A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***HEL16T008 AlamarBlue Assay***
Subtitle	
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NILU	HEALTH EFFECTS LABORATORY **Standard Operating Procedure**	
AlamarBlue® assay		
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1. INTRODUCTION

The AlamarBlue® Assay is performed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. The assay may also be used to establish relative cytotoxicity of agents within various chemical classes. A variety of methods have been developed to measure cell proliferation in whole population of cells. These include the detection of antigens by immunohistochemistry, measurement of DNA synthesis, and quantification of the reducing environment of the cells. The latter relates to the fact that when cells are metabolising they maintain a reducing environment within their cytosol and this reduced state can be measured spectrophotometrically through the conversion of fluorometric/colorimetic REDOX indicators.

1. PRINCIPLE

The AlamarBlue® dye is a redox indicator that yields a colorimetric change and a fluorescent signal in response to metabolic activity. It is a proven safe and non-toxic dye used for quantitative analysis of cell viability and cell proliferation, for cytokine bioassays and *in vitro* cytotoxicity studies. After intracellular uptake, resazurin (oxidised form) is converted to resorufin (reduced form) due to the reducing environment of the cytosol in the cells. Resorufin produces very bright red fluorescence (Figure 1).

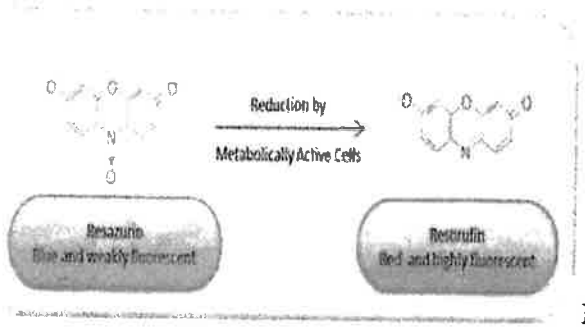

Figure 1. AlamarBlue assay® principle

Viable cells with metabolic activity continuously convert resazurin to resorufin, thereby generating a fluorescence signal that can be quantified as a measure of viability—and cytotoxicity. 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. The assay incorporates an oxidation-reduction (REDOX) indicators. 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.

A disadvantage of this assay is that it is not a direct cell counting technique such as heamocytometry, and the fluorescence or absorbance signal can be affected by both changes in cell number and cell metabolism. Also some nanoparticles (NPs) can interfere with the colorimetric readout (control with the NPs without cells should always be included when testing toxicity of NPs to check for interference).

The amount of fluorescence or absorbance is proportional to the number of living cells and corresponds to the cells metabolic activity. Damaged and/or non-viable cells have lower innate metabolic activity and thus generate a proportionally weaker signal than healthy cells.

2. MATERIAL AND REAGENT

(Vendors and products are suggestions, not mandatory)

Cells (adherent or suspension cells)
Cell culture dishes and plates
Cell culture medium Heat- inactivated Horse Serum, (H1138, Invitrogen)
Penicillin-Streptomycin (15140-122, Invitrogen)
Other supplements of culture medium required for the specific type of cell line (e.g. antibiotics, nonessential amino-acids, sodium pyruvate, etc.)
Trypsin-EDTA (59429C, Sigma)
Phosphate Buffer Saline 1X PBS (Invitrogen, 10010-049)
Countess® Cell Counting Chamber Slides and trypan blue (0.4%) kit (C10228, Invitrogen)
AlamarBlue® (DAL1025/ Invitrogen)
A 96 Dark flat bottom plate (well black polystyrene microplates (VWR))
Multichannel Disposable Solution Basins (VWR)

Distilled water
Ethanol

3. EQUIPMENTS

HEL11E037 FLUOstar OPTIMA (BMG LABTECH, Germany)
HEL11E020 Laminar Flow Hood
HEL11E023 Light microscope Leica
HEL11E044 Finn Pipettes
HEL11E022 Cell Counter Countess / Bürker chamber with cover glass 22x22 mm

4. PREPARATION PROCEDURES

Medium and culture conditions

Cells are cultivated in complete culture medium and incubated in culture dishes or flasks in humidified atmosphere at 37°C, 5% CO_2 as described in independent SOPs for cultivation.

Exposure conditions and treatment with test substance and controls

Cells are exposed to the test substance and controls as described in HEL11B002.

Alamar Blue® preparation

AlamarBlue® is a ready to use reagent from Invitrogen. The staining solution should be removed from the fridge and kept at room temperature to equilibrate before use.

5. PROCEDURE

1. Expose the cells in 96 well plates.
   Generally, expose two wells with cells for each treatment.
2. At end of exposure, remove media and wash twice in PBS or media
3. Add 200 µl medium with 10% AlamarBlue® to each well
   Remember control wells without cells (only medium with AlamarBlue®)

4. Incubate the plate for minimum 1 - 4 h at 37°C, 5% CO_2

   Longer incubation times may be used for higher sensitivity
5. At the end of incubation, transfer from each well 40 µl x 4 into 4 different wells in a flat bottom 96-wells black polystyrene microplates (it should be 40 µl in each well) (4 replica readings pr well; 8 replica reading per concentration). Remember to put replica treatment 1 and 2 into separate plates.
6. Read fluorescence or absorbance at appropriate wavelengths in the FLUOstar OPTIMA microplate plate reader. Excitation 530 nm, emission 590 nm.

Note 1. You may need to determine the seeding density and incubation time for each cell type.
Note 2. Be sure to include appropriate assay controls. To minimize experimental errors, make measurements from a minimum of 4 and up to 10 replicates per well with treated cells, plus 4-10 replicates for the control and no-cell control samples.
Note 3. Use a multichannel pipette and basins when you work with 96 well plate format for more practicability.
Note 4. Optionally, to stop the reaction you can add 50 µl 3% SDS directly to 100 µL of cells in alamarBlue® reagent.

6. EVALUATION, DATA ANALYSIS, DATA SHEETS AND DATA PRESENTATION

Results are analyzed by plotting fluorescence intensity (or absorbance) versus compound concentration. While results are linear and quantitative for both fluorescence and absorbance, fluorescence readings provide higher sensitivity.

Subtract the blank (negative control – samples without cells) from all data.

Percentage of cell viability is calculated as the % difference between treated (T) & untreated (UT) control cells based on fluorescence (RFU values) (% reduction of AlamarBlue) according to the equation:

$$\% \text{ Difference between T \& UT} = \frac{\text{Experimental RFU value with test compound x 100}}{\text{Untreated Control RFU value}}$$

Apply HEL16T008FORMb for calculations.

7. INTERPRETATION OF RESULTS

Criteria for determining a cytotoxic result are:

- Metabolic activity of cells treated with test substance is reduced at least by 20% compared to untreated cells.
- A concentration-related reduced metabolic activity
- Reproducible results

Biological relevance of the results needs to be considered first. Statistical methods may be used as an aid in evaluating the test results. Statistical significance will not be the only determining factor for cytotoxicity. A test substance, for which the results do not meet the above criteria, is considered non-cytotoxic under the assay conditions.

Positive results in an *in vitro* mammalian cytotoxicity test indicate that the test substance induces cytotoxic effect in the cultured mammalian cells used. A positive concentration response that is reproducible is most meaningful. Negative results indicate that, under the test conditions, the test substance does not induce cytotoxicity in the cultured mammalian cells used.

8. RECORDING OF TEST PERFORMANCE

Performance of the test is recorded on test performance sheet template HEL16T008FORMa where raw data are documented from start to the end.

9. INPORTANT ASPECTS

AlamarBlue® staining solution should be stored in the dark, since the compound is light sensitive. The product may be stored for 12 months at room temperature. The 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® is warmed to 37°C and shaken to ensure all components are completely in solution before use.

10. REFERENCES

1. Ahmed SA, Gogal RM Jr, Walsh JE. *A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. J Immunol Metho 1994;170:211-224.*
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