THE 4th GENERATION
NON RADIOACTIVE
CELL PROLIFERATION &
CYTOTOXICITY ASSAY

EZ4U (*NONRADIOACTIVE CELL PROLIFERATION AND CYTOXICITY)*

ELISA KIT

NONRADIOACTIVE CELL PROLIFERATION AND CYTOTOXICITY ASSAY.
CAT. NO. BI-5000 10 X 96 DETEREMINATIONS

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

rev.no. 301004 (replacing 020604)

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1) INTRODUCTION

Proliferation assays are widely used in cell biology for the study of growth factors, cytokines, nutrients and for the screening of cytotoxic or chemotherapeutic agents. There are several ways to determine the number of cells either by microscopic inspection, or by the use of an electronic particle counter, indirectly by measuring the incorporation of radioactive precursors, quantitating total protein with chromogenic dyes, or by measuring metabolic activity of cellular enzymes. The most common assay for cell proliferation is the incorporation of 3H-thymidine into cellular DNA. The 3H-thymidine assay is, however, labour intensive as it requires the removal of excess, unincorporated label by using some method of cell harvesting before measurement. In 1956, the first paper was published on the use of tetrazolium salts as indicators of cell viability. The method was based on the finding that living cells are capable to reduce slightly or uncoloured tetrazolium salts into intensely coloured formazan derivatives. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death.

This method therefore provides an excellent tool for the discrimination of living and death cells. However, the early tetrazolium salts did have some disadvantages, such as the insolubility of the resulting formazan products. Time and labour consuming resolubilisation procedures were necessary, including repipetting and mixing, or the application of hazardous solubilisers. This necessary post assay treatment, however, irreversibly terminated cell proliferation and thus made it impossible to prolong incubation in order to achieve an increase in sensitivity or continue cell culture. These inconveniences led to the development of non-toxic tetrazolium salts which yield soluble reduction products. Although the assay procedure was made easier by these soluble dyes, in practice the use was limited due to the instability of the formazan dye and a relatively low absorbance of the end product as compared to the classical MTT assay.

The BIOMEDICA research department has solved both problems and created an easy to use, rapid and reliable non-isotopic cell proliferation assay. For convenience, we have made it highly compatible with the standard thymidine incorporation assay. Therefore, no changes are required in the setup of the test and in the "labelling" procedure. Furthermore, there is no need for the removal of culture medium before or after the addition of the chromogenic substrate and neither solubilisation nor harvesting procedures are necessary. The work performed by BIOMEDICA resulted in an assay which combines the best of the thymidine and MTT methods, namely: accuracy, speed, reliability and ease of use. Also, according to our data achieved so far, the chromophore appears to be non-toxic. A double labelling with EZ4U and a radioactive nucleotide to obtain more information about cell viability and DNA content is now feasible.

2) CONTENTS OF THE KIT

CONT	KIT COMPONENTS	QUANTITY
SUB	Substrate, lyophilised	10 vials
ACT	Activator solution, ready to use	1 x 30 ml
	Packaqe insert	1 piece

3) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

Microtiterplate reader equipped with a 450 nm or 492 nm filter (see Fig.1). We recommend the use of a 620 nm to 690 nm reference wavelength, which is beneficial but not absolutely necessary.

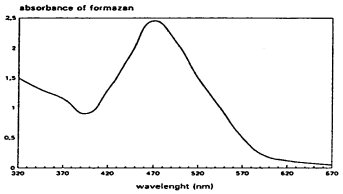

Fig. 1. Absorption spectrum of Formazan.

4) REAGENTS AND SAMPLE PREPARATION

One vial SUB in this EZ4U (Nonradioactive Cell Proliferation and Cytoxicity) ELISA Assay kit contains the amount of substrate sufficient for one 96 well plate with 200 µl of cell culture medium/well. If more substrate is needed, combine the dissolved substrates prior to pulsing.

Dissolve the SUB (substrate) in 2.5 ml ACT (activator). Prewarm this solution to 37°C prior to addition. If necessary, warm up the substrate vial in your hand while mixing with activator.
This procedure yields a straw-coloured solution.

The mixed substrate is designed for immediate use only and should not be stored.

5) GENERAL CONSIDERATIONS FOR SETTING UP ASSAYS WITH EZ4U

The assay set-up is performed in a manner similar to the standard 3H-thymidine incorporation method. Instead of pulsing with tritiated nucleotide, 20 µl of dye solution is added to 200 µl sample. Incubation time is dependent on the metabolic capacity of the cells. Usually 2 to 5 hours of incubation at 37°C are sufficient to yield a significant increase in color intensity. As different cells vary in their ability to convert the yellow colored tetrazolium compound to its red formazan derivative, we recommend testing every new cell-line's metabolic capacity as described in Fig.2. After incubation, the plate is removed from the incubator and gently mixed by tipping the plate at all four sides. To avoid increased standard deviations, the plate must be shaken before reading the optical density.
The absorbance is measured by a microplate-reader, set at 450 nm or 492 nm with 620 nm as a reference. The reference absorbance at 620 nm (or any wavelength between 620-690 nm) is used to correct for nonspecific background values, caused by cell debris, fingerprints, or other potential interferences. However, the reference may be omitted without significant changes in the accuracy of the assay.

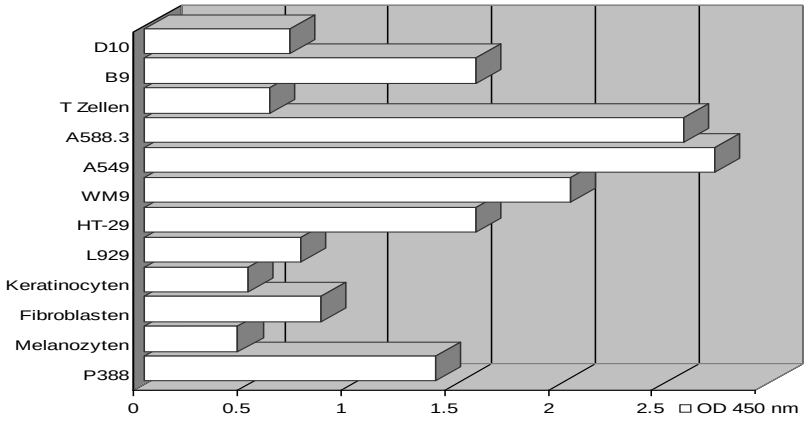

Fig. 2. Different metabolic capacity of various cell lines.

$3x10^3$ cells/well were cultivated in 200 µl RPMI 1640. Following a cultivation period of 3 days, 25 µl of the dye substrate were added to each well. Optical density was recorded after 4 hours, showing significant differences in the metabolic capacity of the various cell lines.

6) IMPORTANT CONSIDERATIONS

- The substrate is not sterile. If sterile conditions are demanded, the solubilised ready to use dye substrate must be sterile filtered. (A minor turbidity prior filtration interferes neither with the filtration, nor with the assay performance).
- Due to the high sensitivity of this test, it is advisable to use as little cells as possible. Otherwise the occurrence of a non linear titration-curve may be possible.
- To achieve reproducible time kinetics in color development, equilibrate cell cultures at 37°C.
- Do not prolong incubation times without pretesting, this might result in an increased background without improved sensitivity.
- The use of a reference wavelength of 620 nm (which is subtracted from the values obtained at 450 or 492 nm) is not absolutely necessary, but increases the performance of the test.
- The chromophore appears to be non-toxic and therefore prolongation of cell culture is possible after removal of the formazane derivative.

7) ASSAY PROTOCOL

All reagents and samples in this EZ4U, (Nonradioactive Cell Proliferation and Cytoxicity) ELISA Assay kit must be at room temperature (18-26°C) before use in the assay.
Add 200 µl cell culture into respective wells.
Add 20 µl SUB (substrate) into each well, swirl gently.
Incubate at 37°C for 2-5 hours, depending on the metabolic capacity of the cells.
If no microplate reader with shaking plate carrier is available, mix the plate on a vibrating platform or by tipping with the fingers.
Read absorbance at 450 nm or 492 nm, with 620 nm as reference.
For the most accurate results, absorbance from a substrate blank in assay medium without cells should be subtracted from all other values.

8) TECHNICAL HINTS

- Do not mix or substitute reagents with those from other lots or sources.
- Do not mix stoppers and caps from different reagents or use reagents between lots.
- Do not use reagents beyond expiration date.
- Protect reagents from direct sunlight.
- Avoid foaming when mixing reagents.

9) PRECAUTIONS

- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Avoid all contact with the reagents by using gloves.

10) LITERATURE

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SYMBOLS

Expiry date / Verfallsdatum / Date de péremption / Data di scadenza /Fecha de caducidad / Data de validade / Uiterste gebruiksdatum / Udløbsdato / Utgångsdatum / Termin Ważności / Lejárati idő / Doba exspirácie / Doba exspirace

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