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A study of the reproducibility of the MTT test

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The MTT test has been widely used as a rapid and sensitive method for screening anticancer drugs as well as for the assessment of cytotoxicity of materials. The reproducibility of the MTT test has been studied in this paper in three ways. First, the reproducibility of MTT assay itself has been investigated. The comparisons were performed within the plate, between plates as well as between flasks of the cultured cells. The Student's *t*-test was used to analyse the data and statistically significant differences were found for all the groups compared. Second, the influence of random sampling was investigated. Statistically significant differences were found with non-random sampling but no differences were found with random sampling. The third part examined the stability of the optical density of the solution read by a spectrophotometer and its dependence on temperature. The stability of the optical density was examined at room temperature and 4 °C. Plates were maintained at these temperatures between readings. The optical density of dissolved formazan solution was compared by analysing the result with ONEWAY and statistically significant differences were found for the group of data at room temperature while no differences were shown for the group of data at 4 °C.

1. Introduction

The MTT (3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyl tetrazolium bromide)-based colorimetric assay was originally described by Mosmann [1] as a useful method for the measurement of *in vitro* cytotoxicity and cell proliferation. The numbers of viable cells growing in microtitre wells can be read by an automatic microplate scanning spectrophotometer, which offers major advantages in speed, simplicity, cost and safety [2]. This method has been widely used in the assessment of anticancer drugs [3, 4] and has recently been applied as a screening test for the biocompatibility evaluation of materials. The MTT assay has been demonstrated as a sensitive, precise, convenient, rapid and economical test method by many studies [1, 2, 5, 4, 6, 7]. Denizot [2] reported that the MTT assay is very much faster than a [^{3}H]thymidine uptake assay. He pointed out that the MTT assay depends both on the number of cells present and on the mitochondrial activity per cell. The sensitivity of the MTT was relevant to the cell types, e.g. they found that IL-2-dependent cell line CTL-2 gave less colour than LB 3. Although for many cell types both assays give similar results, other cells may give a reduced sensitivity with MTT. Ferrai *et al.* [7] claimed that the MTT assay was a useful alternative to ^{3}H-TdR release assay for quantifying macrophage cytotoxicity. Heo *et al.* [6] also found that the MTT assay was more sensitive than the ^{51}Cr release assay. It is reported that the mitochondrial enzyme succinate-dehydrogenase within viable cells is able to cleave the tetrazolium salt MTT into a blue coloured product (formazan) [2] which is soluble in iso-propanol. It is proposed that the amount of formazan produced is proportional to the number of viable cells present [5, 7]. However, Page *et al.* [8] claimed that non-viable cells could also reduce the tetrazolium and emphasized that care was required when using this assay for a short-term cytotoxicity assay. He reported that some antimitotic drugs such as daunorubicin are cytotoxic mainly because they intercalate into DNA and cause cell death over longer periods.

Although the MTT assay is now routinely used as a screening test method to investigate the biocompatibility of the biomaterials in many laboratories we have obtained some confusing results in our laboratory that lead us to question the accuracy and reproducibility of the procedure. The MTT assay has been shown to be useful for the examination of materials that show considerable toxicity. However, in biomaterials research today we are often interested in the difference in response of materials that only show a very mild level of toxicity. It is therefore important to establish a technique which is very sensitive and can pick up these small variations. This study was designed to examine the procedure for the MTT assay systematically to determine ways to increase the accuracy and validity of the test.

Three different parts of the procedure were investigated:

1. The reproducibility of the MTT assay within the plate, between plates and between the flasks of cultured cells.
2. Non-random or random sampling.
3. The stability of the optical density of dissolved formazan solution and its dependence on temperature.

2. Materials and method

96-well microtitre plates were used throughout the

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study. Twelve wells were taken as the number of samples in each group.

2.1. Cell line

The established cell line L929 (obtained from Flow lab, Irvine, Scotland) was used in this study and maintained in growth medium 199 supplemented with 5% foetal calf serum, 50 Iu/ml penicillin and 50 μg/ml streptomycin. A routine subculture was used to maintain the cell line. The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. They grew to confluence after one week's incubation and the monolayer was then harvested by trypsinization.

For part one, cells were diluted 1 + 5 following the routine procedure using culture medium as mentioned above and seeded in the microtitre plates. For part two, the cell suspension was counted using a Coulter Counter before it was diluted into two cell concentrations, 1 + 7 (dilution 1) and 1 + 9 (dilution 2). They were thoroughly mixed with the medium to ensure even distribution and to minimize cell cloning, then 200 μl of each concentration was seeded into the wells of microtitre plates using a multipipette. The cells were maintained in the incubator immediately after cell seeding.

2.2. MTT colorimetric assay

The medium was aspirated and replaced with fresh medium after 48 h, and the cells were maintained in the incubator for another 24 h. For part one, pure culture medium and 50% medium were used. For part two, only culture medium was used.

MTT was dissolved at a concentration of 1 mg/ml in a solution containing 50% PBS and 50% growth medium without phenol red. The growth medium in the microtitre plates was removed by inverting, flicking and blotting the plate before 50 μl of MTT solution was added to each well. The plate was incubated for 4 h at 37 °C, then the MTT solution was removed by carefully inverting, flicking and blotting the plates. 100 μl of iso-propanol was then added to each well, and the plate gently shaken by hand in order to ensure dissolution of the blue formazan. The plates were returned to the incubator for another 5–10 min before examination. The optical density of each well was measured using an automatic microplate reader (MR700, Dynatech Lab Inc, Europe) with a 670 nm reference wavelength and 570 nm test wavelength.

2.2.1. The reproducibility of the MTT assay within the plate, between plates and between the flasks of cultured cells

The cells were diluted in either full strength medium or 50% medium diluted with double distilled water. The reason for the 50% medium was that water is often used as an extractant for the test materials and is therefore used as a negative control.

The cells were seeded into the microtitre plates using the standard procedure. To examine the reproducibility within a plate or between plates all the cells came from the same flask. To examine the reproducibility between flasks, two flasks of cells were compared within the plate as well as between plates. The data were analyzed using the Student's *t*-test and each sample of 12 wells were made up of a pair of consecutive columns of wells.

The standard curve. Six cell dilution were chosen: 1 + 3, 1 + 5, 1 + 7, 1 + 9, 1 + 11 and 1 + 14 using the same culture medium. The cells were counted before dilution and the MTT procedure was the same as above.

2.2.2. Random sampling versus non-random sampling

Full strength medium was used for this part and the cells were seeded in order across the plate from left to right as normal. The cells were subcultured from a confluent monolayer and counted in a coulter counter (Model "7", Coulter Electronics Ltd, England) before being diluted 1 + 7 (dilution 1) and 1 + 9 (dilution 2). Each dilution was used in a separate plate.

In the first instance the sampling data were collected in the standard way with each sample being made up of 12 wells in a pair of columns (i.e. group 1 being made up of column 1 and 2, group 2 columns 3 and 4 and so on). Secondly the same data was analysed using the random sampling procedure, that is the columns in each sample were chosen randomly. The table of random sampling number was used and resulted in group 1 being column 1 and 10, group 2 being column 4 and 6, group 3 being column 2 and 7, group 4 being column 5 and 8, group 5 being column 3 and 9.

The data were analysed by one-way statistical analysis using the statistical computer package SPSS^x.

2.2.3. The stability of the fomazan solution

At the end of the MTT assay the colour of the formazan solution is read in a spectrophotometer. It has been noticed that the colour will change with time. This part of the study investigated the change in the optical density of plates over one hour. Two temperatures were chosen in the test, room temperature and 4 °C. Three plates were studied, two with the cells of dilution 1 and one with the cells of dilution 2. Two of the plates (dilution 1 and 2) were stored at room temperature for the hour, the other (dilution 1) was stored in the refrigerator at 4 °C and only taken out briefly to be read four times at 15–20 min intervals. The data were analysed statistically by ONEWAY with the same statistical package.

3. Results

3.1. The reproducibility of the MTT assay within the plate, between plates and between the flasks of cultured cells

3.1.1. Comparison within a plate

As shown in Tables I and II, both concentrations of medium had statistically significant differences

TABLE I Comparison of reproducibility with full strength medium

Samples	Means	Standard deviation	t value	P value
1	1.8030	0.0890	3.5187	< 0.05
2	1.6476	0.1248		

TABLE II Comparison of reproducibility with 50% medium + 50% D.D. water

Samples	Means	Standard deviation	t value	P value
1	1.1873	0.1131	3.6647	< 0.05
2	1.0358	0.0878		

TABLE III Comparison of reproducibility between plates with 50% medium + 50% D.D. water

Plates	Means	Standard deviation	t value	P value
1	1.4870	0.3022	0.476	> 0.05
2	1.5343	0.1636		
2	1.5343	0.1636	2.272	< 0.05
3	1.6976	0.1878		

($P < 0.05$) with a sample size of 12, indicating that reproducibility within a plate was poor.

3.1.2. Comparison between plates

Three plates were chosen. For the group of data from pure culture medium, most of the optical density values were read as 'over' by the microplate reader, which means the cell number is larger than the reading ability of the machine. Therefore this part of the result was not analysed. The result for 50% medium, with a sample size of 12 are shown in Table III.

Two student t-tests were performed for the comparison of the three plates with 50% medium. One showed no statistically significant difference (> 0.05) while the other had differences (< 0.05). This suggests that the reproducibility between plates is not always achieved.

3.1.3. Comparison between flasks

The experiment was performed within a plate as well as between plates. Again there were too many 'over' values in the full strength medium so that data were not used in the analysis.

Two Student t-tests were run for both comparisons (Tables IV and V) for the 50% medium (sample size = 12). As can be seen, three groups were shown to be statistically significantly different ($P < 0.05$) and only one showed no difference ($P > 0.05$). This indicated that the number of cells coming from different flasks varies greatly even though the flasks were kept in the same environment for the same time.

TABLE IV Comparison between flasks within a plate (50% medium + 50% D.D. water)

Means	Standard deviation	t value	P value
1.8030	0.0890	0.596	> 0.05
1.7760	0.1304		
1.7760	0.1304	2.465	< 0.05
1.6476	0.1248		

TABLE V Comparison of flask reproducibility between plates (50% medium + 50% D.D. water)

Means	Standard deviation	t value	P value
1.1794	0.2322	4.327	< 0.05
1.5343	0.1636		
1.5002	0.1033	3.1913	< 0.05
1.6979	0.1818		

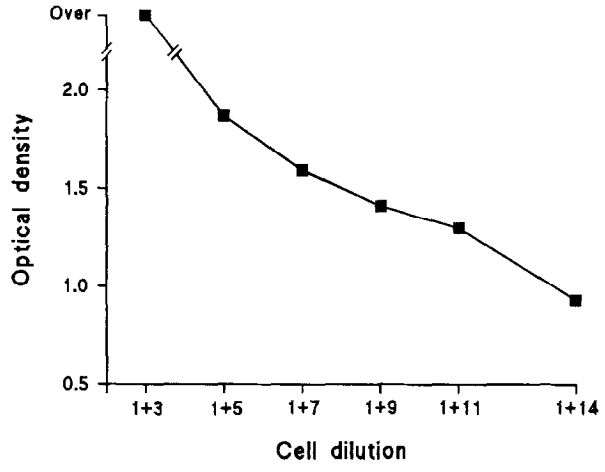

Figure 1 The standard curve of the cell L929.

3.2. Random sampling versus non-random sampling

3.2.1. The standard curve

Since there were too many cells as shown above a standard curve was carried out in order to establish the correct concentration of the cells. Fig. 1 shows the result. Cell dilutions of 1 + 7 and 1 + 9 were therefore chosen for this part of study.

3.2.2. Procedure one—non random procedure

The number of cells in each well for dilution 1 was about $5.5 \times 10^4/0.2$ ml medium. All data were analysed using one-way analysis of variance by SPSS^x. The difference between samples was further analysed using the Duncan method. The result showed that there were statistically significant differences among the five samples ($P < 0.001$, Table VI) although further analysis proved that there were no differences only between group 1 and group 2 or between group 4 and group 5 as shown in Table VII.

The same analysis was also done for the group of dilution 2. The number of cells in each well for dilution

TABLE VI Comparison of non-random sampling for dilution 1 by ONEWAY

Samples	Means	Standard deviation	*F* value	*P* value
1	1.7888	0.1289		
2	1.7962	0.1242		= 0.0000
3	1.6928	0.0991	14.3496	< 0.001
4	1.6015	0.0426		
5	1.5623	0.0596		

TABLE VII Result of comparison between samples for dilution 1 by Duncan

Means of sample	Samples	5	4	3	1
1.5623	5				
1.6015	4				
1.6928	3	*	*		
1.7888	1	*	*	*	
1.7962	2	*	*	*	

* Statistically significant differences between samples

TABLE VIII Comparison of non-random sampling for dilution 2 by ONEWAY

Samples	Means	Standard deviation	*F* value	*P* value
1	1.5865	0.1021		
2	1.5043	0.1043		= 0.0000
3	1.4678	0.0991	9.7932	< 0.001
4	1.4124	0.0331		
5	1.3924	0.0681		

TABLE IX Result of comparison between samples for dilution 2 by Duncan

Means of sample	Samples	5	4	3	2
1.3924	5				
1.4124	4				
1.4678	3	*			
1.5043	2	*	*		
1.5865	1	*	*	*	*

* Statistically significant differences between samples

2 was about $1.8 \times 10^4/0.2$ ml medium. Significant differences were again found among the five samples ($P < 0.001$, Table VIII). Further analysis by the Duncan method showed that there were no differences between group 2 and group 3, between group 3 and group 4 or between 4 and group 5 (Table IX).

3.2.3. Procedure 2—random procedure

Five samples were chosen randomly within plate. The data from the same two cell dilutions were examined with the same statistical analysis. The result showed that there were no statistically significant differences among the five samples ($P > 0.05$, Tables X and XI).

TABLE X Comparison of random sampling for dilution 1 by ONEWAY

Samples	Means	Standard deviation	*F* value	*P* value
1	1.6374	0.1051		
2	1.6758	0.0768		= 0.3291
3	1.7347	0.1621	1.1815	> 0.05
4	1.6627	0.0672		
5	1.7183	0.1835		

TABLE XI Comparison of random sampling for dilution 2 by ONEWAY

Samples	Means	Standard deviation	*F* value	*P* value
1	1.4456	0.1099		
2	1.4459	0.0783		= 0.3417
3	1.5254	0.1382	1.1528	> 0.05
4	1.4498	0.0985		
5	1.4825	0.1018		

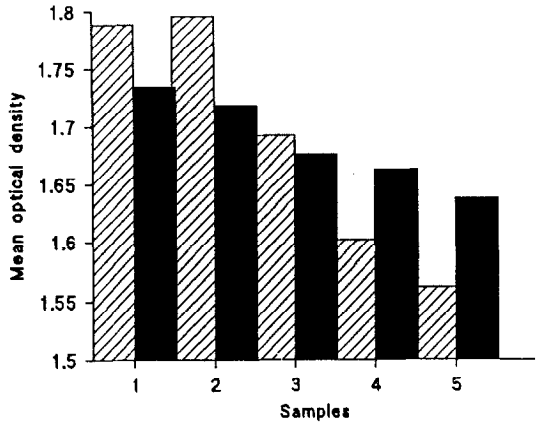

Figure 2 Comparison between the two procedures for five sample means of dilution 1 (▨ standard method; ■ random procedure).

Figs 2 and 3 show the differences of the sampling means for both procedures. It is clearly shown that the sampling means of the random procedure are more even than those of the non random procedure.

3.3. The stability of the dissolved formazan solution:

For the study at room temperature, two plates of dilution 1 and 2 were read. As can be seen from Tables XII and XV, statistically significant differences ($P < 0.05$) were found. On the other hand, no statistically significant differences were shown in the data at 4 °C ($P > 0.05$, Table XVI) in which only one plate of dilution 1 was used. At room temperature, even though no statistically significant differences were found between the third and the fourth reading for dilution 1 and 2, and between the second and third reading for dilution 2, other differences were shown and this indicated that the colour of the dissolved formazan solution was not stable at room temperature. It is also interesting to observe from this part of study that the optical density is decreasing for both

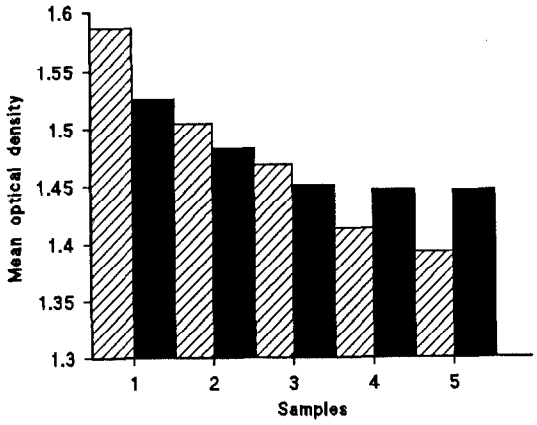

Figure 3 Comparison between the two procedures of five sample means for dilution 2 (▨ standard method; ■ random procedure).

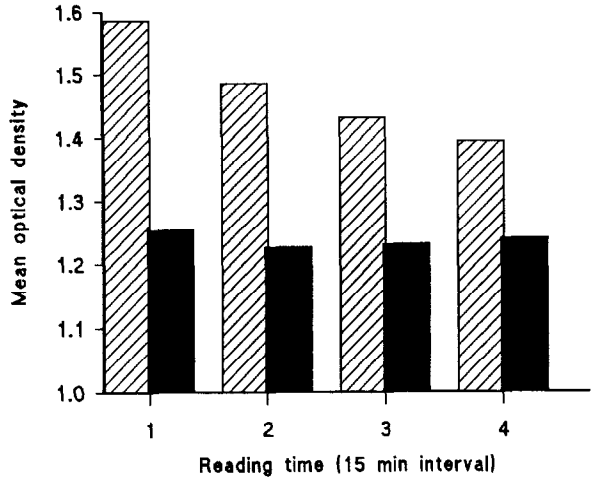

Figure 4 Comparison between solutions kept at two temperatures (▨ plate at room temperature; ■ plate at 4 °C).

TABLE XII Comparison of stability of solution for dilution 1 by ONEWAY

Time of reading	Means	Standard deviation	*F* value	*P* value
1st	1.7888	0.1289		
2nd	1.5809	0.0893	27.5696	< 0.001
3rd	1.4908	0.0877		
4th	1.4443	0.0911		

TABLE XIII Comparison between samples for dilution 1 by Duncan

Means	Times of reading	4th	3rd	2nd
1.4443	4th			
1.4908	3rd			
1.5809	2nd	*	*	
1.7888	1st	*	*	*

* Statistically significant differences between samples

dilution 1 and 2 with time at room temperature which could be confused with an increase in toxicity. Fig. 4 shows the comparison of these two groups of data for dilution 1. It is noticed that the mean optical densities at 4 °C are more even than those at room temperature.

TABLE XIV Comparison of stability of solution at room temperature for dilution 2 by ONEWAY

Time of reading	Means	Standard deviation	*F* value	*P* value
1st	1.5865	0.1021		
2nd	1.4854	0.0793	11.9008	< 0.001
3rd	1.4313	0.0755		
4th	1.3942	0.0761		

TABLE XV Comparison between samples at room temperature for dilution 2 by Duncan

Means	Times of reading	4th	3rd	2nd
1.3942	4th			
1.4313	3rd			
1.4854	2nd	*		
1.5865	1st	*	*	*

* Statistically significant differences between samples

TABLE XVI Comparison of stability of solution at 4 °C for dilution 1 by ONEWAY

Time of reading	Means	Standard deviation	*F* value	*P* value
1st	1.2543	0.1053		= 0.9334
2nd	1.2268	0.1146	0.1434	> 0.05
3rd	1.2313	0.1119		
4th	1.2400	0.1126		

4. Discussion

Since the MTT assay has been widely used as a screening test to evaluate the cytotoxicity of biomaterials *in vitro*, it is important to study its reproducibility in order to improve our experimental validity and accuracy.

From the first part of the study with the sample number used, it was demonstrated that the reproducibility of the MTT test was not good either within a plate, or between plates or between flasks of cells although they were maintained in the same environment for the same time. This indicates that the cell number changes significantly and is the main factor influencing the reproducibility of the MTT assay. It is therefore important to consider how to control the cell number in this test.

It is also noticed in part one that standard deviations varied considerably, with the largest value of 0.3022 (Table III) and the smallest value of 0.0878 (Table II). The standard deviation is an index to show the range of variation in the observed values and is often used to express the precision or accuracy of the experiments. The less the standard deviation, the greater the accuracy of the test and also the lower the standard error of the means, which indicates less error of sampling. Since the standard deviation varied very broadly this indicated that the accuracy of the test was not very good in this part.

Based on the problem met in the first part, a standard curve as shown in Fig. 1, was established in

order to determine the correct dilution of cells. It was decided that dilution of 1+7 and 1+9 were two cell concentrations to be used in the latter part of studies.

From the result of the second part, we can see that when the same data were analysed by two different procedures, different results were obtained for both cell dilutions. It was shown that statistically significant differences were measured among the samples when following the non random procedure, that is sampling by order from left to right across the plate (Table VI and VIII). On the other hand, none of the differences were found when following the random sampling procedure (Tables X and XI). This indicates that the design of the test procedure is a crucial factor in the test reproducibility. Therefore it is necessary to follow the random procedure to improve the validity of the assay and to obtain good reproducibility of the MTT test.

As mentioned above, the number of cells seeded in each well is difficult to control. In our experience, it is essential that the cells are mixed as thoroughly as possible before seeding into the microplates to minimize the variation in cell number and increase the accuracy of the test. From Tables VI–XI, we can see that the precision was improved and the standard deviations were less than 0.200 in the second part of test. However, there was still a variation in cell number observed in this study. For both cell dilutions, it was found that the mean cell number for each pair of columns decreased from left to right across the plates (Tables VI and VIII). This may be due to an increase in cell cloning at the start of cell seeding process. In this case, if the random sampling procedure was not used to choose the test groups, it would be difficult to attribute any statistically significant differences either to the variation in cell number or a factor such as toxicity of the biomaterials being tested. Therefore in order to prevent such mistaken conclusion it is recommended that the random sampling procedure is followed.

The third part of the study showed that the length of time before the plate was read and the temperature of the environment had a significant effect on the optical density reading. At room temperature, the optical density changed significantly over an hour ($P < 0.05$, Tables XII and XIV) while at 4 °C the optical density remained more stable and no significant differences were observed over an hour ($P > 0.05$, Table XVI). This indicated that the colour of the solution was not stable at room temperature. Besides, it is interesting to note that the optical density is decreasing with time (shown in Tables XII and XIV). In this case, if the plates are stored at room temperature for too long the optical density obtained may be less than the real value, which means that the cytotoxicity of the test materials is possibly misread. It could be concluded that the test material was more toxic than it really is. In addition to standardizing the time until the plate is read in the spectrophotometer, it is also suggested that the plates are stored in a refrigerator at 4 °C until they can be read.

5. Conclusion

The reproducibility of the MTT assay was studied systematically in this paper. The reproducibility of the MTT assay, using the sample size reported, was not good for the comparison within a plate, between plates or between flasks in the first part of the study. The cell number was found to be the main factor influencing the test results.

In order to solve this problem, two sampling procedures were performed in the second part; a non-random and random procedure. The reproducibility of the MTT assay was significantly improved by using the random sampling procedure. Therefore it is recommended to use the random sampling procedure to prevent the cell number influence and to increase the validity of the test.

The dissolved formazan solution has been proved to be unstable at room temperature. It is recommended not only to standardize the time before the plates are read in the spectrophotometer but also to store the plates at 4 °C if several plates need to be read at the same time.

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