4: Sample/1250 cells/SC-GEMM 1
   Tube 5: Sample/2500 cells/SC-GEMM 1
   Tube 6: Sample/5000 cells/SC-GEMM1
   The same number of tubes will be required for the reference standard.
4. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.9mL of the SC-HPP 2 Master mix into Tubes 1-3 for the sample and Tubes 7-9 for the reference standard.
5. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.9mL of the SC-GEMM 1 Master mix into Tubes 4-6 for the sample and Tubes 10-12 for the reference standard.
6. Starting at the lowest cell concentration, dispense 0.1mL of each dilution into sample Tubes 1-3 containing the SC-HPP 1 Master Mix. Similarly, starting with the lowest sample dilution, dispense 0.1mL of each dilution into Tubes 4-6

for the SC-GEMM 1 Master Mix.

7. Repeat the same procedure for the reference standard cells for Tubes 7-9 for SC-HPP 2 and Tubes 10-12 for SC-GEMM 1.
8. Cap the tubes and mix gently on a vortex mixer.
9. Remove the sterile, individually wrapped, 96-well plate from the assay kit box.
10. Perform the following dispensing into the 96-well plate using a calibrated pipette, preferably an electronic pipette with repeat function, making sure to change the pipette tip for each cell dilution. In addition, dispense all reagents into the bottom of the well, not on the sides of the well.

    Sample cells, SC-HPP2
    Tube 1: Dispense 0.1mL into wells A1 to H1
    Tube 2: Dispense 0.1mL into wells A2 to H2
    Tube 3: Dispense 0.1mL into wells A3 to H3
    Sample cells, SC-GEMM 1
    Tube 4: Dispense 0.1mL into wells A4 to H4
    Tube 5: Dispense 0.1mL into wells A5 to H5
    Tube 6: Dispense 0.1mL into wells A6 to H6
    Reference standard cells, SC-HPP 2
    Tube 7: Dispense 0.1mL into wells A7 to H7
    Tube 7: Dispense 0.1mL into wells A8 to H8
    Tube 9: Dispense 0.1mL into wells A8 to H9
    Reference standard cells, SC-GEMM 1
    Tube 10: Dispense 0.1mL into wells A10 to H10
    Tube 11: Dispense 0.1mL into wells A11 to H11
    Tube 12: Dispense 0.1mL into wells A12 to H12
11. The 12 cell suspensions each plated at 8 replicate wells will fill up the 96-well plate.
12. After replacing the lid, transfer the 96-well plate to a humidified container (see **Section 11**).
13. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the producing of free radicals.
14. Incubate the cells for 5 days. For greater sensitivity, the incubation time can be extended to 7 days. The ATP concentration should increase 2-3 fold from day 5 to day 7. However, this may occur at the expense of slightly greater variation between the replicates.

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 (Section 13). The instrument should be setup and prepared for use prior to any of the following steps being performed.***
- ***Please refer to* Section 12 *for recommendations and tips prior to starting this part of the procedure. In particular, please refer to* Section 12 *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 (highly recommended), 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 15**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

Both HALO® RS and HALO® SC-IPS Assay Kits include 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

PROTOCOL 1: For bone marrow and umbilical cord blood, use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 23**. ATP concentrations should be within this ATP standard curve range. If they are found to be higher, perform Protocol 2.

PROTOCOL 2: For most mobilized peripheral blood products and purified cells (e.g. $CD34^+$ cells), it is recommended to use the low and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 24**.
It should be noted that not all patients mobilized in the same manner and some may not mobilize at all. In this case, use Protocol 1.
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 15**.

It is important that both the sample and RS ATP values are within the limits of the ATP standard curve, otherwise the interpolation of Relative Luminescence Unit (RLU) values from the luminescence plate reader into ATP concentrations will not be accurate. In some cases, cell proliferation could be greater than 3µM ATP. If 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% 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, 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 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. Alternatively, transfer the 96-well plate directly to the luminescence plate reader without the lid and close the draw of the instrument to incubate for 10 min in the dark.
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.

11. Recommendations and Tips Prior To Using HALO® RS and HALO® SC-IPS.

(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

Both HALO® RS and HALO® SC-IPS are setup using 8 replicates for each point. This is of statistical significance. 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

These assays have been designed to be performed using 8 replicate wells/point. The best plate configuration is therefore in columns across the plate.

(iv) 96-Well Plates Provided

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

Results can be obtained after 5 days of cell incubation. This point in time represents th beginning of exponential cell proliferation growth. To obtain greater sensitivity, the incubation time can be extended to 7 days, at which point, stem cell proliferation will be 2-3 times higher than at 5 days. However, this might occur with a slight increase in coefficients of variation (CV).

12. 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.***
- ***HALO® QC includes solid white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from Preferred Cell Systems™.***

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove the ATP standard, 1 set of ATP controls and the ATP-Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23˚C.
- Sufficient ATP standard, controls and ATP-ER are supplied to perform 1 standard curve 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 -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 ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10mL.

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.

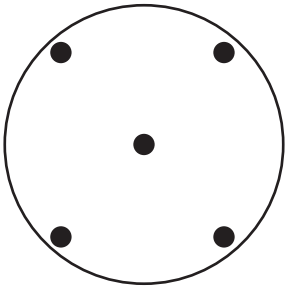
Mixing the Contents of 96-well Plate

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



13. 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.**

14. How to Analyze the Results

HALO® RS and HALO® SC-IPS provide an instrument-based, non-subjective, quantitative readout of hematopoietic stem cell total proliferation ability and potential of the cells being tested. This cannot be obtained using a colony-forming unit (CFU) assay. In fact, the CFU assay is not a cell proliferation assay. It is also extremely insensitive and inaccurate when used for hematopoietic stem cell populations. Since HALO® RS and HALO® SC-IPS measure cell proliferation and proliferation occurs prior to differentiation, these assays are not only completed more rapidly than a CFU assay, but can also predict CFU differentiation results. This is because there is a direct correlation between HALO® QC and the CFU assay.

Most hematopoietic cellular therapy procedures use cryopreserved cells. HALO® RS and HALO® SC-IPS can be used on fresh or cryopreserved cells. If testing fresh cells, it should be emphasized that results produced will not reflect the actual viability or proliferation ability and potential after thawing frozen cells; proliferation ability will be 2-3 fold lower a cryopreserved sample than a fresh sample.

The strength or potency of a sample cannot be performed using a non-validated assay. Assay validation can occur when controls and standards are used. It is, therefore, highly recommended that the user 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 should be compared with those provided in **Section 15** below. Providing the results are within the ranges specified in **Section 15**, 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 potency can NOT be determined with a single value; a dose response is required.

Analyzing the Results

To determine if an in-house RS can be considered similar to or better than the frozen cells provided with the HALO® RS kit or to determine if a sample of umbilical cord blood, bone marrow or mobilized peripheral blood indicates that the unit of cells might be used for transplantation purposes, it is necessary to (1), convert the RLU values obtained from the plate reader into standardized ATP concentrations and (2) plot the data.

To convert RLU values into standardized ATP concentrations see **Section 13** of this manual.

Once all RLU values have been converted to ATP concentrations (µM), the results are plotted as follows:

1. If the instrument software does not allow graphing, use a third-party software (e.g. Excel, Graph Pad Prism) to graph the raw data with cell dose on the X-axis and mean ATP concentration (µM)/well on the Y-axis.
2. The data are plotted for both stem cell populations (SC-HPP 2 and SC-GEMM 1) for the sample and the RS. A total of 4 cell dose response curves will therefore be plotted.
3. A linear regression curve fit for each stem cell dose response is performed and the slope of the dose response curve calculated.
4. Calculate the potency ratio for SC-HPP 2 by dividing the slope of the sample SC-HPP 2 stem cell dose response by that for the slope of the RS SC-HPP 2.
5. Similarly, calculate the potency ratio for SC-GEMM 1 by dividing the slope of the sample SC-GEMM 1 stem cell dose response by that for the slope of the RS, SC-GEMM 1 response.

Interpreting the Results

HALO® RS and HALO® SC-IPS are used to measure two basic parameters of cell proliferation:

- Proliferation ability, which is the amount of stem cell proliferation, in standardized ATP units (µM), at a specific cell dose. Proliferation ability of stem cells is equivalent to stem cell quality.
- Proliferation potential is the capacity of stem cells to proliferate and is determined by the slope of the stem cell dose response curve.

The combination of these two parameters provide all the information needed to interpret the results obtained. By performing a cell dose response for both stem cell populations from the sample and the RS, both stem cell proliferation ability and potential are determined simultaneously. In addition, when comparing the sample with the RS or even with a TNC fraction, several other parameters are defined:

- The slope of the more primitive stem cell population (SC-HPP 2) should be greater than that of SC-GEMM 1, since more primitive stem cells have a greater proliferation potential than mature stem cells. This identifies one stem cell population from another. Thus, the identity of the stem cell can be defined.
- When comparing a less fractionated cell suspension with that of a more highly fractionated or even purified cell population, the overall ATP concentration for all cell doses will be lower for the less fractionated than for the greater or purified population. In short, this defines the purity of the sample.
- Finally, the slope of the stem cell dose response curve is also a direct measure of its proliferation potential. The steeper the slope, the more primitive the stem cell population, the greater its potency. Thus, proliferation potential not only defines stem cell identity, but also strength or potency.

Having this information , it is now possible for the user to interpret the data provided by HALO® RS and HALO® SC-IPS. For example:

1. The ATP concentration produced at a specific cell dose for each stem cell population, usually 5,000 cells/well, should be at least 2-3 x greater than the lowest ATP concentration indicating unsustainable stem cell proliferation. This value is ~0.04µM ATP (**Section 15**). Therefore, the ATP concentration produced by each stem cell population should be about 0.12µM. The ATP concentration at this cell dose is defined as the proliferation ability or stem cell quality. Thus, the stem cell quality of the sample must be equal to or greater than the required value for the stem cells to be considered "high quality".
2. The potency ratio of the reference standard is always consider to be 1 (one).
3. If the potency ratio for each stem cell population measured is greater than 1, the sample exhibits a potency greater the RS. This means that fewer sample cells are required to produce the same response as the RS for the specific stem cell population. If stem cell "quality" and potency are greater than that for the RS, the unit of cells might be considered for use, providing other factors (e.g. sufficient cell number, sufficient $CD34^+$ number are also obtained).
4. If the sample potency ratio of each stem cell population is less than 1, the sample exhibits a potency ratio less than the RS. This means that more cells will be required to produce a similar response to that of the RS. If stem cell "quality" is also below the minimum level, the unit of cells being considered should probably not be used, even if other paremeters, (e.g. cell number, $CD34^+$ number) might indicate otherwise. Do not forget that the ATP concentration being measure is also a metabolic viability measurement. If the ATP concentration is indicating that the cells might not or will not sustain proliferation they will be non-viable and neither the cell number nor the $CD34^+$ number will override this indication.

These are the best and the worst scenarios. Most other scenarios will lie in between. Some will indicate high quality, but low potency ratio and visa versa. It is therefore important that other parameters, such as the MNC and $CD34^+$ count, are also taken into account.

15. HALO® QC Assay Measurement Assurance and Validation Parameters

If HALO® 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 ± 15%(*)	Min / Max	%CV (where applicable)
0.01µM ATP	0.0099µM ATP	0.00972 - 0.0114	0.009 - 0.01	2.34%
0.03µM ATP	0.029µM ATP	0.285 - 0.0336	0.028 - 0.03	1.67%
0.05µM ATP	0.0497µM ATP	0.0486 - 0.0571	0.048 - 0.051	1.57%
0.01µM ATP	0.1026µM ATP	0.1003 - 0.118	0.099 - 0.107	1.96%
0.3µM ATP	0.317µM ATP	0.310 - 0.364	0.302 - 0.325	1.51%
0.5µM ATP	0.5023µM ATP	0.491 - 0.578	0.491 - 0.515	1.19%

Expected Parameter	Observed Value	Mean ± 15%(*)	Min / Max	%CV (where applicable)
1.0µM ATP	1.048µM ATP	1.024 - 1.205	0.977 - 1.117	3.7%
3.0µM ATP	2.722µM 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, (0.05µM ATP	0.0487µM ATP	0.0476 - 0.0560	0.042 - 0.063	6.79%
High control 0.7µM ATP	0.725	0.710 - 0.836	0.655 - 0.904	5.35%
Extra high control (1.75µM 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: ~0.04µM. 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: ~0.01µM. 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 3µM, 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 => 4 logs
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- 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 comparison): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

16. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 15)

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 be 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 =< 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 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-existant, 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.
- *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.

17. References

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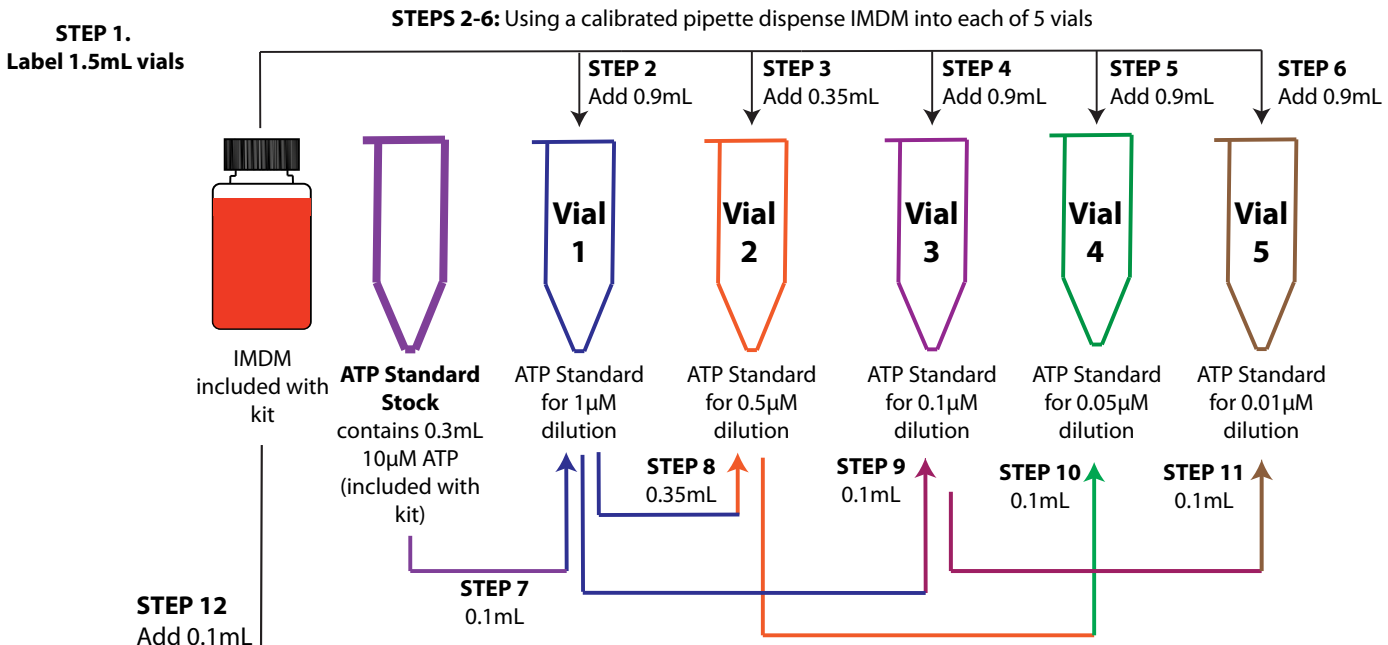
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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

Reagents & Materials Needed
1. 1.5mL vials or similar (not included)
2. IMDM (included)
3. ATP Standard (included)
4. ATP Controls (included)
5. Non-sterile, 96-well plate (included)

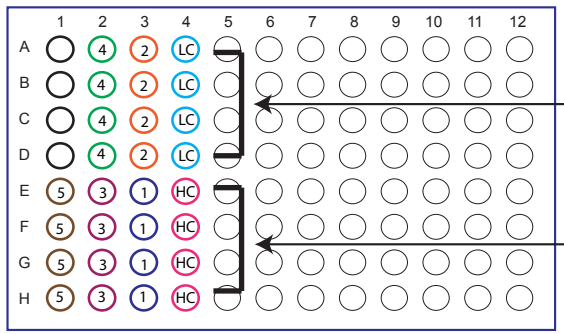
TIPS

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



STEP 12
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 13: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 14: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 15: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 16: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 17: Add 0.1mL from **Vial 1** into wells E3-H3

STEP 18: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

STEP 19: HIGH CONTROL (HC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from high control to wells E4-H4

STEP 20: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 21: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 22: Transfer 96-well plate to luminescence plate reader
STEP 23: Incubate in the dark for 2 minutes and measure luminescence

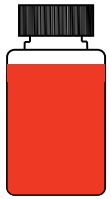
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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

STEP 1.
Label 1.5mL vials

STEPS 2-6: Using a calibrated pipette dispense IMDM into each of 5 vials

STEP 6 Add 0.7mL
STEP 2 Add 0.4mL
STEP 3 Add 0.9mL
STEP 4 Add 0.9mL
STEP 5 Add 0.9mL



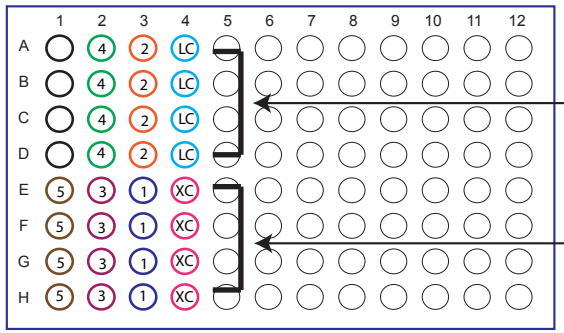
IMDM included with kit

Vial 1 — ATP Standard contains 0.3mL 10µM ATP (included with kit)
Vial 2 — ATP Standard for 1µM dilution
Vial 3 — ATP Standard for 0.3µM dilution
Vial 4 — ATP Standard for 0.1µM dilution
Vial 5 — ATP Standard for 0.03µM dilution

STEP 7 0.2mL
STEP 8 0.1mL
STEP 9 0.1mL
STEP 10 0.1mL

STEP 11
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 12: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 13: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 14: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 15: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 16: Add 0.1mL from **Vial 1** into wells E3-H3

LC

STEP 17: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

XC

STEP 18: EXTRA HIGH CONTROL (XC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from extra high control to wells E4-H4

Reagents & Materials Needed
1. 1.5mL vials or similar (not included)
2. IMDM (included)
3. ATP Standard (included)
4. ATP Controls (included)
5. Non-sterile, 96-well plate (included)

TIPS

> Use calibrated pipettes throughout.
> Vortex thoroughly between each dilution.
> Change tips between each dilution.
> Follow color coding.
> A 0.01µM ATP Standard can be made from the Vial 4 and added to the plate.

STEP 19: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 20: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 21: Transfer 96-well plate to luminescence plate reader
STEP 22: Incubate in the dark for 2 minutes and measure luminescence