



TOXICITY STUDIES

GROWTH ASSAY IN CELL CULTURES

NEUTRAL RED UPTAKE ASSAY (NRU)

Laboratory: INTERLAB Group

Test substance: Biological material

Reference (Chacón): 61 a

Reference (Interlab): T 0263 C

Submitted by: Rafael Chacón

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Assay date: 12.03.1993





I. INTRODUCTION

The objective of this assay is to determine the effects of an extract of the sample submitted on the active growth of a cell culture. To measure these effects, the Neutral Red Uptake assay (NRU) is used (Borenfreund, E and Puerner, JA [1984] J. Tissue Cult. Methods 9: 7-9).

I.a. Sample preparation

The sample of biological material submitted by R. Chacón was treated by a lysis and cryofracture procedure clearly specified by the client. The result of this process is a protein-rich liquid called FR-91 by the client.

This product is that used in the cell culture growth assays.

I.b. Cell line

In this assay we have used, upon petition by the client, the cell line known as HIG-82. This is a cell line of immortalized cells from rabbit synovial capsule, which are therefore synoviocytes.

The cell line HIG-82 was obtained from the American Type Culture Collection (ATCC, ref. CRL 1832) in an ampule frozen in dry ice, and was subcultured according to the supplier's instructions.

I.c. Principle of the method

The principle of the NRU technique is based on the exposure in a microtiter plate of a culture of the chosen cell line in logarithmic growth phase to the substance under assay. After a variable contact period (according to the characteristics of the sample and the precise design of the assay; in this case it was established at 48 hours), the normal growth medium is removed and substituted with medium containing a dye which penetrates selectively in living cells. In this manner, it is possible to quantify the number of viable cells by correlation with the amount of dye present.

I.d. Methodology

1. The 96-well microtiter plates are seeded with 200 μ l of a synoviocyte suspension at a variable density according to the client's request, usually less than 1×10^5 cells/ml.

The culture medium used is DMEM completed with 10% fetal calf serum (FCS) and 2% glutamine. All the media and additives used are from ICN/Flow.

2. Incubate 24 h at 37°C in a 5% CO₂ atmosphere.





3. Four amounts of sample are added in sextuplicate:
2 μ l, 5 μ l, 10 μ l and 15 μ l of the extract

For the quantities of 2 and 5 μ l, 10 μ l of 1/5 and 1/2 dilutions were added, respectively, due to accuracy problems with pipetting quantities less than 10 μ l.

As positive controls, 1 % SDS was included, as well as an alternative which varied according to the client's request and which is marked with an asterisk (*).

4. After 48 hours contact, incubating at 37°C in 5% CO₂, the medium is removed by aspiration and 200 μ l of medium containing 50 μ g Neutral Red are added.

5. Incubate 3 h at 37°C in 5% CO₂.

6. Remove medium and wash with 200 μ l fixing solution (1% CaCl₂/1% formaldehyde).

7. Add 200 μ l/well destaining solution (50% ethanol, 1% acetic acid). Mix for 10 min at room temperature.

8. Read in a microplate reader at 540 nm.

The experimental variation coefficient (VC) calculated for the method for the tests carried out is 7%, although it varies as a function of the position of the column assayed in the microplate.





TABLE II. NEUTRAL RED UPTAKE: RESULTS

Cell line: HIG-82

Reference sample: T 0263 C

	C-	2	5	10	15	C+	*
	0.479	0.489	0.528	0.540	0.619	0.076	0.279
	0.465	0.510	0.530	0.621	0.610	0.079	0.290
	0.595	0.470	0.555	0.593	0.595	0.080	0.256
	0.420	0.418	0.526	0.530	0.570	0.082	0.272
	0.493	0.515	0.532	0.511	0.580	0.084	0.200
	0.461	0.473	0.418	0.585	610	0.081	0.230
X	0.485	0.479	0.514	0.563	0.597	0.080	0.253
CV %	12.1	7.3	9.4	7.6	3.2	3.4	13.0

*: Phenol 5 g/l

Maximum increase relative to the control (%): 23

III. CONCLUSION

A considerable increase in cell growth was observed which could be correlated to the sample dose, achieving a maximum value at 15 μ l/well.

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