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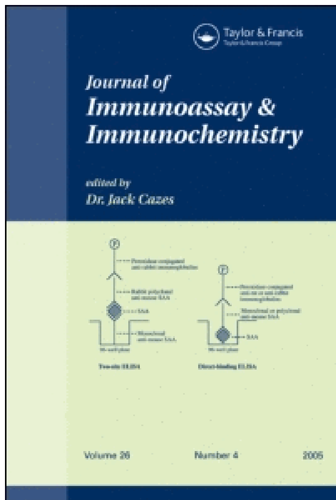


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
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**To cite this Article** Gao, Zu-hua , Briggs, William A. , Rose, Noel R. and Burdick, James F.(1998) 'Use of a Simple Light Absorbance Assay to Measure Lymphocyte Proliferation', Journal of Immunoassay and Immunochemistry, 19: 2, 129 — 143
**To link to this Article: DOI:** 10.1080/01971529808005477
**URL:** http://dx.doi.org/10.1080/01971529808005477

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JOURNAL OF IMMUNOASSAY, 19(2&3), 129–143 (1998)

# USE OF A SIMPLE LIGHT ABSORBANCE ASSAY TO MEASURE LYMPHOCYTE PROLIFERATION

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## ABSTRACT

The proliferative response of human lymphocytes to stimuli such as foreign histocompatibility antigens or mitogens is generally assessed by measuring the amount of tritiated thymidine which the cells incorporate in culture. In this paper, the possibility of assessing lymphocyte proliferation and viability by an empirical assay, using measurement of light absorbance on a ELISA reader in the yellow wave length (450 nm/air-550 nm/air), has been studied. The correlation of these measurements with a colormetric viability assay using MTS/PMS, with tritiated thymidine incorporation and with trypan blue exclusion viability counting, was determined. The results showed that the light absorbance assay correlate well with cell proliferation during 48-120 hours culture period and with cell viability after a 72 hours culture period. The MTS/PMS colormetric assay as well as trypan blue exclusion cell counting confirmed that the light absorbance assay was not merely caused by dead cells. This data confirm that the light absorbance assay is sufficiently sensitive to low levels of proliferation to allow detection of such responses at least as effectively as thymidine incorporation. The light absorbance assay procedure avoids the expense, time and hazards associated with scintillation counting, and is simple to perform without the necessity for reagents and preparative steps required by other assays.

(Key words: Cell proliferation, Thymidine, Mixed lymphocyte culture, Light absorption)



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## INTRODUCTION

The lymphocyte proliferative response to alloantigens, to mitogens, to growth factors or to triggering by monoclonal antibodies frequently involves measuring cellular proliferative response and is usually based on measuring the incorporation of tritiated thymidine ($^3$H thymidine) by cells as they enter synthesis phase. However, this method is subject to several hazards, i.e. generation of radioactive waste with a relative long half-life, and toxicity of the scintillation fluid (1). It is also work intensive and time consuming.

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During the last decade, numerous methods have been proposed which permit a nonradioactive assessment of cell proliferation. These include the incorporation of BrdU (2), the estimation of argyrophilic proteins of nuclear organizer regions (AgNORs) (3), DNA staining with fluorescence dyes (4), the assay of ATP bioluminescence (5) and ,more recently, the ELISA method by detection of the antigen that is recognized by the Ki-67-equivalent monoclonal antibody MIB1 (6). These techniques require the extraction of DNA, binding antibody to solid phase, or the preparation of conjugated anti-BrdU and therefore limited in general usefulness for the measurement of cell proliferation.

Since the initial report by Mosmann (7), in numerous studies on a wide variety of cell types, the validity of using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) tetrazolium dye to assay cell viability and proliferation has been well documented (8, 9, 10). However, the MTT assay also

has drawbacks, the primary one being the aqueous insolubility of the formazan end product. Among various modifications of the MTT assay aimed at overcoming this problem, a MTT analogue, termed MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), with the co-addition of PMS (phenazine methosulfate), was recently shown to be applicable for assaying cell proliferation and viability (11, 12).

In 1991, Dr. Zinsstag and his colleagues noticed that within the pH range of 7.0-7.5, the extinction values of phenol red at 560 nm correlated linearly with the pH of the medium. Cell proliferation in tissue culture increases acidity and therefore turns the media color to yellow. In the present investigation, the empirical validity of monitoring lymphocyte proliferation and cell viability by means of simple light absorbance assay at the yellow wave length of 450 nm/air and deducting the OD at 550 nm/air as the reference signal is demonstrated by analyzing the correlation with the MTS/PMS colormetric assay, $^3$H thymidine incorporation and trypan blue exclusion cell counting.

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## MATERIALS AND METHODS

### Human Peripheral blood sample

Peripheral blood samples from 21 dialysis patients with end stage renal diseases for steroid inhibition test were obtained from Division of Nephrology, Department of Medicine, The Johns Hopkins Hospital. Among them, male 12, female 9, age 17 to 55. Peripheral blood samples from 26 patients after kidney

transplantation for mixed lymphocyte reaction test were obtained from Division of Transplantation, Department of Surgery, The Johns Hopkins Hospital. Among them, male 11, female 15, age 17 to 55.

**Mixed lymphocyte reaction ( MLR)**

Lymphocytes prepared by Percoll sedimentation were cultured in 100 μl aliquots at a concentration of $2x10^6$ cells/ml in triplicate wells. 100 μl of allostimulator cells were added at a concentration of $10^6$ cells/ml. Phytohemagglutinin (PHA, GIBCO BRL) dissolved in tissue culture medium (TCM, RPMI supplemented with 10% human AB serum, 2 mM glutamine, 0.25 nM HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin) in a concentration of 15: 1000 (v / v ) acted as the positive control stimulator and TCM acted as the negative control. Cells were cultured at 37°C in humidified incubators containing 5% $CO_2$-95% air.

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**Steroid inhibition test (SIT)**

Patient's lymphocytes were prepared as for MLR in 96 well plates. PHA in a concentration of 15: 1000 (v / v ) was added in 100 μl aliquot into these wells as stimulator. Prednisolone (P) and methylprednisolone (MP) (Sigma) were dissolved in pure alcohol, and diluted from $10^{-5}$ to $10^{-8}$ M by TCM. 5 μl of P and MP were added, respectively, into the PHA stimulated wells. Cells were cultured under the same conditions as MLR.

**$^{3}$H Thymidine incorporation**

After 3 days culture for SIT and 5 days culture for MLR, 1 μci of $^{3}$H thymidine was added to each well. After 16 hours of culture, the cells were harvested with Titertek cell harvester, the filter discs removed and transferred to scintillant fluid for counting in Beckmann LS 6000 scintillation counter.

**MTS/PMS colormetric assay**

MTS. $3H_2O$ (obtained from Dr. T.C. Owen, University of South Florida) was dissolved in distilled water to obtain a concentration of 6.39 mM and filter sterilized. PMS (Aldrich) was dissolved into 21 times concentrated Dullbecco's phosphate-buffered saline (DPBS, GIBCO BRL) to obtain a concentration of 6.93 mM. The working mixture was pH 7.3, 6 mM MTS.$3H_2O$/0.33 mM PMS, and buffer/salt concentrations as in common DPBS. Twenty microliter of the MTS/PMS solution were added to culture wells containing 2-3x$10^{6}$ lymphocytes in 225 μl culture medium. The final concentration obtained was 0.49 mM MTS/0.027 mM PMS. After 4 hours culture, the amount of reduced formazan produced was assayed by measuring the optical density at 570 nm using the DYNATECH MR5000 ELISA plate reader.

**Light absorbance assay**

At day 3 for SIT and day 5 for MLR, before adding $^{3}$H thymidine, the plates were read by the DYNATECH MR5000 ELISA plate reader at a wave length of 450 nm/air-550 nm/air.

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## Comparison of light absorbance assay, MTS/PMS colormetric assay, $^3H$ thymidine incorporation and cell viability

Five identical plates were set up at the same time. Plates were read after 24, 48, 72, 96 and 120 hours of culture. At these times, MTS/PMS assay, $^3H$ thymidine incorporation and trypan blue exclusion hand counting were executed respectively.

## Data analysis

OD values of light absorbance assay, MTS/PMS colormetric assay, cpm from $^3H$ thymidine incorporation and number of viable cells obtained from 21 SIT patients and 26 MLC patients were plotted, the best curve fit and the correlation coefficient was calculated by the SYSTAT Pearson correlation coefficient analysis system.

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# RESULTS

## Correlation of light absorbance assay and $^3H$ thymidine incorporation in MLR and SIT

As shown in figure 1 and figure 2, curves of OD values of light absorbance assay in both SIT and alloantigen stimulated MLR show the same pattern as the $^3H$ thymidine incorporation cpm values. The Pearson correlation test showed significant positive correlation between the OD and cpm values in the two experiments ($r=0.964$, $P<0.0005$). Figure 3 show a linear correlation between

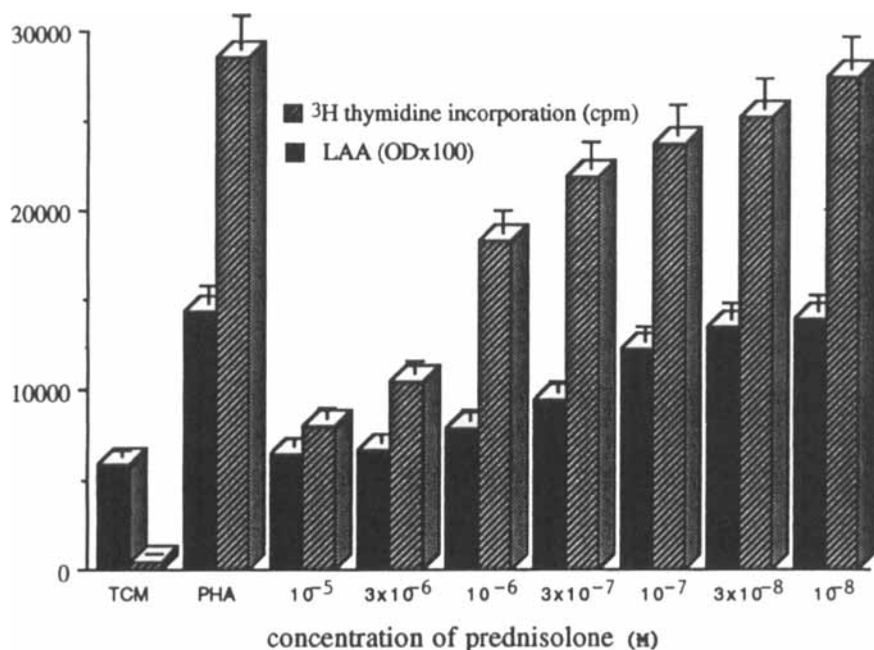


**Figure 1. Comparison of light absorbance assay with $^3$H thymidine incorporation in steroid inhibition test.**

PHA stimulated lymphocyte proliferation was inhibited by different concentrations of prednisolone. TCM and PHA stimulated MLR act as negative and positive control. At day 3, plates were read by light absorbance assay and harvested for the measurement of $^3$H thymidine incorporation. Data are expressed as mean±SD of triplicate culture wells in three different patients.

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the light absorbance assay OD and $^3$H thymidine incorporation cpm values from both experiments.

**Comparison of the light absorbance assay, MTS/PMS colormetric assay, $^3$H thymidine incorporation and cell viability during culture**

During 48 to 120 hours culture, both the light absorbance assay and MTS/PMS colormetric assay correlated well with $^3$H thymidine incorporation

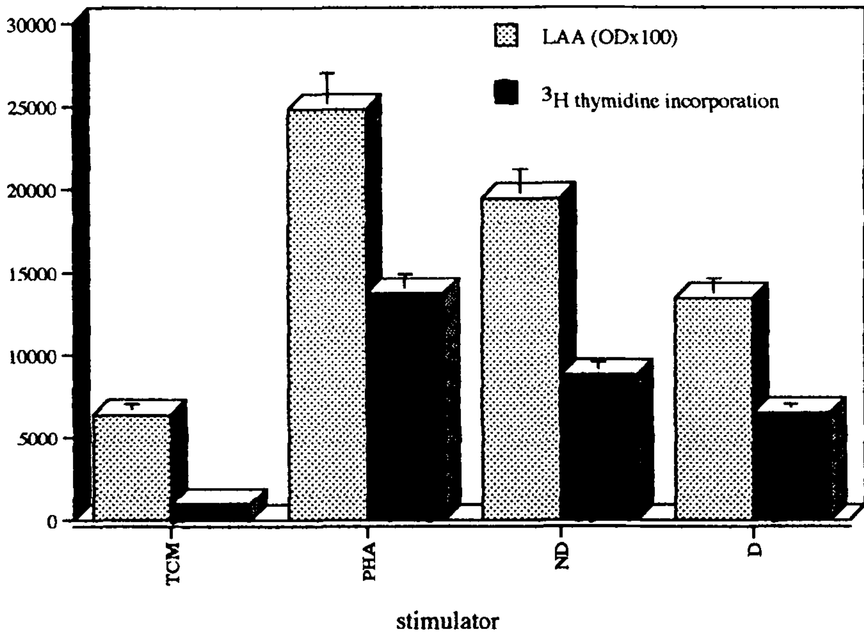


**Figure 2. Comparison of light absorbance assay with $^{3}$H thymidine incorporation in MLR.**

Patients' lymphocytes were stimulated by irradiated (5000 rads) allogenic lymphocytes depleted or not depleted of antigen presenting cells (D, ND) as stimulators and TCM/PHA as negative/positive control. At day 5, plates were read by light absorbance assay and harvested for measurement of $^{3}$H thymidine incorporation. Data are expressed as mean±SD of triplicate culture wells in three different patients.

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which represents cell proliferation ($P<0.01$). After 72 hours in culture, both light absorbance assay and MTS/PMS colormetric assay not only correlated well with cell $^{3}$H thymidine incorporation, but also correlated well with cell viability ($P<0.01$). By reading the plate every 12 hours, we found that the peak of both PHA and alloantigen stimulated lymphocyte proliferation can be reached after 84

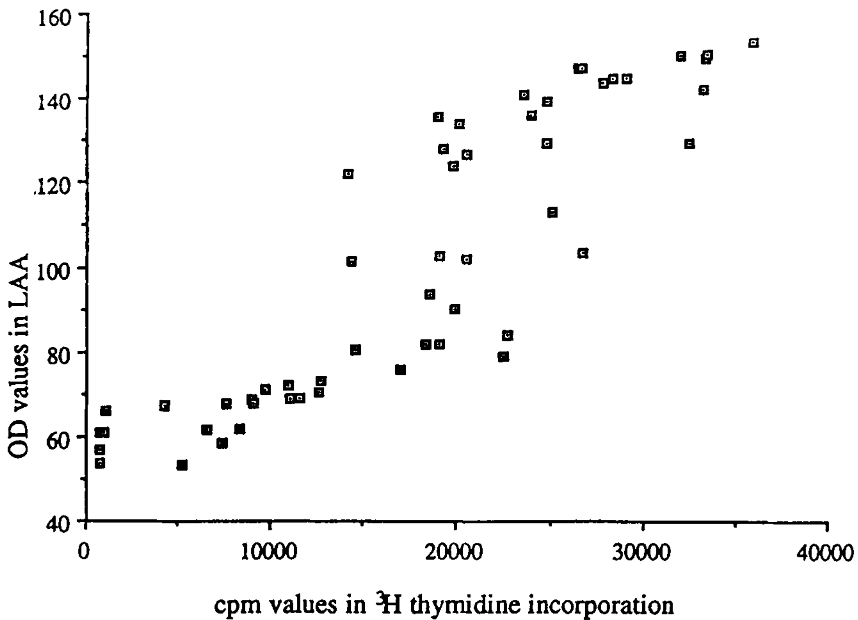


**Figure 3. Correlation of light absorbance assay with $^{3}$H thymidine incorporation.**

Culture plates from three steroid inhibition test (27 test in triplicate) were read at day 3, and six mixed lymphocyte reaction (24 tests in triplicate) were read at day 5. $^{3}$H thymidine incorporation were measured immediately after plate reading. Each symbol represents the mean of triplicate culture wells.

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hours culture (Figure 4). A good correlation between light absorbance assay and $^{3}$H thymidine incorporation can be achieved at around 84 hours.

## Threshold for number of cells measurable by the light absorbance assay

Responder cells from patients were mixed with TCM in double dilution and stimulated with PHA. TCM alone act as the background. As shown in figure 5, a positive reading above background represents a detection threshold for

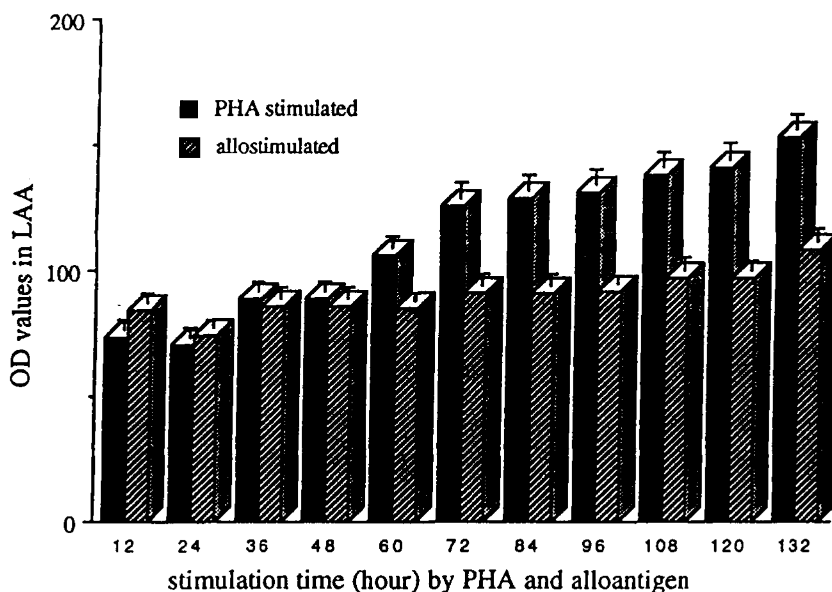


**Figure 4. Time dependence of light absorbance assay**

PHA and alloantigen-stimulated lymphocyte culture plates were read by light absorbance assay ever 12 hours. Responder cells were from the same donor. Data are expressed as mean±SD of 18 culture wells in each group on the same culture plate.

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number of cells whose proliferation to this stimulus is measurable at approximately 7800 cells/ml.

## DISCUSSION

It is generally accepted that cell stimulation induces the activation of the $Na^+$-$H^+$ exchanger on the cell membrane which extrudes proton from the cell (14,15). The migration of $H^+$ to the extracellular environment results in the pH reduction of the culture media. RPMI 1640 (GIBCO), which contains 0.0125 mM

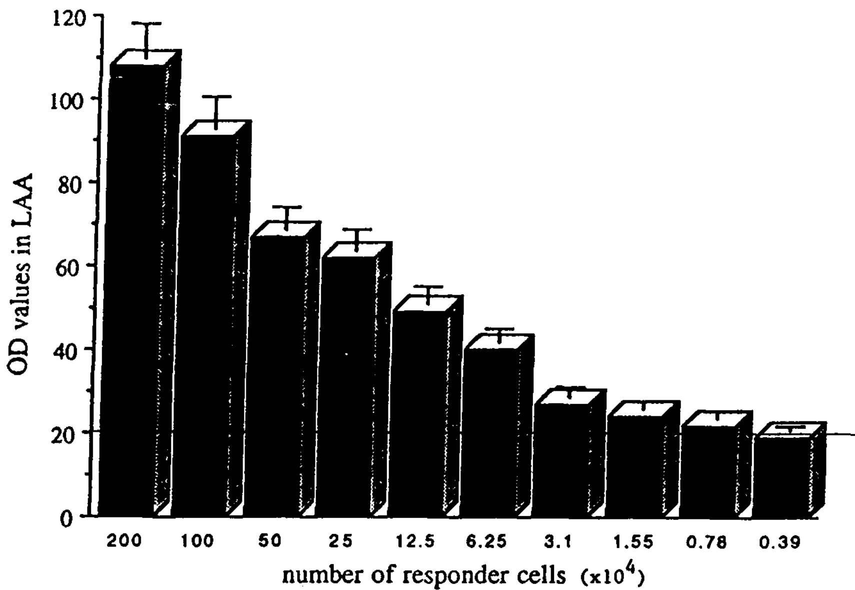


**Figure 5. Threshold for number of cells measurable by the light absorbance assay**

Responder lymphocytes were diluted in double dilution's and stimulated by PHA. TCM alone act as the background. After culture for three days, the plates were read by light absorbance assay. Data are expressed as mean ± SD of six culture wells on the same culture plate. The horizontal line shows the background value.

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phenol red, was used in the SIT and allostimulated MLR as the culture media. The transition interval of phenol red is red at pH 8.2 to yellow at pH 6.8. Decreased pH resulting from cell proliferation caused the media color changes from red to yellow during the culture period. We noticed by eye that after three to five days of culture, the culture media changed from pink to yellow, paralleling the results of $^{3}H$ thymidine incorporation. Measurement of the OD of the yellow

color is done by reading the plate at its maximum absorption wavelength (450 nm/air) and subtracting the reference signal (550 nm/air) to eliminate systematic errors.

The data presented here clearly show that cell proliferation and cell viability can be measured by direct OD reading in an ELISA reader at a wavelength of 450 nm/air-550 nm/air (the light absorbance assay). The OD values after 72 hours of culture correlated closely with the cpm value measured by $^{3}$H thymidine incorporation and with the OD values by MTS/PMS colormetric assay. We also noticed that after 10-15 minutes at room temperature (22°C, 100% air), the OD values decreased presumably due to decrement of $CO_2$ in the solution with time out of the incubator. Although this OD reduction does not affect their correlation with the final cpm value in $^{3}$H thymidine incorporation, we suggest that the plate to be read within a standard time after removal from the incubator. We usually read the plate within 10 minutes.

The light absorbance assay and MTS/PMS colormetric assays measure cell proliferation at the end of the assay, whereas, $^{3}$H thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. This should be kept in mind for specific applications, e.g. for the purpose of distinguishing between death, survival and proliferation.

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The light absorbance assay described in this paper shares with $^3$H thymidine incorporation assay the advantages of precise quantitation and compatibility with computer analysis programs. This assay can measure as few as 7800 proliferating lymphocytes. Its main advantages are simplicity and speed. No special reagents are needed. A 96-well plate can be processed within 90 seconds. No radioisotopes are used, and no scintillation counter or gamma counter is needed. This advantage is partially offset by the requirement for a plate reader, but the scanning rate of a typical machine allows a single plate reader to handle very large numbers of samples. Therefore, this light absorbance assay represents a practical and attractive alternative to $^3$H thymidine incorporation for the measurement of cell proliferation and cell viability.

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