ImmunoLight™ MLC

A Mixed Lymphocyte Culture (MLC) Assay Incorporating an Absorbance/Colorimetric 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

Preferred Cell Systems™

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1. Limitations of the Assay and Precautions

1. *ImmunoLight™ MLC is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *ImmunoLight™ 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 MLR 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 (^{3}H-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.

ImmunoLight™ MLC incorporates a tetrazolium salt called WST-1, which is cleaved by a complex cellular mechanism primarily at the cell surface, to form a soluble formazan that is measured in an absorbance plate reader between 420nm and 480nm. The reaction is dependent on the metabolic activity and therefore the metabolic viability of the cells.

3. Use and Availability

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

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

ImmunoLight™-MLC Assays Available

Catalog Nos.	No. of Samples* for 1-Way MLC	No. of Samples* for 2-Way MLC	No. of Plates/Kit
KM3-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 ImmunoLight™ MLC assay kits can also be obtained in bulk. Please contact Preferred Cell Systems™ for more information.

IMPORTANT:
ImmunoLight™ MLC is for research use only and has not been approved for clinical diagnostic use.

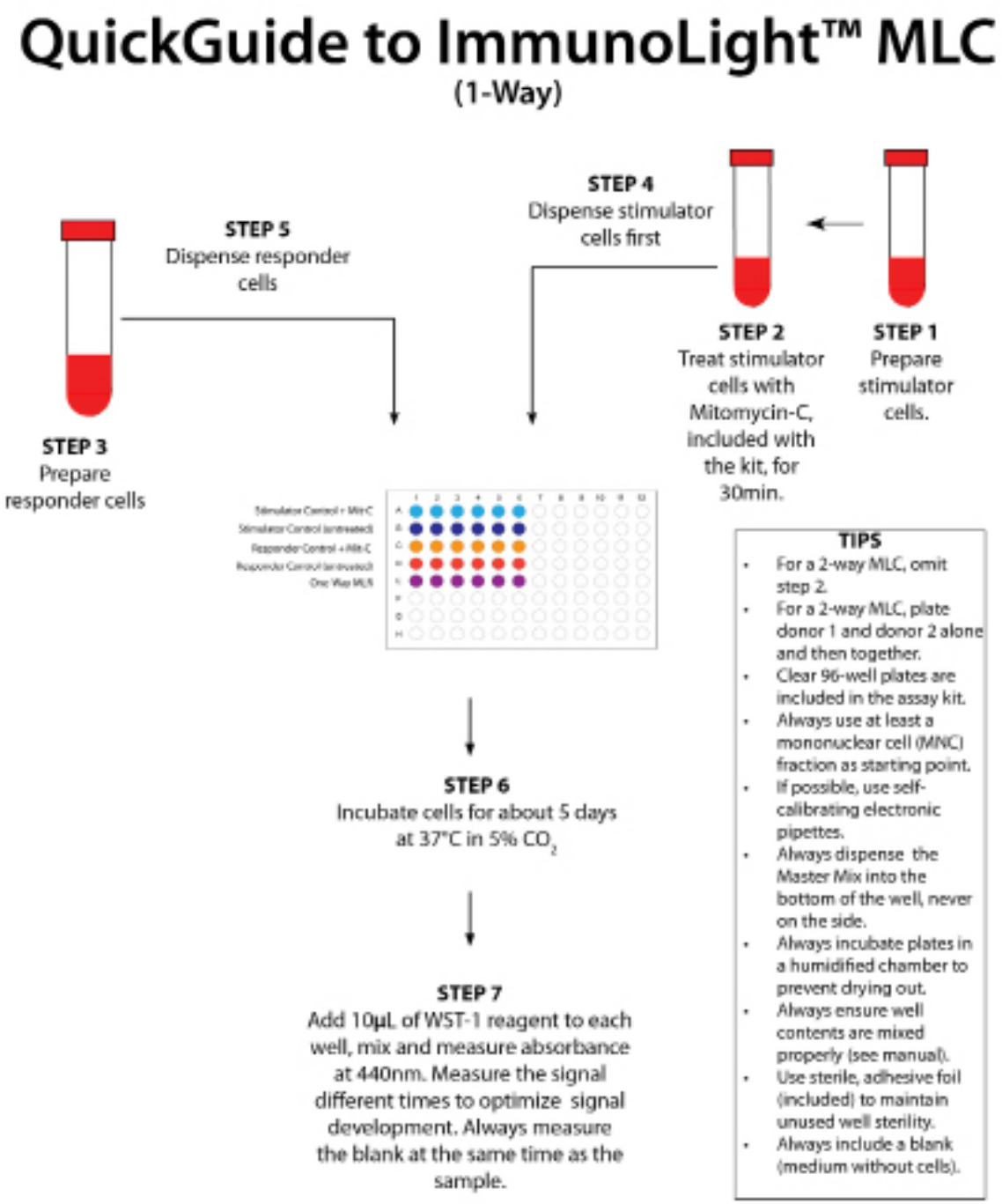
4. Principle of ImmunoLight™ MLC and the WST-1 Absorbance Readout

ImmunoLIGHT™-MLC is a colorimetric/absorbance, 1- or 2-way mixed lymphocyte culture *in vitro* assay. For a 1-way MLC, mitomycin-C, proliferation-inhibited stimulator cells are allowed to settle in 96-well clear plates provided with the kit. Responder cells are then added and the cultures incubated for a period of time to allow potential induced proliferation to occur if the stimulator and responder cells are not compatible. For a 2-way MLC, stimulator and responder cells are added without the former being proliferation-inhibited. To ensure that the mixed lymphocyte reaction occurs as expected, specific controls must be included. All cultures are setup in a total volume of 0.1mL/well.

Once the incubation period has elapsed, 10µL of the ready-to-use WST-1 reagent is added to each well, mixed and further incubated.

WST-1 is a colorimetric/absorbance in vitro assay. It incorporates a customized reagent from Roche. The reagent contains a tetrazolium compound salt, WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). In the presence of an electron coupling reagent (succinate-tetrazolium reductase system), WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. When added to metabolically viable cells WST-1 is reduced. This bioreduction is largely dependent on the glycolytic production of NADPH in viable cells. The cells are incubated with WST-1 for 1-4 hours. Longer incubation periods may be required. This is especially important for cells exhibiting low growth. The plate can be removed from the incubator at different times to measure optimal absorbance. The absorbance is measured between 420-480 nm (max. absorption at about 440 nm) in a 96-well plate reader. A background with medium, but no cells should be included and subtracted from the sample absorbance. A reference wavelength of more than 600nm can also be used.

5. QuickGuide to ImmunoGlo™ MLC (Figure 1)



6. Kit Contents and Storage

ImmunoLight™-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	WST-1 Reagent	Store at -20°C, protected from light. Once thawed, the reagent may be stored at 4°C, protected from light, for up to 4 weeks. The reagent may become viscous. Warm reagent for 2-10min at 37ºC. For longer storage periods, store at -20ºC.
4	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
5	1 x Sterile, clear, 96-well plates for cell culture	Can be kept with other kit components
	Technical manual.	Download from the Preferred Cell Systems, ImmunoLight™ 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% CO_2 (minimum requirement) and 5% O_2 (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-IGSF-100 for 100mL; M-IGSF-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 ImmunoLight™ MLC Protocol

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

Performing ImmunoLight™ 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 MLR.
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% CO_2.
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. ImmunoLight™-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.

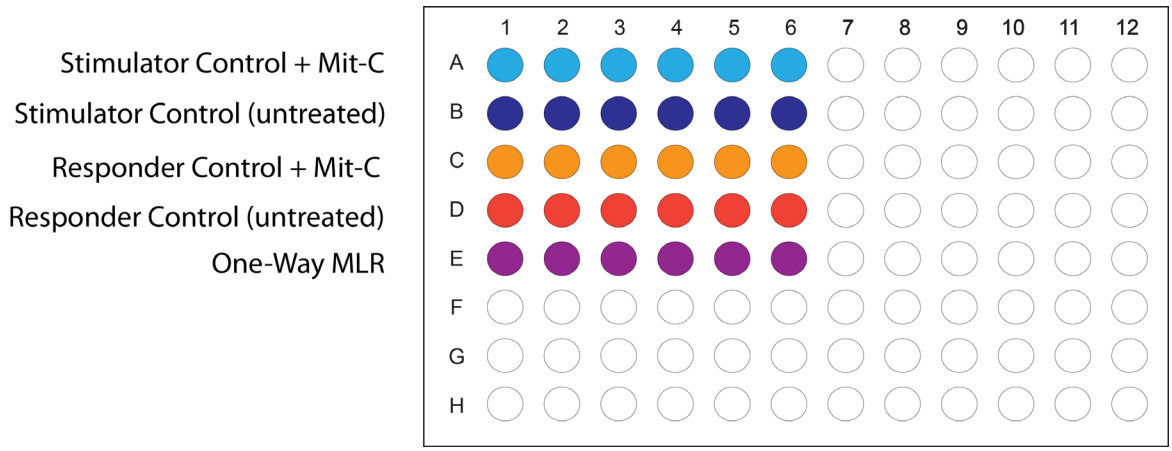
Use calibrated pipettes and sterile tips throughout.

A. One-Way MLC

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

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 x 10^5 cells/well, then the stimulator + Mit-C and responder cells will both have to be 2 x 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.

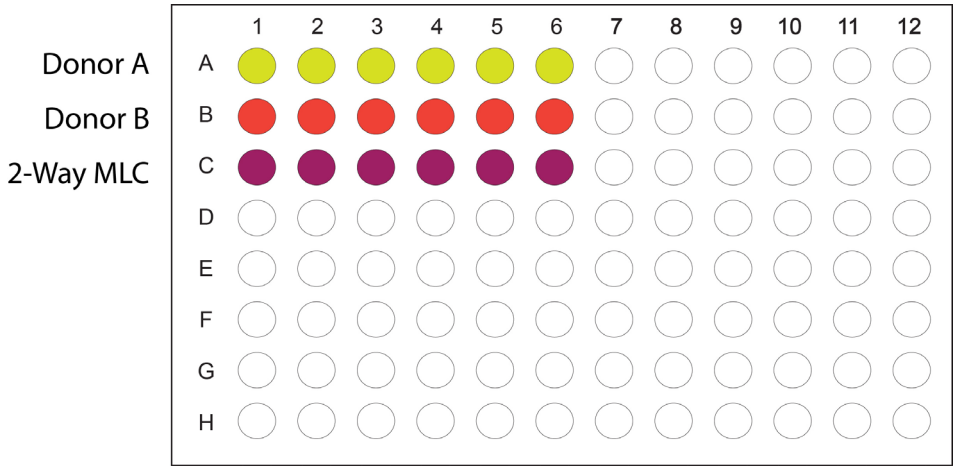
9. Remember to include growth medium-only wells as the blank.
10. Transfer the 96-well plate to a 37°C fully humidified incubator containing an atmosphere of 5% CO_2 and, if possible, 5% O_2.
11. Culture the cells for 5 days. This time period may vary depending on the cells being studied and the species 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 MLR 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 x 10^5 cells/well, then the stimulator and responder cells will both have to be 2 x 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% CO_2 and, if possible, 5% O_2.
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 WST-1

Perform the following steps for sample measurement.

1. Remove the WST-1 reagent from the freezer and thaw at room temperature. If precipitation or turbidity is observed upon thawing, warm the reagent to 37ºC for 2-10 min and mix gently to dissolve the precipitates. Centrifugation is not recommended because the working concentration would decrease.
2. Remove both 96-well plates from the incubator.
3. If only part of the plate has been used for the MLC, transfer the plate to a hood/laminar air flow bench and attach the sterile, adhesive film from the ImmunoLight™ MLC assay kit box as follows:
   (a) Remove the lid from the 96-well plate.
   (b) Remove the backing from the adhesive foil.
   (c) Carefully layer the foil over the plate so that it covers all of the wells and press down the foil over each well.
   (d) Using a sharp blade or knife, cut between the wells that contain the cultures.
   (e) Gently peel away the adhesive foil from the wells containing the cultures.
   (f) This will leave the remaining foil over the unused wells, which will remain sterile for later use.
4. Using a calibrated pipette, add 10µL of the WST-1 reagent to each well, including the wells used for the blanks. Mix gently using the same pipette tip. After each addition, make sure to change the pipette to avoid any carry-over from one well to another.
5. Once the WST-1 reagent has been added to all culture wells, replace the lid and transfer the 96-well plate to the incubator.
6. Incubate the cells for 1-4 hours. Longer incubation times may be necessary. At any time during the color development, the plate may be removed and the absorbance measured at about 440nm. NOTE: Make sure to measure the blank and subtract the value from the sample absorbance value (see below).
7. The absorbance can be plotted against incubation time to determine the optimized incubation time for future assays.
8. NOTE that the absorbance for the blank will increase with time. At 0 hr, the absorbance will be about 0.1 absorbance units. After 4hr, the absorbance will be between 0.2 and 0.3 absorbance units.

9. Recommendations and Tips Prior to Using ImmunoLIGHT™ 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 time 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. Troubleshooting

High Coefficients of Variation (%CV)
Coefficients of variation (%CV) should be =< 15%. The percent coefficient of variation is calculated as standard deviation/mean x 100. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Small volumes are dispensed and the use of instruments that have not been calibrated correctly or have not be calibrated for a long period of time, can lead to high CVs.
- Insufficient mixing of components prior to and during plating should be performed. Use

repeater pipettes where possible. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix reagents.
- Perform a minimum of 6 replicates per point.

Inadequate Cell Culture

- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to adding the cells to the master mix. Ensure that the viability is high prior to culture. If using dye exclusion viability, cells should exhibit approx. 85% viability.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly as indicated in Section 6 of this manual.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Section 9 (iv) and below).
- *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 about 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 clear 96-well plates can be viewed under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in color and/or probably cloudy in appearance. Cell cultures that demonstrate high proliferation will also 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 values.

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.

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

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Website: www.preferred-cell-systems.com

Preferred Cell Systems™

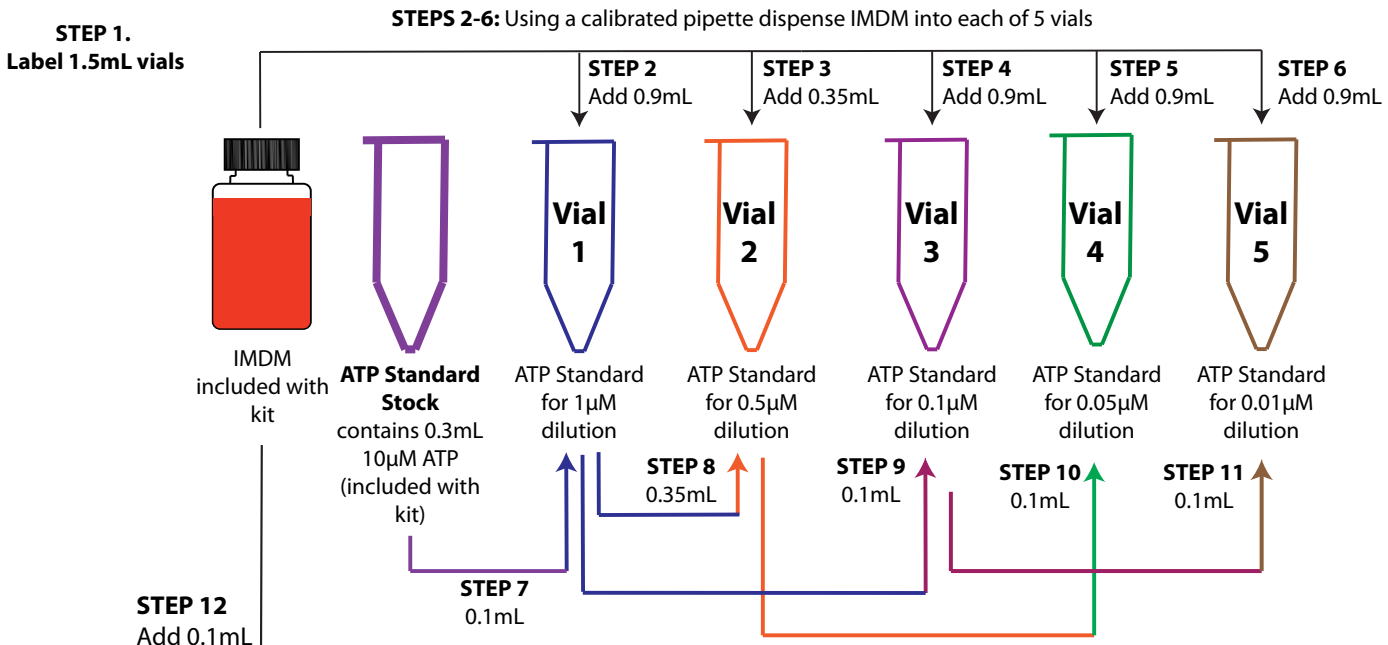
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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)

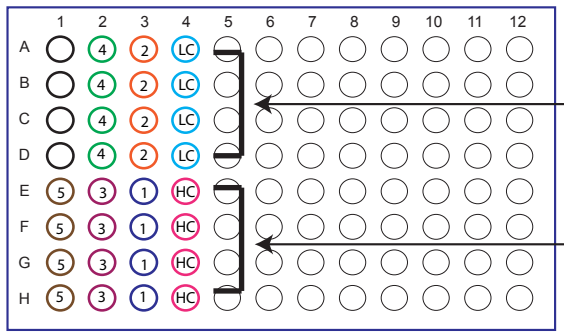
TIPS

> 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

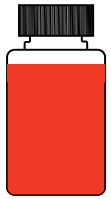
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



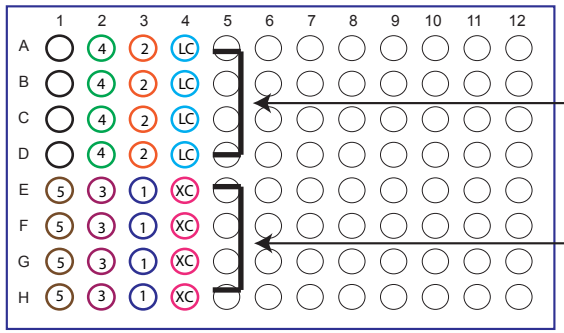
IMDM included with kit

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