Alamar Blue Assay

Alamar blue is a reagent and accompanying assay used to quantify cellular metabolic activity. Taken at a single time point, it can be used to determine the concentration of viable cells in a sample. Taken at two or more time points of the same or similar samples, the assay can be used to measure cell growth kinetics – the growth of a cell population over time.

The reagent solution contains the non-fluorescent blue-colored molecule resazurin, which, when chemically reduced (a metabolic activity of cells), turns into the highly fluorescent red-colored resorufin. Both the color change and the change in fluorescence allow for the direct measurement of cell metabolic activity (and thus viable cell concentration) via absorbance or fluorescence measurements in a plate reader.


For fluorescence measurements, a direct linear correlation between fluorescence and viable cell concentration can be created through the fluorescence measurement of samples of known cell concentration. The equation of the line created can then be applied to the fluoresences of samples of unknown concentrations.

Because of variability between measurements taken on different days, each set of samples tested requires its own equation to be determined. Because cell growth on each fiber is not expected to exceed 100,000 cells, samples of 20, 50, and 100 thousand cells are used to create the equations.

- **Sterilize** the hood working area.
- Gather the following materials:
  - Cell culture dish containing cells at approximately 60% confluence or higher
  - Alamar blue reagent
    - Located at 4 °C in refrigerator 5, bottom shelf, S308
  - Media container and any intermediate media transfer container
  - Sterile PBS container
  - Trypsin container
  - Electric pipette holder
  - 10 mL pipettes (should already be in hood)
  - Ultra-sterilized Pasteur pipettes (should already be in hood)
  - Hemocytometer and cell counter
  - P20 and tips
  - P200 and tips
Prepare wells of known cell concentration to create the standard curve

- In a six well plate, reserve three wells for known cell concentrations of 20, 50, and 100 thousand cells.
  - Lift, count, and seed cells according to the protocol “Cell Splitting and Counting”
  - To calculate the volume of resuspended cell solution to add to each well, use the following formula:
    \[
    \text{volume of cells to add} = \frac{\text{seeding density [example, 20,000 cells]}}{\# \text{ of cells/mL}}
    \]
    Where “\# of cells/mL” is the number of cells counted from the starting plate IF the cells were resuspended in 1 mL after being spun down. If the resuspension volume was more than 1 mL, divide the number of cells counted by the volume in mLs.
    - For example, if 124 cells were counted from a 3 mL sample, divide 124x10^4 cells by 3. This number is how many cells/mL are present in your sample.

- Add 2 mL of media to each of the three wells.
- Add 200 µL of the Alamar blue reagent to each well.
- Gently swirl the plate to ensure adequate distribution of the reagent.
- Incubate for 5.5 to 6 hours.
- Cells left over may be seeded back into cell culture plates for subsequent use.

Clean-up during incubation

- Ensure that the following have been put away:
  - Alamar blue reagent bottle (4 °C in refrigerator 5, bottom shelf, S308)
  - Media bottle (refrigerator)
  - PBS bottle (cabinet above centrifuge)
  - Trypsin tube (refrigerator)
  - Bag of sterile plates (drawer)
  - Pipettes P1000, P200, P20 (rack beside hood)
  - Electric pipette holder (plugged in or returned to rack on center counter)
  - Cell counter (drawer to right of microscope)

- Ensure that the following have been sterilized and disposed of into the autoclave bin and any leftover media/cells or other fluid has been aspirated:
  - Old plates/wells
  - 15 mL Centrifuge tube

- The following can be disposed of into the autoclave bin without the need for sterilization:
- Pasteur pipettes (into the sharps jar)
- Pipette tips
- Glass pipettes

- **Sterilize and clean** the following:
  - Hemocytometer (drawer to right of microscope)
    - Spray actual hemocytometer and coverslide liberally with ethanol; carefully wipe each dry with a Kimwipe before replacing into the box.
  - Aspiration tube
    - Remove and dispose of Pasteur pipette in autoclave bin. With vacuum still on, place end of hose onto the hole of an ethanol squeeze bottle, allowing the ethanol to pump through the tubes connecting to the vacuum. Make sure that the tube under the hood is clear of any “pink” fluid before turning off the vacuum.

- **Sterilize** the surface of the hood again and organize objects inside so it looks neat.
- Ensure that the following are **turned off**:
  - Vacuum under hood
  - Microscope
  - Water bath
  - Hood fluorescent light

- **DO NOT close the hood window or turn off the blower.**

### Prepare the fibers for Alamar blue analysis
- Using sterile, dry tweezers, move each fiber into a new 24 well plate.
  - This is done to ensure that cells adhered to the plastic well plate are not included in the assay
- Add 500 µL of media to each well. In addition, add 500 µL of media to an empty well, to be used as a negative control.
- Add 50 µL of Alamar blue reagent to each well (including the control).
- Gently swirl the plate to ensure adequate distribution of the reagent.
- Incubate for 5.5 to 6 hours.

### Create and read plates for analysis
- Turn on the plate reader and computer in S305.
- Draw out a picture of the 96 well plate to determine where samples will be located.
  These include the unknown fiber samples and the positive and negative controls.
- Remove 150 µL from each sample per well. Ensure that you use at least two duplicates.
- Keep plate covered in tin foil before being read.
- Take the plate to S305 for reading. When placing the plate in the drawer, **DO NOT use the purple plate adapter.**
- On the computer, open SoftMax Pro 5.4.4 from the desktop.
- Under “Protocols” mouse over “LTU protocols_u” and select “Alomar_blue_assay.”
- In “Untitled 1,” click the “template” button.
• Assign spaces as knowns, unknowns, and controls.
• Click the “settings” button.
  o Change “Read mode” to fluorescence; make sure the “read from bottom” is checked.
  o Change the number of wavelengths to 1 at excitation 545 nm and emission at 590 nm.
• Verify that the plate is in the machine and press “Read” on the computer.
• After the reading, save the data in a folder labeled with the day’s date.
• After saving as a .pda (SoftMax Pro file), under “File” mouse over “Import/Export” and click “Export…”
  o Export to the same folder but as a .txt file. This file can be opened in any computer through the program notepad.

Final clean-up
• Turn off the computer and plate reader.
• Rinse out samples from 96 well plate using tap and then distilled water. Leave the plate to dry upside-down on paper towel near the sink by the plate reader.
• Back in S306, aspirate out remaining Alamar blue solution and dispose of plates into the autoclave bin.
• Return any pipettes used to the rack beside the hood.
• Dispose of used Pasteur pipettes in the sharps jar in the autoclave bin.
• Sterilize and clean the aspiration tube as per instructions above.
• Sterilize the surface of the hood again and organize objects inside so it looks neat.
• Ensure that the following are turned off:
  o Vacuum under hood
  o Microscope
  o Water bath
  o Hood fluorescent light
• DO NOT close the hood window or turn off the blower.