

---

---

**Nanotechnologies — 5-(and  
6)-Chloromethyl-2',7' Dichloro-  
dihydrofluorescein diacetate (CM-  
H2DCF-DA) assay for evaluating  
nanoparticle-induced intracellular  
reactive oxygen species (ROS)  
production in RAW 264.7 macrophage  
cell line**

*Nanotechnologies — Essai au diacétate de 5-(et 6)- Chlorométhyle  
-2',7' Dichloro-dihydro-fluorescéine (CM-H2DCF-DA) pour l'évaluation  
de la génération intracellulaire d'espèces réactives à l'oxygène  
induites par les nanoparticules sur la lignée souche 264.7 de  
macrophages*

This document is a preview generated by EBS



**COPYRIGHT PROTECTED DOCUMENT**

© ISO 2016, Published in Switzerland

All rights reserved. Unless otherwise specified, no part of this publication may be reproduced or utilized otherwise in any form or by any means, electronic or mechanical, including photocopying, or posting on the internet or an intranet, without prior written permission. Permission can be requested from either ISO at the address below or ISO's member body in the country of the requester.

ISO copyright office  
Ch. de Blandonnet 8 • CP 401  
CH-1214 Vernier, Geneva, Switzerland  
Tel. +41 22 749 01 11  
Fax +41 22 749 09 47  
copyright@iso.org  
www.iso.org

# Contents

Page

<b>Foreword</b>	<b>iv</b>
<b>Introduction</b>	<b>v</b>
<b>1 Scope</b>	<b>1</b>
<b>2 Normative references</b>	<b>1</b>
<b>3 Terms and definitions</b>	<b>1</b>
<b>4 Symbols and abbreviated terms</b>	<b>3</b>
<b>5 Materials</b>	<b>3</b>
<b>6 Technical equipment</b>	<b>5</b>
<b>7 Nanoparticle sample preparation</b>	<b>5</b>
<b>8 Preparations</b>	<b>6</b>
8.1 General	6
8.2 Flow cytometry calibration	7
8.3 Experimental culture medium	7
8.4 Reagent preparation	7
8.5 Preparation of cell stock culture	7
8.6 Preparing culture for experiments	7
8.7 Verification of healthy cell growth	8
8.8 Evaluation of nanoparticle interference	9
8.9 Control preparation	9
8.9.1 General	9
8.9.2 Control description	9
8.9.3 Sin-1 stock solution preparation (1 mM)	10
<b>9 Evaluation of nanoparticle impact on ROS generation in cells</b>	<b>10</b>
9.1 Prepare cells in the 24 well plates	10
9.2 Dose the cells with nanoparticles and controls	10
9.3 Expose the cells to CM-H <sub>2</sub> DCF-DA Assay	11
9.4 Incubate the cells with CM-H <sub>2</sub> DCF-DA	12
9.5 Flow cytometry analysis	12
<b>10 Data analysis and results</b>	<b>12</b>
<b>Annex A (informative) Alternate cell lines</b>	<b>14</b>
<b>Annex B (informative) Alternate fluorescence characterization techniques</b>	<b>15</b>
<b>Annex C (informative) Suspension preparation and characterization</b>	<b>16</b>
<b>Annex D (informative) Example experimental data from RAW 264.7</b>	<b>17</b>
<b>Bibliography</b>	<b>20</b>

## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see [www.iso.org/directives](http://www.iso.org/directives)).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see [www.iso.org/patents](http://www.iso.org/patents)).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: [www.iso.org/iso/foreword.html](http://www.iso.org/iso/foreword.html).

The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

## Introduction

The field of nanotechnology continues to advance rapidly through the development of new materials, products and applications. At the same time, many questions have been raised relating to the potential impact on human health and on the environment of some of these materials. Internationally, a large programme of research is underway to better understand and quantify potential hazards. Also, the chemicals used to coat the surface of nanoparticles in processing or in products can affect the interactions of nanoparticles with cells, even more so due to their large surface to volume ratio. Thus, there is a need for reliable fast screening methods to determine the potential toxicity aspects of nanoparticles with characterization of chemical functionalization on nanoparticles.

It is likely that monitoring biological response of cellular model systems to nanoparticle exposure can provide insight into the “modes-of-action” of nanoparticles and which of them may need to be further investigated for risk assessment.

In 2008, a number of international researchers concluded that some published results of nanomaterial toxicity could not be replicated across laboratories and that accurate and reproducible nanotoxicology tests were needed. As a result of this, the International Alliance for NanoEHS Harmonization (IANH) was formed with the goal of developing testing protocols that would accurately assess toxicity and biological interactions of nanoparticles in cellular systems and that these be reproducible in any laboratory. The IANH performed round robin characterization of particle size distributions in liquid suspensions and *in vitro* interactions of nanomaterials with cells with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 5-(and-6)-chloromethyl-2',7'-Dichloro-dihydro-fluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCF-DA), and propidium iodide assays. The IANH identified a number of factors that increased variability and developed techniques to reduce variability.

Oxidative stress, which leads to DNA damage, is a primary driver leading to the accumulation of mutations which occurs in living organisms, so it is important to assess whether nanoparticles can induce reactive oxygen species in living cells.

This document is a method to assess potential nanoparticle induced radical oxygen species (ROS) generation in cells through *in vitro* measurements. Although multiple techniques are used for determining generation of oxygen radicals in cells, the CM-H<sub>2</sub>DCF-DA has been used in round robin testing to evaluate ROS generation in mouse macrophages (RAW 264.7). The CM-DCF-DA assay provides a general measure of oxidative stress rather than detecting specific oxygen radicals or reactive species. [4] While this assay has not been evaluated in a broad range of cells, it does provide insight into the potential for ROS generation in macrophages which may play an important role in scavenging particles from the body.

The CM-DCF-DA assay has a margin of error even when controls are used and a number of factors could produce false negatives. [4] The CM-H<sub>2</sub>DCF-DA assay is not optimal for detecting all ROS species, such as the superoxide anion and hydroxyl radical which have short half-lives. In addition, measurement using cytometry should be performed quickly after cells have been exposed in the assay, because DCFH and DCF can leach from cells or the DCFH can be oxidized. Also, the CM-H<sub>2</sub>DCF-DA assay is deactivated in serum, so cells should be washed to remove serum and cells could be lost in this process resulting in a potential false negative. Furthermore, some nanoparticles may interact with DCFH and partially quench fluorescence. Thus, negative ROS results with this assay may not be conclusive. ISO/TS 18827 utilizes electron spin resonance (ESR) to detect the presence of ROS species in cells and differentiate between the different reactive oxygen species without interference.

In addition, there are several factors that could produce false-positive results. [5] Some nanoparticles and dead cells can fluoresce. Some nanoparticles can catalytically interact with CM-H<sub>2</sub>DCF-DA or the assay components can preferentially adsorb on the surface of the particle. [5] In order to establish true positives, controls should be established to characterize nanoparticles alone under test conditions, as well as distinguish dead cell fluorescence from live cells with ROS.

Furthermore, due to light-induced auto-oxidation, CM-H<sub>2</sub>DCF-DA solutions at any concentration should be protected from light and air by storing in the dark in a sealed container filled with nitrogen gas or argon.

Thus, the CM-H<sub>2</sub>DCF-DA assay may be applicable to only particular cell lines and nanoparticles and outcomes should be confirmed by additional assays (see [Annex A](#) for alternate cell lines). In particular, as a number of factors could lead to false negatives, or positives, other tests should be pursued and a positive result should be confirmed to not be caused by interference.

Controls are needed to determine a baseline of fluorescence of unexposed cells, determine whether cells are affected by non-toxic nanoparticles and also to demonstrate that known ROS generating chemicals and nanoparticles produced ROS which could be determined under assay conditions. Furthermore, it is important to determine whether nanoparticles interfere with the fluorescence of the assay and potentially invalidate assessment of nanoparticle induced ROS generation in cells. Controlled experiments could be performed with cells exposed to Sin-1 with varying concentrations of nanoparticles present to determine whether the nanoparticles quench the fluorescence.

NOTE This assay is considered to be a screening assay that rapidly provides information about a nanoparticle interaction with a cellular system. Although screening type assays are critical for use in evaluating nanoparticle effects on cells, it is important that interpretation of the results be verified with other ROS and related cellular assays.

# Nanotechnologies — 5-(and 6)-Chloromethyl-2',7' Dichloro-dihydrofluorescein diacetate (CM-H2DCF-DA) assay for evaluating nanoparticle-induced intracellular reactive oxygen species (ROS) production in RAW 264.7 macrophage cell line

## 1 Scope

This document describes how to test and evaluate results obtained from *in vitro* ROS generation in RAW 264.7 macrophage cells exposed to nano-objects, nanoparticles, their aggregates and agglomerates using the CM-H<sub>2</sub>DCFDA assay.

The protocol in this document is limited to use of a 24 well plate so if other plates were to be used, volumes would need to be adjusted and the protocol steps validated to ensure confidence in the test results.

## 2 Normative references

There are no normative references in this document.

## 3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2, ISO 10993-5 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <http://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

### 3.1

#### **agglomerate**

collection of weakly bound particles or *aggregates* (3.2) or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces (for example, van der Waals forces) or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4]

### 3.2

#### **aggregate**

particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces (for example, covalent bonds) or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.