alamarBlue® Assay

U.S. Patent No. 5,501,959

Indications for Use

The alamarBlue® Assay is designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. The bioassay may also be used to establish relative cytotoxicity of agents within various chemical classes (3). The toxicologist can establish baseline data for predicting the toxicity of related novel agents by comparing such baseline data with known *in-vivo* toxicity.

The assay is simple to perform since the indicator is water soluble, thus eliminating the washing/fixing and extraction steps required in other commonly used cell proliferation assays.

Product Description

The alamarBlue® Assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth (6).

The specific (fluorometric/colorimetric) REDOX indicator incorporated into alamarBlue® has been carefully selected because of several properties. First, the REDOX indicator exhibits both fluorescence and colorimetric change in the appropriate oxidation-reduction range relating to cellular metabolic reduction. Second, the REDOX indicator is demonstrated to be minimally toxic to living cells. Third, the REDOX indicator produces a clear, stable distinct change which is easy to interpret. The REDOX indicator has no current or past indication of carcinogenic capacity.

As cells being tested grow, innate metabolic activity results in a chemical reduction of alamarBlue®. Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form.

Experiments performed at Alamar suggest that reduction of alamarBlue® requires uptake by the cells. To test this hypothesis, we grew A549 cells to confluency in T25 flasks using RPMI 1640. The media was then removed from two flasks containing cells and replaced with fresh media. Fresh media was also added to a sterile flask containing no cells to serve as a negative control. All flasks were then re-incubated at 37°C, 5% CO_2 for four hours.

At the end of the four hour incubation, alamarBlue® was added to each flask. There was no immediate color change in any flasks upon addition. In one of the flasks containing cells, the media was left in contact with the cell layer, while the other T flask was turned over so that the media was not in contact with the cell layer. All flasks were then incubated for 1 hour at 37° and rechecked for color change.

If alamarBlue® reduction occurred simply from the reduction of the external medium, we would expect the flask in which the media was in contact with the cells and the flask in which media was no longer in contact with the cells to exhibit the same amount of reduction. This was not the case. The flask where the media was not in contact with the monolayer following addition of alamarBlue® displayed no color change from the blue of the negative control flask. The flask

where the cells were in contact with the monolayer was very pink, indicating a higher percentage of reduction. This seems to indicate uptake by the cells is required for reduction of alamarBlue®.

We have analyzed the possible interaction of alamarBlue® in cellular respiration by looking at the relative redox potential of the various components of the biological respiration chain. These are presented in Table 1, which includes the oxidation – reduction potentials for alamarBlue® and MTT and where they interact with the system.

Table 1. Oxidation reduction potentials in the electron-transport system.

Half-reaction		E^1o(mV)pH7.0 25°C
$O_2 + 4H^+ + 4e^-$	$2H_2O$	+820
alamarBlue®$_{ox}$ + 2H + $2e^-$	alamarBlue®$_{RED}$	+380
cytochromes$_{ox}$ + $1e^-$	cytochromes$_{RED}$	+290 to +80
$MTT_{ox} + 2H^+ + 2e^-$	MTT_{RED}	-110
$FMN + 2H^+ + 2e^-$	$FMNH_2$	-210
$FAD + 2H^+ + 2e^-$	$FADH_2$	-220
$NAD + 2H^+ + 2e^-$	$NADH + H^+$	-320
$NADP + 2H^+ + 2e^-$	$NADPH + H^+$	-320

A substance on the left side of one of the half reactions can be expected to oxidize a substance on the right side only if the latter has a more negative E_0 than the former. Using this rule, it is evident that MTT will be reduced by $FMNH_2$, $FADH_s$, NADH, NADPH, but will not be reduced by cytochromes. It is further evident that alamarBlue® will be reduced not only be each of these, but by the cytochromes as well.

The importance of this lies in the fact that the flow of electrons may be interrupted by the introduction of an artificial electron-acceptor (redox indicator) with an oxidation-reduction potential intermediate between those of any two members of the electron transport chain. Thus, whenever a substrate is oxidized in the presence of a tetrazolium salt (MTT), the released electrons will not be transported through the usual sequence of cytochromes, but will be trapped. This shuts down the respiratory chain. alamarBlue®, on the other hand, is intermediate only between final reduction of O_2 and cytochrome oxidase (Cyt.a_3). alamarBlue® is reduced by the removal of oxygen and its replacement by hydrogen. alamarBlue® may substitute for molecular oxygen for any of the oxidoreductases which routinely utilize molecular oxygen as an electron acceptor.

Data may be collected using either fluorescence – based or absorbance – based instrumentation. Fluorescence is monitored at 530-560nm excitation wavelength and 590nm emission wavelength (Fig. 1a). Absorbance is monitored at 570nm and 600nm (Figure 1b).

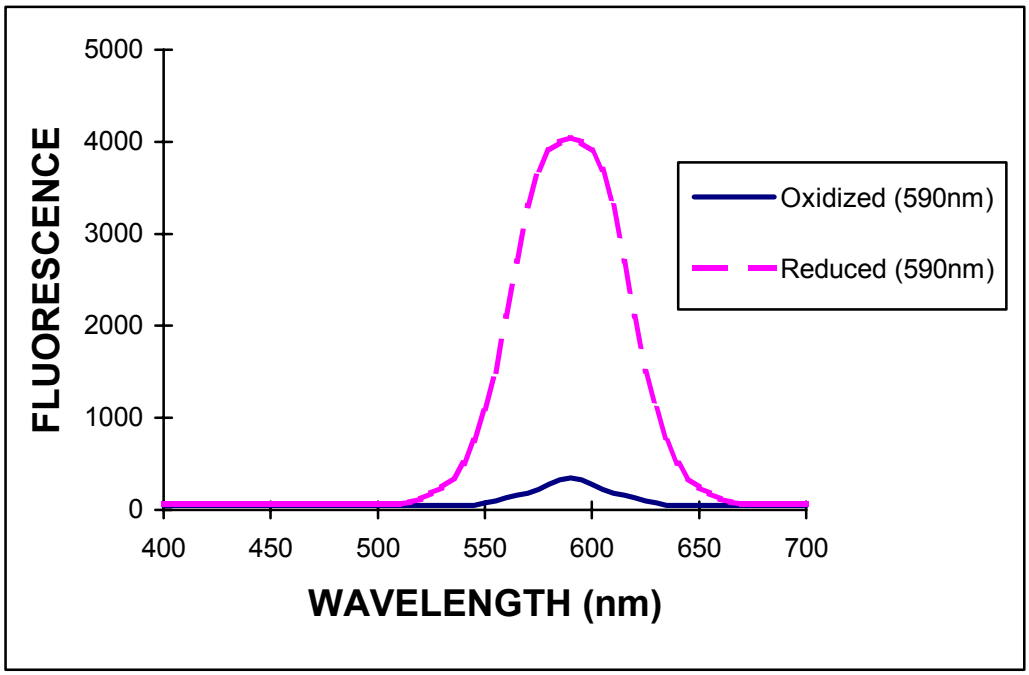


Figure 1a. alamarBlue® Fluorescence Emission Spectra

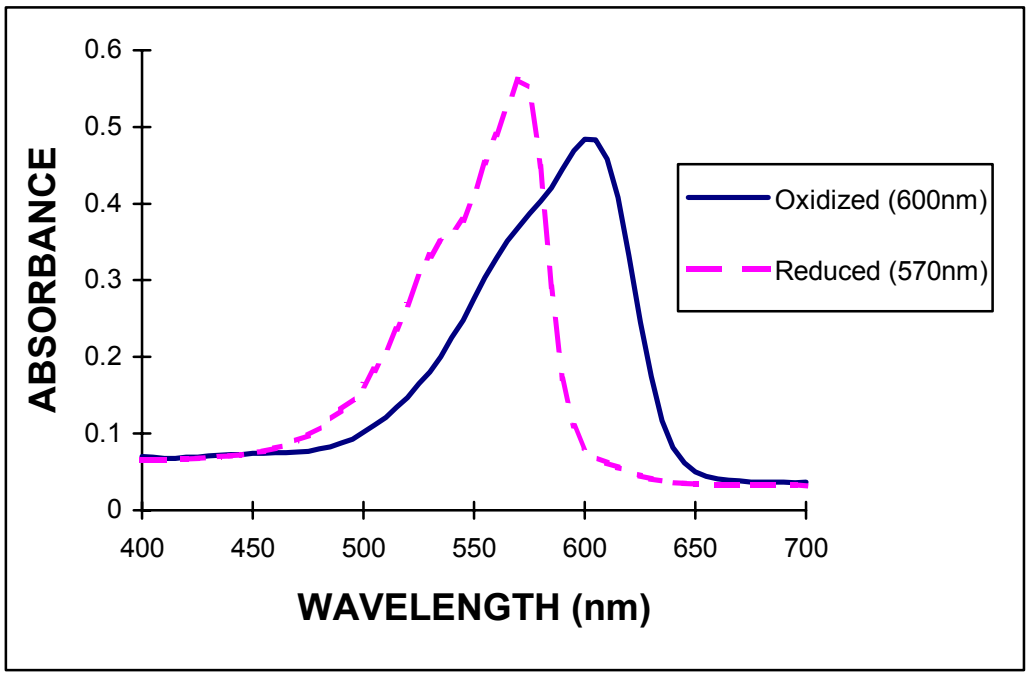


Figure 1b. alamarBlue® Absorbance

Advantages

The alamarBlue® Assay offers many advantages over conventional cell or radioactively-labelled incorporation assays:

Features	Benefits
Fluorescent/Colorimetric	Allows Choice of detection method
Water soluble	No extraction required
Works on suspension or attached cell lines	No centrifugation required
Fewer steps	Time saving/easily adaptable to automation
Stable	Allows for continuous cell Growth monitoring, kinetic Studies, incubation time of days
Non-toxic to cells	Less likely to interfere with Normal metabolism
Non-toxic to technician	Safe, disposable, less regulation

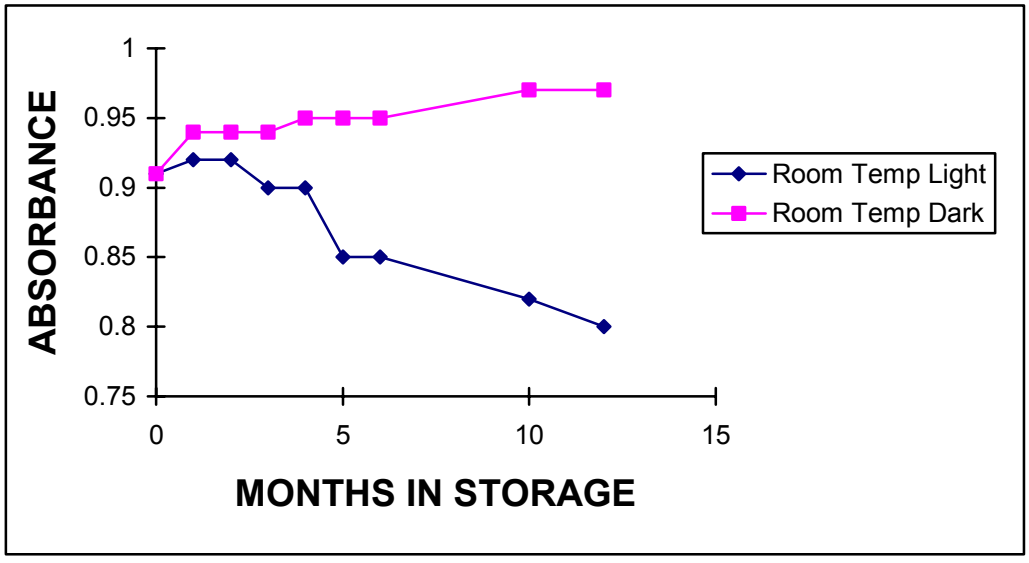
Storage Conditions

alamarBlue® should be stored in the dark, since the compound is light sensitive (Table 2). The product may be stored for 12 months at room temperature. This expiration date is given on the product label. If shelf life beyond 12 months is desired, storage at 2-8°C increases shelf life to 20 months. alamarBlue® may also be frozen at -70°C indefinitely. Because the indicator is a multicomponent solution, it is recommended that frozen alamarBlue® be warmed to 37°C and shaken to ensure all components are completely in solution.

Table 2. alamarBlue® Stability

Average absorbance at 600 nm is presented for each month tested. alamarBlue® was packaged in amber bottles. Light exposure was continuous at a level of approximately 100 lumens. Measurements were made on a Perkin Elmer UV/VIS Spectrophotometer Lamda 2 model with a 1 cm path length.

	MONTH								
Storage Condition	0	1	2	3	4	5	6	10	12
Room Temp Light	0.909	0.925	0.925	0.896	0.901	0.853	0.853	0.820	0.805
Room Temp Dark	0.909	0.944	0.937	0.944	0.949	0.954	0.954	0.966	0.966



Quality Control Testing Method for alamarBlue®

Materials and Equipment:

- pH 7.4, 0.1M potassium phosphate buffer
- 10 ml test tube
- pipettor capable of accurately dispensing 0.4 ml
- plate reader with one of the following filters: 540, 570, 600, 630nm
- Dynatech flat bottom plate

Procedure

1. Pipette 0.4 ml of alamarBlue® into a test tube.
2. Dilute to 10 mls with phosphate buffer.
3. Mix well.
4. Pipette 100 μl into each well of a clear, flatbottom microblate (note: there will be enough solution to fill 10 columns in the plate).
5. Read absorbance at appropriate wavelenths.

*Expected Results:

Wavelength (nm)	Average Absorbance (Standard Deviation)
540	0.145 (0.002)
570	0.225 (0.003)
600	0.313 (0.004)
630	0.116 (0.002)

***Absorbance Values may be affected by the type of plate (whether round or flat bottom) and the plate manufacturer.**

The reduced form (red) of alamarBlue® is very unstable in water. For this reason, it is difficult to recommend a standard test for the reduced form. However, the reduced form is very stable in media. To determine the absorbance/fluorescence to be expected from the reduced form (red) for a particular experiment, it is suggested that 1X alamarBlue® be made up in the media intended for use in an autoclavable container. Reduce this preparation by autoclaving for 15 minutes. Remove from the autoclave and allow to cool to room temperature. Swirl the solution several times and pipette 100 μl into the wells of a flat bottom microtiter plate. Measure the absorbance at the proper wavelenths.

Table 3 a-b presents the absorbance values for the oxidized and reduced forms of the indicator in several commonly used culture media. No QC protocol is recommended for fluorescence since fluorescence units are arbitrary and the scale used varies widely from one instrument to another. From table 3 a-b it should be apparent that the reduced form of alamarBlue® is highly fluorescent. When attempting to measure very small changes in reduction, fluorescence measurements will produce greater sensititvity.

Table 3a-b. Absorbance values for oxidized/reduced forms of alamarBlue® for commonly used culture media.

Powdered media was obtained from Sigma and prepared according to their instructions. All media contained phenol red. The required amount of sodium bicarbonate was added to each media, and each was pH adjusted to 7.4 with 1N HCl or 1N NaOH. alamarBlue® was added to each media which was then split into 2 samples. One sample of each media was autoclaved for 15 minutes to produce the reduced form. The medias were dispensed into a flat bottom Dynatech plate (100 μl per well) and absorbance read at 540, 570, 600, 630nm on a Cambridge Technologies plate reader. Fluorescence measurements were made on a Cambridge Technologies, Inc. (Watertown, MA) Model 7620 Microplate fluorometer – settings were: bottom reading, light source setting 12, no max AFU, excitation: 530, emission: 560, gain/16.

a. ABSORBANCE VALUES

Powdered Media	Sigma Product #	Wavelength (nm) 540 OX	540 RED	570 OX	570 RED	600 OX	600 RED	630 OX	630 RED
BME EBSS	B9638	0.610	1.207	0.853	1.502	0.845	0.244	0.261	0.177
BME HBS	B9763	0.468	1.087	0.705	1.403	0.817	0.154	0.254	0.097
McCoy's 5A	M4892	0.520	1.133	0.740	1.421	0.756	0.250	0.236	0.183
MEM EBSS	M0268	0.582	1.186	0.819	1.483	0.820	0.235	0.252	0.168
MEM HBSS	M4642	0.480	1.066	0.713	1.383	0.811	0.145	0.251	0.088
Nut Mix F-10	N6635	0.361	0.784	0.583	1.117	0.798	0.138	0.248	0.091
Nut Mix F-12	N6760	0.374	0.796	0.604	1.135	0.822	0.137	0.255	0.085
RPMI 1640	R6504	0.431	0.928	0.659	1.250	0.795	0.161	0.248	0.101

b. FLUORESCENCE VALUES

Powdered Media	Sigma Product#	Fluorescence Units Oxidized	Reduced
BME EBSS	B9638	1926	55676
BME HBS	B9763	3840	60256
McCoy's 5A	M4892	2640	50545
MEM EBSS	M0268	2377	54493
MEM HBSS	M4642	4194	59202
Nut Mix F-10	N6635	2472	70092
Nut Mix F-12	N6760	5232	68132
RPMI 1640	R6504	6472	58796

General Procedure for Determining Length of Incubation Time and Plating Density for a Cell Line

The two variables which most affect the response of cells to alamarBlue® are length of incubation time and number of cells plated. It is recommended that the plating density and incubation time be determined for each cell line using the following procedure:

1. Harvest cells which are in log phase growth stage and determine cell count. Plate cells at various densities, some dilutions being above and below the cell density expected to be used.

2. Aseptically add alamarBlue® in an amount equal to 10% of the culture volume.

3. Return cultures to incubator. Remove the plate and measure fluorescence/ absorbance each hour following plating for the first 6-8 hours. It is also recommended that the plate remain in incubation overnight and measurements made the following day at 24 hours. Two kinds of information can be obtained from this data: (1) for any given incubation time selected, the range in cell density can be determined for which there is a linear response relating cell numbers to alamarBlue® reduction, and (2) for any given cell density selected, the maximum incubation time can be determined in which the control cells turn the indicator from the oxidized (blue) form to the fully reduced (red) form.

4. Measure absorbance at a wavelength of 570nm and 600nm. Or, measure fluorescence with excitation wavelength at 530-560nm and emission wavelength at 590 nm.

5. To generate the graph for (1), plot the log of cell density on the x-axis and reduction of alamarBlue® from absorbance (using equation 5 to be discussed in calculations for absorbance) or fluorescence on the y-axis (Fig.2). To generate the graph for (2), plot the number of hours incubated on the x-axis and reduction of alamarBlue® on the y-axis (Fig. 3). A sample data set and the resulting graphs are presented below.

Sample Data Set

		a549 cells/ml				**p388 cells/ml**			
Time	Blue in Media	500	1000	5000	10000	500	1000	5000	10000
t0	0.336	0.336	0.338	0.348	0.372	0.342	0.334	0.332	0.328
t2	0.334	0.339	0.352	0.432	0.540	0.340	0.333	0.335	0.335
t4	0.333	0.346	0.365	0.489	0.590	0.339	0.332	0.339	0.346
t5.5	0.321	0.344	0.366	0.511	0.573	0.331	0.325	0.335	0.344
t20	0.322	0.438	0.510	0.518	0.486	0.332	0.328	0.381	0.434

Absorbance values at 570nm after blanking with media only. a549 is a monolayer culture, p338 is a suspension cell line.

Time	Blue in Media	a549 cells/ml				p388 cells/ml			
		500	1000	5000	10000	500	1000	5000	10000
t0	0.441	0.439	0.443	0.459	0.496	0.451	0.438	0.433	0.422
t2	0.440	0.425	0.421	0.349	0.267	0.448	0.435	0.422	0.404
t4	0.432	0.411	0.397	0.265	0.162	0.444	0.432	0.414	0.391
t5.5	0.424	0.397	0.377	0.211	0.135	0.435	0.424	0.404	0.377
t20	0.412	0.271	0.180	0.102	0.112	0.423	0.415	0.337	0.253

Absorbance values at 600nm after blanking with media only. a549 is a monolayer culture, p338 is a suspension cell line.

Using absorbance data from the sample data set, percent reduction of alamarBlue® was calculated using equation 5:

Time	a549 cells/ml				p388 cells/ml			
	500	1000	5000	10000	500	1000	5000	10000
t0	6.3	6.1	6.0	5.7	5.9	6.0	6.3	7.0
t2	8.6	11.5	35.4	65.7	5.9	6.3	8.3	10.6
t4	11.9	17.3	57.6	89.9	6.3	6.6	10.2	14.5
t5.5	13.6	20.4	70.0	91.8	6.1	6.4	10.9	16.2
t20	49.6	76.2	88.3	80.7	8.1	8.4	29.5	51.3

These values are plotted to produce figures 2-3.

Fig. 2A. A549

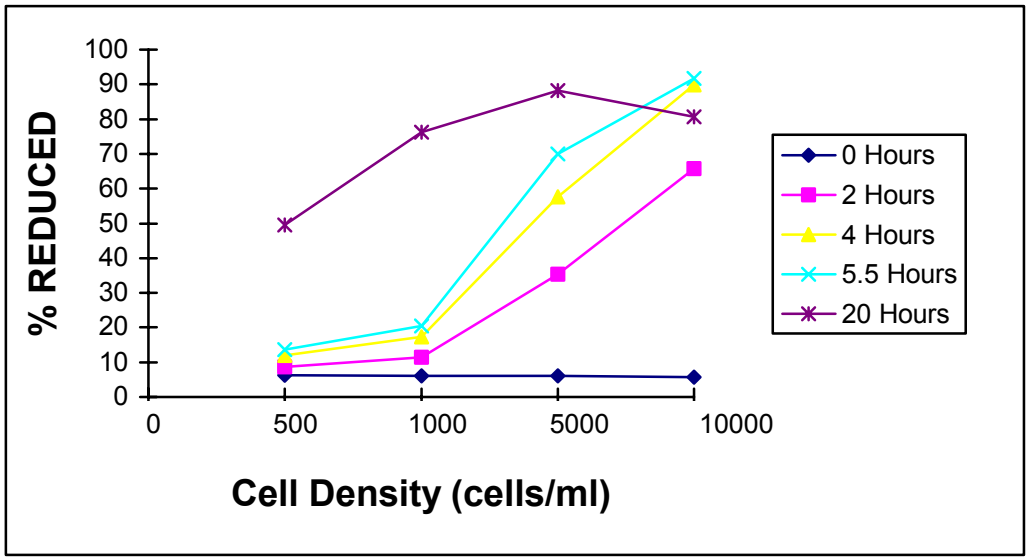


Fig. 2B. P388

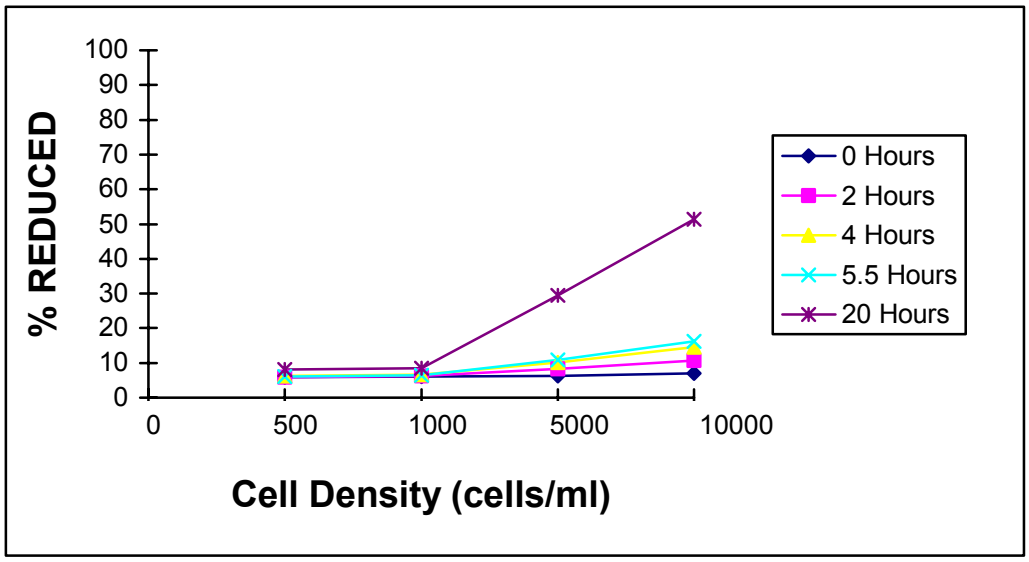


Fig. 3A. A549

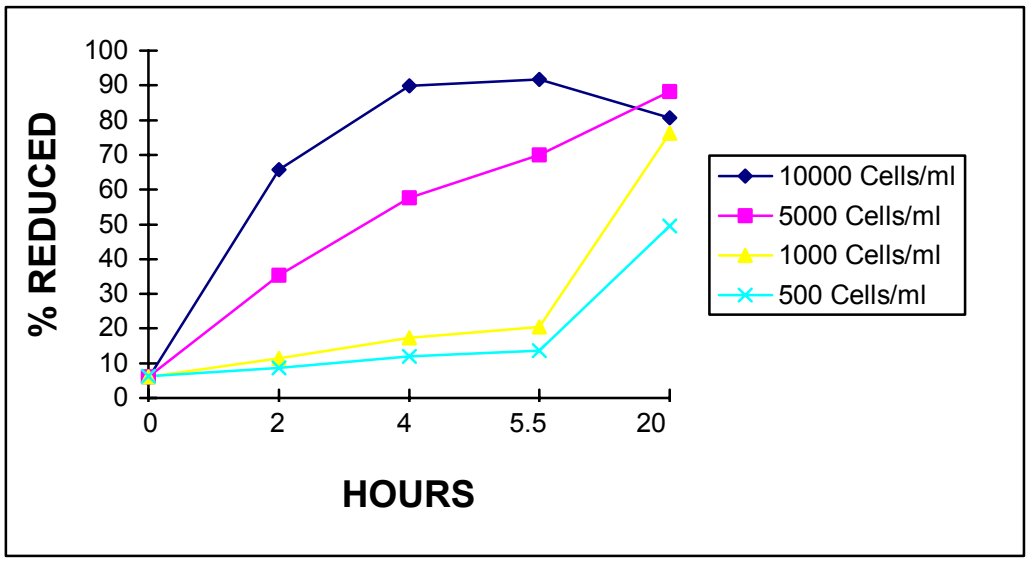
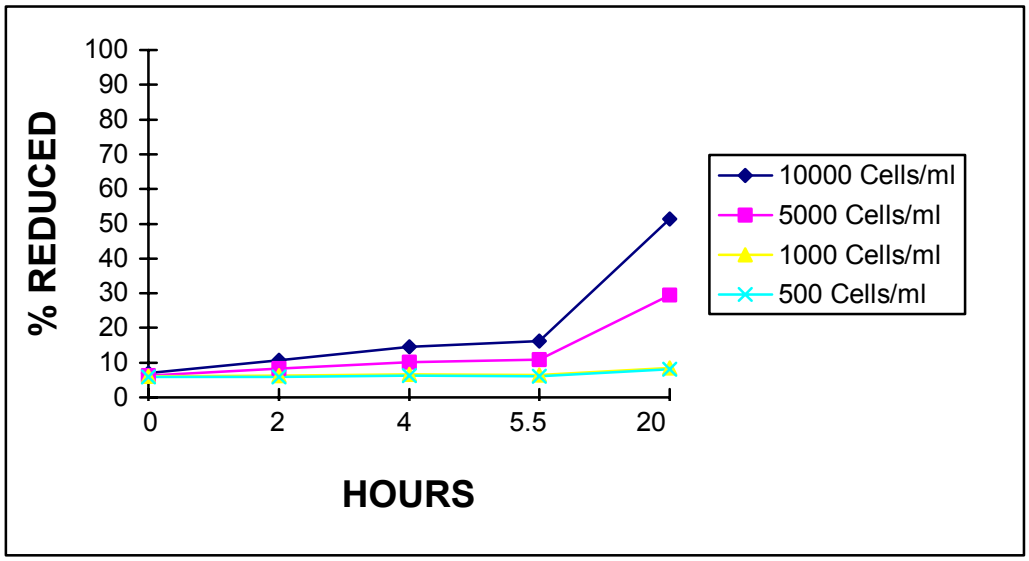


Fig. 3B. P388



From fig. 2A, if, for example, the desired incubation time with alamarBlue® were 4 hours, any plating density from 500 to 10,000 cells/ml could be used and expected to produce a reaction within the linear range for alamarBlue® for that incubation period. However, if the intent is to incubate for 20 hours, the reaction could only be expected to be within the linear range if plated at 500-1000 cells/ml for this cell line.

On the other hand, with P388, even with an initial plating density up to 10,000 cells per well, data is within range and alamarBlue® has only been reduced by 50% at that point. This indicates cells could be incubated with alamarBlue®, even when plated at this high density for up to 2 days. If a shortened exposure is desired, then the initial plating density should be increased. If the goal is to continue the experiment for more than 2 days, the initial plating density should be decreased. Similar information is gained from examination of fig. 3a-b.

Note

With high cell numbers or extended incubation time (days), you will reach a point where the red form stops increasing and begins to decline. The absorbance/fluorescence level drops with a corresponding clearing of the red color. This is demonstrated in Fig. 3A when plated at 10,000 cells/ml and incubated for more than 4 hours.

Microbial contaminants will also reduce alamarBlue® and will yield erroneous results if contaminated cultures are tested by this method.

In-house studies indicated samples with protein concentrations equivalent to 10% fetal bovine serum did not interfere with the assay. However, Page et. al. (7) indicated serum may cause some quenching of fluorescence and recommended using the same serum concentration in controls to take this into account. Geogan et. al. (5) tested the effects of varying concentrations of fetal bovine serum (FBS), bovine serum albumin (BSA), and polyvinylpyrrolidone in the alamarBlue® assay. They found that increasing concentrations of FBS and BSA did affect the assay. However, they provide a method to test your test matrix for effects due to these

compounds and provide a straightforward method of calculation to correct for any such effects. This method can be applied to determine the effect of any additive in your test media matrix, including the test chemicals themselves.

There is no interference from the presence of phenol red in the growth medium. The presence of phenol red merely shifts the values approximately 0.03 units higher (see table 4).

Table 4. Effect of phenol red on absorbance values at 570 nm.

Absorbance value for various levels reduction of alamarBlue® in RPMI 1640 w/MOPS with and without phenol red, pH 7.0. 100 µl per well, Dynatech flat bottom plate.

		% REDUCED				
	Media	Blue	10	30	60	90	Red
RPMI 1640	0.032	0.47	0.52	0.61	0.73	0.85	0.88
RPMI 1640 w/ phenol red	0.061	0.53	0.54	0.64	0.76	0.88	0.91

Effect of Storage of Plates on Measurements

Many investigators find that they may not be able to read plates on the day an experiment is performed. It is recommended that plates be refrigerated and read within 1 – 3 days. Plates can be wrapped in foil or plastic wrap to prevent evaporation. Table 5 presents absorbance data for the oxidized (blue) and reduced (red) forms of alamarBlue® for plates which were read on day 1, stored overnight refrigerated and read again on days 2 and 3. Data is presented for some different plate types and plate manufacturers to illustrate the effect these variable have on absorbance values obtained.

Table 6 gives the fluorescence values for the same plate. If plates are stored refrigerated and fluorescence measurements are being used, keep in mind that fluorescence measurements are influenced by temperature (see Table 7). If measurements are normally taken at 37°C, then plates should be warmed to that temperature before reading.

Table 5. Effect of Storage Plates on Absorbance
100 µl of RPMI 1640 w/MOPS 7.0 no phenol red.

		ABSORBANCE							
		BLUE (Oxidized)					RED (Reduced)		
		540nm	570nm	600nm	630nm		540nm	570nm	600nm	630nm
Dynatech	Day	(.003)	(.005)	(.007)	(.003)		(.020)	(.027)	(.002)	(0.0)
1										
Flat Bottom		.298	.496	.708	.236		.693	1.017	.126	.075
	Day	(.003)	(.004)	(.006)	(.002)		(.020)	(.027)	(.008)	(.009)
2										
		.294	.484	.692	.227		.697	1.018	.164	.118
	Day	(.003)	(.006)	(.008)	(.003)		(.010)	(.024)	(.008)	(.009)
3										
		.296	.486	.691	.231		.734	1.038	.199	.149

Table 5. Effect of Storage Plates on Absorbance (Con't)
100 µl of RPMI 1640 w/MOPS 7.0 no phenol red.

		ABSORBANCE							
		BLUE (Oxidized)					RED (Reduced)		
Corning 1	Day	(.002)	(.004)	(.005)	(.002)		(.020)	(.024)	(.003)	(.004)
Flat Bottom		.210	.335	.474	.169		.530	.772	.137	.105
	Day	(.002)	(.003)	(.004)	(.001)		(.020)	(.027)	(.004)	(.004)
2		.210	.329	.458	.161		.580	.822	.193	.159
	Day	(.002)	(.003)	(.005)	(.002)		(.020)	(.035)	(.004)	(.003)
3		.200	.322	.444	.160		.600	.823	.210	.172
Corning 1	Day	(.001)	(.002)	(.002)	(.001)		(.014)	(.018)	(.002)	(0.0)
Round Bottom		.380	.635	.913	.300		.870	1.266	.151	.084
	Day	(.002)	(.003)	(.004)	(.002)		(.011)	(.014)	(.002)	(.002)
2		.390	.641	.914	.295		.850	1.241	.146	.083
	Day	(.004)	(.006)	(.008)	(.004)		(.017)	(.021)	(.007)	(.006)
3		.390	.646	.916	.302		.860	1.237	.159	.094

Standard deviations are in parentheses (calculated for n=8)

Table 6. Effect of Storage of Plates on Fluorescence
Measurements made at room temperature (22°C)

	Fluorescence Units					
	BLUE (Oxidized)			RED (Reduced)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Dynatech Flat Bottom	(7.4) 188	(49.6) 265	(42.8) 277	(123) 5821	(127) 5973	(157) 5867
Corning Flat Bottom	(4) 160	(6) 184	(10) 206	(101) 5065	(101) 5124	(130) 4904
Corning Round Bottom	(4.1) 185	(16) 250	(43) 289	(61) 6048	(219) 5904	(110) 5481

Standard Deviation in parentheses (calculated for n = 8).

Table 7. Effect of Temperature on Fluorescence
RPMI 1640 w/MOPS pH 7.0 no phenol red, 100 µl per well, Dynatech flat bottom.

Temp.	Fluorescence
37°C	(171.1) 5216
22°C	(157.6) 5867
4°C	(192.1) 6881

General Discussion of Calculations when Using Absorbance

It is clear from Fig. 1b that there is considerable overlap in the absorption spectra of the oxidized and reduced forms of alamarBlue®. When there is no region in which just one component absorbs, it is still possible to determine the two substances by making measurements of two wavelengths (8). The two components must have different powers of light absorption at some points in the spectrum. Since absorbance is directly proportional to the product of the molar extinction coefficient and concentration, a pair of simultaneous equations may be obtained from which the two unknown concentrations may be determined:

1. $C_{RED}(\varepsilon_{RED})\lambda_1 + C_{OX}(\varepsilon_{OX})\lambda_1 = A\lambda_1$

2. $C_{RED}(\varepsilon_{RED})\lambda_2 + C_{OX}(\varepsilon_{OX})\lambda_2 = A\lambda_2$

To solve for the concentration of each component:

3. $$C_{RED} = \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX}) A\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1(\varepsilon_{OX})\lambda_2 - (\varepsilon_{OX})\lambda_1(\varepsilon_{RED})\lambda_2}$$

4. $$C_{OX} = \frac{(\varepsilon_{RED})\lambda_1 A\lambda_2 - (\varepsilon_{RED})\lambda_2 A\lambda_1}{(\varepsilon_{RED})\lambda_1(\varepsilon_{OX})\lambda_2 - (\varepsilon_{OX})\lambda_1(\varepsilon_{RED})\lambda_2}$$

To determine the percent reduction of alamarBlue®:

$$\% \text{ Reduced} = \frac{C_{RED} \text{ Test Well}}{C_{OX} \text{ Negative Control Well}}$$

5. $$= \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1} \times 100$$

To calculate the percent difference in reduction between treated and control cells in cytotoxicity/proliferation assays:

6. $$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}}{(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}} \times 100$$

Where

- C_{RED} = concentration of reduced form alamarBlue® (RED)
- C_{OX} = oxidized form of alamarBlue® (BLUE)
- ε_{OX} = molar extinction coefficient of alamarBlue oxidized form (BLUE)
- ε_{RED} = molar extinction coefficient of alamarBlue reduced form (RED)
- A = absorbance of test wells
- A' = absorbance of negative control well. The negative control well should contain media + alamarBlue but no cells.
- A° = absorbance of positive growth control well
- λ_1 = 570nm (540nm may also be used)
- λ_2 = 600nm (630 may also be used)

The key equations to use are (5) and (6), equation 5 for calculating the percent reduction from the blue oxidized form and equation 6 for calculating percent difference between treated and control cells in cytotoxicity/proliferation experiments. Equations 1-4 are included only for completeness of the discussion.

Blanking of the plate reader should be done with a well containing media only. Table 3 contains the necessary values for solving the equations stated above. Table 4 lists typical absorbance values for different percent reduction of alamarBlue®.

TABLE 3. Molar Extinction Coefficients for alamarBlue®.

Wavelength (λ)	ε_{RED}	ε_{OX}
540nm	104,395	47,619
570nm	155,677	80,586
600nm	14,652	117,216
630nm	5,494	34,798

TABLE 4. Typical Absorbance Values for Microplate Reader. Test wells contain 100 µl RPMI 1640 w/MOPS with no phenol red at pH 7.0 with alamarBlue®, containing the reduced form present in known amounts.

	Wavelength (nm)			
% Reduced alamarBlue®	540	570	600	630
0 Fully Oxidized (Blue)	0.29	0.47	0.67	0.22
10	0.32	0.52	0.62	0.21
20	0.35	0.56	0.57	0.19
30	0.38	0.61	0.50	0.18
40	0.41	0.64	0.45	0.16
50	0.45	0.69	0.39	0.14
60	0.48	0.73	0.33	0.13
70	0.51	0.77	0.28	0.11
80	0.54	0.81	0.23	0.10
90	0.57	0.85	0.16	0.08
100 Fully Reduced (Red)	0.60	0.88	0.10	0.06
Media	0.03	0.03	0.03	0.03

Example Calculation 1:

$\lambda_1 = 570$, $\lambda_2 = 600$
$(\varepsilon_{OX})\lambda_2 = 117{,}216$
$(\varepsilon_{OX})\lambda_1 = 80{,}586$
$(\varepsilon_{RED})\lambda_1 = 155{,}677$
$(\varepsilon_{RED})\lambda_2 = 14{,}652$
$A\lambda_1 = 0.61$ Observed absorbance reading for test well
$A\lambda_2 = 0.42$ Observed absorbance reading for test well
$A'\lambda_2 = 0.64$ Observed absorbance reading for negative control well
$A'\lambda_1 = 0.44$ Observed absorbance reading for negative control well

To calculate percent reduced using equation 5:

$$\text{Percent reduced} = \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2}{(\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1} \times 100$$

$$= \frac{(117{,}216)(.61) - (80{,}586)(.42)}{(155{,}677)(.64) - (14{,}652)(.44)} \times 100$$

$$= \frac{71{,}502 - 33{,}846}{99{,}633 - 6{,}447}$$

$$= \frac{37{,}656}{93{,}186} = 0.404 \times 100 = 40\%$$

Example Calculation 2:

$\lambda_1 = 570$, $\lambda_2 = 600$
$(\varepsilon_{OX})\lambda_2 = 117{,}216$
$(\varepsilon_{OX})\lambda_1 = 80{,}586$
$(\varepsilon_{RED})\lambda_1 = 155{,}677$
$(\varepsilon_{RED})\lambda_2 = 14{,}652$
$A\lambda_1 = 0.65$ Observed absorbance reading for test well
$A\lambda_2 = 0.36$ Observed absorbance reading for test well
$A°\lambda_1 = 0.78$ Observed absorbance reading for positive control well
$A°\lambda_2 = 0.19$ Observed absorbance reading for positive control well

To calculate the percent difference in reduction between treated and control cells in cytotoxicity/proliferation assays using equation 6:

Percent difference in reduction

$$= \frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}}{(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}} \times 100$$

$$= \frac{(117{,}216)(.65) - (80{,}586)(.36)}{(117{,}216)(.78) - (80{,}586)(.19)} \times 100$$

$$= \frac{76{,}190 - 29{,}011}{91{,}428 - 15{,}311} \times 100$$

$$= \frac{47{,}179}{76{,}117} \times 100 = 62\%$$

This would indicate that the amount of reduction in the test well is only 62% of that in the control well, or put another way, that growth in the test well is inhibited by 38% when compared to that of the control.

Simplified Method to Correct for Overlap of Oxidized/Reduced Forms of alamarBlue® to Determine Amount of alamarBlue® with other Filters

In the first version of this package insert, the suggested calculation for determining the amount of reduced alamarBlue® was simply $Absorbance_{570} - Absorbance_{600}$. This works well when most of the test wells are fully reacted, i.e. fully oxidized (blue) or fully reduced (red). In this situation, there is no correction needed for the shoulder of the oxidized substrate at 570 (Fig. 1b), because in fully reduced wells there is no oxidized substrate present.

Apparently this was not the situation for many alamarBlue® users, and the need for a correction for the contribution of the oxidized substrate present at 570nm was pointed out (4). Thus, the second revision of this package insert derived the molar extinction coefficients and a method for calculating the amount of reduced alamarBlue® for the most commonly encountered standard filters found in plate readers.

Since the release of this revision, it has become apparent from our users that not enough filters were considered. Many times users have requested a method to determine coefficients for their particular filters, for example, using a 565nm filter with a 610nm filter. Deriving molar extinction coefficients is a very tedious method subject to numerous possibilities for introducing error. Known amounts of reduced and non-reduced alamarBlue® must be prepared in a series of ratios of reduced/oxidized forms and absorbance measurements made for the wavelengths to be tested. These steps are subject to dilution errors as well as errors which may be caused by not having the reduced form 100% reduced.

From the personal communication of Dr. Geier (4) and in independently derived calculations by Goegan et. al. (5) a very simple and accurate method for deriving the amount of reduced alamarBlue® present is given. This simple calculation can be used for any filter combination.

<u>The method is as follows:</u>

1. Make up alamarBlue® as directed in the package insert (1 to 10 dilution in 100 μl media).
2. Measure the absorbance at the lower wavelength filter and at the higher wavelength filter.
3. Measure the absorbance of 100 μl media only at the wavelengths in Step 2.
4. Subtract the absorbance values of media only (Step 3) from the absorbance values of alamarBlue® in media (Step 2). This gives the absorbance of alamarBlue® in media – absorbance of media only. Call this AO_{LW} = absorbance of oxidized form at lower wavelength, and AO_{HW} = absorbance of oxidized form at higher wavelength.
5. Calculate correction factor: R_O. $R_O = AO_{LW} / AO_{HW}$.
6. To calculate the percent of reduced alamarBlue®:
   $AR_{LW} = A_{LW} – (A_{HW} \times R_O) \times 100$
   This replaces equation 5 (page 14).
   The replacement for equation 6 (page 14) to calculate the percent difference between treated and control cells in cytotoxicity/proliferation assays then becomes:

$$\text{Percent difference in reduction} = \frac{A_{LW} – (A_{HW} \times R_O) \text{ for test well}}{A_{LW} - (A_{HW} \times R_O) \text{ positive growth control}} \times 100$$

An example of these calculations when the filters are 570nm and 600nm is given below (4):

Sample	Abs 570	Abs 600
10% alamarBlue® in 100 μl medi	0.728	0.969
100 μl media	0.082	0.037
Abs 10% AB in media - Abs. media only	0.646	0.932

$R_O = AO_{570}/AO_{600}$
$R_O = 0.646/0.932 = 0.693$

AO_{570} = absorbance of oxidized form at 570nm.
AO_{600} = absorbance of oxidized form at 600nm.

This same correction factor was independently derived by Geogan et. al. (5), in their equations. 7 and 1.

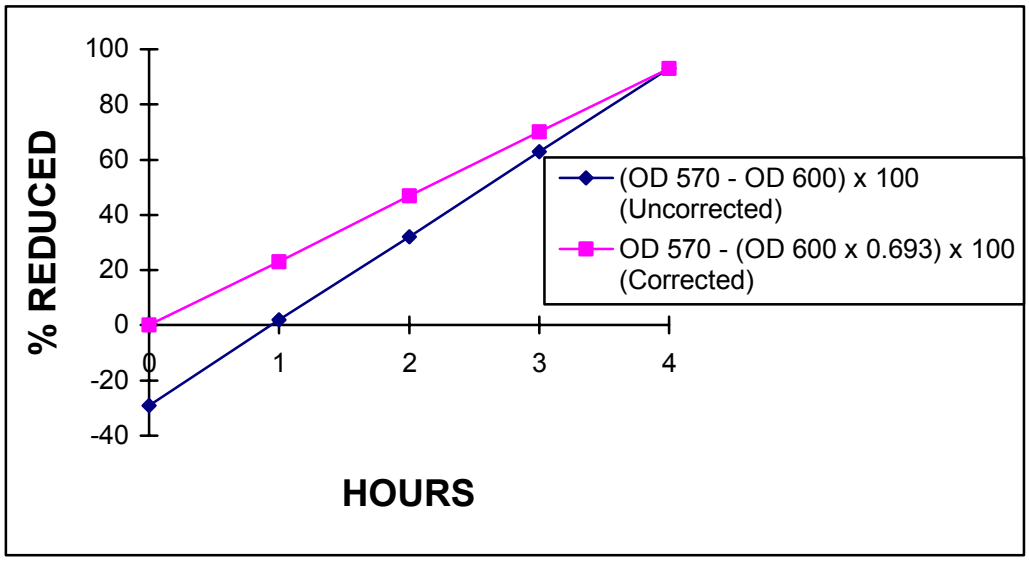
To calculate amount reduced alamarBlue®:

$AR_{570} = A_{570} – (A_{600} X R_O)$ where A_{570} and A_{600} are sample absorbances minus the media blank.

Example Assay:

Time Point		Abs 570 Test Well	Abs 600 Test Well	Abs 570 – Abs 600 X100	Abs 570 – (Abs 600)(0.693) x100
(fully oxidized)	0	0.65	0.93	- 28	0
	1	0.72	0.70	2	23
	2	0.79	0.47	32	46
	3	0.86	0.23	63	70
(fully reduced)	4	0.93	0.00	93	93

These data points are plotted below:



As can be noted from the graph there was a difference between the corrected and uncorrected reduced absorbance. It should also be noted that lack of correction leads to negative absorbances. This may account for the unexplained negative absorbances calculated by some users.

For users with different filter pairs the only change will be in R_O whose calculation is explained in the above method and, of course, taking the absorbance measurements of the test samples with those filters.

alamarBlue® Reduction Curves

Examples of reduction curves are included to demonstrate the usefulness of the alamarBlue® assay for measuring cell proliferation. (Figure 4)

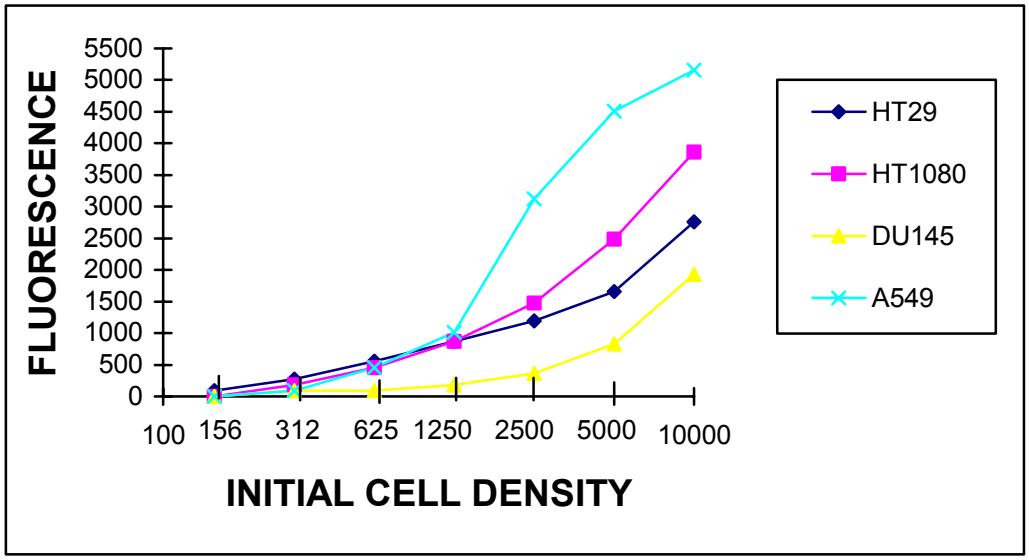


Figure 4. Detection of Cell Growth of Four Cell Lines Using alamarBlue®

alamarBlue® is especially well suited for kinetic studies that involve monitoring cell growth for extended exposure periods. The stability of alamarBlue® allows the investigator to add the indicator at the beginning of the experiment and continue to follow reduction by the cells for several days. (Figure 5)

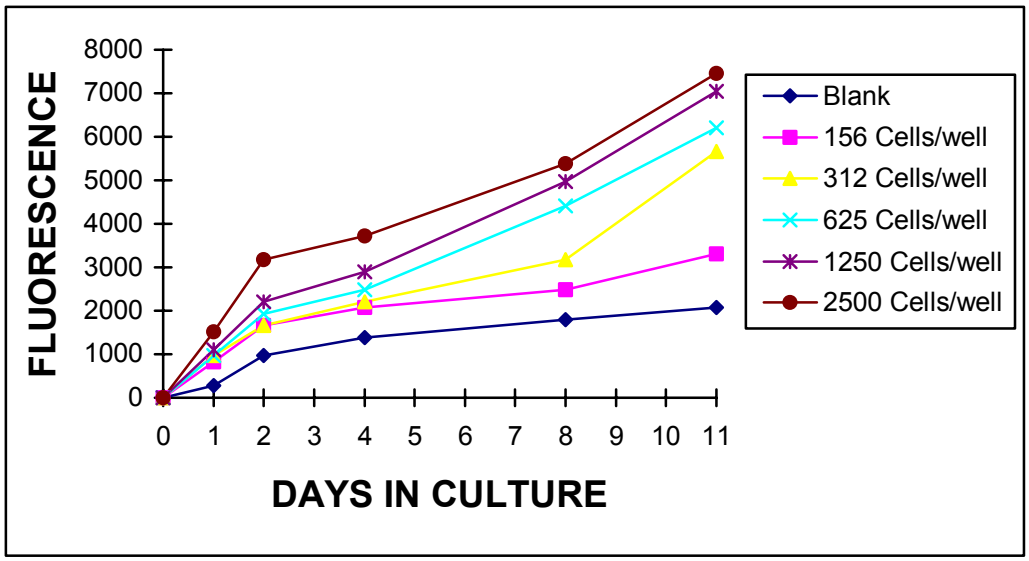


Figure 5. Continuous Incubation of Cell Line A549 with alamarBlue®

Example Procedure of Cytotoxicity Assay

Preparation of Cells for Testing

1. Harvest an appropriate cell line by trypsinization and subsequent trypsin inhibitor treatment.

2. Centrifuge cells, re-suspend in growth medium and count.

3. Calculate the total cell number and adjust to 1 x 10^4 cell/ml. This is a suggested cell density which has worked in our studies with cancer cell lines. Refer to the article by Alley et. al. (2) for suggestions on plating densities and growth medium when working with cancer cell lines and chemotherapeutic agents.

4. Add 250µl of cell suspension to each well. Incubate at 37°C in 5% CO_2 atmosphere for the number of days required for the particular cell line to be in log phase (usually 3 days).

Exposing Cells to Test Agents

1. Prepare appropriate dilutions of test agent in growth media.

2. Aspirate spent growth medium from the wells and add 250µl of each dilution of test agent to the wells.

3. Cover, then return to the incubator for 2 days.

4. After incubation, add 25µl of the indicator to each well. Incubate panels for an additional 3 hours. Panels may then be read spectrophotometrically (absorbance at 570nm and 600nm) or spectrofluorometrically (excitation, 530-560nm; emission, 590nm).

Data Analysis

Fluoresence:

1. Calculate percent of untreated control with the following formula:

$$\frac{\text{FI 590 of test agent dilution}}{\text{FI 590 of untreated control}} \times 100$$

2. Use semi-log graph paper and plot the percent of untreated control for each dilution of a given test agent on the y-axis versus the concentration of the test agent on the x-axis.

3. Determine the LD50 endpoint from the graph by reading from where the 50 percent point intercepts the Dose Response Curve to the concentration along the x-axis. That concentration is the LD50 value. (Figure 6)

Absorbance:

1. Calculate percent of untreated control with the following formula:

$$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}}{(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}} \times 100$$

or

$$\frac{A_{LW} - (A_{HW} \times R_O) \text{ test well}}{A_{LW} - (A_{HW} \times R_O) \text{ positive growth control}} \times 100$$

2. Use semi-log graph paper and plot the percent of untreated control for each dilution of a given test agent on the y-axis versus the concentration of test agent on the x-axis.

3. Determine the LD50 endpoint from the graph by reading from where the 50 percent point intercepts the Dose Response Curve to the concentration along the x-axis. That concentration is the LD50 value. (Figure 6)

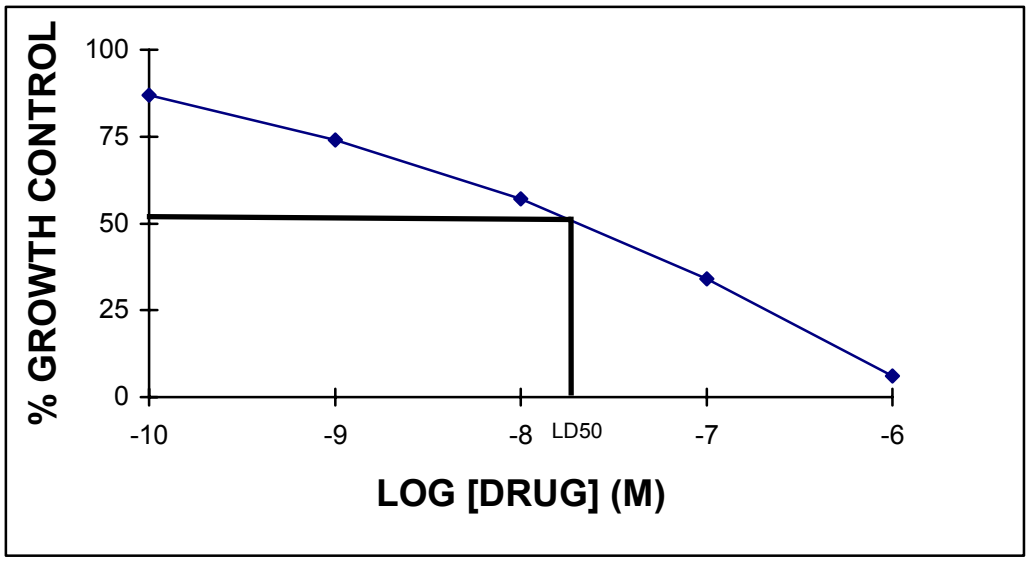


Figure 6. Determination of Doxorubicin LD50 Using alamarBlue®

Applications

Current users of MTT, XTT or neutral red uptake in proliferation/cytotoxicity assays can substitute alamarBlue® for each of these tests (3,7). This substitution can be done at the time point when you would normally add MTT, XTT or neutral red.

Figures 7 – 9 are examples from comparison tests with each of these methods that have been conducted at Alamar Biosciences, Inc.

alamarBlue® has also been shown to be a rapid and simple non-radioactive assay alternative to the [^{3}H] thymidine incorporation assay (1).

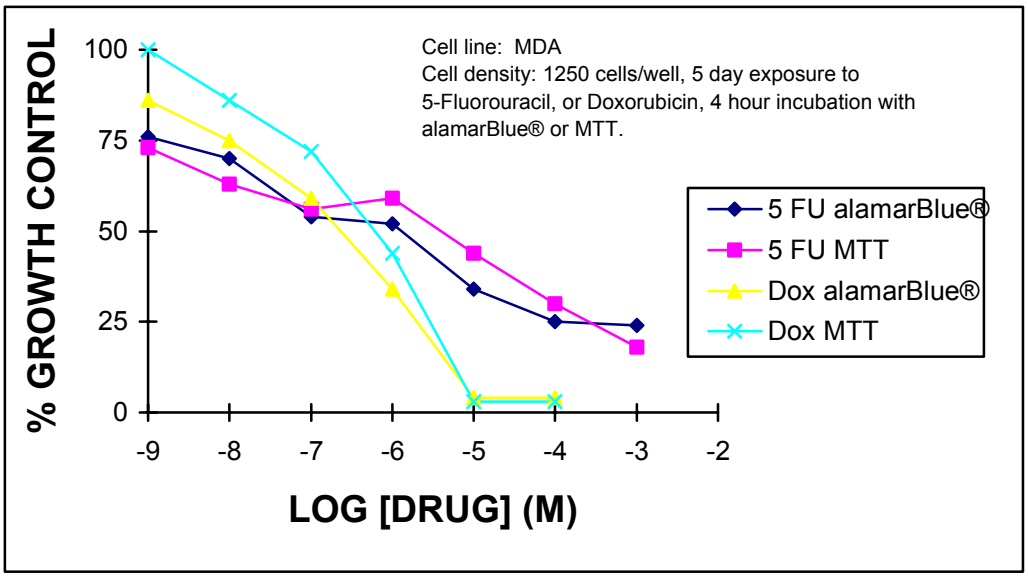


Figure 7. Comparison of LD50 Using alamarBlue® and MTT

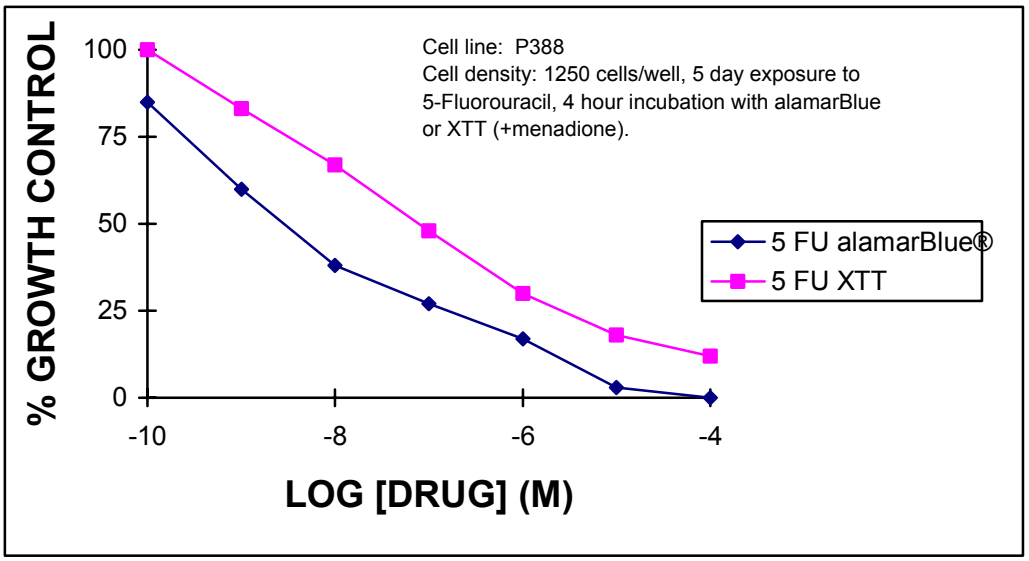


Figure 8. Comparison of LD50 Using alamarBlue and XTT LD50 for alamarBlue® derived from fluorescence measurements.

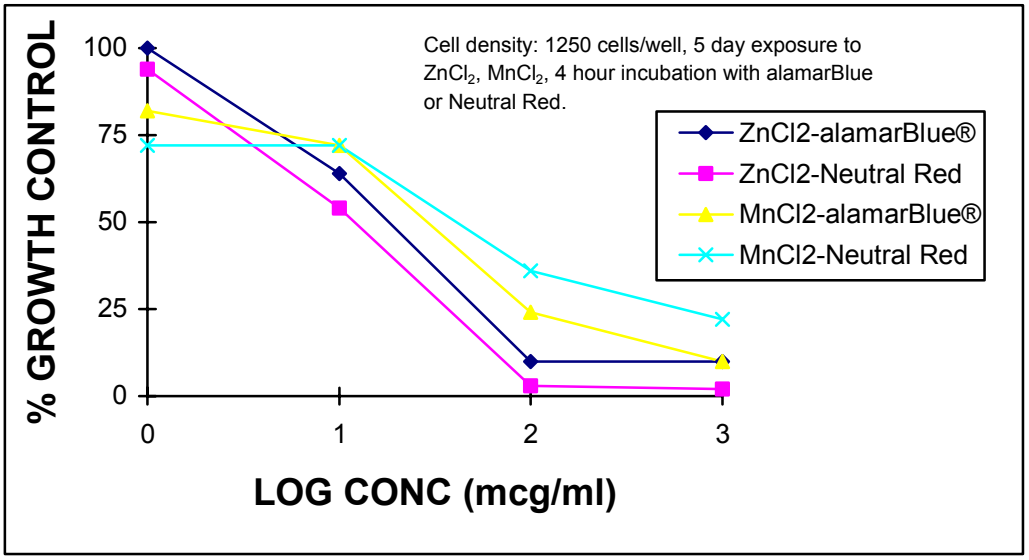


Figure 9. Comparison of LD50 Using alamarBlue® and Neutral Red for Abrasives Applied to Human Epidermal Keratinocytes LD50 for alamarBlue is derived from fluorescence measurements.

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Ordering Information

To place an order or for more information, please call 800-955-6288.

alamarBlue can be ordered in the following sizes:

Product Number	Description
DAL1010	alamarBlue (1X) 10 ml bottle
DAL1025	alamarBlue (1X) 25 ml bottle
DAL1100	alamarBlue (1X) 100 ml bottle