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A NEW CYTOCOLORIMETRIC ASSAY USING PHOSPHATASE ACTIVITY FOR MEASURING CELLULAR FUNCTIONS

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ABSTRACT

In this study cellular phosphatase activity, referred to as enzyme developed color (EDC) assay, was used for an *in vitro* assessment of cell proliferation and cytotoxicity. Optimum conditions were established and found to be 10×10^3 cells / well at 37°C and 60 min incubation (for developing color). Under the same conditions a direct correlation between optical density (OD) and cell number was observed. Thus, the ODs for a cell line, Ish at 100, 50, 25 and 10 $\times 10^3$ cells/well were 1.08 ± 0.05, 0.59 ± 0.02, 0.33 ± 0.02, and 0.17 ± 0.01 respectively ($r=0.995$, $p<0.001$). When EDC was compared with another colorimetric assay, i.e. MTT, the results showed a direct correlation with $r=0.995$, $p<0.001$.

Repeating the experiment with live and fixed tumor cells showed similar results. Thus, for the Ish line, the ODs at 50 and 10 $\times 10^3$ cells/well for fixed and live cells were 0.66 ± 0.01, 0.18 ± 0.01 and 0.64 ± 0.03 ($p>0.05$), 0.19 ± 0.01 ($p>0.05$), respectively. The assay was also shown to be suitable for the measurement of cell cytotoxicity and compared well with the MTT assay.

These findings indicated that a simple, rapid and economical EDC assay could be used to investigate various cellular functions. The main advantage of EDC is it's suitability to use stored cells. This provides flexibility for testing samples stored over a long period in order to limit inter-experimental variations.

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INTRODUCTION

The use of *in vitro* tissue culture has proven invaluable for investigation of various cellular functions. In many cases the initial screening of pharmacological agents in clinical use had been carried out in cell culture models. These *in vitro* techniques include simple cell counting, trypan blue dye exclusion technique,[1] FACs analysis,[2] incorporation of radioactive thymidine[3] and the non-

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radioactive thymidine analogue bromodeoxyuridine.[2,4]

In our previous study,[5] we modified the colorimetric technique originally described by Mosmann[6] for estimating tumor cell viability after their exposure to various chemicals. In addition, the use of this technique was extended[5] to replace the conventional radioactive thymidine assay used for cell proliferation and chromium release assay for cytotoxicity studies.[7,8]

Techniques based on phosphatase activity have long been used for measurement of serum placental alkaline phosphatase (PLAP) in patients suspected of testicular cancer.[9] The alkaline phosphatases (ALP) are members of non-specific phosphomonoesterases capable of hydrolyzing a range of phosphate monoesters[10] as well as hydrolysis of orthophosphate esters, inorganic pyrophosphate and also carry out transphosphorylation activity.[11] Though the precise role of ALPs are to be elucidated, it is believed that they are involved in the transport of specific substances from plasma membranes, and participate in hydrolysis of intracellular metabolites. The placental isoform has been reported to act as the Fc receptor for IgG, hence facilitating the transport of Ig into the fetus, from the maternal circulation. In addition, ALP have also been reported to play an important role in the process of wound healing. [11-13]

The aim of this study was to investigate whether it was possible to use ALP activity for the measurement of cellular functions.

MATERIALS AND METHODS

Development of cell lines

Two of the lines used for this study were in house established tumor lines, i.e. Wil and Fen from transitional bladder tumors.[14] They were established from fresh tumor biopsies by finely cutting the tissue. After passage through a sieve with the aid of a sterile syringe plunger and washing, cells were cultured overnight at 37°C in RPMI, containing 10% v/v fetal calf serum (FCS, Sigma). The non-adherent cells and cell debris were removed and replaced by fresh medium. Adherent cells were fed until confluence (1-2×10^6/25cm^2 flask) and were expanded by trypsinization and seeded at a lower cell number (0.5×10^6/25cm^2 flask). Other cell lines and their sources have previously been reported by Nouri et al.[8] For JEG3 see Rinke De Wit.[15] Tera II, Ep 2102, T47D and T24 lines were obtained from ATCC.

Enzyme developed color (EDC) technique

Tumor cells were trypsinized, counted and dispensed onto flat-bottomed microtiter plates (100 µL/well) at varying cell numbers. They were incubated under various conditions and after the termination of incubation (see below) the supernatants were removed by flicking the plate. The cells

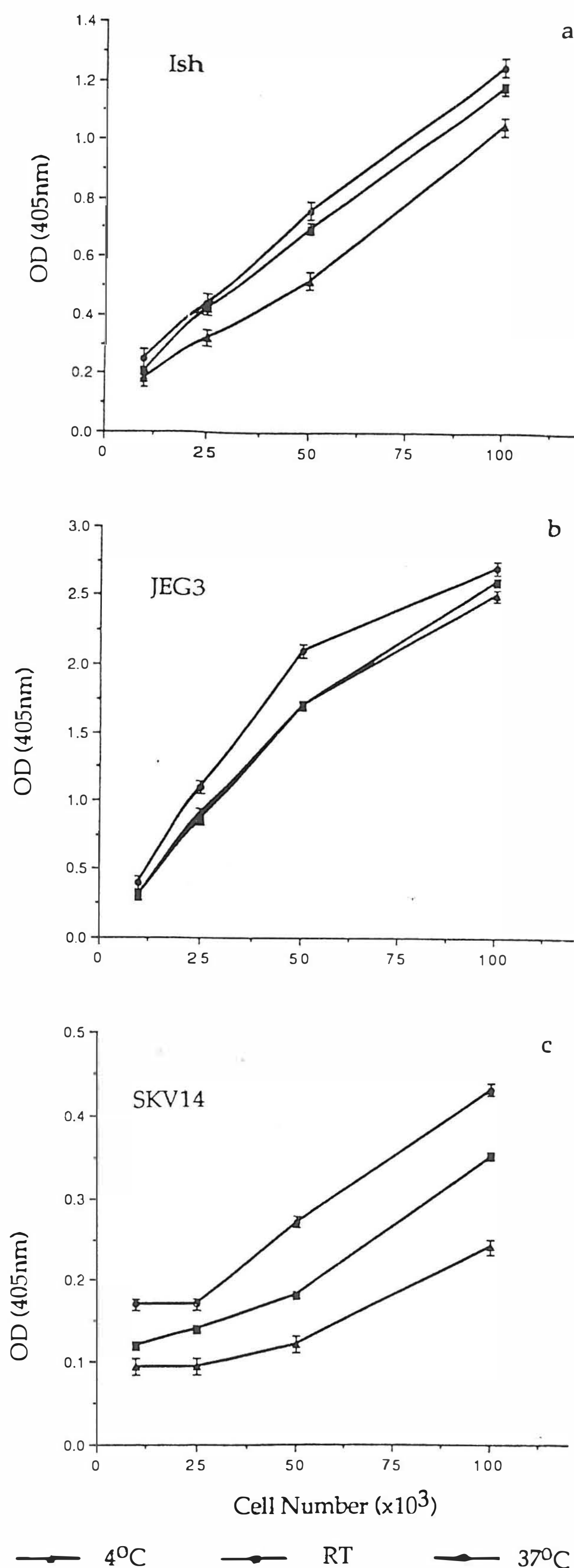

Fig. 1. Optimization of incubation conditions.

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Table I. Optical densities of various cell lines using EDC assay.

Cell line	25×10^3 cells/well		25×10^3 cells/well	
	30 min	60 min	30 min	60 min
Testis				
Tera II	1.72 ±0.01	2.59 ±0.01	0.64 ±0.01	0.95 ±0.10
EP2102	>3	>3	1.98 ±0.04	2.78 ±0.10
Bladder				
Wil	0.10 ±0.01	0.11 ±0.01	0.09 ±0.01	0.09 ±0.01
Fen	0.11 ±0.01	0.13 ±0.01	0.09 ±0.01	0.10 ±0.01
T24	0.10 ±0.01	0.08 ±0.01	0.04 ±0.01	0.04 ±0.01
Others				
Ish	0.41 ±0.02	0.44 ±0.06	0.17 ±0.01	0.18 ±0.01
JEG3	0.29 ±0.02	0.66 ±0.06	0.14 ±0.01	0.23 ±0.03
T47D	0.10 ±0.01	0.11 ±0.01	0.09 ±0.01	0.09 ±0.01

Results are presented as mean ±SD of optical densities (OD).

Table II. Qualitative assessment of ADC and EDC assays.

Lines	ADC	EDC
Testis		
Tera I	-	++++
Tera II	-	++++
Ep2102	-	++++
Bladder		
Wil	-	±
T24	-	-
5637	-	-
Fen	-	-
Others		
Ish (endometrium)	+++ ±*	++ ±
JEG3 (choriocarcinoma)	+++*	+++

Qualitative results of EDC (after 60 min at 37°C) and ADC assay are expressed as strong positive (++++) to negative (-). Values denoted by * indicate cases where up to 30% of the cells showed strong positivity.

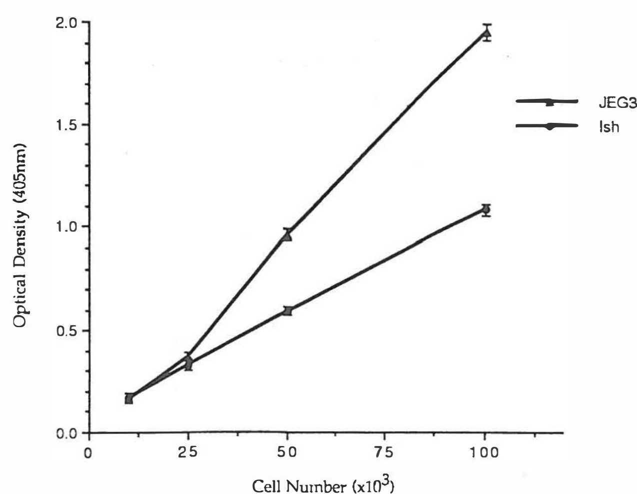

Fig. 2. Correlation between cell number and optical density in EDC assay. Results are plotted as OD against cell number.

were then washed with PBS + NaN_3 (0.02% v/v) and following flicking, 100 μL of phosphate substrate buffer (24.5 mg $MgCl_2$, 48 mL of diethanolamine adjusted to pH 9.8 in a total volume of 500 mL) containing 1 mg/mL of p-nitrophenyl phosphate, disodium substrate tablet (P104-105, Sigma) was added to each well. The plates were incubated for varying lengths of time and under different temperatures. At the termination of the experiment, the optical density (OD) was measured using an ELISA reader at 405 nm.

MTT assay

MTT assay was carried out as described by Hussain.[5] Tumor cells were trypsinized, counted and dispensed onto flat-bottomed microtiter plates (100 μL/well) at varying cell numbers. They were incubated under various conditions and after the termination of incubation 10 μL of MTT (5 mg/mL, Sigma) was added and incubated for three hrs at 37°C. The supernatants were removed and replaced with 100 μL/well of isopropanol containing HCl (0.04 M) and the ODs were measured. The cytotoxicity assay was carried out by adding interleukin-2 (Cetus, 100 U/mL/2×10^6) activated peripheral blood mononuclear cells (LAK) at various effector to target ratios. The cell mixtures were incubated for 4 hrs before washing the cells with RPMI + 2% FCS. This was followed by the addition of 100 μL of RPMI plus 10 μL of MTT for a further 3 hr. The content of each well was then discarded and 100 μL/well of acidified isopropanol was added and incubated for 30 min after which the ODs were determined.

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Table III. Optical densities of various cell lines using EDC and MTT assays.

	Tera II		Ish		JEG3	
Cell no. ($\times 10^3$/well)	MTT	EDC	MTT	EDC	MTT	EDC
50	0.84±0.09	>3	0.85±0.03	0.62±0.06	0.78±0.01	1.09±0.05
25	0.62±0.05	2.05±0.09	0.63±0.03	0.36±0.03	0.53±0.01	0.65±0.05
10	0.37±0.01	0.82±0.04	0.38±0.01	0.20±0.01	0.33±0.02	0.26±0.02

Results are presented as mean ±SD of optical densities. The coefficient of correlation and p values between the results of MTT and EDC assays in all the cases were $r>0.995$ and $p<0.001$.

Percent specific killing was calculated as follows:

$$\%\text{Control} = \frac{\text{Test OD-OD of effector cells alone}}{\text{OD of target cells alone}} \times 100$$

A similar approach was used for measuring killing activity using EDC assay except, instead of adding MTT, p-nitrophenyl phosphate buffer was added to the remaining tumor cells and incubated for 1hr at 37°C.

Immunocytochemistry

Tumor cell lines were dispensed onto sterile multi-well microscope slides and incubated for 24 hr after which they were washed in PBS + NaN_3, air dried and fixed in acetone. After hydration in PBS the peroxidase-anti-peroxidase (PAP) technique previously described by Nouri et al.[16] was used. This detection system is referred to as antibody developed color (ADC) assay.

Table IV. Effects of drugs on tumor cells as detected by MTT and EDC assays.

Cell Line		MTT	EDC
JEG3	Cis	35%	46%
	Vin	13%	69%
Ish	Cis	28%	48%
	Vin	21%	71%
T47D	Cis	47%	61%
	Vin	30%	57%

Results are represented as percent inhibition. In all the cases, cells were treated with drugs for 48 hrs before the assays. Cis denotes cisplatin (5 µg/mL) and Vin denotes vinblastin (0.05 µg/mL).

RESULTS

Optimizing time and temperature conditions in EDC assay

Three cell lines were cultured in three separate microtiter plates and incubated, one at 4°C, one at room temperature and one at 37°C. The results are presented in Fig. 1 (a, b and c). As can be seen, in all the cases and conditions, there was a direct correlation between cell number and OD. Thus, ODs (10×10^3 cells/well) for JEG3 cells at 4°C, room temperature and 37°C were 0.30±0.03, 0.32±0.02and0.42±0.03,respectively. The corresponding values for Ish and SKV14 lines were 0.17±0.001, 0.20±0.001, 0.27±0.002 and 0.10±0.01, 0.12±0.001 and 0.17± 0.001. Based on these observations, incubation of cells at 37°C for 60 min to develop color were taken to be appropriate and used for the subsequent experiments. In the case of the SKV14 line, because of low OD, the incubation time had to be increased to make accurate measurement possible (results not shown).

Correlation between cell number and optical density

Tumor cell lines at varying cell numbers were used for EDC assay. As can be seen in Fig. 2, there was a dose related increase in ODs as the cell number increased. Thus, ODs for cell line JEG3 at 10, 25, 50 and 100×10^3 cells/well were 0.16±0.01, 0.37±0.02, 0.96±0.03 and 1.95±0.01, respectively ($r=0.995$, $p<0.001$). A similar pattern was seen for other cell lines (Fig. 2). Based on these results 10×10^3 cells/well was considered to be sufficient for developing OD for accurate measurement and used in the subsequent experiments.

EDC comparative study for different tumor lines

The results in Table I show the relative ODs for various cell lines using EDC assay. As can be seen, the ODs for Tera II and Ep2102 lines were all greater than 0.95±0.10 whereas under the same conditions the maximum values for the bladder lines were 0.09±0.01. These results indicated that the ODs for the testis lines were much greater than all the other tumor lines tested.

When the cell-free supernatants from the above experiment were screened for the presence of PLAP using the standard ELISA technique, no positivity was detected even in the case of testis lines (results not shown), indicating that phosphatases are not secreted by cells and are most probably cell bound.

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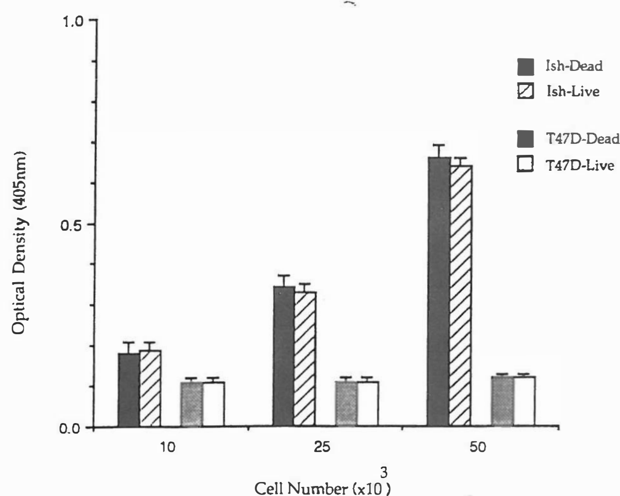

Fig. 3. Effects of drying on EDC activity. Results of optical density are plotted against cell number

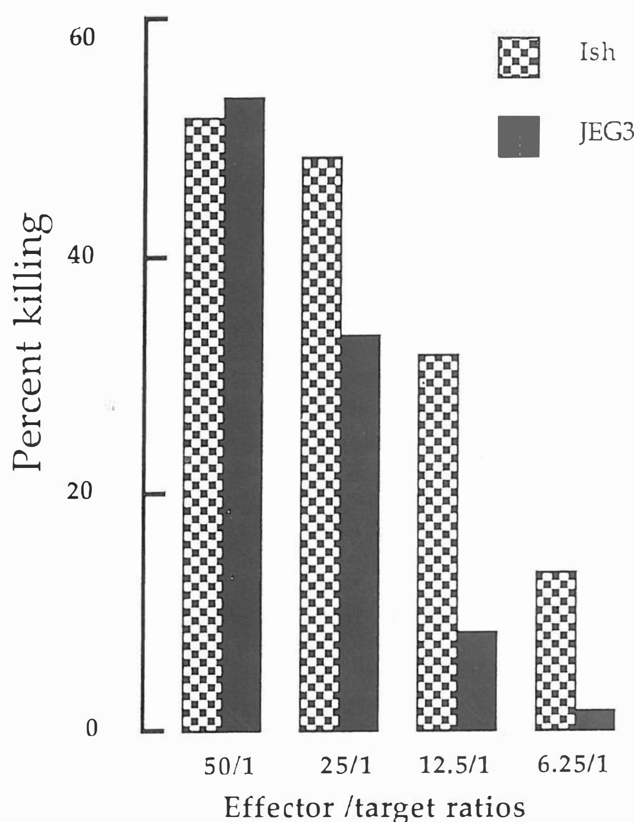

Fig. 4. Tumor cell killing by IL-2-activated MNCs against two tumor targets using EDC assay.

Qualitative assessment of cellular phosphatase using EDC and ADC assays

Various cell lines were used to assess the qualitative difference in cellular phosphatase activity as measured by EDC and ADC assays (using antibody specific for the placental isoform of ALP). As can be seen from Table II, testis lines showed strong positivity when tested with EDC assay but were negative with ADC. In the case of the bladder lines, both measurements showed negativity whilst in the case of Ish and JEG3 lines they were both positive. These results indicated that testis tumor lines showed strong ALP activity only when measured by enzyme-mediated rather than antibody-mediated assay.

Effects of drying of cells on ALP activity

In order to establish whether dried cells could be used in EDC assay, two cell lines were used to set up to identical plates containing 10, 25 and 50×10^3 cells/well. From one, the medium was removed and after washing, left to air dry for 2 hrs. After discarding the medium and washing the second plate, phosphatase substrate was added plates and incubated at 37°C for 60 min. As can be seen from Fig. 3 in the case of T47D the ODs at 10, 25, and 50×10^3 cells/well for air dried and live cells were 0.11±0.01, 0.11±0.01 and 0.12±0.01 and 0.10±0.01, 0.11±0.01 and 0.12±0.01, respectively. The corresponding values for Ish cell line were 0.18±0.01, 0.34±0.01 and 0.66±0.01 and 0.19±0.01, 0.33±0.03 and 0.64±0.03. These results demonstrated that both live and fixed cells could be used in EDC assay. The use of stored samples could be important for minimizing inter-experimental errors where there are a large number of samples collected over a long period of time.

Comparison between EDC and MTT assays

In our previous studies,[5] MTT assay was reported to be a reliable method for measuring cell proliferation and cytotoxicity. In this experiment three tumor lines were used to investigate whether the results from EDC and MTT assays were comparable. As can be seen from Table III, there was a good degree of concordance between the cell number and OD. The coefficient of correlation in all the cases were greater than 0.995, $p<0.001$. These results indicated that the results of the EDC assay could be as sensitive as the MTT assay when measuring tumor cell number.

In a separate experiment, three tumor lines cultured in two plates were exposed to two drugs, cisplatin and vinblastin (concentrations had been previously determined to give 20% to 50% inhibition using MTT assay) and after 48 hr of incubation one plate was assessed with MTT assay and the other with EDC assay. As can be seen from Table IV, both drugs showed inhibitory activity in MTT as well as EDC assays; however, the degree of inhibition tended to be higher for EDC assay. Thus, the percent inhibition by cisplatin (5 µg/mL) for MTT and EDC assays for JEG3, Ish and T47D lines were 35% and 46% ($p>0.05$), 28% and 48% ($p<0.01$) and 47% and 61% ($p=0.03$), respectively. The respective values for vinblastin (0.05 µg/mL) were 13%

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and 69% ($p<0.002$), 21% and 71% ($p<0.001$) and 30% and 57% ($p=0.02$). These results indicated that the EDC assay was at least as sensitive as the MTT assay for assessing the cytotoxic activity of various agents.

Cytotoxic activity of LAK cells as determined by EDC assay

The use of EDC assay as a cytotoxicity test was investigated using LAK cells as effector cells and the results are presented in Fig.4. As can be seen, there was a direct correlation between the effector to target ratios and percent killing. Thus, the percent killing for Ish line at 50/1, 25/1, 12.5/1 and 6.25/1 E/T ratios was 53%, 48%, 30% and 14% respectively. The corresponding values for JEG3 cell line were 55%, 34%, 8% and 4%. These results indicated that it was possible to extend the use of EDC assay for measuring cytotoxicity.

DISCUSSION

The findings of this investigation can be summarized as follows:

1. Under optimized conditions, ther results of EDC showed a direct correlation between cell number and OD.
2. There was a good concordance between the results of EDC and MTT assays.
3. EDC assay could be used for assessing fixed cells as well as LAK cytotoxic activity.

These results showed that a simple EDC assay could be used for various *in vitro* tests. In addition, the flexibility of the assay for using fixed cells makes it more versatile for testing samples collected over a long period.

Two issues have recently been attracting a great deal of interest, first the cost of research programs and second the increase in background radioactivity in the environment. The latter has encouraged the development of techniques which do not rely on radioactive reagents. Our previous efforts in this area[5] resulted in the modification of the MTT colorimetric assay originally reported by Mosmann.[6] Careful studies showed that the technique was sensitive enough to replace the ^{3}H-thymidine incorporation and chromium release assay which have been used as a "gold standard" for the measurement of cell proliferation and cytotoxicity respectively. In our present study we demonstrated that there was a good correlation between the results of EDC and MTT assays with "r" values of greater than 0.995 and $p<0.001$.

The uptake of radioactive thymidine by dividing cells has long been the method of choice for measurement of cell proliferation.[3] However, the technique is rather time consuming and apart from handling radioactive reagents it requires a sophisticated counting machine which may not be available in small research units. There have been various techniques for assessing cell proliferation and cytotoxicity, the simplest being the counting of cells under a microscope. However, the major problem with this has been the time required for completing a simple screening test and hence has not attracted major enthusiasm. Dye exclusion trypan blue test is the modified version of the counting technique except that the number of cells taking up the dye, i.e. dead cells are counted.[1] The incorporation of bromodeoxyuridine (BrdU), the non-radioactive thymidine analogue to detect cell proliferation has proven to be more environmentally friendly[16] but similarly time consuming.

We suggest that the EDC technique could be used for assessing *in vitro* cellular functions where the end point is the assessment of the number of surviving cells. The main advantage of this assay over the others is the rapidity and simplicity as well as the fact that it is economically very favorable. The suitability of the EDC assay using dead cells is particularly interesting in experiments where the number of samples are either large or the study is completed over a long period and frequent screening may be uneconomical or cause unacceptable inter-experimental error.

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