SciForschen
Open HUB for Scientific Research
ISSN 2576-5833
Journal of Biochemistry and Analytical Studies
Short Communication Volume: 1.1 Open Access

Basic Condition to Formazan Improve Sensitivity of the MTT Colorimetric Assay Dye

Mariarita Perri, Maria Cristina Caroleo and Erika Cione*

Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Italy

***Corresponding author:** Erika Cione, Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Italy, Tel: 39-0984-493193; Fax: 39-0984-493107; **E-mail:** erika.cione@unical.it

Received date: 30 Mar 2016; **Accepted date:** 10 May 2016; **Published date:** 16 May 2016.

Citation: Perri M, Caroleo MC, Cione E (2016) Basic Condition to Formazan Improve Sensitivity of the MTT Colorimetric Assay Dye. J Biochem Analyt Stud 1(1): doi http://dx.doi.org/10.16966/2576-5833.104

Abstract

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay is able to discriminate living cells; the signal generated is dependent on the degree of activation of the cells. In this paper, we show how the tetrazolium dye procedure conducted on a limited number of cells ($1x10^3$) is enhanced under basic condition. Moreover, the basic condition generate a more stable dye where only one peak is highlighted at 545 nm, abolishingbackgrounds. A solution of NaOH 7N was usedas formazan enhancer. The basic milieu turns the formazan dye deriving form low number of cells from brown to intense purple and provides a stable and more sensitive way, leading to high effectiveness of the assay. This strategy can be a useful tool for spectrophotometer analysis where the limit of detection of formazan dye is a problem and a plate reader is not available.

Keywords: Tetrazolium dye; MTT; Sodium Hydroxide

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay, originally described by Mosmann, has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation and the method was successively modified for better resolution [1-3]. For survival and proliferation determination, other methods, such as [^{3}H]-thymidine uptake (^{3}H-TdR) or trypan blue are available but actually, the MTT assay replaced both. The signal generated is dependent by the capability of a mitochondrial dehydrogenase of living cells, when cytochrome *c* is not released, to produce formazan from tetrazolium salt [4,5]. However, the original technique has several technical limitations, namely a less than optimal sensitivity, a variable background due to protein precipitation on adding an organic solvent to dissolve the blue formazan product, and a low solubility of the product. These problems have been overcome by several modifications: i) avoidance of serum and phenol red in the incubation medium; ii) use higher concentration of MTT, iii) elimination of the medium containing MTT after the reaction and subsequent use of pure propanol, isopropanol or ethanol to rapidly solubilize the formazan crystals and iv) use of a more judicious reference wavelength in a dual wavelength spectrophotometer [3]. Due to these modifications the reliability and sensitivity of the test have been increased to the point where it can replace the [^{3}H] thymidine uptake assay, in many cases, to measure cell proliferation or survival in growth factor or cytotoxicity assays. The results can be read on a micro-plate reader or in a standard spectrophotometer. It is worth to note that MTT assay is very easy and main advantages are: i) elimination of radioactive compounds; ii) reproducibility; iii) performance and quantification facility and iv) rapidity. However, there is a further technical limitation, due to the variability among read peaks [5-8]. Here, we introduced a further modification to the tetrazolium dye technique, using basic condition (NaOH 7 N) that yields an improved stability and sensitivity of the dye. To verify the stability (after reduction by cells in formazan) the absorbance was monitored in a range of wavelength between 400 and 700 nm. Briefly, MTT was dissolved in a stock solution: 5 mg/ml MTT (Sigma) in RPMI-1640, DMEM or DMEM/F-12 without phenol red and serum (Gibco, Invitrogen). This solution was filtered through a 0.2 µm filter and stored at 4°C. For the assay 1:40 dilution of the 5 mg/ml stock was used in a volume of 1.0 ml for each well of 24-wells plate. 1×10^3 cells were incubated at 37°C for 120 minutes. As a first step, the converted dye is solubilized with 1 ml acidic isopropanol/0.04 N HCl, pipetted up and down several times to assure that the converted dye dissolves completely and then transferred into a 1.5 ml eppendorf tube and centrifuge at 13,000 rpm for 2 minutes. The supernatant is transferred into a new eppendorf tube and 25 µl Sodium Hydroxide (NaOH) 7 N were added to the dye solution, vortexed for 10 secs and centrifuged at 13,000 rpm for 2 minutes. Conversely, to the method found for determination of protein concentration, namely WST-8 formazan (Dojindo), in which MTT is reduced to formazan by basic condition, our method is based on the capability of the already reduced formazan to produce, under basic condition, higher intensity color that become more stable when monitoring the absorbance [9]. We observed that the formazan dye is brown at acidic pH, and turns to intense purple when the pH is 9.5 with a maximum wavelength peak at 545 nm; the solution is stable for more than one week at room temperature. As we can see in the Figure 1A-B, under the conditions used, only one peak is highlighted at 545 nm. Further, in order to evaluate the sensitivity of the modified dye, the MTT-formazan (Sigma) was used as reference standard and with the formazan enhancer a 1/10 of the initial quantity was detected with a comparable absorbance value. In the absence of NaOH 7N no peaks were detected in presence of MTT-formazan (Figure 1C) 50 µg or (Figure 1D) 500 µg in a range of 400-700 nm wavelenght. The absorbance of the converted dye was detected with an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences) in plastic cuvettes. According to the capability of the formazan to turn to intense purple after exposition to a strong base milieu, the now abolished variability among read peaks makes the MTT assay more attractive even if laboratories cannot use plate reader supply.

Acknowledgments

This work was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Italia). The authors thanks Dr D. Sturino for english revision.

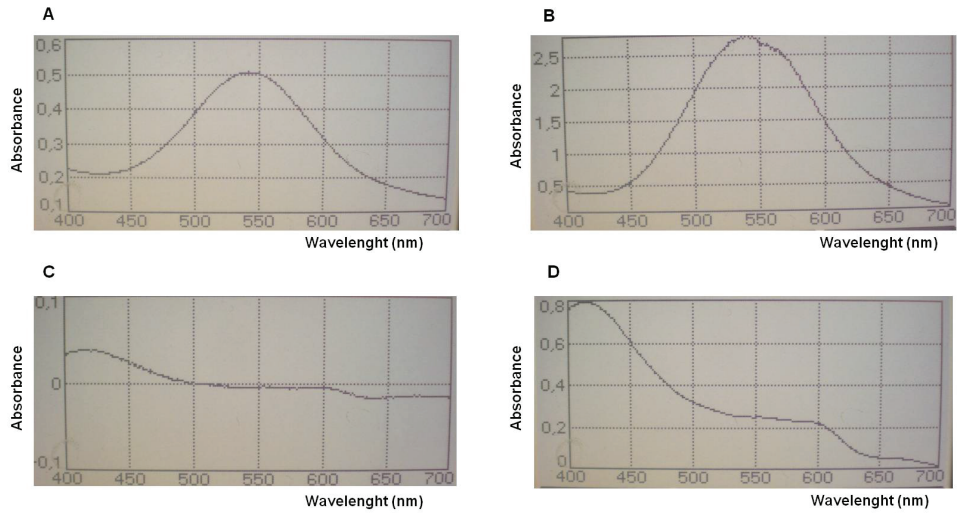

Figure 1: Basic conditions to Formazan improve sensitivity of the MTT assay dye. The enhancer NaOH 7N was added in presence of MTT-formazan (A) 50 µg or (B) 500 µgand a well defined peak was highlighted at 545 nm wavelenght. In the absence of NaOH 7N no peaks were detected in presence of MTT-formazan (C) 50 µg or (D) 500 µg in a range of 400-700 nm wavelength.

References

1. Ferrari M, Fornasiero MC, Isetta AM (1990) MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. J Immunol Methods 131: 165-172.
2. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55-63.
3. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89: 271-277.
4. Kok SH, Gambari R, Chui CH, Lau FY, Cheng GY, et al. (2007) Paradoxical proliferative potential of iron (II) sulphate on cancer cells after the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Int J Mol Med 19: 971-975.
5. Cione E, Tucci P, Senatore V, Perri M, Trombino S, et al. (2008) Synthesized esters of ferulic acid induce release of cytochrome c from rat testes mitochondria. J Bioenerg Biomembr 40: 19-26.
6. Zhao XY, Zhao LY, Zheng QS, Su JL, Guan H, et al. (2008) Chymase induces profibrotic response via transforming growth factor-beta1/Smad activation in rat cardiac fibroblasts. Mol Cell Biochem 310: 159-166.
7. Sippel RS, Carpenter JE, Kunnimalaiyaan M, Lagerholm S, Chen H (2003) Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells. Am J Physiol Gastrointest Liver Physiol 285: 245-254.
8. Van Gompel JJ, Kunnimalaiyaan M, Holen K, Chen H (2005) ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells. Mol Cancer Ther 4: 910-917.
9. Theiszová M, Jantová S, Dragúnová J, Grznárová P, Palou M (2003) Comparison the cytotoxicity of hydroxyapatite measured by direct cell counting and MTT test in murine fibroblast NIH-3T3 cells. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149: 393-396.