

ImmunoGlo™ MLC

A Mixed Lymphocyte Culture (MLC) Assay
Incorporating an Standardized ATP Bioluminescence
Readout

Technical Manual

(Version 7-19)

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

For *In Vitro* Research Use Only
Not for Clinical Diagnostic Use

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Preferred Cell Systems™

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1. Limitations of the Assay and Precautions

1. *ImmunoGlo™ MLC is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *ImmunoGlo™ MLC 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. Safety data sheets (SDS) are included in each literature packet.*

2. Introduction

Under normal, steady-state conditions, immune cells demonstrate little or no proliferation. When stimulated, however, different types of immune cells can exhibit different degrees of proliferation activity. The proliferation activity will be dependent upon the type of inducer, concentration and any co-stimulators that might be present.

A specific type of immune reaction occurs when peripheral blood lymphocytes from different donors are mixed together in different proportions and cultured together for several days. The histocompatibility complex present on cells detects whether the donors are compatible. If the cells from each donor are compatible, little or no reaction will occur. If the cells are not compatible, stimulation will occur resulting in a dramatic increase in cell proliferation. This type of reaction is called a mixed lymphocyte reaction (MLR) and is performed in a mixed lymphocyte culture (MLC).

There are two types of MLC. In a one-way (1-way) MLC, the lymphocytes of one individual are inactivated by first treating the cells with mitomycin-C or radiation to inhibit proliferation. If the donors are incompatible, the cells from the untreated donor react to the foreign histocompatibility antigens resulting in cell proliferation. In a two-way (2-way) MLC, the cells from both donors are left untreated and can stimulate each other to proliferate. Under these conditions, however, the direction of the stimulation will not be obvious. A MLC is usually used to examine T-lymphocyte helper cells (T_H cells, CD4) or to generate cytotoxic T-lymphocytes (CTLs). The MLR is also used to test the compatibility of donor and patient for cell transplantation purposes.

Immune or lymphocyte proliferation has traditionally been measured using a radioactive marker, usually tritiated thymidine (3H -Tdr), or more recently a non-radioactive marker that incorporates into the cell's DNA, such as bromodeoxyuridine (BrdU) which might be detected using a colorimetric (absorbance) or fluorescence readout. Other markers include EdU and CFSE. However, a radioactive marker has usually been the method of choice because of the high sensitivity, despite the use of a hazardous compound that also involves regulated waste removal.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of viability, functionality and cell proliferation. The amount of iATP produced by a cell correlates directly with its functional status. The most sensitive non-radioactive readout to measure cell proliferation is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used for ImmunoGlo™ MLC.

All ATP bioluminescence assays from Preferred Cell Systems™ include reagents to calibrate and standardize the assay so that results can be compared over time.

ImmunoGlo™ MLC is easy to learn and rapid to use and can replace all other methods for measuring lymphocyte proliferation.

3. Use and Availability

ImmunoGlo™-MLC is a research tool to measure a 1- or 2-way stimulation of lymphocytes due to histocompatibility differences.

ImmunoGlo™-MLC is used with human peripheral blood lymphocytes or purified immune cell populations. For research purposes, animal tissues can also be used.

ImmunoGlo™-MLC Assays Available

Catalog Nos.	No. of Samples* for 1-Way MLC	No. of Samples* for 2-Way MLC	No. of Plates/Kit
KM1-MLC-1	3	5	1

* Based on performing 6 replicates/sample and incorporating 4 controls for a 1-way and 2 controls for a 2-way MLC.

Please note that ImmunoGlo™ MLC assay kits can also be obtained in bulk. Please contact Preferred Cell Systems™ for more information.

IMPORTANT:

ImmunoGlo™ MLC is for research use only and has not been approved for clinical diagnostic use.

4. Principle of ATP Bioluminescence Assays

ImmunoGlo™ MLC is an ATP bioluminescence assay. The fundamental concept underlying this assay is the measurement of the cell's chemical energy in the form of intracellular ATP (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When cells are stimulated to proliferate, 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 type and concentration of the stimulator cells.
- The plated cell concentration.

Cells are cultured for defined period of time. When the culture period has elapsed, a single ATP-Enumeration Reagent (ATP-ER) 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 for 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 plate luminometer as relative luminescence units (RLU). To calibrate and standardize the assay, an ATP standard and controls are

provided. **Performing the ATP standard curve and controls prior to measuring samples is highly recommended for the assay, since it provides the user with the information that the assay is functioning correctly. Calibrating and standardizing the assay has the following important advantages:**

1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure that the instrument is working correctly as well as the reagents.
3. Allows the investigator to compare measurement assurance parameters with those in Section 12 of this technical manual. All results for both the ATP standard curve and controls must lie within the ranges stated in Section 12. This will allow the investigator to proceed with sample measurement and have the confidence that the results will be trustworthy.
4. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (μM).
5. Performing the ATP standard curve allows results to be compared over time.

The ATP standard curve and controls need only to be measured once on the day samples are to be processed.

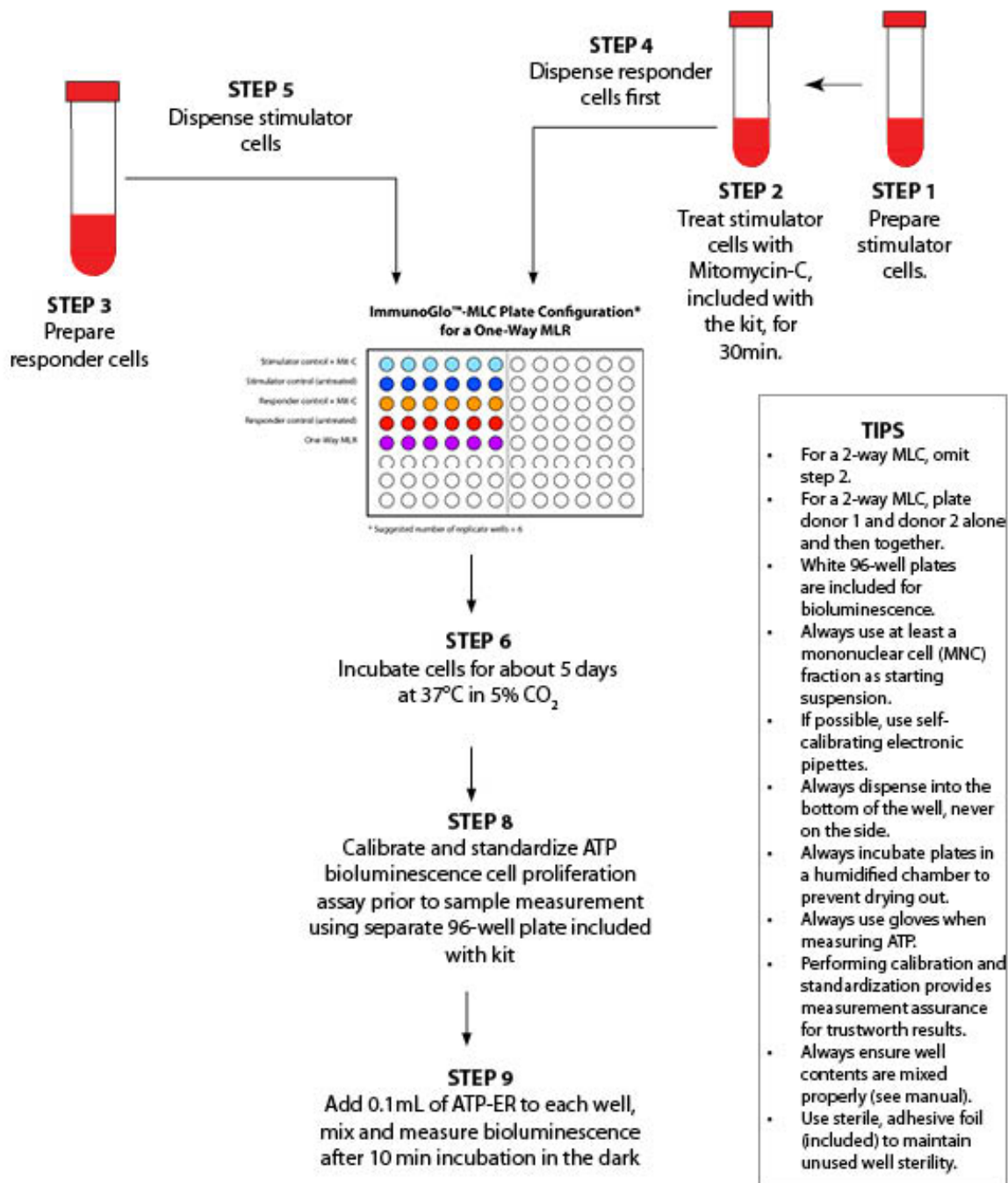
DO NOT use results from an ATP standard curve or controls performed on one day for samples processed on another day.

To convert non-standardized RLU values to standardized ATP concentrations, please see Section 11 of this Technical Manual.

NOTES

5. QuickGuide to ImmunoGlo™ MLC (Figure 1)

QuickGuide to ImmunoGlo™ MLC (1-Way)



6. Kit Contents and Storage

ImmunoGlo™-MLC 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	Mitomycin-C (solid substance). Add 1mL of PBS to each vial just prior to use.	-20°C until used. 1-2 weeks dissolved. Keep at 2-8°C in the dark.
2	Base ImmunoGro™ Medium for cell culture without growth factors and cell dilutions (if needed) and containing antibiotics (gentamicin, streptomycin, penicillin and neomycin)	-20°C until used; max. 1 year. Store for 2 months at 2-4°C
3	Iscove's Modified Dulbecco's Medium (IMDM) for dilution of the ATP standard only. NOT FOR CELL CULTURE.	-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	1 x Sterile, clear, 96-well plates for cell culture	Can be kept with other kit components
9	1 x Non-sterile, solid white, 96-well plates for ATP standard curve determination.	Can be kept with other kit components
	Technical manual.	Download from the Preferred Cell Systems, ImmunoGlo™ MLC webpage

Exact volumes of the kit reagents and supplies are provided on a separate sheet included with this assay kit.

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 has an expiry date on the box. 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 purchased from Preferred Cell Systems™.

7. Equipment, Supplies and Reagents Required, But Not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Luminescence plate reader (LB962 CentroLIA/pc from Berthold Technologies and available from Preferred Cell Systems™).
3. Sterile, capped, plastic tubes (5mL, 10mL, 50mL)
4. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette

7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO₂ (minimum requirement) and 5% O₂ (preferable).
10. Hemocytometer or electronic cell counter to determine cell concentration.
11. Flow cytometer or hemocytometer for determining viability. A flow cytometer might also be required to determine the proportion of immune cell types in a cell suspension.

Reagents

1. Additional Base ImmunoGro™ Low-Serum Medium. For cell dilutions and cell culture without growth factors, e.g. to measure background controls (Catalog Number: M-IG-100 for 100mL; M-IG-500 for 500mL).
2. Sterile Phosphate Buffered Saline (PBS)
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. Lymphoprep).
5. ACK Lysis buffer (Cat. No. K-Lysis-100, Preferred Cell Systems™, Inc)
6. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.

8. The ImmunoGlo™ MLC Protocol

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

Performing ImmunoGlo™ MLC is a 4-step process for a 1-way MLC and 3-step process for a 2-way MLC.

Step 1 – Cell preparation.

Step 2 -- Treatment of stimulator cells with mitomycin-C for a 1-way MLC.

Step 3 – Cell culture and incubation in the 96-well plate.

Step 4 – Absorbance measurement.

Steps 1 to 3 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

A MLC assay is usually performed using human peripheral blood lymphocytes, but cells from other species can also be used. It is best, however, to start with a mononuclear cell (MNC) fraction that has been significantly depleted of red blood cells (RBCs). Depletion of erythrocytes is essential since they can interfere with the assay when present at high concentrations (hematocrits > 10%) and cause false positive results.

It is recommended to use density gradient centrifugation using the manufacturer's protocol.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated

method such as flow cytometer using 7-AAD or another vital stain.

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

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

2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to the desired working cell concentration. This will usually be 10-100 fold greater than the final cell concentration/well. For cell culture, the optimal cell concentration/well should be determined using a cell dose response.

STEP 2. Treatment of Cells with Mitomycin-C (Mit-C) for a 1-Way MLC

IMPORTANT: Mitomycin-C is a toxic compound. Handle with care. Use laboratory gloves and universal protective clothing.

NOTE: Mitomycin-C decomposes rapidly. Please follow storage conditions on Page 5.

This step is only required for a 1-way MLC. If a 2-way MLC is required, omit this step and continue to Step 3.

For a 1-way MLC, one cell sample acts as the stimulator, while the other acts as the responder. Although it is usual to treat the stimulator cells (whichever donor is designated the stimulator) with an inhibitor of proliferation prior to use, it is recommended to also treat an aliquot of the responder cells as a control. This is shown in Fig. 1. The cells are treated with mitomycin-C prior to use to inhibit proliferation. The following is a suggested protocol for mitomycin-C - treated cells.

1. Prepare cell suspensions so that after treatment and 2-3 cell washing steps, sufficient cells will be available for controls and MLC.
2. Transfer an aliquot of the cells to a sterile tube.
3. Mitomycin-C (included with this kit) is dissolved in physiological saline or PBS. Prepare a 10 fold working dilution so that when added to the target cell suspension the final mitomycin-C concentration will be 25µg/mL. For example, add 1.0mL of sterile PBS or saline to each vial containing 250µg of mitomycin-C. This produces a solution of 250µg/mL. When added at 10% of the total volume, the final concentration is 25µg/mL.
4. Add Mitomycin-C working concentration to the cells.
5. Incubate for 30 minutes at 37°C in a fully humidified atmosphere containing 5% CO₂.
6. After the incubation time has elapsed, remove the tube from the incubator and add 5-10mL of sterile physiological saline or PBS to dilute the agent and centrifuge the cells at 200 x g for 10 minutes at room temperature.
7. Remove and discard the supernatant.

8. Resuspend the cells in another 5-10 mL of saline or PBS and centrifuge again under the same conditions as in Step 6.
9. If necessary, Steps 7 and 8 can be repeated for a third cell wash. Note that with each centrifugation step, approx. 10% of the cells will be lost.
10. After the last wash, resuspend the cells in a volume of ImmunoGro™ medium (provided).
11. Perform a nucleated cell count and, if required, a viability determination.
12. Adjust the cell concentrations to the required working concentration that is 20 times (20 x) the final concentration/well.

STEP 3. ImmunoGlo™-MLC - Cell Culture Protocol

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

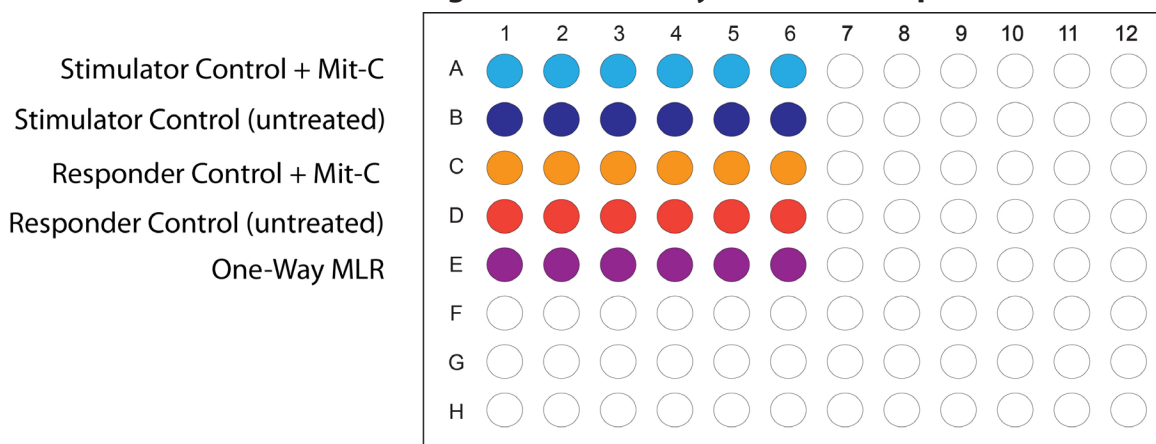
Perform all cell cultures under sterile conditions in a biosafety cabinet.

Use calibrated pipettes and sterile tips throughout.

A. One-Way MLC

Figure 2 shows the suggested 96-well plate configuration for a 1-way MLR and includes four controls. It may be necessary to perform a cell titration curve to obtain optimal cell concentrations.

FIGURE 2. Plate Configuration for 1-Way MLC with 6 Replicates



1. Remove the sterile 96-well plates from the assay kit box and allow to attain room temperature.
2. Prepare the stimulator and responder cells ± Mit-C at the required working concentration, equivalent to twenty times (20 x) the final concentration/well. For example, if the final concentration is to be 1×10^5 cells/well, then the stimulator + Mit-C and responder cells will both have to be 2×10^6 cells/mL.
3. Dispense 0.05mL of culture medium into all control wells. See Fig. 2.
4. Dispense 0.05mL of the stimulator control cells treated with Mit-C into each replicate well. Figure 2 shows the number of replicate wells. This could be reduced to 4 replicate wells. Addition of

0.05mL of the cell suspension to 0.05mL of culture medium will dilute the cells to the final cell concentration.

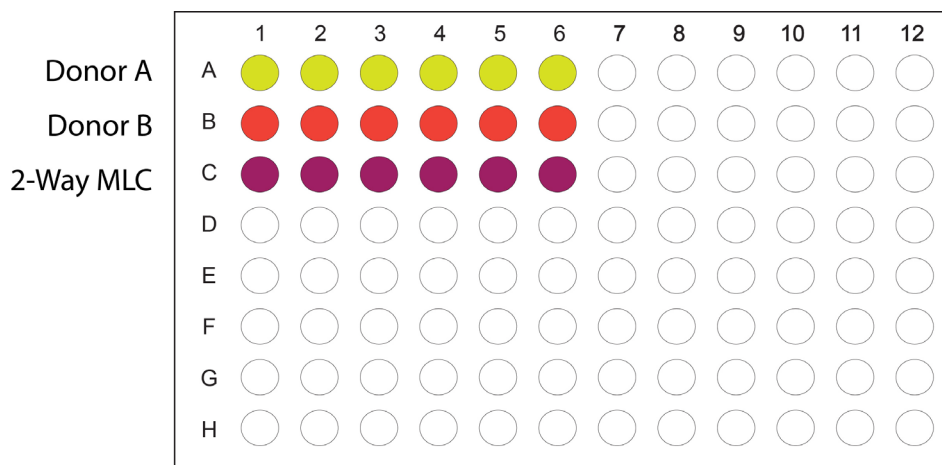
5. Dispense 0.05mL of the untreated stimulator cells into the next row (B) of replicate wells.
6. Dispense 0.05mL of responder control cells treated with Mit-C into the third row (C) of replicate wells.
7. Dispense 0.05mL of the untreated responder cells into the fourth row (D) of replicate wells.
8. Finally, dispense 0.05mL of the Mit-C - treated stimulator cells into each replicate well followed by 0.05mL of the untreated responder cells into the same replicate wells (row E). Each of these wells will now contain a total of 0.1mL diluted to the same concentration as the controls.
9. Remember to include growth medium-only wells as the blank.
10. Transfer the 96-well plate to a 37°C fully humidified incubator containing an atmosphere of 5% CO₂ and, if possible, 5% O₂.
11. Culture the cells for 5 days. This time period may vary depending on the cells being studied and the species being used.

B. Two-Way MLC

NOTE: A background with no cells should also be included as a blank. Subtract the blank absorbance from the sample absorbance.

Figure 3 shows the suggested 96-well plate configuration for a 2-way MLC and includes 2 controls.

FIGURE 3. Plate Configuration for 2-Way MLC with 6 Replicates



A 2-way MLC does not use mitomycin-C - treated cells. The two donors are used to stimulate each other.

1. Remove the sterile 96-well plates from the assay kit box and allow to attain room temperature.
2. Prepare the stimulator and responder cells at the required working concentration, equivalent to twenty times (20 x) the final concentration/well. For example, if the final concentration is to be 1×10^5 cells/well, then the stimulator and responder cells will both have to be 2×10^6 cells/mL.
3. Dispense 0.05mL of culture medium into all control wells. See Fig. 2.
4. Dispense 0.05mL of Donor A into replicate wells (row A). Fig. 3 shows that 6 replicate wells are

recommended, although this can be reduced to 4.

5. Dispense 0.05mL of Donor B into the next row of replicate wells (row B).
6. Dispense 0.05mL of Donor A cells into the third row (C) of replicate wells followed by 0.05mL of Donor B cells into the same replicate wells. The resulting dilution with a total volume of 0.1mL will produce the same final concentration as the controls.
7. Remember to dispense 0.1mL of growth medium into the same number of replicate wells used for the samples. This will be the blank.
8. Transfer the 96-well plate to a 37°C fully humidified incubator containing an atmosphere of 5% CO₂ and, if possible, 5% O₂.
9. Culture the cells for 5 days. This time period may vary depending on the cells being studied and the species being used.

STEP 4 – Measurement of Lymphocyte Proliferation using ATP Bioluminescence

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 REFER TO SECTION 11 ON HOW TO SETUP THE PLATE LUMINOMETER. The instrument should be setup and prepared for use prior to any of the following steps being performed.***
- ***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 in the wells.***
- ***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.***

It is highly recommended to standardize the assay prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

ImmunoGlo™ 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

PROTOCOL 1: If it is expected that the cells have a low proliferation ability, use the low and high calibration controls and perform an ATP standard curve from 0.01 μM to 1 μM. **See Page 21.**

PROTOCOL 2: If it is expected that the cells have a high proliferation ability, use the low and extra high calibration controls and perform an ATP standard curve from 0.03 μM to 3 μM. **See Page 22.**

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. If Protocol 2 has been used and values are not as high as 0.03 μM ATP, perform Protocol 1. In some cases, cell proliferation could be greater than 3 μM ATP. If ATP values from the samples are greater than 3 μM, 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 0.1 mL of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 10.
4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. Preferred Cell Systems™ does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

9. Recommendations and Tips Prior to Using ImmunoGlo™ MLC.

- (i) Always perform a cell dose response to determine the optimal cell concentration to use.
- (ii) **Number of Replicates Performed**
The number of replicates/sample is arbitrary. For statistical purposes, 6 replicates/sample are recommended. 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**
Performing 6 replicates/well means that the samples can be plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6.
- (iv) **Number of Replicates**
The recommended number of replicates per sample is 6. This will help reduce high coefficients of variation (CVs) and provide statistical relevance if outliers are encountered. The number of replicates can be reduced, but this might severely reduce statistical relevance of the assay.
- (v) **96-Well Plates Provided**
The reagents have been optimized to work with the 96-well plate(s) provided. Other plates can be used. However, cell growth and absorbance output can be seriously affected and the assay kit warranty will be void. Additional plates can be purchased from HemoGenix® if required.
- (vi) **Humidity Chamber**
If cell incubation times are greater than 3 days, a humidity chamber is recommended due sample volume evaporation. Even fully humidified incubators do not keep the humidity level high enough to keep the sample from evaporating. This usually results in so-called “edge effects”. A humidity chamber can be assembled using plastic lunch boxes or other plastic ware available from a supermarket or discount stores. Holes must be made in the lid to enable adequate gas exchange. Disposable serological pipettes are cut to an appropriate length to fill the bottom of the container. Distilled/deionized water is poured into the container to just below the level of the pipettes. This allows for adequate water to keep the humidity high without the plates sitting in water. Please contact HemoGenix® for further information about assembling and using humidity chambers.

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.

- *ImmunoGlo™ 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 Solutions™.*

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove the ATP standard, 1 set of ATP controls and the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23°C.
- Enough ATP standard and monitoring reagent is supplied to perform 2 standard curves and controls for each sterile plate provided. Additional ATP standards and controls can be obtained from Preferred Cell Solutions™.
- 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 Enumeration 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 (µl) required = 0.1mL x (number of wells used + 24 (background, ATP dose response wells and ATP controls)).

ATP Standard Curve

Depending on the size of the kit purchased, non-sterile, 96-well plates have been included to perform an ATP standard curve prior to processing the sample cultures. Performing an ATP standard curve and controls on each day samples are processed is highly recommended.

- 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 results to be compared with the measurement assurance criteria in Section 12 of this Technical Manual. Results should be within the ranges provided. For technical support, please contact Preferred Cell Systems™.
- 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.
- It allows results to be compared over time, thereby providing valuable historical data.

Adhesive Plate Covering Film

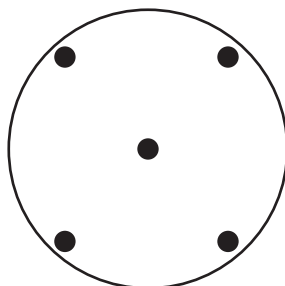
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with the film to ensure sterility.

Mixing the Contents of 96-well Plate

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 4. 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.

12. ImmunoGlo™ MLC Assay Measurement Assurance and Validation Parameters

If ImmunoGlo™ has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in the Table below. 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, contact Preferred Cell Systems for help.

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%
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%

Expected Parameter	Observed Value	Mean \pm 15% ^(*)	Min / Max	%CV (where applicable)
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 most immune cells: \sim 0.04 μ M.
- ATP value below which cells are not metabolically viable: \sim 0.01 μ M.
- 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 medium provided in the kit 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

ImmunoGlo™ MLC 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): \sim 95%.
- High throughput capability (Z-Factor): $>$ 0.76 (lowest possible value, 0.5; highest possible value, 1).

13. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 12)

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 12. 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.
- *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 (0.1 mL), 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.

Ordering Information

Toll free: 1-888-436-6869

Tel: (719) 264-6251

Fax: (719) 264-6253

Email: info@preferred-cell-systems.com

Order online at preferred-cell-systems.com

Technical Support

Tel: (719) 264-6251

Email: info@preferred-cell-systems.com

Preferred Cell Systems™

1485 Garden of the Gods Road

Suite 152

Colorado Springs, CO 80907

U.S.A.

Website: www.preferred-cell-systems.com

ImmunoGlo™ MLC was designed and developed by Preferred Cell Systems™.

The ATP readout used for ImmunoGlo™ MLC is protected by U.S. patents

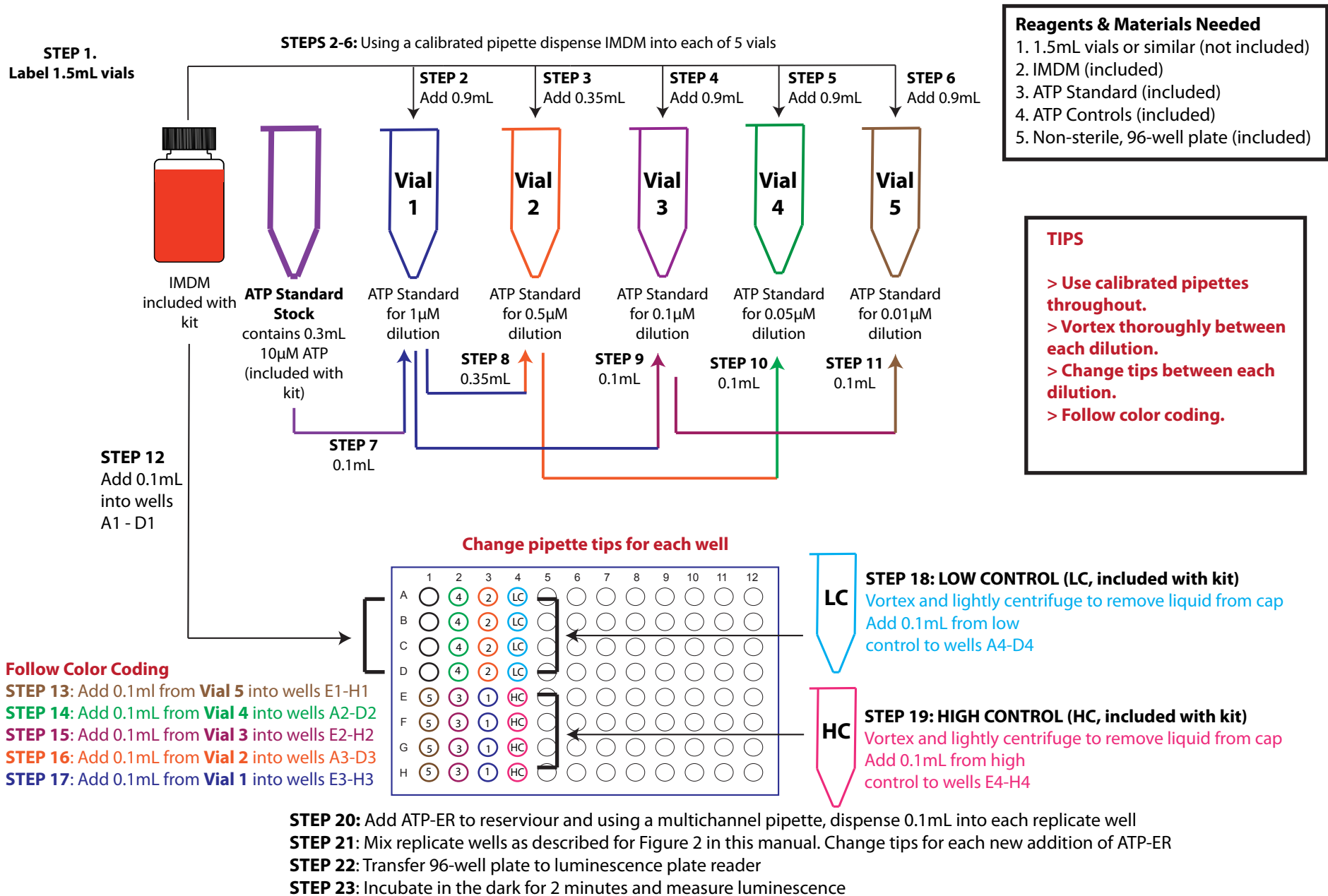
7,354,729, 7,354,730, 7,666,615, 7,709,258, 7,883,861, 7,700,354.

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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

