User Guide

Orangu™

Cell Counting Solution

Cat OR01

Version 2.2

Contents

Orangu™ Cell Counting Solution

Product components

Table 1. Orangu™ range of products.

Product	Catalogue number
Orangu™ solution, 5 ml	OR01-500
Orangu™ solution, 2 x 5 ml	OR01-1000

Storage

Store at 4°C. Orangu™ has a shelf life of at least six months from receipt.
If you are not planning on using Orangu™ within 2 months from receipt, aliquot the solution and store it at -20°C.
Please note that this product is light sensitive and should be protected from light until use.

Equipment and materials required but not supplied with these reagents

- Absorbance microplate reader fitted with 450 nm filter
- 96-well plate (or multiwell plate of choice)
- CO_2 incubator
- Pipettes

Introduction and assay principle

Orangu™ is a cell proliferation and cytotoxicity assay which utilizes WST-8, a water-soluble tetrazolium salt, which produces a formazan orange dye upon reduction in the presence of an electron mediator (Figures 1 and 2).

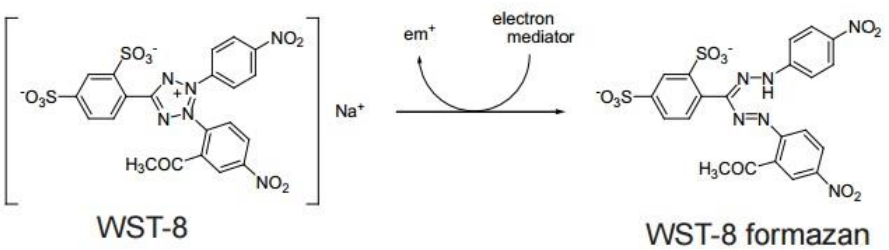

Figure 1. Structures of WST-8 and WST-8 formazan.

Orangu™ is a one-bottle solution; no premixing of components is required. Orangu™ provides a sensitive colorimetric assay for the determination of the number of viable cells in cell proliferation and cytotoxicity assays.

The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of live cells and the length of incubation.

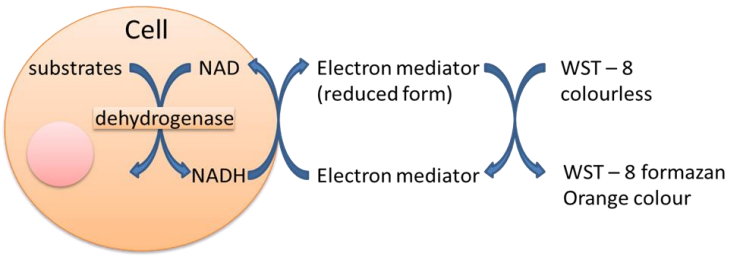

Figure 2. Principle of the cell viability detection with Orangu™.

Orangu™ data correlates very well with the [^{3}H]-thymidine incorporation assay. Detection sensitivity using Orangu™ is higher than assays using other tetrazolium salts (such as MTT, XTT, MTS or WST-1) as it relies on most of dehydrogenase activities that happens in a cell. However, other methods will rely only on mitochondrial dehydrogenase.

Procedure

If further culture of the cells is required following Orangu™ assay, please filter the solution through a 0.2 µm membrane to ensure sterility.

Assay optimisation for first-time users: Orangu™ assay is based on a proportional relationship between absorption and viable cell numbers. If you are using this assay for the first time, an optimisation step is recommended as the assay will depend on your cell type.

General protocol

1. Inoculate cell suspension (100 µl/well) in a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (e.g. at 37°C, 5% CO_2) to equilibrate.

2. Add 10 µl of Orangu™ solution to each well of the plate.
   Do not introduce bubbles to the wells - they interfere with the absorbance reading.

3. Incubate the plate for 1 – 4 hours in the humidified incubator.
   More than 1 hour in the incubator allow a higher signal.

4. Measure the absorbance at 450 nm using a microplate reader. To measure the absorbance later (within 24 hours), add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it in the dark at room temperature.

Cell proliferation and cytotoxicity assay

1. Dispense 100 µl of cell suspension (5 x 10^3 cells/well) in a 96-well plate. Pre-incubate the plate for 24 hours in a humidified incubator (e.g. at 37°C, 5% CO_2).
   If you are working with floating type cells, we recommend to use a V bottom plate.

2. Add 10 µl of various concentrations of substances to be tested to the plate.

3. Incubate the plate for an appropriate length of time (e.g. 6, 12, 24 or 48 hours) in the incubator.

4. Add 10 µl of Orangu™ solution to each well of the plate. Be careful not to introduce bubbles to the wells, since they interfere with the absorbance reading.

5. Incubate the plate for 1 – 4 hours in the humidified incubator.
Incubation periods will depend on cell number and type, longer incubations may be required for cells with weaker signal, e.g. leukocytes.

6. Measure the absorbance at 450 nm using a microplate reader. To measure the absorbance later, add 10 µl of 1% w/v SDS or 0.1 M HCl to each well, cover the plate and store it with protection from light at room temperature. No absorbance change should be observed for 24 hours.

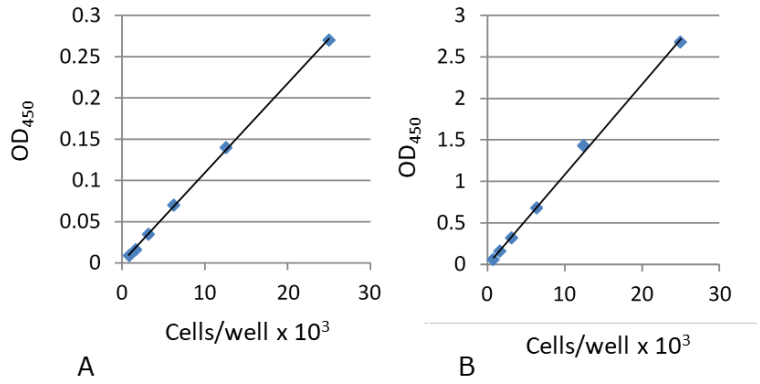

Figure 3. Standard curves for (A) HL60 and (B) HeLa cells.

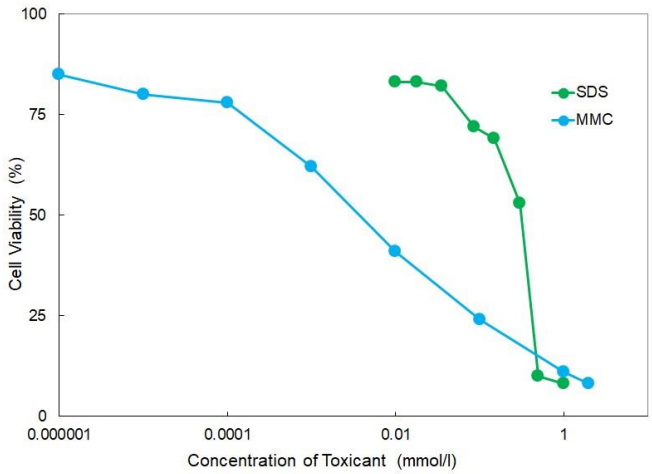

Figure 4. Toxicological test of chemicals using Orangu™.

Cell line:	HeLa
Culture medium:	MEM, 10% FBS
Chemicals:	Mitomycin-C (MMC) light blue; Dodecylsulfate, sodium salt (SDS) dark blue
Incubation:	37°C, 5% CO_2, 2 hours
Detection:	450 nm
Reference:	650 nm

Notes on use of Orangu™

- The Orangu™ assay is based on the detection of dehydrogenase activity in viable cells. Conditions that affect dehydrogenase activity in viable cells may lead to a discrepancy between the actual viable cell number and the cell number determined using the Orangu™ assay.
- WST-8 may react with reducing agents to generate WST-8 formazan. Measure the background absorbance if reducing agents are used in cytotoxicity assays or cell proliferation assays.
- Take care not to introduce any bubbles into the wells – they may interfere with the absorbance reading.
- Culture media containing phenol red can be used with this kit for cell viability assays.
- Membrane filtration is recommended for the sterilization of the Orangu™ solution, if necessary.
- The incubation time varies by the type and number of cells in a well. For example, please see data in Figure 3 comparing HL60 and HeLa cells. Leukocytes give weak coloration, thus a long incubation time (up to 4 hours) or a large number of cells (~1 x 10^5 cells/well) may be necessary.
- The cytotoxicity of this kit is very low. Further color development is possible after reading the absorbance.
- Other cell proliferation assays, such as neutral red or crystal violet can be used after the Orangu™ assay on the same samples.
- Measure and subtract the absorbance at 600 nm or higher from that for a highly turbid cell suspension.

Troubleshooting

How many cells should there be in a well?

- For adherent cells, at least 1×10^3 cells are necessary per well (100 µl medium). For leukocytes, at least 2.5×10^3 cells are necessary per well (100 µl medium) because of low sensitivity. The recommended maximum number of cells per well for the 96-well plate is 2.5×10^4. If a 24-well or 6-well plate is used for this assay, calculate the number of cells per well accordingly, and adjust the volume of the Orangu™ solution in a well to 10% of the total volume.

Is Orangu™ toxic to cells?

- Since the toxicity of Orangu™ is very low, the same cells can be used for other cell proliferation assays such as DNA fluorometric assays after the Orangu™ assay is completed.

Does phenol red affect the assay?

- No. The absorption value of phenol red in a culture medium can be removed by subtracting the absorption value of a blank solution from the absorption value of each well. Therefore, a medium containing phenol red is usable for the Orangu™ assay.

Does Orangu™ stain viable cells?

- No. Since WST-8 and its formazan dye are highly water-soluble, Orangu™ cannot be utilized for cell staining purpose.

I do not have a 450 nm filter. What other filters can I use?

- You can use filters with an absorbance between 430 and 490 nm, even though 450 nm filter gives the best sensitivity.

References

- M. Ishiyama et al. Talanta 44: 1299–1305. 1997
- M. Tominaga et al. Analytical Communications 36: 47-50. 1999

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