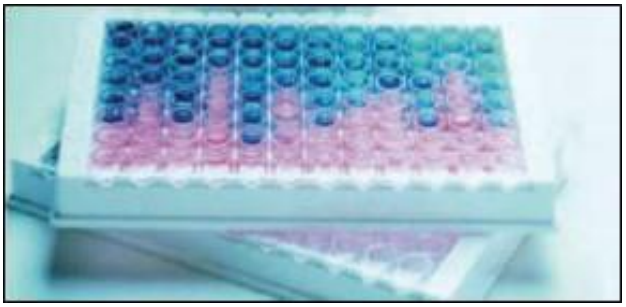
alamarBlue

Technical Datasheet



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alamarBlue

Volume / Quantity: BUF012A – 25 ml
BUF012B – 100 ml

Product Form: Liquid

Preservatives / Stabilizers: None present

Product Description:
alamarBlue is an indicator dye which incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to the chemical reduction of growth medium, resulting from cell growth. The alamarBlue assay is designed to quantitatively measure the proliferation of various human and animal cell lines, bacteria and fungi.

Indications for Use:

- The bioassay can be used to establish proliferation or relative cytotoxicity.
- Baseline data for predicting the toxicity of related novel agents can be compared to baseline data with known *in-vivo* toxicity.
- alamarBlue is for use between pH6.8 and pH7.4.

Product Principle:

- Growing cells cause a chemical reduction of alamarBlue.
- Continued growth maintains a reduced environment. (fluorescent, red)
- Inhibition of growth maintains an oxidized environment. (non-fluorescent, blue)
- Data may be collected using either fluorescence-based or absorbance-based instrumentation.
- Fluorescence is monitored at 530-560nm excitation wavelength and 590nm emission wavelength.
- Absorbance is monitored at 570nm and 600nm.

Shelf life: See datasheet.

Storage and Stability: See datasheet.

This product is light sensitive and should be stored in the dark

Health and Safety information:
The REDOX indicator in alamarBlue has no current or past indication of carcinogenic capacity.
(A full Health and Safety assessment is available upon request)

Manufactured for Bio-Rad by Trek Diagnostic System. U.S. patent 5,501,959.

Please visit bio-rad-antibodies.com/alamarBlue for more:

- **Calculate results online**
- **Example calculations**
- **References**
- **Frequently asked questions**

QC test for alamarBlue

Materials and Equipment:

- 0.1M potassium phosphate buffer, pH7.4
- 10ml test tube
- Pipette capable of accurately dispensing 0.4ml
- Plate reader with the filters 570nm and 600nm. (Alternative filters can be used – see bottom of page).
- Dynatech flat bottom plate

Procedure:

1. Shake alamarBlue to mix before use
2. Pipette 0.4ml of alamarBlue into a test tube
3. Dilute to 10ml with phosphate buffer
4. Mix well
5. Pipette 100µl into each well of a clear, flat bottom microplate
6. Read absorbance at appropriate wavelengths

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)

Expected results for Quality Control (QC) test – using Dynatech flat bottomed plates.

N.B. Absorbance values may be affected by the type of plate (whether round or flat bottom) and the plate manufacturer (See Appendix 4 for further information about the effect of plates on absorbance).

No QC protocol is recommended for fluorescence since fluorescence units are arbitrary and the scale used varies widely from one instrument to another.

For an indication of the absorbance and fluorescence values for the fully oxidized and reduced forms of alamarBlue with different media, please visit the Frequently Asked Questions section found at: bio-rad-antibodies.com/alamarBlue.

Alternative wavelengths

Although it is preferable to use the recommended wavelengths, a correction factor can be calculated allowing the use of one alternative filter (page 8).

Recommended	Alternative
570	540
600	630

570nm and 600nm are the recommended wavelengths; only one wavelength may be altered for its respective alternative wavelength.

Method and calculations for alamarBlue

Method for determining optimum length of incubation and plating density.

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, above and below the cell density expected to be used.
2. Aseptically add alamarBlue in an amount equal to 10% of the volume in the well.
3. Return plates 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 be made the following day at 24 hours. Two kinds of information can be obtained from this data:
   (i) for any given incubation time selected, the range in cell density relating cell number to alamarBlue reduction can be determined by a linear response;
   (ii) for any given cell density selected, the incubation time can be determined as the time taken for the control cells to 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 an excitation wavelength at 530-560nm and emission wavelength at 590nm.
5. Calculate the percentage reduction of alamarBlue at each cell density or incubation period.

   a. Equation 1 calculates percentage reduction of alamarBlue using absorbance

Percentage reduction of alamarBlue ***(equation 1)***

$$= \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where: O1 = molar extinction coefficient (E) of oxidized alamarBlue (Blue) at 570nm*
O2= E of oxidized alamarBlue at 600nm*
R1 = E of reduced alamarBlue (Red) at 570nm
R2= E of reduced alamarBlue at 600nm
A1 = absorbance of test wells at 570nm
A2 = absorbance of test wells at 600nm
N1 = absorbance of negative control well (media plus alamarBlue but no cells) at 570nm

N2 = absorbance of negative control well (media plus alamarBlue but no cells) at 600nm
* Only one appropriate substitute wavelength may be used.

Wavelength	Reduced (R)	Oxidized (O)
540nm	104395	47619
570nm	155677	80586
600nm	14652	117216
630nm	5494	34798

Molar extinction coefficients for alamarBlue at different wavelengths

b) To calculate percentage reduction of alamarBlue using fluorescence, use equation 2

$$\text{Percentage reduction of alamarBlue (}\textit{equation 2}\text{)} = \frac{\text{Fl 590 of test agent} - \text{Fl 590 untreated control}}{\text{Fl 590 of 100\% reduced alamarBlue}^{TM} - \text{Fl 590 untreated control}} \times 100$$

Where: Fl 590 = Fluorescent Intensity at 590nm emission (560nm excitation).

6. Plot a graph of percentage reduction of alamarBlue at each cell density or incubation period.
   a) For experiments to determine optimum cell density, plot log of cell density on the x axis, and percentage reduction on the y axis;
   b) For experiments to determine the optimum incubation time, plot the number of hours on the x axis, and the percentage reduction on the y axis.
   c) Use the graphs to determine optimum cell density or incubation period.
   (Example data is given in appendix 1)

General method for measuring cytotoxicity or proliferation using alamarBlue

1. Harvest cells which are in the log phase of growth and determine cell count. Adjust the cell count to $1x10^4$ cells/ml (suggested cell density). The optimum cell density may vary between cell types.
2. Plate cells and expose to test agent as determined by researcher.
   For determining the effect of a test agent on cell growth, ensure correct controls are included e.g. stimulated vs. unstimulated cells.
3. Mix by shaking, then aseptically add alamarBlue in an amount equal to 10% of the volume in the well.
4. Incubate cultures with alamarBlue for 4 - 8 hours. N.B. The optimum incubation time may vary between cell types.
5. Measure cytotoxicity or proliferation using spectrophotometry of fluorescence.

Method for measuring cytotoxicity or proliferation using alamarBlue by spectrophotometry.

6. Measure absorbance at wavelengths of 570nm and 600nm after required incubation. Use a blank of media only.

To calculate the percent difference in reduction between treated and control cells in cytotoxicity and proliferation assays (Equation 3):

$$\text{Percentage difference between treated and control cells (}\textit{equation 3}\text{)} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

Where: O1 = molar extinction coefficient (E) of oxidized alamarBlue (Blue) at 570nm*
O2= E of oxidized alamarBlue at 600nm*
A1 = absorbance of test wells at 570nm
A2 = absorbance of test wells at 600nm
P1 = absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 570nm
P2 = absorbance of positive growth control well (cells plus alamarBlue but no test agent) at 600nm
* Only one appropriate substitute wavelength may be used.

Wavelength	Reduced (R)	Oxidized (O)
540nm	104395	47619
570nm	155677	80586
600nm	14652	117216
630nm	5494	34798

Molar extinction coefficients for alamarBlue at different wavelengths

Alternatively, it may be useful to calculate the percent reduction of alamarBlue (Equation 1):

$$\text{Percentage reduction of alamarBlue } \textit{(equation 1)} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \times 100$$

Where: O1 = molar extinction coefficient (E) of oxidized alamarBlue (Blue) at 570nm*
O2= E of oxidized alamarBlue at 600nm*
R1 = E of reduced alamarBlue (Red) at 570nm
R2= E of reduced alamarBlue at 600nm
A1 = absorbance of test wells at 570nm
A2 = absorbance of test wells at 600nm
N1 = absorbance of negative control well (media plus alamarBlue but no cells) at 570nm
N2 = absorbance of negative control well (media plus alamarBlue but no cells) at 600nm
* Only one appropriate substitute wavelength may be used.

For an Excel calculator and example calculations, please visit bio-rad-antibodies.com/alamarBlue.

General method for measuring cytotoxicity or proliferation using alamarBlue by fluorescence.

6. Read fluorescence at excitation 560nm, emission 590nm.

To calculate percent difference in reduction between treated and control cells in cytotoxicity/ proliferation assays use the following formula (Equation 4):

Percentage difference between treated and control cells (*equation 4)*	**=**	**FI 590 of test agent** / **FI 590 of untreated control**	**x 100**

Where: FI 590 = Fluorescent Intensity at 590nm emission (560nm excitation).

If required, equation 2 can be used to find the percentage reduction of alamarBlue using fluorescence:

Percentage reduction of alamarBlue (*equation 2)*	**=**	**FI 590 of test agent – FI 590 untreated control** / **FI 590 of 100% reduced alamarBlue™ – FI 590 untreated control**	**x 100**

Where: FI 590 = Fluorescent Intensity at 590nm emission (560nm excitation).

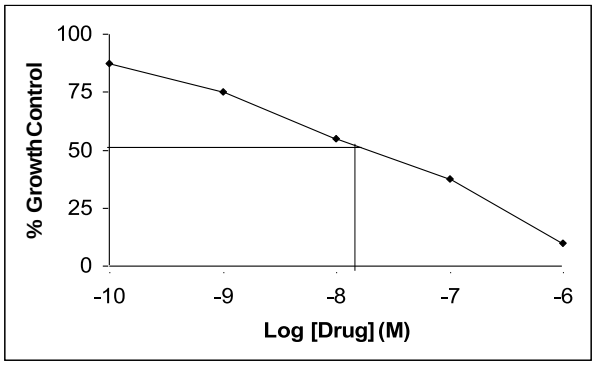
For equation 2, it is necessary to include the fluorescence value for alamarBlue in its fully reduced form. To produce the 100% reduced form of alamarBlue, simply autoclave a sample containing media and alamarBlue for 15 minutes. Since fluorescence units are arbitrary and may therefore vary depending upon instrument set up, it is important that users determine the fluorescence reading for 100% reduction using the same media and instrument as for their samples.

Worked calculations and Frequently Asked Question at bio-rad-antibodies.com/alamarBlue.

Method to determine LD50 using alamarBlue.

Use semi-log graph paper to plot the percent of untreated control (using equation 5) for each dilution of a given test agent on the y-axis vs. the concentration of the test agent on the x-axis.

To determine the LD50 endpoint from the graph, read from where the 50 percent point intercepts the Dose Response Curve to the concentration along the x-axis. This concentration is the LD50 value.



Determination of Doxorubicin LD50 using alamarBlue

Calculations for using alamarBlue in Spectrophotometry with different filters.

1. Make up alamarBlue (AB) as directed in the package insert (1/10 dilution in 100µl media).
2. Measure the absorbance of alamarBlue in media at the lower wavelength (LW) filter and at the higher wavelength (HW) filter.
3. Measure the absorbance of 100ul media only (blank) at the two wavelengths.
4. Subtract the absorbance values of media only from the absorbance values of alamarBlue in media.

AO_{LW} = Absorbance of AB in media – Absorbance of media only

AO_{HW} = Absorbance of AB in media – Absorbance of media only

Where: AO_{LW} = absorbance of oxidized form at the lower wavelength
AO_{HW} = absorbance of oxidized form at the higher wavelength

5. Calculate correction factor (R_0):

Correction factor for different filters:

$$R_0 = AO_{LW}/AO_{HW}$$

6. Calculate percentage difference in reduction between treated and control cells in cytotoxicity/proliferation assays:

Percentage difference in reduction $= \frac{A_{LW} - (A_{HW} \times R_0) \text{ for test well}}{A_{LW} - (A_{HW} \times R_0) \text{ for control well}} \times 100$

(Equation 5)

Where: A_{LW} = absorbance at lower wavelength minus the media blank
A_{HW} = absorbance at higher wavelength minus the media blank
R_0 = Correction factor (calculated in step 5)

7. If the alternative calculation to find the percent reduction of alamarBlue is required, use the following equation:

Percentage reduction of alamarBlue $= [\, A_{LW} - (A_{HW} \times R_0) \,] \times 100$

(Equation 6)

alamarBlue Appendix

Appendix 1: Worked example to determine optimum cell density and incubation time for A549 cells, using alamarBlue and spectrophotometry.

The cell line A549 is a monolayer culture. The method given on page 4 was followed to find the percentage reduction of alamarBlue after incubation with A549 cells at different densities and for different incubation periods.

Absorbance values at 570nm and 600nm after blanking with media only for A549 cells.

Time (hours)	Absorbance values at 570nm					Absorbance values at 600nm				
	Blue In Media	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml	Blue In Media	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml
0	0.336	0.336	0.338	0.348	0.372	0.441	0.439	0.443	0.459	0.496
2	0.334	0.339	0.352	0.432	0.540	0.440	0.425	0.421	0.349	0.267
4	0.333	0.346	0.365	0.489	0.590	0.432	0.411	0.397	0.265	0.162
5.5	0.321	0.344	0.366	0.511	0.573	0.424	0.397	0.377	0.211	0.135
20	0.322	0.438	0.510	0.518	0.486	0.412	0.271	0.180	0.102	0.112

Using absorbance data from the sample data set, percent reduction of alamarBlue was calculated using equation 1:

Percentage reduction of alamarBlue after incubation with A549 cells at different cell densities and incubation periods.

Time (hours)	Percentage reduction of alamarBlue			
	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml
0	6.3	6.1	6.0	5.7
2	8.6	11.5	35.4	65.7
4	11.9	17.3	57.7	89.9
5.5	13.6	20.4	70.0	91.8
20	49.6	76.2	88.3	80.7

These values are plotted to produce Figures 1a & 1b.

Figure 1a

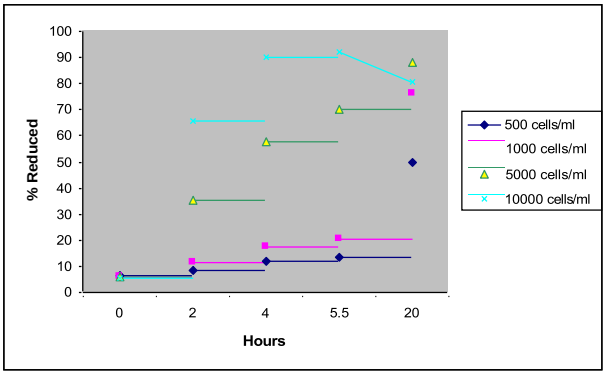
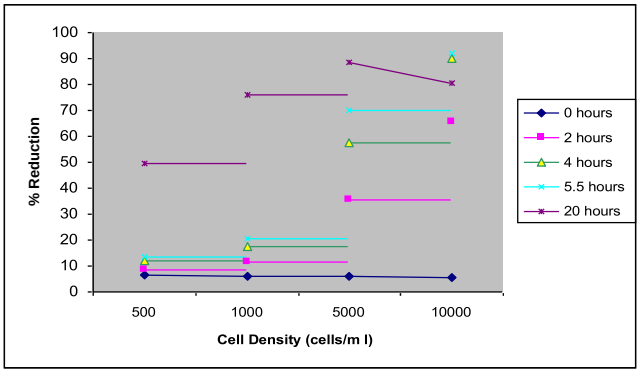


Figure 1b



Graphs showing percentage of alamarBlue reduction for A549 cells with different incubation periods (Figure 1a) and at different initial cell densities (Figure 1b).

Please refer to the Worked Example section of bio-rad-antibodies.com/alamarBlue for notes to accompany these graphs.

Appendix 2: Worked example to determine optimum cell density and incubation time for P388 cells, using alamarBlue and spectrophotometry.

The cell line P388 is a suspension cell line. The method given on page 4 was followed to find the percentage reduction of alamarBlue after incubation with P388 cells at different densities and for different incubation periods.

Absorbance values at 570nm and 600nm after blanking with media only for P388 cells.

Time (hours)	Absorbance values at 570nm					Absorbance values at 600nm				
	Blue In Media	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml	Blue In Media	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml
0	0.336	0.342	0.334	0.332	0.328	0.441	0.451	0.438	0.433	0.422
2	0.334	0.340	0.333	0.335	0.335	0.440	0.448	0.435	0.422	0.404
4	0.333	0.339	0.332	0.339	0.346	0.432	0.444	0.432	0.414	0.391
5.5	0.321	0.331	0.325	0.335	0.344	0.424	0.435	0.424	0.404	0.377
20	0.322	0.332	0.328	0.381	0.434	0.412	0.423	0.415	0.337	0.253

Using absorbance data from the sample data set, percent reduction of alamarBlue was calculated using equation 1:

Percentage reduction of alamarBlue for P388 cells at a range of cell densities following different incubation periods

Time (hours)	Percentage reduction of alamarBlue			
	500 cells/ml	1000 cells/ml	5000 cells/ml	10000 cells/ml
0	5.9	6.0	6.3	7.0
2	5.9	6.3	8.3	10.6
4	6.3	6.6	10.2	14.5
5.5	6.1	6.4	10.9	16.2
20	8.1	8.4	29.5	51.3

These values are plotted to produce Figures 2a & 2b.

Figure 2a

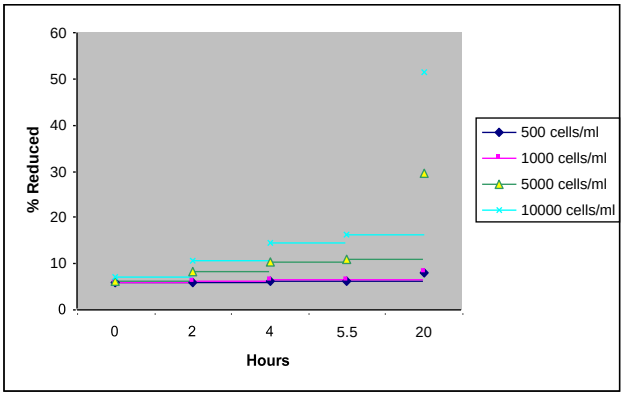
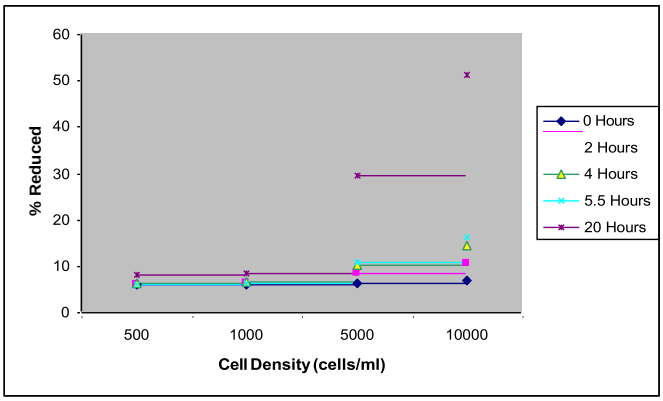


Figure 2b



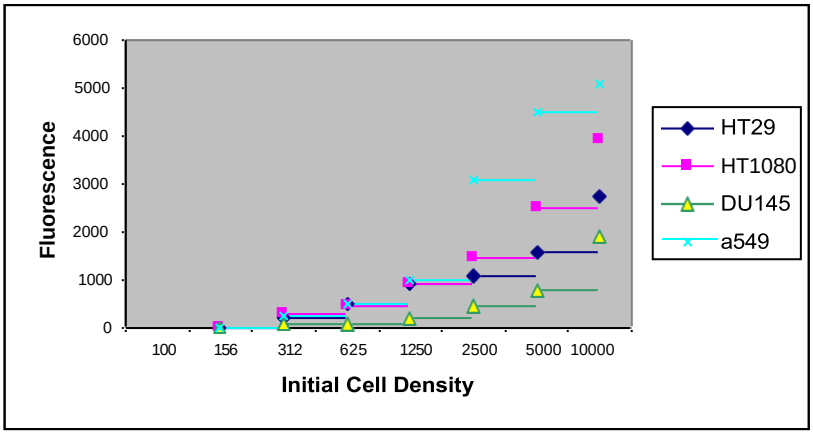
Graphs showing percentage of alamarBlue reduction for P388 cells with different incubation periods (Figure 2a) and at different initial cell densities (Figure 2b).

Please refer to the Worked Example section of bio-rad-antibodies.com/alamarBlue for notes to accompany these graphs.

Appendix 3: Example alamarBlue reduction curves for four cell lines using fluorescence.

Examples of reduction curves are included to demonstrate the usefulness of the alamarBlue assay for measuring cell proliferation.

Detection of cell growth of four cell lines using alamarBlue.



Appendix 4: Effect of storage plates on spectrophotometric readings.

The absorbance readings of alamarBlue may be affected by the type of plates which are used for experiments. A series of absorbance readings were taken for the oxidized and reduced forms of alamarBlue using a range of different types of plates. Following the readings on day 1, plates were covered in foil and refrigerated prior to further readings on days 2 & 3. See Frequently Asked Question 11 at bio-rad-antibodies.com/alamarBlue for more information about storing plates prior to collecting data.

Plate Type	Day	Absorbance							
		BLUE (Oxidized)				RED (Reduced)			
		540nm	570nm	600nm	630nm	540nm	570nm	600nm	630nm
Dynatech Flat Bottom	Day 1	(0.003) 0.298	(0.005) 0.496	(0.007) 0.708	(0.003) 0.236	(0.020) 0.693	(0.027) 1.017	(0.002) 0.126	(0.000) 0.075
	Day 2	(0.003) 0.294	(0.004) 0.484	(0.006) 0.692	(0.002) 0.227	(0.020) 0.697	(0.027) 1.018	(0.008) 0.164	(0.009) 0.118
	Day 3	(0.003) 0.296	(0.006) 0.486	(0.008) 0.691	(0.003) 0.231	(0.010) 0.734	(0.024) 1.038	(0.008) 0.199	(0.009) 0.149
Corning Flat Bottom	Day 1	(0.002) 0.210	(0.004) 0.335	(0.005) 0.474	(0.002) 0.169	(0.020) 0.530	(0.024) 0.772	(0.003) 0.137	(0.004) 0.105
	Day 2	(0.002) 0.210	(0.003) 0.329	(0.004) 0.458	(0.001) 0.161	(0.020) 0.580	(0.027) 0.822	(0.004) 0.193	(0.004) 0.159
	Day 3	(0.002) 0.200	(0.003) 0.322	(0.005) 0.444	(0.002) 0.160	(0.020) 0.600	(0.035) 0.823	(0.004) 0.210	(0.003) 0.172
Corning Round Bottom	Day 1	(0.001) 0.380	(0.002) 0.635	(0.002) 0.913	(0.001) 0.300	(0.014) 0.870	(0.018) 1.266	(0.002) 0.151	(0.000) 0.084
	Day 2	(0.002) 0.390	(0.003) 0.641	(0.004) 0.914	(0.002) 0.295	(0.011) 0.850	(0.014) 1.241	(0.002) 0.146	(0.002) 0.083
	Day 3	(0.004) 0.390	(0.006) 0.646	(0.008) 0.916	(0.004) 0.302	(0.017) 0.860	(0.021) 1.237	(0.007) 0.159	(0.006) 0.094

Absorbance of alamarBlue oxidized and reduced forms for a range of plates, including those refrigerated prior to data collection. 100ul of RPMI 1640 with MOPS pH7.0, no phenol red. Standard deviations are in parentheses (calculated for n=8).