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A high-throughput end-point assay for viable mammalian cell estimation

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Abstract

A single wavelength colorimetric microplate-based assay was developed using non-cytotoxic dye resazurin for the estimation of viable cell concentrations of Chinese hamster ovary (CHO) and hybridoma cells. Experimental results showed variations in pH and temperature caused by cell cultivation and assay operations were well tolerated. Cell concentrations can be effectively determined in the range of 10^5–10^7 cells ml^{-1} using a microplate reader at the wavelength of 605 nm. This assay can be performed in a high-throughput manner such that a large number of cell culture samples can be screened within a relatively short time frame. When used together with a cell culture system of high-throughput format, it may have potential utilities in applications such as cell culture medium formulation and optimization.

Abbreviations: CHO – Chinese hamster ovary; SFM – Serum-free medium

Introduction

Medium optimization plays an important role in the phase of process development during the commercialization of biological products made by large scale mammalian cell cultures (Glacken et al. 1986; Xie and Wang 1994; Zhou and Hu 1994; Zhou et al. 1995; Zhou et al. 1997). Since the efficiency of a process has a direct impact on the economy of manufacturing and eventually determines the economic viability of a biological product, it is essential for a manufacturing process to be run at a high performance when a drug is produced at a commercial scale. Obtaining high producer cells by cloning and improving product yields by optimizing medium compositions are the common approaches used together in order to maximize process efficiencies. However, because of the high component complexities of media and the slow growth of mammalian cells, optimization of cell culture media is often considered as an expensive, time-consuming and labor-intensive step. Since the concentration of viable cells is one of the key process variables in cell cultures (Xie and Wang 1994; Shen et al. 1994; Zhou et al. 1995; Shen et al. 1996; Zhou et al. 1997), it is anticipated that the availability of an efficient and cost-effective method, which is capable of handling a large number of cell culture samples for the estimation of viable cells, will significantly improve the efficiency and cost-saving of medium optimization processes.

Since its introduction in 1993, the non-cytotoxic dye resazurin, also called Alamar Blue, has been widely used in assays to monitor the proliferation of mammalian cells and microbes (Fields and

Lancaster 1993; Rasmussen 1999; Rasmussen and Nicolaisen 1999; Batchelor and Zhou 2004). Upon enzymatic conversion in mitochondria, resazurin is reduced to resorufin which is subsequently released into the growth media from the cells. Spectrophotometric and fluorometric changes of both resazurin and resorufin in the media can therefore be measured as indicators of cellular activities and proliferation of cells. However, fluorometric detection has been the preferred choice because of its better sensitivity than that of spectrophotometric detection. In combination with fluorometric detection, resazurin method was reportedly able to achieve a sensitivity of measurement comparable to that of the [^{3}H]thymidine assay (Ahmed et al. 1994; Desaulniers et al. 1998). Besides the absence of radioactivities, resazurin method can also avoid the multiple steps of handling such as addition and removal of liquids, cell lysis, and dissolution of formazan crystals formed, as happened to other non-radioactive methods using 5-bromo-deoxyuridine (BrdU) (Huong et al. 1991), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dipbenyltetrazolium bromide) (Mosmann 1983; Gazzano-Santoro et al. 1997; Abate 1998; Mshana 1998), XTT (3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate) (Roehm et al. 1991) and enzyme activities (Putnam et al. 2002; Batchelor and Zhou 2004). The above advantages certainly have made resazurin assay an attractive alternative for automated high-throughput operations. Although tremendous efforts have been made to develop spectrophotometric methods such as dual wavelength spectrometry (Ahmed et al. 1994; Goegan et al. 1995; Zhi-Jun et al. 1997; Degnim et al. 1998; Putnam et al. 2002), the lack of precision and added complexity of calculations may significantly limit the efficiency and effectiveness of a high-throughput method. Trypan blue exclusion is a commonly used method for the assessment of viable cells in laboratories (Freshney 1987). However, there is no question that it suffers from low efficiency, incapability of high-throughput, and human errors between operators.

In this paper, results of developing a high-throughput end-point assay are presented to estimate viable Chinese hamster ovary (CHO) and hybridoma cells in the range of 10^5–10^7 cells ml^{-1} using spectrophotometric detection at 605 nm. This assay is considered to have potential applications in the formulation and optimization of serum-free cell culture media.

Materials and methods

Materials

A serum-free cell culture medium (SFM) was prepared by supplementing insulin (1 mg l^{-1}), transferrin (1 mg l^{-1}), bovine serum albumin (100 mg l^{-1}) and sodium selenite (10^{-8} M) to Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (Invitrogen, Carlsbad, CA, USA) and filtered through 0.2 μm membranes before use. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 96 well cell culture microplates were purchased from Corning (New York, USA).

Resazurin stock solution (1.2 mM) was prepared by dissolving resazurin in 20 mM phosphate buffered saline followed by 0.2 μm membrane filtration. Stock solution was diluted to 200 μM before use.

Cell lines and stock cultures

A dihydrofolate reductase deficient Chinese hamster ovary (CHO dhfr$^-$) cell line was purchased from ATCC (Manassas, VA, USA). A murine hybridoma cell line, JJ-1, secreting monoclonal antibodies (MAb) against human Tissue Factor (hTF) was kindly provided by Dr Xintai Zhao, Shanghai Cancer Institute. Both cell lines were individually adapted to suspension cultures in above SFM and passaged routinely. Before being used in the following experiments, cells were harvested by centrifugation and counted with a hemocytometer according to trypan blue dye exclusion method after resuspension in fresh SFM. Experiments were carried out in above SFM for both cell lines except being specified.

Effects of pH and temperature on resazurin absorbance

To simulate the pH conditions culture media may encounter, buffer solutions of pH 5.0, 5.5, 6.0 (20 mM citric buffer), 6.5, 7.0, 7.5, 8.0 (20 mM

phosphate buffer), 8.5 and 9.0 (20 mM Tris–HCl) were prepared. Aliquots of 100 μl in volume were added in quadruplicates to a 96 well microplate. With corresponding buffers as blanks, absorbance at 605 nm was read on microplate reader (Bio-Rad Microplate Reader 550) following the addition of 20 μl resazurin solution to each well and mixing.

To test the effects of temperature on the absorbance reading of resazurin, 5, 10, 20, 30, 40 and 50 μl of resazurin solution were added in quadruplicates to three 96 well microplates. SFM was added to make the total volume in each well to be 100 μl, Microplates were incubated at 4, 25 and 37 °C for 20 min respectively, followed by immediate absorbance reading at 605 nm.

Background determination of cells in microplates

CHO and JJ-1 cells were harvested from stock cultures, counted after trypan blue staining, and prepared to make suspensions of 10^7 cells ml^{-1} in SFM. Cell suspensions of 4.88×10^3, 9.77×10^3, 1.95×10^4, 3.91×10^4, 7.81×10^4, 1.56×10^5, 3.13×10^5, 6.25×10^5, 1.25×10^6, 2.5×10^6, and 5.0×10^6 cells ml^{-1} were further made by two-fold serial dilutions. To measure the background absorbance by the cells, aliquots of 100 μl in volume were added to each column of microplates followed by absorbance reading.

Dynamic measurements of absorbance reductions in microplates

To measure the dynamic responses in absorbance reduction as functions of cell concentration, suspensions of CHO and JJ-1 cells were prepared as 7.81×10^4, 1.56×10^5, 3.125×10^5, 6.25×10^5, 1.25×10^6 (low concentration range) and 1.25×10^5, 2.5×10^6, 5.0×10^6, 1.0×10^7 cells ml^{-1} (high concentration range) in SFM after harvest from stock cultures. Aliquots of 100 μl were added to each column of four separate microplates. Resazurin solution of 30 or 20 μl was added to each well containing CHO or JJ-1 cells, respectively. After mixing, absorbance at 605 nm was read at time intervals of 0, 20, 60, 90, 120, 180, 240 min for low concentration range or 0, 15, 20, 30, 90 min for high concentration range. Microplates were incubated in a 37 °C CO_2 incubator between readings.

Determination of viable cell concentrations of CHO and JJ-1 cells grown in microplates

To two rows of a cell culture microplate, aliquots of 100 μl CHO or JJ-1 of 10^5 cells ml^{-1} in SFM were inoculated. Totally five microplates were inoculated for each cell line. Microplates were incubated in a 37 °C CO_2 incubator. One plate was taken for assay after every 24 h. Cells in one row were suspended for counting with trypan blue method, and another row was used for the measurement of absorbance reduction after reaction for 90 min in a 37 °C CO_2 incubator following the addition of 20 μl of resazurin solution and mixing. Viable cell concentration was estimated using standard curves prepared along with experiments using cells from the stock cultures.

Cell culture medium and component concentration screening

SFM containing 50 mM isoleucine or 100 × Fe complex were two-fold serially diluted and aliquots of 100 μl were added to microplates in triplicates. Then each well was inoculated with 10 μl of JJ-1 of 10^6 cells ml^{-1} in SFM. Microplates were incubated for 48 h in a 37 °C CO_2 incubator. Absorbance reading at 605 nm was carried out after the addition of 20 μl of resazurin solution and mixing. Microplates were incubated in a 37 °C CO_2 incubator between readings.

Aliquots of 100 μl of eight different formulations of SFMs for JJ-1 cells were added to each well in a microplate in triplicates. Each well was then inoculated with 10 μl of JJ-1 of 10^6 cells ml^{-1} in SFM. Microplates were incubated for 48 h in a 37 °C CO_2 incubator. After 48 h, 20 μl of resazurin solution was added to each well and mixed before absorbance reading at 605 nm. Microplates were incubated in a 37 °C CO_2 incubator between readings.

To quantify cell concentrations, standard curves between absorbance reduction at 605 nm and cell concentration were made along with experiments using cells from the stock culture of JJ-1 cells.

Results

Effects of pH and temperature on resazurin absorbance

Because the formation of lactic acid is culture condition dependent, pH variations among cultures may become an important issue in the development of a high-throughput colorimetric assay. Moreover, the evaporation of CO_2 is unavoidable during the reading of microplates which is normally executed outside CO_2 incubators, resulting in pH shifts towards more alkaline conditions. Experimental results showed no significant variations in spectrometric absorbance of resazurin at 605 nm from pH 6.5 to pH 9.0. Because the pH conditions of mammalian cell cultures are rarely run below 6.5, experimental results confirmed the tolerance of this assay to the pH conditions during incubation and microplate reading.

At the three temperature conditions tested, 4, 25 and 37 °C, almost identical absorbance readings were detected in response to resazurin concentration, confirming the tolerance of spectrometric reading of resazurin at 605 nm to temperature changes between 37 °C incubation and ambient temperature of microplate reading.

Background absorbance by CHO and JJ-1 cells

For both cell lines, there existed slow increases in absorbance but readings were lower than 0.1 if the cell concentrations were less than 10^6 cells ml^{-1}. However, background absorbance increased dramatically as the cell concentrations increased beyond 10^6 cells ml^{-1}. It was also noted that the JJ-1 cells exhibited a stronger background than CHO cells, probably due to their differences in size and roughness of cell surfaces.

Dynamic measurements of absorbance reductions in microplates

In suspension cultures of mammalian cells, concentrations of viable cells are generally maintained in the range of 10^5–10^7 cells ml^{-1} (Xie and Wang 1994; Zhou et al. 1995). Based on the observation that the background absorbance changed with cell concentration, it was rational to divide cell concentration into two overlapping ranges, 7.8×10^4–1.25×10^6 and 1.25×10^6–1.0×10^7 cells ml^{-1}, and the dynamic relationship between absorbance reduction and cell concentration was investigated individually for each concentration range. Results are presented as absorbance reduction between time zero and time t $[A_0 - A_t]$ (ΔOD_{605}) vs. the concentration of viable cells in wells.

For both cell lines, absorbance reduction increased with reaction time in both concentration ranges tested (Figures 1a, 1b, 2a and 2b). At lower cell concentrations (Figures 1a and 2a), reaction time of 1 h did not produce signals strong enough for all cell concentrations. Satisfactory signals

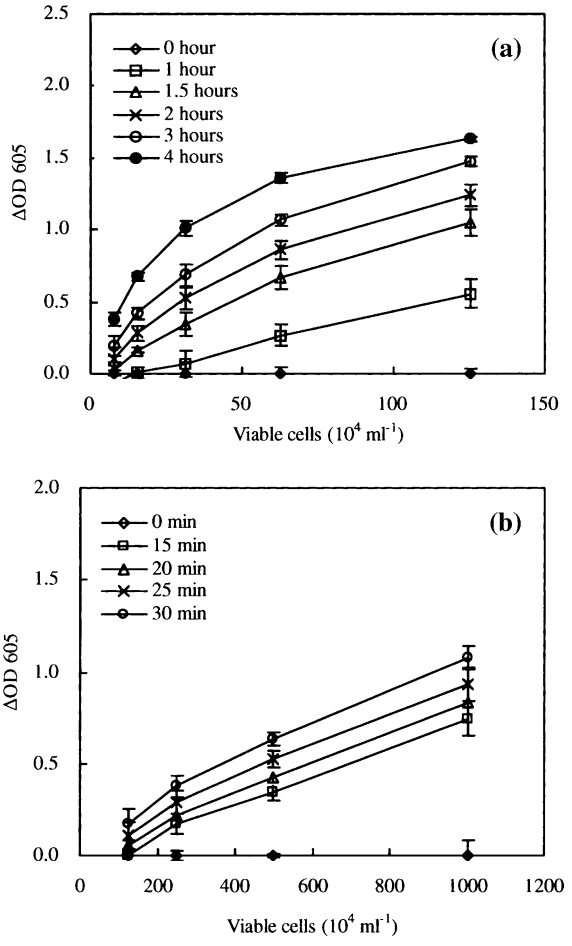

Figure 1. Absorbance reduction of resazurin as a function of viable cell concentration of CHO and reaction time. (a) 7.8×10^4–1.25×10^6 ml^{-1}. (b) 6.25×10^5–1.0×10^7 ml^{-1}. Each point was the mean of absorbance readings of eight wells.

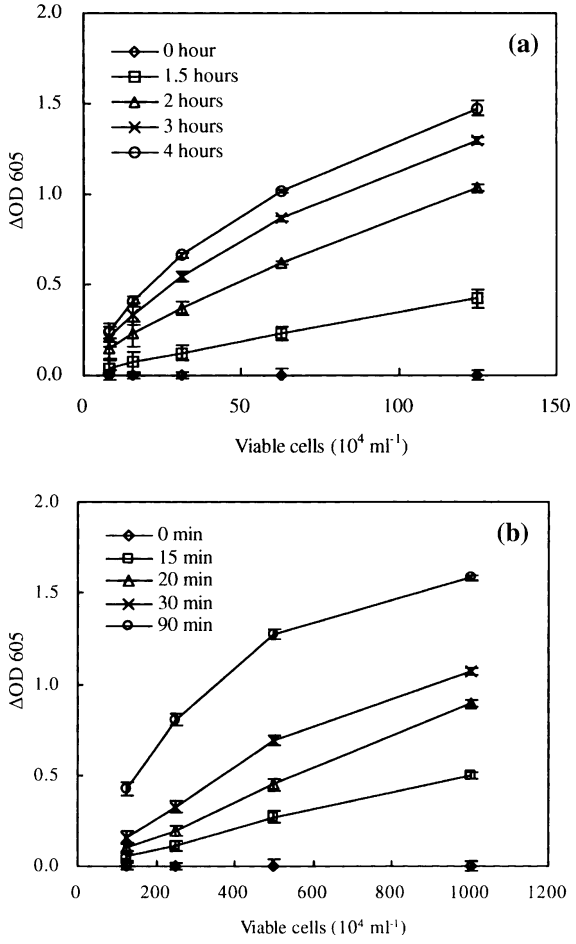

Figure 2. Absorbance reduction of resazurin as a function of viable cell concentration of JJ-1 and reaction time. (a) 7.8×10^4–1.25×10^6 ml^{-1}. (b) 6.25×10^5–1.0×10^7 ml^{-1}. Each point was the mean of absorbance readings of eight wells.

were produced when time of reaction was extended to 2, 3 and 4 h however. In general, the curves changed from a pattern of linearity to a pattern of saturation curves as reaction time became longer, indicating the possible limitation of resazurin. For CHO cells, a reaction time of 25–30 min gave satisfactory signal reading, while a longer reaction time of 30–90 min was considered more appropriate for JJ-1 cells (Figures lb and 2b).

Comparison between resazurin and trypan blue methods for cells grown in microplates

The growth of CHO and JJ-1 cells in microplates were monitored using trypan blue and resazurin methods respectively (Figures 3a and b). For both cell lines, standard curves of 7.8×10^4–1.25×10^6 and 1.25×10^6–1.0×10^7 cells ml^{-1} were prepared to calculate the concentrations of cells. For CHO cells, results shown in Figure 3a indicated a satisfactory correlation between concentrations of viable cells estimated with two methods. In the case of JJ-1 cells (Figures 3b), however, it is also apparent that the resazurin method gave a higher estimation than trypan blue staining ranging from 0 to 40%. The exact reason underlying this phenomenon is to be determined but may be caused by their difference in mechanism (Freshney 1987; Fields and Lancaster 1993). The former relies on the exclusion of negatively charged dye trypan blue by the intact cell membranes of viable cells,

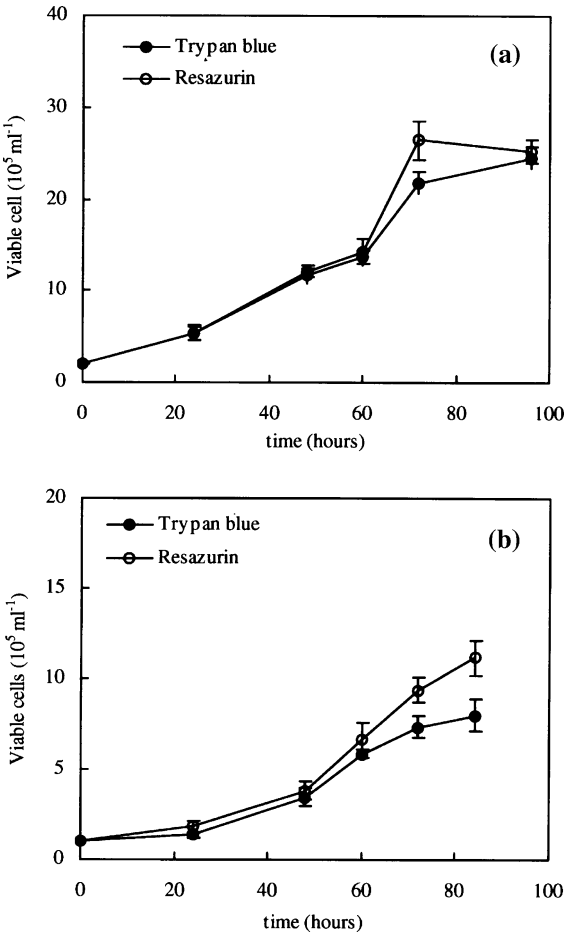

Figure 3. Determination of viable cell concentration assessments of JJ-1 and CHO cells grown in microplates. (a) CHO cells. (b) JJ-1 cells. Each point was the mean of absorbance readings of eight wells.

while the latter measures the reduction activity of resazurin by mitochondrial enzymes. It is speculated that mitochondrial activities associated with the dead cells as assessed with trypan blue might have contributed to the conversion of resazurin. Further studies are needed to understand this phenomenon in order to further implement this assay.

Medium component concentration and formulation screening

Using hybridoma JJ-1 cells, three examples are presented here to demonstrate the utilities of this assay in culture medium formulation and optimization activities. With the yield of viable cells as an indicator of the performance of media, isoleucine and an iron complex in SFM were titrated in certain ranges of concentrations. As the results indicate (Figures 4a and b), isoleucine and the iron complex exhibited inhibitory effects on the growth of hybridoma cells when their concentrations increased to certain levels. Figure 5 demonstrates another capability of this assay screening different existing formulations.

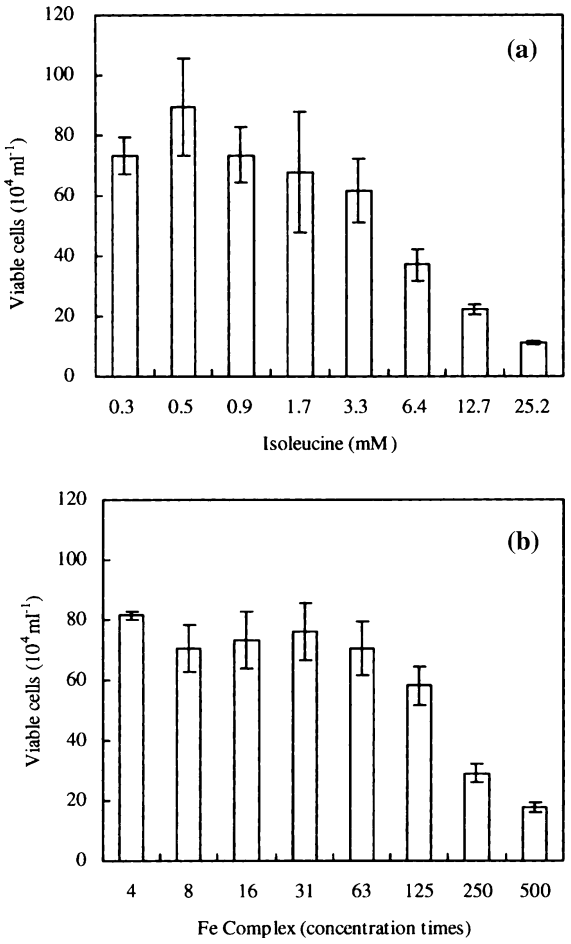

Figure 4. Concentration titration of medium components of JJ-1 cells. (a) Isoleucine in SFM. (b) Fe complex in SFM. Each point was the mean of absorbance readings of three wells.

Discussion

Optimization of cell culture media is known to be costly, labor intensive and time consuming. A thorough investigation requires a tremendous amount of work, screening components, concentration ranges and component combinations. Using the non-cytotoxic dye resazurin, we developed a single wavelength end-point colorimetric 96 well microplate-based assay for the estimation of viable cell concentrations of mammalian cells. Examination of environmental factors indicated that this assay can well tolerate pH variations among different cultures and temperature shifts during assays. Detailed procedures can be implemented by optimizing the concentration of resazurin used and the time of reaction based on the profile of background absorption by the cells and

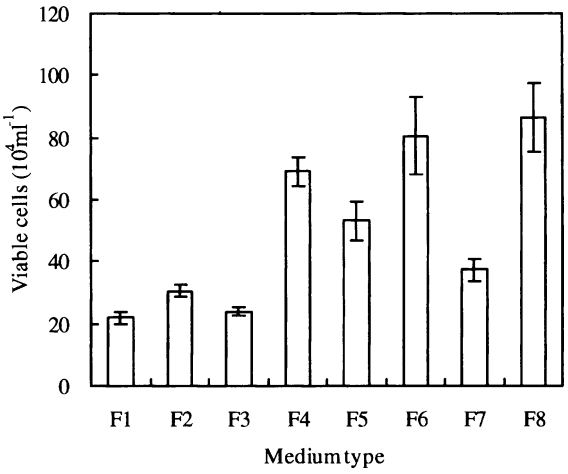

Figure 5. Formulation screening of SFMs for JJ-1 cells. Eight different SFM formulations were tested including two commercially available SFMs. Each point was the mean of absorbance readings of three wells. Fl: DMEM; F2: DMEM/F12; F3: RPMI1640; F4: HyClone; F5: Gibco; F6-8: SFMs formulated in this laboratory.

the range of cell concentrations studied. The level of viable cells in cultures could be either represented by absorbance reduction at 605 nm or concentration with the help of standard curves of cells. In this study, a two-step strategy was applied to measure cell concentration ranging from 10^5 to 10^7 cells ml^{-1}.

Another factor should be evaluated is the formation of resofurin which contributes to the absorbance measurement at the end of assay (A_t) at 605 nm. The molar extinction coefficients (ε) of resazurin and resofurin were obtained from spectral scanning (Shimadzu UV-260) as 2.398 and 0.586 $M^{-1}\,cm^{-1}$, respectively. Assuming that there is no resofurin initially present in resazurin solution and that resazurin is enzymatically converted to resofurin in equal molar ratio, the following relationships exist in cuvettes of 1 cm light path length:

$$A_0 = \varepsilon_{\text{resazurin}} C_{\text{resazurin},0} \tag{1}$$

$$A_t = \varepsilon_{\text{resazurin}} C_{\text{resazurin},t} + \varepsilon_{\text{resofurin}} C_{\text{resofurin},t} \tag{2}$$

$$C_{\text{resazurin},0} = C_{\text{resazurin},t} + C_{\text{resofurin},t} \tag{3}$$

where $C_{\text{resazurin},0}$, $C_{\text{resazurin,t}}$ and $C_{\text{resofurin,t}}$ are the concentrations of resazurin and resofurin at time zero and t. The following equation can be derived

$$\begin{aligned} A_0 - A_t &= \varepsilon_{\text{resazurin}} C_{\text{resazurin},0} \\ &\quad - \varepsilon_{\text{resazurin}} C_{\text{resazurin},t} - \varepsilon_{\text{resofurin}} C_{\text{resofurin},t} \\ &= (\varepsilon_{\text{resazurin}} - \varepsilon_{\text{resofurin}})(C_{\text{resazurin},0} - C_{\text{resazurin},t}) \end{aligned} \tag{4}$$

Therefore we may conclude that, although the formation of resofurin leads to interference to the measurement of A_t, the absorbance reduction $[A_0 - A_t]$ at 605 nm is still in direct proportion to $(C_{\text{resazurin},0} - C_{\text{resazurin},t})$, i.e. the metabolic activity of mitochondria, except the absorbance values measured are approximately 25% ($\varepsilon_{\text{resofurin}}/\varepsilon_{\text{resazurin}}$) lower than without the interference.

The essential goal of this assay is to measure viable cell concentrations in a high-throughput manner so that a large number of cultures can be screened within a relatively short time frame. When used together with a cell culture system of high-throughput format, it may have great utilities in applications such as formulation and optimization of cell culture media.

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References

Abate G., Mshana R.N. and Miorner H. 1998. Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in Mycobacterium tuberculosis. Int. J. Tuberc. Lung Dis. 2(12): 1011–1016.

Ahmed S.A., Gogal R.M. and Walsh J.E. 1994. A new and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. J. Immunol. Methods 170: 211–224.

Batchelor R.H. and Zhou M. 2004. Use of cellular glucose-6-phosphate dehydrogenase for cell quantitation: applications in cytotoxicity and apoptosis assays. Anal. Biochem. 329: 35–42.

Degnim A.C., Morrow S.E., Ku J., Zar H.A. and Nakayama D.K. 1998. Nitric oxide inhibits peroxide-mediated endothelial toxicity. J. Surg. Res. 75: 127–134.

Desaulniers D., Leingartner K., Zacharewski T. and Foster W.G. 1998. Optimization of an MCF7-E3 cell proliferation assay and effects of environmental pollutants and industrial chemicals. Toxicol. in Vitro 12: 409–422.

Fields R.D. and Lancaster M.V. 1993. Dual attribute continuous monitoring of cell proliferation/cytotoxicity. Am. Biotechnol. Lab. 11: 48–50.

Freshney R.I. 1987. Culture of Animal Cells: A Manual of Basic Techniques (2nd ed.). Alan R. Liss, Inc., New York.

Gazzano-Santoro H., Ralph P., Ryskamp T.C., Chen A.B. and Mukku V.R. 1997. A non-radioactive complement-dependent cytotoxicity assay for anti-CD20 monoclonal antibodies. J. Immunol. Methods 202: 163–171.

Glacken M.W., Fleischaker R.J. and Sinskey A.J. 1986. Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. Biotechnol. Bioeng. 28: 1376–1389.

Goegan P., Johnson G. and Vincent R. 1995. Effects of serum protein and colloids on the AlarmaBlue assay in cell cultures. Toxic. in Vitro 9(3): 257–266.

Huong P.L.T., Kolk A.H.J., Eggelte T.A., Verstijnen C.P.H.J., Gilis H. and Hendriks J.T. 1991. Measurement of antigen

specific lymphocyte proliferation using 5-bromodeoxyuridine incorporation. J. Immunol. Methods 140: 243–248.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55–63.

Mshana R.N., Tadesse G., Abate G. and Miorner H. 1998. Use of 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant Mycobacterium tuberculosis. J. Clin. Microbiol. 36(5): 1214–1219.

Putnam K.P., Bombick D.W. and Doolittle D.J. 2002. Evaluation of eight in vitro assays for assessing the cytotoxicity of cigarette smoke condensate. Toxic. in Vitro 16: 599–607.

Rasmussen E.S. 1999. Use of fluorescent redox indicators to evaluate cell proliferation and viability. In Vitro Mol. Toxicol. 12: 47–58.

Rasmussen E.S. and Nicolaisen G.M. 1999. Stability of resazurin in buffers and mammalian cell culture media. In Vitro Mol. Toxicol. 12: 195–202.

Roehm N.W., Rodgers G.H., Hatfield S.M. and Glasebrook A.L. 1991. An improved colorimetric assay for cell proliferation and viability utilizing tetrazolium salt XTT. J. Immunol. Methods 142: 257–265.

Shen B.Q., Clarke M.F. and Palsson B.O. 1996. Kinetics of retroviral production from the amphotrophic ΨCRIP murine producer cell line. Cytotechnol. 22: 185–195.

Shen B.Q., Greenfield P.F. and Reid S. 1994. Hybridoma cells in a protein-free medium within a composite gel perfusion bioreactor. Cytotechnol. 16: 51–58.

Xie L. and Wang D.I.C. 1994. Fed-batch cultivation of animal cells using different medium design concepts and feeding strategies. Biotechnol. Bioeng. 43: 1175–1189.

Zhi-Jun Y., Sriranganathan N., Vaught T., Arastu S.K. and Ahmed S.A. 1997. A dye-based lymphocyte proliferation assay that permits multiple immunological analyses: mRNA, cytogenetic, apoptosis, and immunophenotyping studies. J. Immunol. Methods 210: 25–39.

Zhou W. and Hu W.-S. 1994. On-line characterization of a hybridoma cell culture process. Biotechnol. Bioeng. 44: 170–177.

Zhou W., Rehm J., Europa A. and Hu W.-S. 1997. Alteration of mammalian cell metabolism by dynamic nutrient feeding. Cytotechnology 24: 99–108.

Zhou W., Rehm J. and Hu W.-S. 1995. High viable cell concentration fed-batch cultures of hybridoma cells through online nutrient feeding. Biotechnol. Bioeng. 46: 579–587.