

## Preliminary study on neutral red uptake assay as an alternative method for eye irritation test

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### Abstract

**Objective:** To study the possibility of Balb/c 3T3 neutral red uptake (NRU) phototoxicity assay as an alternative method for Draize eye irritation test with animal.

**Methods:** 23 reference chemicals and 26 cosmetic products were assessed in the present study and an acute eye irritation/corrosion test was conducted for the cosmetic products. In vivo and in vitro results of eye irritation were compared.

**Results:** The coefficient of agreement between in vivo and in vitro results obtained with 23 reference chemicals was 0.542; it reached 0.737 when acids, alkalis and amines were excluded from the reference chemicals; the Spearman's rank correlation coefficient between two sets of 3T3 NRU results for 23 reference chemicals was 0.988, with a coefficient of agreement of 0.847; the coefficient of agreement between in vivo and in vitro results obtained with tested cosmetic products was 0.570.

**Conclusion:** Compared with Draize test, 3T3 NRU assay demonstrated good predictive capacity, reproducibility and reliability, indicating the practical application of 3T3 NRU assay to eye irritation evaluation as a screening approach.

**Keywords:** alternative method, eye irritation test, neutral red uptake

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The Draize rabbit test has continued to be the method of choice for the regulatory toxicology safety assessment of eye irritation hazard of cosmetic products and ingredients in many countries since 1940s<sup>[1]</sup>. However, the increase in animal use has drawn the public attention to issues such as animal welfare and 3Rs (reduction of the number of animals used, refinement of techniques and procedures to reduce pain and distress, replacement of animal techniques with non-animal techniques)<sup>[2]</sup> in recent years. European Union ("EU") stipulated in EU cosmetics directive 2003/15/EC (7th amendment to Directive 76/768/EEC) a ban of animal use in toxicity and allergenic reaction tests for assessment of cosmetic products in EU countries by 2009 and a further ban of animal use in safety evaluation of cosmetic products and ingredients by 2013 with an aim to ban the import and sale of cosmetics involving the use of animal-based assessment methods in member states in a stepwise manner<sup>[3]</sup>. Therefore, there is an imperative for reduction, refinement and replacement of animal use in toxicological safety evaluation of cosmetics. Since eye irritation caused by exogenous chemical irritants usually involves damage in cornea,

conjunctive epithelium and endothelial cells, it can be predicted based on endpoint observations of changes in cell activity, membrane integrity, release of cytosolic enzymes and metabolic disorder, etc. The present study was intended to establish a 3T3 NRU cytotoxicity assay using Balb/c 3T3 as the test object and the neutral red uptake ("NRU") cytotoxicity as the end point. Meanwhile, acute eye irritation / corrosion tests and evaluations<sup>[4]</sup> were conducted for 23 reference chemicals which had a known degree of eye irritation and 26 personal care products including hair dyes, shampoos and conditioners which had an unknown degree of eye irritation in accordance with the Hygienic Standard for Cosmetics, 2002 by Ministry of Health ("MOH"). The in vivo and in vitro results were compared and analyzed to study the feasibility of the 3T3 NRU assay to replace the animal-based eye irritation test and provide a scientific basis for safety evaluation of cosmetics using in vitro eye irritation methods.

### 1. Materials and methods

#### 1.1. Test substances

23 chemical substances from 11 categories with

Draize results recorded in the Reference Chemicals Data Bank (1999) of Organization for Economic Cooperation and Development ("OECD")<sup>[5]</sup> were selected for the 3T3 NRU cytotoxicity assay, including 3 cationic surfactants, 1 anionic surfactant, 3 non-ionic surfactants, 3 acids, 1 alkali, 2 alcohols, 2 esters, 3 amines, 3 inorganic salts, 1 organic salt and 1 ketone (all purity guaranteed). The evaluation of eye irritation intensity included all severity ratings from non-/mildly irritant to strongly irritant. Cosmetic products under evaluation were test samples registered with Center for Disease Control and Prevention of Guangdong Province ("CDCP") from January 2006 to January 2007, 26 varieties in total, including shampoos, hair conditioners and hair dyes and these cosmetic products were classified as non-/slightly irritant, mildly irritant, irritant/corrosive respectively.

### 1.2. Cells

Balb/c 3T3 clone A31 cells were supplied by Shanghai Institute for Biological Sciences and cultured at  $37.0 \pm 0.5^\circ\text{C}$ , relative humidity 60%,  $\text{CO}_2$  5.0% in phenol-red-free DMEM (Dulbecco's modified Eagle's medium) medium containing 10% calf serum, 4mM L-glutamine, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin.

### 1.3. Test animal

Healthy adult New Zealand white rabbits (2500  $\pm$  200g) were selected. There was no limitation on whether female or male rabbits should be used. Animals were subject to 3 days of quarantine and adaptation before test in rabbit room prepared in accordance with requirements for cosmetics evaluation. Rabbits were fed with regular feedstuff and supplied with filtered pure water during the adaptation period and the test process. There was no limitation on the amount of water consumed.

### 1.4. Apparatus

OLYMPUS-CK2 inverted phase contrast microscope, SHELLAB  $\text{CO}_2$  incubator, VL-5BS clean bench (Taipei), Sunnyvala VERSA microplate reader (United States).

### 1.5. Test methods

#### 1.5.1. 3T3-NRU cytotoxicity assay<sup>[6]</sup>

3T3 cells in culture medium were inoculated into the 96-well plate, 100 $\mu\text{l}$  each well, at  $1 \times 10^5$  cells/ml. The cells were removed from the incubator after 24  $\pm$  2 hours of inoculation and the culture medium was replaced. The freshly prepared culture medium, 100 $\mu\text{l}$  each well, was added to the blank control group and the negative control group, while the test substance solution, 100 $\mu\text{l}$  each well, was added to the test group. Each test substance was measured at eight concentrations respectively, 6 parallel wells each level. Meanwhile, a positive control group was set up with sodium dodecyl sulphate ("SDS"). After 24  $\pm$  0.5 hours of inoculation, the culture medium was

removed and the plate was rinsed 3 times with PBS, 200 $\mu\text{l}$  each well, and then washed. The medium containing neutral red (50 $\mu\text{g}/\text{ml}$ ) was added, 200 $\mu\text{l}$  each well. After 3 hours of culture, the medium was removed and the plate was rinsed 2 times with PBS, 200 $\mu\text{l}$  each well. The desorbing solution, 100 $\mu\text{l}$  each well, containing 1% glacier acetic acid, 50% ethanol and 49%  $\text{H}_2\text{O}$ , was added and shaken for 15 minutes with a micromixer in a dark place. The absorbance of colored solution was measured at 540nm with a microplate reader. The concentration producing 50% inhibition for neutral red uptake ( $\text{NRU}_{50}$ ,  $\mu\text{g}/\text{ml}$ ) was calculated. The concentrations of  $\geq 1250$ ,  $200 \leq \text{NRU}_{50} < 1250$  and  $< 200$  represented non-/mildly irritant, moderately irritant and strongly irritant (ingredients) respectively, and the concentrations of  $\geq 150$  and  $< 150$  represented non-/mildly irritant and irritant/corrosive respectively (products).

#### 1.5.2 Acute eye irritation / corrosion test<sup>[4]</sup>

Tests were conducted in accordance with MOH Hygienic Standard for Cosmetics, 2002, p96-p98.

#### 1.5.3 Statistical analysis

In order to evaluate the predictive capacity of in vitro methods, the in vitro eye irritation results were compared with those of animal test. Statistical analyses through Pearson's chi-square, McNemar, Gamma and Kappa tests were conducted with SPSS (version 13.0). In addition, the two sets of 3T3 NRU results were analyzed through Spearman's rank correlation, Pearson chi-square, McNemar, Gamma and Kappa tests to identify the reproducibility of the 3T3 NRU assay.

## 2. Results

### 2.1. Dose-effect relationship

The cell survival rates of the reference chemical SDS obtained at eight concentrations were compared, 6 parallel wells each concentration. Test data indicated that the cell survival rate decreased in response to the increase in SDS concentration. As illustrated in Fig. 1,  $F = 1042.182$ ,  $P < 0.001$  in a single factor analysis of variance on results obtained at different concentrations, reflecting a dose-effect relationship featuring lower cell survival rates at higher concentrations of SDS. See Table 1 for details.

### 2.2. Efficacy study

Tests were conducted for 23 selected reference chemicals at eight concentrations, 6 parallel wells each concentration and all the tests were repeated after a month's interval. The results are in Table 2 as below.

Given the three ratings based on results of Draize test, i.e. non-/mildly irritant ( $\text{MMAS} < 25$ ), moderately irritant ( $25 \leq \text{MMAS} < 59$ ) and strongly irritant ( $\text{MMAS} \geq 59$ )<sup>[5]</sup>, 3T3 NRU cytotoxicity assay identified correctly 8 of the 12 substances classified as non-/mildly irritant in Draize test (3 classified

Table 1. Results of 3T3 NRU cytotoxicity assay at different concentrations of SDS (n=6)

Concentration (µg/ml)	Absorbance ( $\bar{x}\pm s$ )	F	P
6.81	0.315±0.012	1042.182	< 0.001
10	0.314±0.007		
14.7	0.288±0.005		
21.5	0.246±0.015		
31.6	0.181±0.004		
46.4	0.118±0.007		
68.1	0.073±0.003		
100	0.059±0.004		

as moderately irritant and 1 as strongly irritant), identified correctly 5 of the 8 substances classified as moderately irritant in Draize test (3 classified as non-/mildly irritant) and identified correctly 8 of the 10 substances classified as strongly irritant in Draize test (2 classified as non-/mildly irritant). The inconsistency in severity ratings was mainly

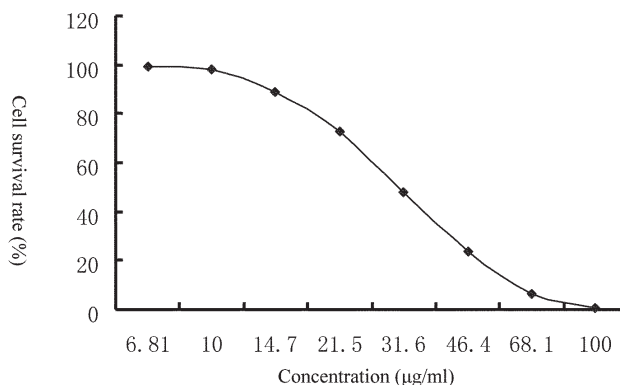


Fig. 1 Results of 3T3 NRU cytotoxicity assay at different concentrations of SDS

found with acids, alkalis and amines. Findings of Pearson's chi-square, McNemar, Gamma and Kappa

Table 2. Results of 3T3 NRU cytotoxicity assay and animal test for reference chemicals

Category	Test substance	Draize test*		1 <sup>st</sup> time		2 <sup>nd</sup> time	
		MMAS	Classification	NRU <sub>50</sub>	Classification	NRU <sub>50</sub>	Classification
Inorganic salt	Ammonium nitrate	18	I	412	II	190	III
	Sodium oxalate	61	III	89	III	115	III
	Sodium perborate	30	II	573	II	403	II
Organic salt	Calcium mercaptoacetate	4.0	I	4651	I	5199	I
Non-ionic surfactant	Tween 20	4.0	I	211	II	197	III
	polyethylene glycol 400	0	I	>10000	I	>10000	I
	Triton-X-100 (5%)	34	II	251	II	240	II
Anionic surfactant	Triton-X-100 (10%)	46	II	356	II	397	II
	SDS (3%)	16	I	1349	I	1572	I
	SDS (15%)	59	III	222	II	151	III
Cationic surfactant	SDS (30%)	60.5	III	124	III	100	III
	Domiphen bromide	96.3	III	22	III	15	III
	Cetrimide (0.1%)	3	I	3155	I	2811	I
Alcohol	Cetrimide (1%)	36	II	450	II	383	II
	Cetrimide (10%)	90	III	29	III	16	III
	Benzalkonium chloride (1%)	56	II	385	II	416	II
	Benzalkonium chloride (5%)	84	III	114	III	135	III
	Benzalkonium chloride (10%)	108	III	88	III	76	III
	Glycerol	2	I	>10000	I	>10000	I
	Ethanol	24	I	>10000	I	>10000	I
	Acetone	56	II	>10000	I	>10000	I
	Aspartic acid	37	II	4286	I	4421	I
	Trichloroacetic acid (3%)	6.7	I	>10000	I	>10000	I
Ester	Trichloroacetic acid (30%)	106	III	5810	I	3209	I
	Glacier acetic acid	68.0	III	2893	I	2259	I
	Ethyl acetate	15	I	>10000	I	>10000	I
Amine	Butyrolactone	43	II	>10000	I	>10000	I
	Diisopropanolamine	23	I	167	III	143	III
	Promethazine	72	III	7.3	III	5.5	III
Alkali	Triethanolamine	0	I	805	II	845	II
	NaOH (1%)	26	II	-	-	-	-
	NaOH (10%)	108	III	-	-	-	-

Note: MMAS – Modified maximum average score

"-" represents no data available.

The present study classified the reference chemicals into 3 severity ratings of eye irritation, i.e. I, II and III, which stood for non-/mildly irritant, moderately irritant and strongly irritant.

[\* Source: Bagley DM, Gardner JR, Holland G, et al. Eye Irritation: Updated Reference Chemicals Data Bank. Toxicology in vitro, 1999, 13(3): 505-510.]

Table 3. Comparison of average classification based on two sets of 3T3 NRU results with classification based on Draize results for reference chemicals

NRU <sub>50</sub> Irritancy classification	Draize irritancy classification			Total
	I	II	III	
I	8	3	2	13
II	3	5	0	8
III	1	0	8	9
Total	12	8	10	30

Note: (Fisher's Exact Test)  $\chi^2 = 18.995$ ,  $P < 0.001$ ; McNemar  $P = 0.846$ ; Gamma = 0.692,  $P = 0.001$ ; Kappa = 0.542,  $P < 0.001$ .

Table 4. Comparison of average classification based on two sets of 3T3 NRU results with classification based on Draize results for reference chemicals (acids, alkalis and amines excluded)

NRU <sub>50</sub> Irritancy classification	Draize irritancy classification			Total
	I	II	III	
I	7	2	0	9
II	2	5	0	7
III	0	0	7	7
Total	9	7	7	23

Note: (Fisher's Exact Test)  $\chi^2 = 23.629$ ,  $P < 0.001$ ; McNemar  $P = 1.000$ ; Gamma = 0.947,  $P < 0.001$ ; Kappa = 0.737,  $P < 0.001$

Table 5. Five repeated tests of 3T3 NRU cytotoxicity assay

Test substance	1 <sup>st</sup> time	2 <sup>nd</sup> time	3 <sup>rd</sup> time	4 <sup>th</sup> time	5 <sup>th</sup> time	$\bar{x} \pm s$	CV
SDS	37	38	38	36	35	36.8±1.30	0.035
PBS buffer	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	-
Promethazine	7.3	5.5	6.8	7.8	6.3	6.74±0.89	0.132
Domiphen bromide	22	15	25	18	20	20±3.80	0.19
Calcium mercaptoacetate	4651	5199	5034	4782	4937	4920±213.6	0.043

Table 6. Comparison of two sets of 3T3 NRU results for reference chemicals

1 <sup>st</sup> time classification	2 <sup>nd</sup> time classification			Total
	I	II	III	
I	13	0	0	13
II	0	6	0	6
III	0	3	8	11
Total	13	9	8	30

Note: (Fisher's Exact Test)  $\chi^2 = 39.853$ ,  $P < 0.001$ ; McNemar  $P = 0.083$ ; Gamma = 1.000,  $P < 0.001$ ; Kappa = 0.847,  $P < 0.001$

tests indicated rank correlation (Gamma = 0.692,  $P = 0.001$ ) and agreement in classification (Kappa = 0.542,  $P < 0.001$ ) between in vivo and in vitro results, as shown in Table 3. The statistic analysis achieved a noticeable increase in correlation and agreement (Gamma = 0.947,  $P < 0.001$ ; Kappa = 0.737,  $P < 0.001$ ) after the data pertinent to acids, alkalis and amines were excluded, as shown in Table 4.

### 2.3. Reproducibility study

#### 2.3.1. 5 reference chemicals

Five repeated tests were conducted for PBS buffer, SDS, promethazine, domiphen bromide and calcium mercaptoacetate at two weeks' interval and NRU<sub>50</sub> ( $\mu\text{g}/\text{ml}$ ) values were calculated respectively. The results indicated that 3T3 NRU cytotoxicity assay provided a good reproducibility, as shown in Table 5.

#### 2.3.2. 23 reference chemicals

As a further demonstration of reproducibility of 3T3 NRU cytotoxicity assay, the present study included a repeated test of all reference chemicals, as shown in Table 1. Comparison of the NRU<sub>50</sub> values and severity ratings obtained from the first time and second time tests indicated that only 3 chemicals were classified as II for the first time and as III for the second time. Spearman's rank correlation analysis showed  $r_s = 0.988$ ,  $P < 0.001$ , as illustrated in Fig.

2; and Pearson's chi-square, McNemar, Gamma and Kappa tests revealed high correlation and agreement between the two sets of 3T3 NRU results (Gamma = 1.000,  $P < 0.001$ ; Kappa = 0.847,  $P < 0.001$ ), as shown in Table 6, indicating good reproducibility of 3T3 NRU cytotoxicity assay.

### 2.4. Applicability study

3T3 NRU tests were conducted on 26 personal care products including shampoos, hair conditioners and hair dyes which had an unknown degree of eye irritation were selected. The results were compared

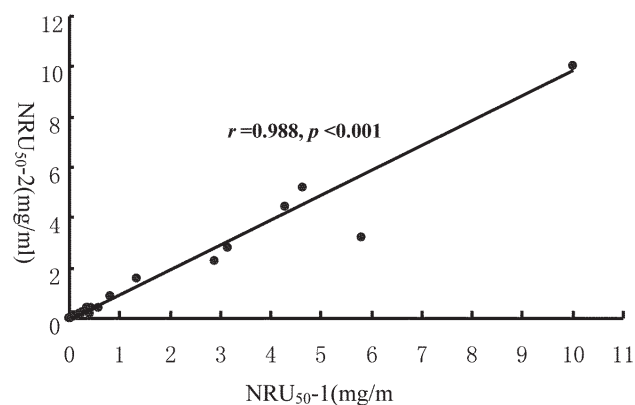


Fig. 2 Comparison of two sets of 3T3 NRU results for 23 reference chemicals

Table 7. Comparison of in vitro results with in vivo results for cosmetic products under evaluation

Category	Product name	Whole-animal test	3T3-NRU assay	
			NRU <sub>50</sub>	Classification
Hair conditioner	Aloe hair conditioner	Non-irritant	355	Non-/mildly irritant
	Hair cream (for normal to dry hair)	Non-irritant	42	Irritant/corrosive
Shampoo	Herbal anti-alopecia shampoo	Slightly irritant	224	Non-/mildly irritant
	Aloe shampoo	Slightly irritant	260	Non-/mildly irritant
	Anti-alopecia shampoo	Mild irritant	152	Non-/mildly irritant
	Color retaining shampoo	Mildly irritant	292	Non-/mildly irritant
	Anti-hair loss nourishing shampoo	Mildly irritant	204	Non-/mildly irritant
	Anti-hair loss shampoo	Mildly irritant	610	Non-/mildly irritant
	Herbal essence shampoo	Mildly irritant	229	Non-/mildly irritant
	Nourishing & anti-dandruff shampoo	Irritant	81	Irritant/corrosive
	Energizing grapefruit shampoo	Irritant	209	Non-/mildly irritant
	Hair dye	Hair coloring cream (golden yellow)	Non-irritant	195
Hair coloring cream (red)		Non-irritant	155	Non-/mildly irritant
Hair coloring cream (brown)		Non-irritant	174	Non-/mildly irritant
Hair coloring cream (brown)		Slightly irritant	117	Irritant/corrosive
Hair coloring cream (natural black)		Slightly irritant	170	Non-/mildly irritant
Hair coloring cream (claret)		Slightly irritant	132	Irritant/corrosive
Coloring oil treatment 3N (color)		Slightly irritant	175	Non-/mildly irritant
Hair coloring cream (yellow)		Mildly irritant	339	Non-/mildly irritant
Coloring oil treatment (dark brown)		Mildly irritant	124	Irritant/corrosive
Coloring oil treatment (brown)		Mildly irritant	192	Non-/mildly irritant
Instant coloring cream (natural black)		Irritant	112	Irritant/corrosive
Hair coloring cream (light red brown)		Irritant	129	Irritant/corrosive
Hair coloring cream (copper brown)		Irritant	119	Irritant/corrosive
Herbal hair coloring cream (chestnut brown)		Corrosive	21	Irritant/corrosive
Hair coloring cream (natural black)		Corrosive	134	Irritant/corrosive

Table 8. Comparison of in vitro results with in vivo results for cosmetic products under evaluation

NRU <sub>50</sub> irritancy	Animal test irritancy		Total
	Mildly-or-less irritant	Moderately-to-strongly irritant	
Mildly-or-less irritant	15	1	16
Moderately-to-strongly irritant	4	6	10
Total	19	7	26

Note: (Fisher's Exact Test)  $\chi^2 = 9.036$ ,  $P = 0.003$ ; McNemar  $P = 0.375$ ; Gamma = 0.915,  $P = 0.002$ ; Kappa = 0.570,  $P = 0.003$ .

with those of the whole-animal eye irritation test to identify the applicability of the in vitro method. The findings are as below:

The study on 26 personal care products indicated that, given the in vivo results, 3T3 NRU assay was able to identify two degrees of eye irritation, that is, mildly-or-less irritant and moderately-to-strongly irritant. In particular, 3T3 NRU identified 15 of 19 mildly-or-less irritant products correctly and 3 of the 4 products inconsistently classified were hair dyes. 3T3 NRU also identified 6 of the 7 moderately-to-strongly irritant products except for 1 shampoo. Statistical analysis through Pearson's chi-square, McNemar, Gamma and Kappa tests revealed the rank correlation (Gamma = 0.915,  $P = 0.002$ ) and agreement on classification (Kappa = 0.570,  $P = 0.003$ ) between the in vivo and in vitro methods.

### 3. Discussion

Eye irritation caused by exogenous chemical irritants usually involves damage in cornea, conjunctive epithelium and endothelial cells. As a result of the difference in pH values of cytolysosome and cytoplasm, the selective uptake of the vital dye neutral red into cellular lysosome will occur, and the amount of uptake is in proportion to the number of living cells in the culture medium. Therefore,

the degree of damage in eye tissue cells caused by chemicals can be simulated using the NRU assay.

3T3-NRU assay is a short-term test system based on a monolayer culture of 3T3 cells with cytotoxicity used as the endpoint for irritancy evaluation of test substances. The present study tested and classified 23 cosmetic ingredients into three severity ratings, i.e. non-/mildly irritating, moderately irritating and strongly irritating, and the results were compared with those of Draize test. 3T3 NRU assay identified 21 of the 30 chemicals correctly. Inconsistency with in vivo results was found in acids, alkalis and alcohols, which could have been caused by pH dilution of acids or alkalis during buffer preparation. Another reason might be changes in potential toxicity of test substances caused by reaction with chemicals in the culture medium or by impact of the buffer, as a result of direct contact between the cultured in vitro cells and the test substances due to lack of in vivo protection mechanism<sup>[7]</sup>. In addition, the volatile nature of alcohols might result in a cytotoxicity reading lower than expected, while the anesthetic effect of alcohols was likely to cause eye damage in whole-animal tests<sup>[8]</sup>. Another statistical analysis was conducted without these substances and a significant increase in correlation and agreement between in vivo and in vitro results was achieved.

The present study also indicated high accuracy in evaluation of strongly eye-irritant substances, i.e. all 7 strongly irritant substances were classified correctly (acids and alkalis excluded). Some previous studies show that the NRU 3T3 assay, in combination with the HET-CAM test, can be used for screening of strongly eye-irritant substances<sup>[9]</sup>. However, in view of the discrepancy between NRU 3T3 results and animal-based results for acids, alkalis and amines, caution should be taken in evaluating multiple varieties of test substances and the scope of application should be carefully identified in practice.

In the present study, the wells at the edge of the 96-well plate were used as the blank control group to avoid the possible "edge effect". Neutral red uptake assay is a good quantitative colorimetric method<sup>[10]</sup>, but the precipitation of the neutral red dye may give rise to noticeable needle-shaped crystal at the bottom of solution, which is nonreversible and has an adverse impact on the results. Therefore, the medium containing neutral red should be centrifugated or filtered before use and it's required to carry out observations during the dyeing process to prevent such precipitation<sup>[11]</sup>.

Results of 3T3 NRU assay are in agreement with in vivo data in terms of eye irritation classification of cosmetic products. This is basically because personal care products like shampoos and hair conditioners are surfactant-based formulas and the cell membranes are surfactant-sensitive. Some previous studies also believe that NRU is an acceptable toxicological endpoint for evaluation of shampoos.

Compared with the traditional animal-based methods, the culture and passage of Balb/c 3T3 cell strains is a laboratory technique relatively easy-to-operate and cost-efficient and the in vitro measurement of cytotoxicity, as a routine test, can provide objective and reliable results<sup>[13]</sup>. Quantitative results can be achieved with 3T3 NRU assay in wide applications, especially in the eye irritation assessment of hair care products, with good predictive capacity and reproducibility in comparison with animal-based methods. It has a good prospect to be used as an effective screening approach for the assessment of eye irritation, but further efforts are necessary to extend the use of this method to a greater number and variety of test substances and to carry out inter-laboratory comparisons and validation studies.

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## Footnotes

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