alamarBlue® Frequently Asked Questions

1. Which cells can I use for alamarBlue® tests?
   alamarBlue® has been successfully used with a wide range of cells including bacteria, yeast, fungi, protozoa and cultured mammalian cells. To date, it has been suitable for use with every cell type tested.

2. Will phenol red affect the measurements for alamarBlue®?
   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 1).

Media	Media absorbance value	Absorbance value for alamarBlue® at various levels of reduction					
		0% reduced	10% reduced	30% reduced	60% reduced	90% reduced	100% reduced
RPMI 1640 without phenol red	0.032	0.47	0.52	0.61	0.73	0.85	0.88
RPMI 1640 with phenol red	0.061	0.53	0.54	0.64	0.76	0.88	0.91

Table 1: Effect of phenol red on absorbance values at 570 nm. Absorbance value for various levels of reduction of alamarBlue® in RPMI 1640 pH 7.0, with MOPS (both with and without phenol red) when using Dynatech flat bottom plates and 100µl per well.

3. Which media can be used with alamarBlue®?
   Most commonly used media are suitable for use with alamarBlue® assays.
   Since the media is used in the blank, and also any other positive or negative control plates, any effects of the media on absorbance or fluorescence should be minimal.
   Refer to Frequently Asked Question 15 for example data for oxidized and reduced forms of alamarBlue® using a range of different media.

4. Will alamarBlue® be toxic to my cells?
   alamarBlue® has undergone extensive testing, and is non toxic to all cell types tested.
   It has also been shown to have neither a positive nor negative effect on cell growth.
   Unlike other cell proliferation redox indicators, alamarBlue® does not interfere with the electron transport chain, and does not affect cell respiration or function.

5. Will microbial contaminants or carrier protein interfere with alamarBlue® measurements?
   alamarBlue® will be reduced by microbial contaminants. Therefore, results from contaminated cultures tested by this method will not be accurate.
   In-house studies indicated samples with protein concentrations equivalent to 10% fetal bovine serum (FBS) did not interfere with the assay. However, Page *et al.* (1993) reported that serum may cause some quenching of fluorescence and recommended using the same serum concentration in controls to take this into account. Goegan *et al.* (1995) tested the effects of varying concentrations of FBS, bovine serum albumin (BSA), and polyvinylpyrrolidone on the alamarBlue® assay. Results showed that increasing concentrations of FBS and BSA did affect the assay. A method and calculation was developed to analyze the test matrix for effects due to these two compounds; allowing the correction of such effects.
   This calculation can then be applied to determine the consequence of any additive test media matrix, including the test chemicals themselves.

6. Why is it necessary to optimize the cell density used for studies with alamarBlue®?
   alamarBlue® measures cell proliferation most accurately when the cells are in the exponential growth phase. If the cell density is too high, cell proliferation will decrease, giving less reduction of the alamarBlue® than would have been expected. At low cell density, the slower growth rate could result in insignificant alamarBlue® reduction. A cell density of 1 x 10e4 cells/ml is generally recommended, which often corresponds to cells being in the exponential phase. However, since cells vary in their proliferation rate, it is impossible to recommend a cell density which is suitable for all experiments. Instead we recommend that customers perform a control experiment to determine the optimum cell density for their studies. It is equally important to optimize the incubation time for alamarBlue®. Please refer to Worked examples for the general method to determine the optimum cell density and incubation time.

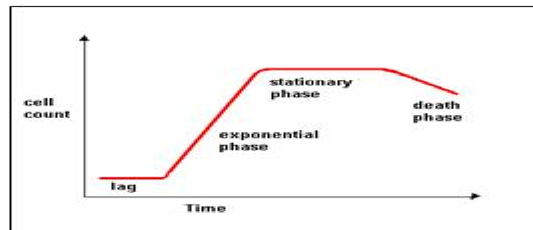


Figure 1: A standard bacterial growth curve.

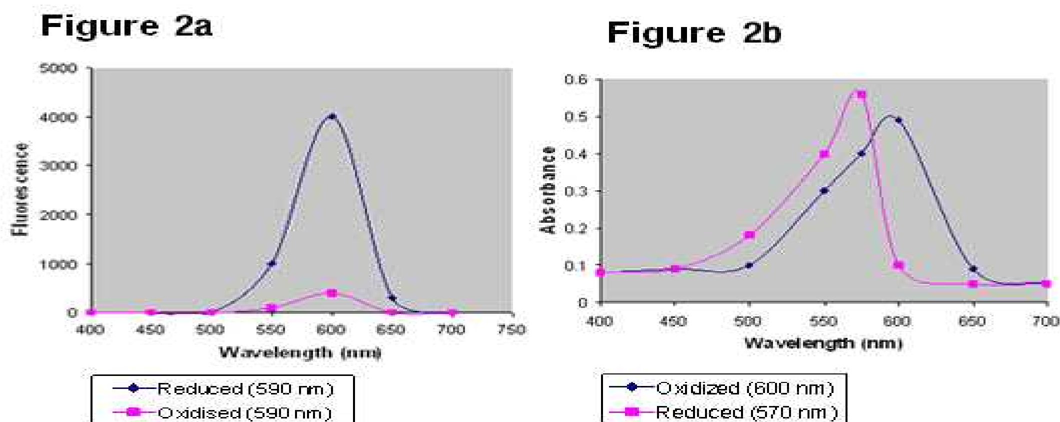
7. There seems to be a decrease in the reduction of alamarBlue® if I use a long incubation time?
   alamarBlue® contains proprietary buffering agents, which maintain the equilibrium between the oxidized and reduced forms of the indicator. If alamarBlue® is used for extended incubation periods, there is a possibility that the buffering agents will reach their maximum buffering efficiency. As this point, the equilibrium between the oxidized and reduced forms of alamarBlue® is disrupted, and a colorless form of the dye may be obtained. This could result in an apparent bleaching of the solution, and a decrease in the amount of the reduced (red) form of alamarBlue®.
   If a decrease in reduction of the solution is observed at a certain point, or if any apparent bleaching is observed, we recommend that the incubation time is shortened. Please refer to Worked example for details for control experiments to optimize incubation time for alamarBlue®.
   Alternatively, if the length of the experiment is longer than the optimal alamarBlue® incubation time, we suggest that an endpoint test is used. This type of experiment is particularly useful for cell proliferation studies over days, weeks and months. For more information, please refer to Frequently Asked Question 8 below.

8. Can I use alamarBlue® to repeatedly measure cell proliferation at intervals of days or weeks?
   alamarBlue® can be used for long term cell proliferation studies where measurements are taken repeatedly. For these types of study we would recommend that aliquots of the cell medium/ suspension are taken at each time point for incubation with alamarBlue® prior to an endpoint test. Please refer to Breinholt, D. *et al*. (1998) for an example of this type of experiment.

9. Is it possible to use different filters than 570 nm and 600 nm for measuring cell growth by absorbance?
   In the general method section of the datasheet, the extinction coefficients are given for filters at 540 nm, 570 nm, 600 nm and 630 nm.
   Rather than deriving molecular extinction coefficients for other filters, which may be subject to error, a simple and accurate method has been devised, to calculate the amount of reduced alamarBlue® present, for any filter combination. (Personal communication of Dr. Geier and in independently derived calculations by Goegan *et. al*. (1995) . For more information, please refer to Example calculations for using different filters in the Methods and Calculations section.

10. Is it better to measure cell cytotoxicity using absorbance or fluorescence?

alamarBlue® reduction is regularly measured using absorbance, which gives good levels of accuracy for most experiments, and is particularly easy to use.
It is generally the case, however, that more sensitive readings can be obtained when fluorescence is used, especially when attempting to measure very small changes in reduction. This is principally because there is little interference from unreduced alamarBlue® on the spectra of reduced alamarBlue® when measured by fluorescence (Figure 2a). When measuring absorption there can be considerable overlap of the oxidized and reduced forms of alamarBlue® (Figure 2b).



Figures 2a &2b: alamarBlue® fluorescence emission spectra and absorbance readings

11. Can I store my plates for measurement at a later date?

If it is not possible to read plates on the day an experiment is performed, plates may be refrigerated and read within 1-3 days. Plates should be wrapped in foil or plastic wrap to prevent evaporation and stored in the dark.
Please refer to Appendix 4 in the technical datasheet for example absorbance data for alamarBlue® plates where readings were taken up to 3 days after the end of incubation.
N.B. If plates are refrigerated and fluorescence measurements are being used, keep in mind that fluorescence measurements are influenced by temperature. If measurements are normally taken at 37°C, then plates should be warmed to that temperature before reading.

12. Will temperature affect the fluorescence readings?

Fluorescence measurements are influenced by temperature. It is recommended that the temperature used for fluorescence readings remains constant, and that if plates are refrigerated they are warmed to that temperature before reading.

Temperature	Fluorescence
37°C	(171.1)
	5216
22°C	(157.6)
	5887
4°C	(192.1)
	6881

Table 2: Effect of temperature on fluorescence. Standard deviations are in parentheses (calculated for n=8). RPMI 1640 with MOPS pH7.0, no phenol red at 100ul per well, using Dynatech flat bottom plate.

13. Can alamarBlue® be used for assays to measure lymphocyte proliferation?

Recent studies have shown that alamarBlue® may be used successfully to measure lymphocyte proliferation, although one study by De Fries, R. *et al.* (1995) has suggested that alamarBlue® may not give such as sensitive reading as the traditional 3H-Thymidine incorporation assays .

14. How can I determine the absorbance or fluorescence for the fully reduced form of alamarBlue®?

The reduced form 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 for a articular 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 before taking the appropriate measurements.

15. What are example absorbance and fluorescence values for the oxidized and reduced forms of alamarBlue®?

Example absorbance values are given in Table 3a and example fluorescent values are give in Table 3b. Since these readings can vary depending on the type of media used, a range of powered media was used. Powdered media was obtained from Sigma and prepared according to their instructions. All media contained phenol red. pH was adjusted to 7.4 with 1N HCI 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 media were dispensed into a flat bottom Dynatech plate (100µl/well)

Powdered Media	Sigma Product code	Wavelength (nm)							
		540		570		600		630	
		OX	RED	OX	RED	OX	RED	OX	RED
BME EBSS	B9638	0.61	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	0.52	1.133	0.74	1.421	0.756	0.25	0.236	0.183
MEM EBSS	M0268	0.582	1.186	0.819	1.483	0.82	0.235	0.252	0.168
MEM HBSS	M4642	0.48	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	1.25	0.795	0.161	0.248	0.101

Table 3a : Absorbance values for oxidized/reduced forms of alamarBlue® for commonly used culture media at different wavelengths.

Powdered Media	Sigma Product Code	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

Table 3b: Fluorescence values for oxidized/reduced forms of alamarBlue® for commonly used culture media. Measurements were taken on a Cambridge Technology, Inc. (Watertown, MA) Model 7620 Microplate. Fluorometer-settings were: bottom reading, light source setting 12, no max AFU; Excitation: 530 nm; Emission: 580 nm, gain/16.