

# ***Current Protocols in Toxicology***

October 8, 2012

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Unit: CPTX 20.8

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1. Unit Introduction: (a) Please confirm the note added at the end of the introduction (taken from the Critical Parameters section).
2. Basic Protocol 1: (a) Materials list: Please note specifically where in the protocol the following items are used: (1) adjustable micropipets, (2) adjustable multi-step pipet, (3) centrifuge, (4) inverted microscope, (5) laminar flow cabinet, (6) repetitive positive displacement dispenser/pipet tip (5-ml combitips), (7) sterile disposable pipets, (8) vortex mixer, (9) 1.5-ml micro test tubes, and (10) 15- and 50-ml tubes. If not used directly within the protocol, please remove from the materials list.  
  
(b) Step 1: How many cells are you culturing? How much medium?  
  
(c) Step 1: Please confirm the cells are incubated at the end of this step.  
  
(d) Step 2: Please note what was originally step 2 was incorporated into step 1 as part of the step and as the annotation. It made better sense to keep this information directly within the step. Please confirm this change.  
  
(e) Step 2: How much PBS do you use for the wash?  
  
(f) Step 2: Please confirm you are trypsinizing for 3 min at room temperature.  
  
(g) Step 3: What is complete culture medium? Is this the RPMI 1640 plus supplements added in step 1 (i.e., glutamine, pen/strep, etc.?). If not, please add additional details for the complete culture medium.  
  
(h) Step 3: Are you counting the cells using trypan blue?  
  
(i) Step 3, annotation: By “passed,” do you mean passaged?  
  
(j) Step 4: Are what point were the cells frozen in liquid nitrogen? Please provide all of the details necessary.
3. Support Protocol 1: (a) Materials list: Please specify what is added to the RPMI 1640 medium to make it complete.  
  
(b) Materials list: Please confirm the materials list is complete as shown.
4. Basic Protocol 2: (a) Materials list: Please confirm the materials list is complete as shown.

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6. Basic Protocol 4: (a) Materials list: Please confirm the materials list is complete as shown.  
  
(b) Materials list: Wash buffer and blocking and dilution buffer: Are you using D-PBS in these buffers?  
  
(c) Step 5: How much wash buffer do you use for each wash?  
  
(d) Step 7: Are you preparing both concentrated and working standards at this point?  
  
(e) Steps 8, 11, 15, & 19: How much wash buffer do you use for each wash?  
  
(f) Steps 22 & 23: Please confirm these steps are performed using an ELISA plate reader.
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# The NCTC 2544 IL-18 Assay for the In Vitro Identification of Contact Allergens

UNIT 20.8

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## ABSTRACT

Assessment of allergenic potency of low-molecular-weight compounds is generally performed using animal models, such as the murine Local Lymph Node Assay (LLNA) and the Guinea Pig Maximization Test (GMT). Progress in understanding the mechanism of skin sensitization, including effects on the production of cytokines by different cell types within the skin, provides the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing. This unit will describe a method for discriminate contact allergens from low-molecular-weight respiratory allergens and irritants, based on the selective induction of intracellular interleukin 18 (IL-18) in the human keratinocyte cell line NCTC 2544. Similar results could also be obtained using primary human keratinocytes or other human keratinocyte cell lines, e.g., HaCaT, HPKII, etc. IL-18 was chosen as this cytokine has been demonstrated to favor Th-1-type immune responses by enhancing the secretion of pro-inflammatory mediators such as TNF- $\alpha$ , IL-8, and IFN- $\gamma$ , and to play a key proximal role in the induction of allergic contact dermatitis. *Curr. Protoc. Toxicol.* 54:20.8.1-20.8.18. © 2012 by John Wiley & Sons, Inc.

Keywords: contact sensitizers • contact dermatitis • keratinocytes • cytokines • in vitro toxicology • alternative method

## INTRODUCTION

IL-18, formerly known as IFN- $\gamma$  inducing factor (IGIF), which belongs to the IL-1 cytokine family, is a potent inducer of IFN- $\gamma$  by activated T-cells (Okamura et al., 1995). IL-12 and IL-18 play important roles in the development of T helper type I (Th1) cells and are synergistic in the induction of IFN- $\gamma$  by T cells (Okamura et al., 1995). IL-18 has been demonstrated to favor Th-1 type immune responses by enhancing the secretion of pro-inflammatory mediators such as TNF- $\alpha$ , IL-8, and IFN- $\gamma$ , and to play a key proximal role in the induction of allergic contact dermatitis (Cumberbatch et al., 2001). Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999). Antonopoulos et al. (2008) demonstrated that IL-18 is a key mediator of Langerhans cell (LC) migration and contact hypersensitivity, acting upstream of IL-1 $\beta$  and TNF- $\alpha$ , suggestive of a central role of IL-18 in the regulation of cutaneous immune responses.

NCTC 2544 is a commercially available epithelial-like skin cell line originating from normal human skin, which possesses robust expression of cytochrome P450-dependent enzymatic activities. We have previously demonstrated that at noncytotoxic concentrations (cell viability >80%, as assessed by the MTT reduction assay), all contact sensitizers, including pre-pro-haptens, induced a dose-related increase in IL-18, whereas irritants (with the exception of sulfamic acid) and respiratory allergens failed to induce IL-18. A total of 33 chemicals were tested, with an overall accuracy of 97% with respect to predicted response of IL-18 based on compound classification, i.e., contact sensitizers vs. irritants and respiratory allergens (Corsini et al., 2009; Galbiati et al., 2011; Teunis et al., 2012). This manuscript details an in vitro test protocol to discriminate contact allergens from irritants and respiratory allergens based on a selective upregulation of

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20.8.1

Supplement 54

**Table 20.8.1** Products

| Products                                                        | Company               | Catalog no./model |
|-----------------------------------------------------------------|-----------------------|-------------------|
| Adjustable micropipet <sup>a</sup>                              | Gilson                |                   |
| Adjustable multi-step pipet <sup>a</sup>                        | Gilson                |                   |
| 5-ml combitips <sup>a</sup>                                     | Eppendorf             | ??0030 069.250??  |
| Sterile disposable pipets <sup>a</sup>                          | Greiner Bio One       |                   |
| Centrifuge <sup>a</sup>                                         | Heareus               | Sepatech          |
| ELISA Plate reader <sup>a</sup>                                 | Molecular Devices     | E max             |
| –20°C freezer <sup>a</sup>                                      | Profile Ge            |                   |
| –80°C freezer <sup>a</sup>                                      | Angelantoni Industrie | Platilab 370      |
| Incubator, 37°C, 5% CO <sub>2</sub> , 95% humidity <sup>a</sup> | Thermo                | 3111              |
| Inverted microscope <sup>a</sup>                                | Wilovert              |                   |
| Laboratory balance (accuracy 0.1 mg) <sup>a</sup>               | Gibertini             | E42               |
| Laminar flow cabinet <sup>a</sup>                               | Gelaire               | BSB 4A            |
| Vortex <sup>a</sup>                                             | Bio Cote              | SA8               |
| Plate sealers <sup>a</sup>                                      | Sigma                 | Z369659           |
| Multiwell pipettor <sup>a</sup>                                 | Sigma                 | Z370800           |
| Water bath <sup>a</sup>                                         | Grant                 | JB Aqua 5 Plus    |
| 1.5-ml micro test tubes <sup>a</sup>                            | Eppendorf             | T9661             |
| 15-ml tubes <sup>a</sup>                                        | Corning               | 430766            |
| 50 ml tubes <sup>a</sup>                                        | Corning               | 430291            |
| 75-cm <sup>2</sup> flask <sup>a</sup>                           | Corning               | 430720            |
| 24-well plate <sup>a</sup>                                      | Corning               | 3526              |
| 96-well plate <sup>a</sup>                                      | Corning               | 3596              |
| 96-well flat-bottom, ELISA plates                               | Corning               | 3369              |

<sup>a</sup>Laboratories are allowed to use their own equivalent materials.

intracellular IL-18 protein in the human-derived keratinocyte cell line NCTC 2544 cells (NCTC) after 24 hr exposure to chemicals, at noncytotoxic concentrations.

This manuscript contains four basic protocols and two support protocols organized in the order of anticipated workflow. Culture techniques for NCTC 2544 cells are described in Basic Protocol 1, followed by Support Protocol 1, which provides guidance for making and diluting compounds in anticipation of initial cytotoxicity testing by the MTT assay (Basic Protocol 2). After determining the upper limit of nontoxic compound concentrations for data collection, treatment parameters and actual quantification of IL-18 protein expression are detailed in Basic Protocols 3 and 4, respectively. Finally, Support Protocol 2 briefly outlines a simple means to quantify protein for normalization.

For a comprehensive list of materials and reagents used throughout this manuscript, with supplier and catalog information, please refer to Tables 1 and 2.

??NOTE: Working with human cells and tissue is inherently dangerous. Working with human cells is recommended to take place in a specially equipped laboratory by people who have had the proper training. The use of biosafety level II cabinets is highly recommended.??

**Table 20.8.2** Reagents

| Reagents                                                        | Company             | Catalog no. |
|-----------------------------------------------------------------|---------------------|-------------|
| Bovine serum albumin <sup>a</sup>                               | Sigma               | A4503       |
| Chloridric acid <sup>a</sup>                                    | Sigma               | 43557-0     |
| DMSO <sup>a</sup>                                               | Sigma               | D5876       |
| Dulbecco's phosphate-buffered saline <sup>a</sup>               | Sigma               | D8537       |
| Fetal bovine serum <sup>a</sup>                                 | Sigma               | F6178       |
| Glutamine <sup>a</sup>                                          | Sigma               | G7513       |
| Isopropanol <sup>a</sup>                                        | Sigma               | I0398       |
| Gentamicin                                                      | Sigma               | G1272       |
| Penicillin / Streptomycin (Pen/step) <sup>a</sup>               | Sigma               | P0781       |
| <i>p</i> -phenylenediamine                                      | Sigma               | P6001       |
| RPMI 1640 medium <sup>a</sup>                                   | Sigma               | R0883       |
| Triton X-100 <sup>a</sup>                                       | Sigma               | X-100       |
| 0.4% Trypan blue <sup>a</sup>                                   | Sigma               | T8154       |
| Trypsin/EDTA <sup>a</sup>                                       | Sigma               | T3924       |
| Anti-human IL-18 monoclonal antibody, 1 mg/ml                   | MBL (sold by Space) | D044-3      |
| Anti-human IL-18 biotinylated monoclonal Ab, 0.5 mg/ml          | MBL (sold by Space) | D045-6      |
| Human recombinant IL-18                                         | MBL (sold by Space) | B001-5      |
| Streptavidin conjugated-polyhorseradish peroxidase <sup>a</sup> | Immunotools         | 31334248    |
| TMB liquid substrate supersensitive for ELISA                   | Sigma               | T4444       |
| 1 M H <sub>2</sub> SO <sub>4</sub> <sup>a</sup>                 | Sigma               | 339741      |
| Bicinchoninic Acid solution                                     | Sigma               | B9643       |
| Copper(II) Sulfate solution                                     | Sigma               | C2284       |

<sup>a</sup>Laboratories are allowed to use their own equivalent materials.

### SELECTIVE UP-REGULATION OF INTRACELLULAR IL-18 BY CONTACT ALLERGENS IN THE HUMAN KERATINOCYTE CELL LINE NCTC 2544

This protocol provides instructions for the basic culture and passaging of NCTC 2544 cells for IL-18 protein analysis following compound exposure. Basic Protocols 2 to 4 contain detailed instructions for using NCTC 2544 cells for contact allergen assays.

#### Materials

Master cell and working cell bank: NCTC 2544 cells can be obtained from Istituto Zooprofilattico di Brescia, Cat. n. BS CL 143 (Via Bianchi, 9 - 25124 Brescia: Tel. +3903022901; Fax: +39030 2425251; e-mail: *info@bs.izs.it*)

RPMI 1640 culture medium

Glutamine

Penicillin/streptomycin (pen/strep)

Gentamicin

Fetal bovine serum (FBS)

Dulbecco's phosphate-buffered saline (D-PBS)

Trypsin/EDTA

0.4% Trypan blue

**BASIC  
PROTOCOL 1**

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### 20.8.3

??Freezing medium: cells are frozen in RPMI-1640, 20% FBS and 10 % glycerol at about  $2 \times 10^6$  cells/ vial in liquid nitrogen (freezing medium is prepared fresh when needed)

75-cm<sup>2</sup> flasks

Hemocytometer (e.g., Burker, Neubauer) or equivalent cell counter

Incubator, 37°C, 5% CO<sub>2</sub>, 95% humidity

24-well plates *or* 96-well plates

??Adjustable micropipets 2-20 µl, 20-200 µl, 200-1000 µl

??Adjustable multi-step pipet

??Centrifuge

??Inverted microscope

??Laminar flow cabinet

??Repetitive positive displacement dispenser/pipet tip (5-ml combitips)

??Sterile disposable pipets

??Vortex mixer

??1.5 ml micro test tubes

??15- and 50-ml tubes

Additional reagents and equipment for counting cells using a hemacytometer and trypan blue staining (Phelan, 2006)

**NOTE:** The handling of this cell line is not very different from any other cell lines. No specific recommendations apply.

1. Culture the NCTC cells in a 75-cm<sup>2</sup> flask containing ?? ml RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin/100 µg/ml streptomycin, gentamicin, 10% fetal bovine serum (heat inactivated by heating at 56°C for 30 min or buy heat-inactivated serum from the supplier). ??Incubate the cells at 37°C, 5% CO<sub>2</sub>, 95% humidity.??

*All culture reagents (i.e., culture medium, PBS) should be warmed to 37°C in a water bath before use.*

2. When 80% confluency is reached, pass the cells (usually by 1:5 dilution) by trypsinization, and maintain them in the culture medium (final volume: 10 ml per flask). Trypsinize the cells by washing them once with ?? ml D-PBS, followed by incubation with 2 ml trypsin/EDTA for ~3 min ??at room temperature??.
3. When the cells detach from the plastic surface, add 2 ml complete culture medium to stop the trypsin activity. Count the cells using a hemacytometer ??and trypan blue staining?? and resuspend them in new flasks or plate them for experiments. Incubate the cells at 37°C, 5% CO<sub>2</sub>, 95% humidity.

*Typically, cells are ??passed?? on Monday and Friday; change the culture medium on Wednesday if it turns yellow.*

4. ??After thawing from liquid nitrogen,?? use the cells for testing after 3 weeks of culture, ~4 to 5 passages.

*Consistent results were obtained culturing cells up to 5 months; longer periods have not been tested. Therefore, cells can be used between 3 weeks after thawing, up to 5 months after thawing.*

5. *Seed the cells for experiments:* The day before the experiment is planned, bring the cells to a density of  $1.5\text{--}2.5 \times 10^5$  viable cells/ml in complete culture medium.
6. Plate 0.5 ml per well in a 24-well plate for IL-18 assessment or 0.1 ml per well in 96-well plate for the MTT assay (Basic Protocol 2).

**GUIDELINES FOR MAKING DILUTIONS OF TEST SUBSTANCES**

Below are general guidelines for working with stock compounds for testing. To minimize waste, the following should be kept in mind for volume calculations: (a) each concentration should be tested in quadruplicate (i.e., four wells per concentration of each compound); (b) PPD (at a final concentration of 60 µg/ml) should be included for each experiment (also in quadruplicate) as a positive control; (c) test each chemical on three separate occasions (different days).

Conduct all treatments under sterile conditions.

**Materials**

Solid and liquid compounds  
Dulbecco's phosphate-buffered Saline (DPBS)  
Dimethyl sulfoxide (DMSO)  
*p*-phenylenediamine (PPD)  
RPMI 1640 culture medium (complete)  
Laboratory balance (accuracy 0.1 mg)

1. For solid compounds, assume that 1 g of solid compound equals 1 ml volume contribution.

*For example, if maximum solubility (either practical or desired) of a compound is 500 mg/ml, weigh 500 mg compound and add 500 µl vehicle to obtain 500 mg/ml.*

2. For liquid compounds, weigh the compound and add vehicle until a total volume of 1 ml is reached.

**IMPORTANT NOTE:** *Treat viscous compounds as solid compounds!*

3. Final concentrations (in culture medium) higher than 1.0 mg/ml will not be tested. Prepare fresh dilutions of each test compound for each experiment. Depending on solubility, dissolve chemicals either in D-PBS or in DMSO [final in-well vehicle concentration: <0.2% (v/v) DMSO].

*Never filter the chemical solutions.*

*Choice of vehicle (PBS or DMSO): Choose the vehicle which results in highest maximum solubility. When maximum solubility is the same for both vehicles, select PBS over DMSO.*

4. Prepare fresh PPD as a positive control for each experiment. Make a 30 mg/ml stock solution in DMSO and dilute stock solution 500× with complete culture medium before use.

**MTT ASSAY FOR ASSESSMENT OF CELL VIABILITY (RANGE FINDING STUDY)**

Prior to initiating treatments, perform two independent MTT experiments to determine the CV80% (i.e., the concentration of the tested chemical resulting in 80% viability). For downstream assays, we suggest that final compound concentrations (in culture medium) not exceed 1 mg/ml. This protocol assumes that NCTC cultures have been established (Basic Protocol 1) and compound selection established, and compound dilutions prepared (Support Protocol 1).

**Materials**

NCTC 2544 cells  
RPMI 1640 culture medium (complete)  
Dulbecco's phosphate-buffered saline (D-PBS)  
Dimethyl sulfoxide (DMSO)

Test chemicals (see Support Protocol 1)  
 MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution  
 (see recipe)  
 Acidic isopropanol (see recipe)  
 Adjustable micropipets 2–20  $\mu\text{l}$ , 20–200  $\mu\text{l}$ , 200–1000  $\mu\text{l}$   
 Adjustable multistep pipet  
 Incubator, 37°C, 5% CO<sub>2</sub>, 95% humidity  
 Paper towels  
 ELISA plate reader equipped with 595-nm and 405- to 450-nm filters

### Day 1

1. Seed the cells in a 96-well plate using 0.1 ml of a cell suspension at  $1.5\text{--}2.5 \times 10^5$  cells/ml per well. See Figure 20.8.1 for an image of NCTC cells at 80% confluency.

*To ensure equal density of cells in all wells, swirl the pipet while adding the cells and gently tap against the side of the plate after adding the cells.*

### Day 2

2. For each of the test chemicals, prepare fresh stock solutions (see Support Protocol 1).
3. For a new chemical, twelve 1:2 serial dilutions (concentrations 1 to 12) are suggested (see Table 20.8.3).

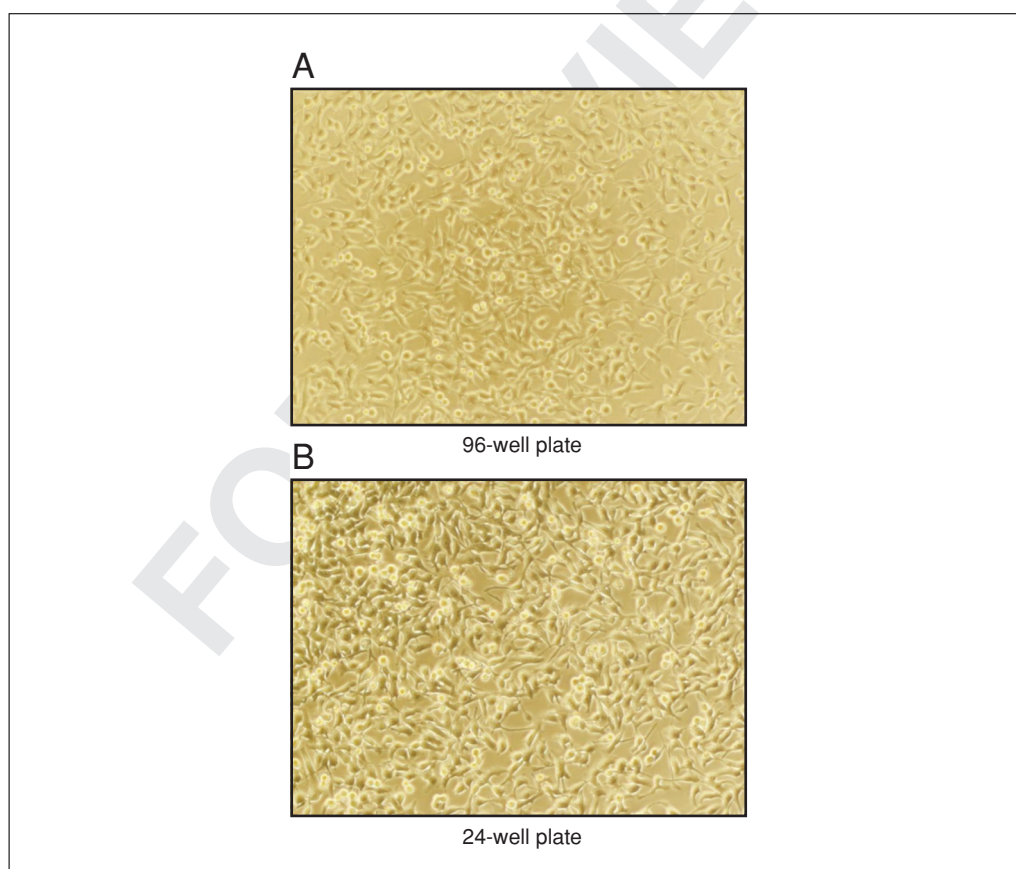


**Figure 20.8.1** Example of an 80% confluent culture of NCTC 2544 cells in a 96-well plate (**A**) and a 24-well plate (**B**). If cells reach confluency at the moment of treatment, the ability to identify contact allergens is lost; therefore, a careful check for the optimal cell density using PPD, as reference contact allergen, is critical. Inversion microscope, magnification 20 $\times$ .

Table 20.8.3 Plate Configuration for MTT Assay<sup>a</sup>

|   | 1       | 2       | 3       | 4       | 5       | 6       | 7       | 8       | 9       | 10      | 11      | 12      |         |
|---|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| A | Chem A  | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| B |         | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| C |         | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| D | Chem B  | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| E |         | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| F |         | Conc 12 | Conc 11 | Conc 10 | Conc 9  | Conc 8  | Conc 7  | Conc 6  | Conc 5  | Conc 4  | Conc 3  | Conc 2  | Conc 1  |
| G | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  | Medium  |
| H | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle | Vehicle |

<sup>a</sup>Medium indicates cells treated with culture medium alone, while vehicle indicates cells treated with 0.2% (v/v) final DMSO concentration or culture medium alone when chemical is dissolved in PBS. A total of two chemicals can be tested in one plate.

4. Prepare working solutions by adding 6 µl of the stock solutions to 3 ml culture medium. Never filter the chemical solutions.
5. For vehicle control, 12 µl DMSO or D-PBS is added to 6 ml culture medium. Discard culture medium and add 0.1 ml per well of culture medium containing increasing concentrations of the tested chemicals or vehicle control.
6. Incubate the cells for 24 hr ± 1 hr at 37°C, 5% CO<sub>2</sub>, 95% humidity.

### Day 3

7. Discard the culture medium by aspiration, add 100 µl MTT solution (0.75 mg/ml final concentration in culture medium) per well and incubate for 3 hr at 37°C. Discard the MTT solution, tap on a paper towel **very carefully** to remove excess MTT and lyse the cells in 0.1 ml acidic isopropanol per well.
8. Mix thoroughly using a multichannel pipet or place on a shaker (gently shaking-plate settings) for 10 min.

NOTE: *Check for homogeneity.*

9. Read OD at 570 nm in a microplate reader.
10. Express the results as % of cell viability versus vehicle cells (100%).
11. *Medium and vehicle:* Calculate the OD mean from the twelve replicates for each plate.

*Tested compound: calculate the OD mean from the three replicates from each tested concentration. The viability % is calculated relative to the mean of vehicle:*

$$\text{Viability}(\%) = \left( \frac{\text{average OD compound}}{\text{average OD vehicle}} \right) \times 100\%$$

12. Calculate the CV80 by linear regression analysis of data.

## BASIC PROTOCOL 3

### CELL TREATMENT FOR INTRACELLULAR IL-18 PRODUCTION

Once treatment conditions have been established (see Basic Protocols 1 and 2), cells may be treated. In this protocol, we describe the general workflow for treating cells and preparing lysates for IL-18 analysis by ELISA (Basic Protocol 4). To determine intracellular IL-18 content, seed NCTC cells in 24-well plates. After overnight adherence, incubate the cells with four increasing concentrations of the selected chemicals or their corresponding vehicles for 24 hr. Refer to Support Protocol 1 for information on preparing compounds for treatments.

#### Materials

NCTC 2544 cells  
 Dimethyl sulfoxide (DMSO)  
 RPMI 1640 culture medium (complete)  
*p*-phenylenediamine (PPD)  
 Dulbecco's phosphate-buffered saline (D-PBS)  
 Triton X-100  
 Ice  
 24-well plate  
 Adjustable micropipets 2-20 µl, 20-200 µl, 200-1000 µl  
 Adjustable multi-step pipet

Incubator, 37°C, 5% CO<sub>2</sub>, 95% humidity  
–80°C freezer

### Day 1

1. Seed the cells in a 24-well plate (0.5 ml of a cell suspension of  $1.5\text{--}2.5 \times 10^5$  cells/ml; until ~80% confluency is reached on day 2). See Figure 20.8.1 for an image of NCTC cells at 80% confluency.

*To ensure equal density of cells in all of the wells, swirl the pipet while adding the cells and gently tap against the side of the plate after adding the cells.*

### Day 2

2. The highest concentration tested corresponds to the one resulting in 80% cell viability as determined during the range finding assay (average of two independent assays).
3. Prepare fresh stock solutions for each of the test chemicals. Use four 1:2 serial dilutions (A–D).
4. Prepare working solutions by adding 6 µl of the stock solution or DMSO to 3 ml culture medium.
5. Prepare freshly a 30 mg/ml PPD stock solution in DMSO for the positive control. Add 6 µl of the 30 mg/ml solution to 3 ml culture medium to obtain a working solution.
6. Discard the culture medium by aspiration and treat cells with 0.5 ml culture medium containing chemical, vehicle, or positive control (see Table 20.8.4).
7. Incubate the cells for 24 hr at 37°C, 5% CO<sub>2</sub>, 95% humidity.

### Day 3

8. After 24 hr of incubation, remove the supernatant by aspiration, wash the monolayer gently once with 0.5 ml D-PBS (37°C) and lyse the cells in 0.25 ml cold D-PBS containing 0.5% Triton X-100.
9. Before harvesting the cell lysates by pipetting up and down, keep the plates on ice for 15 min.
10. Store the cell lysates (the 24-well plate) at –80°C, until assessment of IL-18 (Basic Protocol 4) and total protein (Support Protocol 2).
11. Once thawed, keep the samples on ice to avoid protein degradation as no protease inhibitors are used.

*Avoid multiple freeze-thaw cycles.*

**Table 20.8.4** Plate Configuration for IL-18 Treatment

|                      |                      |                      |                      |                               |                              |
|----------------------|----------------------|----------------------|----------------------|-------------------------------|------------------------------|
| Chemical 1<br>Conc A | Chemical 1<br>Conc B | Chemical 1<br>Conc C | Chemical 1<br>Conc D | Positive control <sup>a</sup> | Vehicle control <sup>b</sup> |
| Chemical 1<br>Conc A | Chemical 1<br>Conc B | Chemical 1<br>Conc C | Chemical 1<br>Conc D | Positive control              | Vehicle control              |
| Chemical 1<br>Conc A | Chemical 1<br>Conc B | Chemical 1<br>Conc C | Chemical 1<br>Conc D | Positive control              | Vehicle control              |
| Chemical 1<br>Conc A | Chemical 1<br>Conc B | Chemical 1<br>Conc C | Chemical 1<br>Conc D | Positive control              | Vehicle control              |

<sup>a</sup>Positive control: PPD 60 µg/ml final concentration.

<sup>b</sup>Vehicle control indicates cells treated with 0.2% (v/v) final DMSO concentration or culture medium alone if chemical is dissolved in PBS.

**Table 20.8.5** Plate Configuration of IL-18 MTT Assay

|   | 1                    | 2                | 3      | 4      | 5      | 6      | 7      | 8      | 9      | 10     | 11 | 12 |
|---|----------------------|------------------|--------|--------|--------|--------|--------|--------|--------|--------|----|----|
| A | Vehicle <sup>a</sup> | PPD <sup>b</sup> | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| B | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| C | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| D | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| E | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| F | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| G | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |
| H | Vehicle              | PPD              | Conc 4 | Conc 3 | Conc 2 | Conc 1 | Conc 4 | Conc 3 | Conc 2 | Conc 1 |    |    |

<sup>a</sup>Vehicle control indicates cells treated with 0.2% (v/v) final DMSO concentration or culture medium alone if chemical is dissolved in PBS. A total of four chemicals can be tested on one plate.

<sup>b</sup>Positive control: PPD (60 µg/ml final concentration).

12. In addition, perform a MTT assay (see Basic Protocol 2) to check for cell viability. Use the same controls and concentrations as used for the IL-18 assay (see Table 20.8.5).

*At least one concentration tested should give a CV of ~80%. If that is not the case, the experiment should be repeated (except for chemicals that did not result in a CV80 during the dose finding experiments).*

#### **BASIC PROTOCOL 4**

#### **DETERMINING INTRACELLULAR IL-18 CONTENT BY ELISA**

Intracellular IL-18 is assessed by sandwich ELISA. This type of ELISA is based on consecutive incubation steps with different antibodies and the samples of interest, resulting in visualization—via a substrate reaction—of the amount of IL-18 protein present in the cell lysates. The detailed steps for this procedure are below; briefly, the process is as follows.

In the first step, the ELISA plate is coated with a coating antibody directed against IL-18 epitope(s). To avoid nonspecific binding of the antibodies, blocking must be performed. Subsequently, samples are added to the plate for incubation, along with a recombinant IL-18 standard curve (all on the same plate). During this incubation step, the IL-18 protein, present in the cell lysates, binds to the coating antibody. Unbound protein is then washed away and the IL-18 protein detected with an IL-18 specific biotinylated antibody. Incubation with streptavidin-HRP allows for binding to biotin, followed by incubation with the TMB substrate, which produces a deep blue color during the enzymatic degradation by HRP. Quantitation is subsequently possibly by measuring optical density (OD) at 595 nm or 655 nm—or, if the substrate reaction is stopped by the addition of sulfuric acid, 450 nm. Results may be expressed as pg/mg of total intracellular protein content, determined by the BCA method (Support Protocol 2).

All standards should be run in duplicate; single points are acceptable for samples. Based on intracellular IL-18 concentration obtained testing, the following prediction model may be applied.

If the fold increase in intracellular IL-18 concentration is statistically significant,  $\geq 1.2$  the chemical is classified as contact sensitizers (R43). If the fold increase in intracellular IL-18 concentration is  $< 1.2$ , the chemical is classified as non-contact sensitizer. The positive control meets the acceptance if the fold increase in intracellular IL-18 is  $> 1.5$  compared to vehicle treated cells. For a given chemical, the same classification must be

obtained in at least two out of three independent experiments. The 1.2-fold increase is intended for at least one of the concentrations tested

### **Materials**

Anti-human IL-18 monoclonal antibody, 1 mg/ml  
Coating buffer: 100 mM carbonate/bicarbonate buffer in water, pH 9.6  
Ice  
Wash buffer: PBS + 0.01% Tween 20  
Blocking and dilution buffer : PBS + 1% BSA  
Concentrated and working standards (see recipes)  
Anti-human IL-18 biotinylated monoclonal Ab, 0.5 mg/ml  
Streptavidin-conjugated polyhorseradish peroxidase (HRP)  
3, 3',5 ,5'-Tetramethylbenzidine (TMB) liquid substrate for ELISA  
Stop solution: 1 M H<sub>2</sub>SO<sub>4</sub> in distilled H<sub>2</sub>O  
Adjustable micropipets: 2-20 µl, 20-200 µl, 200-1000 µl  
Adjustable multi-step pipet  
96-well flat-bottom, ELISA plates  
Plate sealer  
4°C incubator  
Paper towels  
ELISA plate reader equipped with 595-nm and 405- to 450-nm filters  
Additional reagents and equipment for measuring total cell protein (see Support Protocol 2)

#### **Day 1: Coat the wells with capture antibody**

1. Dilute the IL-18 capture antibody (mouse anti-human IL-18 monoclonal antibody) 1:1000 in coating buffer: 10 µl capture antibody + 10 ml coating buffer (final concentration 1 µg/ml).
2. Pipet in each well 100 µl of the diluted IL-18 capture antibody.
3. Seal the 96-well plate and incubate overnight at 4°C.

#### **Day 2: Block the plate with blocking buffer**

4. Bring all reagents (except antibodies) to room temperature and place the plates at room temperature. Thaw the samples and the IL-18 standards (and keep on ice).
5. Remove the capture antibody and wash three times, each time with ?? ml wash buffer. For the last wash, remove any residual buffer by tapping on a paper towel.
6. Block the plate with blocking buffer. Pipet 200 µl blocking buffer into each well. Seal the plate and incubate 1 hr at room temperature.
7. ??Prepare the standards??, quality control samples and dilutions for samples during the 1 hr incubation with the dilution buffer.

*Samples are diluted in such a way that the OD values will be in the linear part of the standard curve. The recombinant IL-18 is diluted in dilution buffer. In Table 20.8.6, an example is given regarding the serial dilution of recombinant IL-18 antibody required for standard IL-18 curve for two ELISA plates.*

8. Remove the blocking buffer and wash three times, each time with ?? ml wash buffer. For the last wash remove any residual buffer by tapping on paper towel.

#### **Incubation of standard dilutions, quality controls and sample dilutions**

9. Pipet 100 µl standards dilutions from 0 pg/ml (dilution buffer) to 1000 pg/ml, and diluted samples per well.

**Table 20.8.6** Dilution of Recombinant IL-18 Antibody Required for Standard IL-18 Curve

| Final conc. IL-18 STD | X µl of rec IL-18 standard at 500 ng/ml | X µl of previous dilution | X µl of dilution buffer |
|-----------------------|-----------------------------------------|---------------------------|-------------------------|
| 1000 pg/ml            | 5 µl                                    |                           | 2495 µl                 |
| 500 pg/ml             | —                                       | 500 µl of the 1000        | 500 µl                  |
| 250 pg/ml             | —                                       | 500 µl of the 500         | 500 µl                  |
| 125 pg/ml             | —                                       | 500 µl of the 250         | 500 µl                  |
| 62,5 pg/ml            | —                                       | 500 µl of the 125         | 500 µl                  |
| 31,25 pg/ml           | —                                       | 500 µl of the 62,5        | 500 µl                  |
| 15,6 pg/ml            | —                                       | 500 µl of the 31,25       | 500 µl                  |

10. Seal the plate and incubate for 2 hr at room temperature.
11. Remove the standard and samples and wash three times, each time with ?? ml wash buffer. For the last wash, remove any residual buffer by tapping on paper towel.

***Incubate with biotin-labeled detection antibody***

12. Prepare the detection antibody (mouse anti human IL-18 biotinylated monoclonal antibody) by diluting the detection antibody at 1:2000 in dilution buffer: 5 µl detection antibody + 10 ml dilution buffer (final concentration 250 pg/ml).
13. Pipet 100 µl detection antibody into each well
14. Seal the plate and incubate for 1 hr at room temperature.

15. Remove the detection antibody and wash three times, each time with ?? ml wash buffer. For the last wash, remove any residual buffer by tapping on a paper towel.

***Incubate with Streptavidin-HRP conjugate***

16. Prepare the streptavidin-HRP by diluting the streptavidin-HRP conjugate 1:5000 in dilution buffer: 2 µl streptavidin-HRP conjugate + 10 ml dilution buffer.
17. Pipet 100 µl streptavidin-HRP conjugate into each well.
18. Seal the plate and incubate 30 min at room temperature.
19. Remove the streptavidin-HRP conjugate and wash three times, each time with ?? ml wash buffer. For the last wash, remove any residual buffer by tapping on paper towel.

***Add TMB substrate solution***

20. Pipet 100 µl TMB substrate solution into each well.
21. Incubate the plate (without plate sealer) ~8 to 20 min at room temperature in the dark.
22. Read the plate, without stopping the reaction, at 595 nm.
23. Alternatively, add 50 µl stop solution into each well. Read absorbance at 450 nm.
24. From the raw data (measured absorbance values at OD 595 or 450 nm) calculate the mean absorbance for each set of duplicate standards and samples.

*R<sup>2</sup> of the standard curve should be >0.95.*

*OD values of the samples should be within the linear part of the standard curve. When samples do not meet this criteria, they should be re-tested, using a different sample dilution. During the ELISA, also re-test the vehicle and positive control.*

### Analyze the data

25. Measure total cell protein from remaining lysate (Support Protocol 2)
26. Express the relative amount of IL-18 protein present in the cell lysates in pg/ml.
27. Express the result as pg/mg of total cell protein as assessed by the BCA protein determination method.
28. Calculate the pg/mg by the following equation:

$$\text{IL-18} = \left[ \frac{\text{IL-18} \left( \frac{\text{pg}}{\text{ml}} \right) \text{ in cell lysate}}{\text{Total protein content} \left( \frac{\text{mg}}{\text{ml}} \right) \text{ in cell lysate}} \right] = \text{pg/mg}$$

29. Include the positive control in each run. The positive control meets the acceptance if the fold increase in intracellular IL-18 is  $> 1.5$  compared to vehicle-treated cells (if the tested chemical is dissolved in PBS or water, the relative control cells for PPD will be the same control cells used for the chemical even if PPD stock solution is dissolved in DMSO. The intracellular IL-18 content of naïve cells or treated with DMSO is very similar).
30. Also, perform an unpaired, independent samples *t*-test with a 95% confidence interval percentage to confirm that the positive control is significantly different compared to the vehicle.

*If the fold increase of at least one chemical concentration in intracellular IL-18 is  $\geq 1.2$ , the chemical is classified as contact sensitizer (R43).*

*The increase in intracellular IL-18 (pg/mg) of the chemical must be statistically significant in the Dunnett's multiple comparison test.*

*If the fold increase in intracellular IL-18 is  $> 1.2$ , the chemical is classified as noncontact sensitizer.*

*For a given chemical, the same classification must be obtained in two out of three independent experiments.*

*A 20% variation among replicates is considered acceptable.*

### TOTAL PROTEIN DETERMINATION

The protein determination of the samples must be performed parallel to the ELISA. Total protein content of the cell lysates is determined by the bicinchoninic acid (BCA) method. The BCA assay is a biochemical assay for the assessment of total protein concentration, similar to Lowry protein assay, Bradford protein assay or biuret reagent. The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to protein concentration, which can then be measured using colorimetric techniques.

#### Materials

- Protein standard curve (1.0 mg/ml  $\rightarrow$  0.04 mg/ml)
- BSA for standard curve
- Lysis buffer (0.5% Triton X-100)
- Bicinchoninic acid solution (BSA; Sigma)
- Copper(II) Sulfate solution (Sigma)
- 96-well plate
- Plate reader with 540- to 560-nm absorbance filter

### SUPPORT PROTOCOL 2

Alternative  
Methodologies in  
Toxicology

### 20.8.13

**Table 20.8.7** Serial Dilution of BSA Required for Standard BSA Curve

| Final conc. BSA | X µl of previous dilution                                  | X µl of PBS containing 0.5% Triton X-100 |
|-----------------|------------------------------------------------------------|------------------------------------------|
| 1 mg/ml         | Dissolve 3 mg BSA in 3 ml PBS containing 0.5% Triton X-100 |                                          |
| 0.8 mg/ml       | 800 µl of the 1.0 mg/ml                                    | + 200 µl                                 |
| 0.6 mg/ml       | 600 µl of the 1.0 mg/ml                                    | + 400 µl                                 |
| 0.4 mg/ml       | 500 µl of the 0.8 mg/ml                                    | + 500 µl                                 |
| 0.2 mg/ml       | 500 µl of the 0.4 mg/ml                                    | + 500 µl                                 |
| 0.1 mg/ml       | 500 µl of the 0.2 mg/ml                                    | + 500 µl                                 |
| 0.04 mg/ml      | 200 µl of the 0.2 mg/ml                                    | + 800 µl                                 |

1. Prepare a standard curve from 1.0 mg/ml → 0.04 mg/ml BSA in PBS containing 0.5% Triton X-100 (lysis buffer).

*Lysis buffer is used to be able to compare samples with standards.*

*In Table 20.8.7 an example is given regarding the serial dilution of BSA required for standard BSA curve for two 96-wells plates. Perform the standard in duplicate.*

2. Prepare a BCA buffer containing 12 ml BCA and 240 µl copper sulfate solution. Store in the dark and use within a couple of hours.
3. Pipet 50 µl standard or undiluted cell lysate per well of a 96-well plate.
4. Add 100 µl BCA buffer per well. Incubate the plate ~30 to 60 min at room temperature in dark. Read absorbance at 540 to 560 nm.

*From the raw data (absorbance values) the analysis program calculates the mean absorbance for each set of duplicate standards and samples. Sample concentration (mg/ml) is then computed in the linear section of the BSA standard curve by using a linear/linear interpolation.*

## REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

### **Acidic isopropanol preparation, 1:24 (v/v)**

Dilute 1 ml of 1 N HCl in 23 ml of isopropanol  
Store up to 1 month at room temperature

### **Concentrated standard**

Reconstitute recombinant human IL-18 25 µg in 250 µl distilled water Vortex gently to mix. Allow the standard to equilibrate for at least 15 min. Divide into 20-µl aliquots in polypropylene tubes (final concentration 100 µg/ml). Store up to 2 years at – 20°C.

### **MTT solution**

Prepare a 7.5 mg/ml solution MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in PBS  
Vortex for 10 min  
Filter with a 0.22-µm syringe filter  
Dilute the MTT solution 1:10 in culture medium before use  
Store up to 15 days at 4°C, protected from light

*continued*

**NOTE:** MTT is light sensitive. Protect from light using aluminum foil. Prewarm to 37°C before use and protect from light until use.

### **Working standard**

Dilute the concentrated IL-18 standard 1: 200 by adding 995 µl distilled water to 5 µl of the 100 µg/ml aliquot (see recipe for concentrated standard).

Divide into 50- µl aliquots (twenty aliquots in total; final concentration 0.5 µg/ml)

Store up to 2 years at –20°C

## **COMMENTARY**

### **Background Information**

Chemical allergy describes adverse health effects, resulting from the stimulation of specific immune response by chemicals. The two most frequent manifestations of chemical-induced allergies are contact hypersensitivity and respiratory sensitization, both of which can have serious impact on the quality of life, and represent a common occupational health problem (Wahlberg, 1996). Contact hypersensitivity can occur as a result of exposure to a wide variety of chemicals and certain drugs (Nethercott and Holness, 1989), cosmetics (De Groot, 1988; Remaut and Thune, 1992; Andersen, 1993), and various metals including nickel (Picardo et al., 1990) and chromium (Nethercott and Holness, 1989). Chemical respiratory allergy, although less common than allergic contact dermatitis, is of considerable significance, not only because asthma resulting from sensitization can be severe, but at times it can also be fatal.

At present, the identification of potential sensitization of chemicals is carried out using animal models, such as the guinea pig maximization test (Magnusson and Kligman, 1969) and the local lymph node assay (Kimber and Basketter, 1992). However, in the screening of new chemicals for human use, it should be very important both from safety and economic point of view to have biological markers to discriminate allergy and irritation events that have different impact on human health. Furthermore, due to the new policy on chemicals (REACH), in the European Union, in vitro methods will play a major role in the near future. Over the last decade, several in vitro methods have been proposed to identify the potential of chemicals to induce skin sensitization (Vandebriel et al., 2005; Ryan et al., 2007), but no accepted in vitro method for the identification of sensitizing chemicals is yet available.

Both skin resident cells, such as keratinocytes (KC), Langerhans cells (LC) and mast cells, and immigrating leucocytes, including T lymphocytes and natural killer cells,

actively participate in contact allergy (Cavani et al., 2007). KC play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, which plays a role in LC migration, and T cell trafficking, through the height of the inflammatory phase with direct interactions with epidermotrophic T cells, through the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T cells (Gober and Gaspari, 2008). Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the KC and skin organotypic culture as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified.

### **Critical Parameters and Troubleshooting**

Compound solubility, chemical reactivity, and metabolic activation may mask the potential allergenicity of some chemicals. It is, however, important to note that NCTC have been demonstrated to possess the phase I activities 7-ethoxycoumarin O-deethylase (ECOD), 7-ethoxyresorufin O-deethylase (EROD), and 7-pentoxeresofurin O-depenthylase (PROD), and the phase II enzymes glutathione S-transferase, aldehyde dehydrogenase and quinine reductase. These enzyme were all expressed and inducible by 3-methylcholantrene (Gelardi et al., 2001).

The measurements are to be performed within the described cytotoxic range (CV80 relative to vehicle treated cells). As maximal test concentration, 1000 µg/ml is used. We chose not to test chemicals at higher concentrations to avoid false-positive response due to unspecific induction of stress response or contaminants in the test chemical preparation.

Critical points identified in the performance of the NCTC assay are the cell density (Corsini

et al., 2009; Galbiati et al., 2011) and the time after thawing when start experiments. If cells reach confluence at the moment of treatment, the ability to identify contact allergens is lost; therefore, a careful check for the optimal cell density using PPD, as reference contact allergen, is critical. In our hands, a cell density of  $1\text{--}2.5 \times 10^5$  cells/ml gave optimal stimulation. Due to the use of different fetal bovine serum, a different cellular doubling time can be expected in the different laboratories. Therefore, we suggest that each laboratory should check for the optimal cell density, using the positive control PPD as a reference compound. Regarding time after thawing, cells should be used between 3 weeks after thawing, up to 5 months after.

In order to avoid protein degradation, it is recommended to keep the samples on ice during the procedure of thawing and plating. Cell lysates should avoid repeated freeze-thaw cycles. Samples should be stored at  $-80^\circ\text{C}$ .

Among experiments, differences in the absolute value of IL-18 were observed, also in vehicle-treated cells. In control cells, historically, the intracellular IL-18 content varied from 300 to 2500 pg/mg. This may be due to differences in the number of cells seeded and adhering to plate wells, to the use of a different cell batch, as well as to differences in the IL-18 ELISA and protein assessment performance, which may result in over- or down-estimation of IL-18 content. For this reason, the stimulation index is calculated. As long as cells do not reach confluency and are used after 3 weeks from thawing, the discriminatory capacity is preserved independently from the absolute value of IL-18.

Working with human cells and tissue is inherently dangerous. Working with human cells is recommended to take place in a specially equipped laboratory by people who have had the proper training. The use of biosafety level II cabinets is highly recommended.

Store chemicals in safety cupboards. Apply special storage conditions, if necessary (e.g., special temperature, protect from light, etc.). Working with chemical substances that are likely to cause irritation and/or sensitization of the skin and respiratory system is inherently dangerous. Chemicals must be handled with maximum care.

Handling waste and disposal of waste must be concerned carefully. Try to avoid overproduction of waste as much as possible, without compromising the quality of the experiments performed. Avoid mixing different chemical waste categories because of dangerous inter-

actions between chemicals and risk for explosion. Dispose of possibly biohazardous material accordingly.

It is highly recommended that the workers involved get special training in safety issues. In some countries it might be necessary to draft a contract for waiving of liability. National and local laws may differ between countries. Each principal investigator is responsible for the correct adherence to the law applicable.

## Anticipated Results

Intracellular IL-18 was investigated after exposure of NCTC cells to contact allergens, respiratory allergens, and irritants. It has been shown that exposure of NCTC cells to contact allergens results in a dose-related induction of intracellular IL-18, whereas exposure to respiratory allergens and irritants fails to induce IL-18 production, indicating the possibility to use IL-18 to identify contact allergens (Corsini et al., 2009; Galbiati et al., 2011). IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization and to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as  $\text{TNF-}\alpha$ , IL-8 and  $\text{IFN-}\gamma$ , (Okamura et al., 1995; Cumberbatch et al., 2001; Antonopoulos et al., 2008). IL-18 has no apparent role in irritant contact dermatitis (Antonopoulos et al., 2008), indicating that the role of IL-18 in contact hypersensitivity is not simply part of a general requirement for IL-18 in skin inflammation. Furthermore, Hartwig et al. (2008) demonstrated using IL-18-deficient mice that the absence of IL-18 does not affect any of the asthma-specific parameters, including allergen-specific IgE, histological changes of the lungs, infiltrating leukocytes, serum cytokine levels and airway hyper responsiveness.

It has been investigated if skin sensitizers could selectively up-regulate this cytokine in human KC and it has been demonstrated that contact sensitizers, including pre/pro-haptens, increase intracellular IL-18 in a dose-related manner. In contrast, irritants and respiratory allergens failed to up-regulate IL-18 (Corsini et al., 2009; Galbiati et al., 2011).

The measure of the release of IL-18 did not offer any advantage, since a passive release of this constitutively expressed cytokine could be observed at high concentrations with both allergens and irritants, likely to be due to a cell damage effect. Also, the evaluation of IL-18 mRNA expression did not provide an additional advantage. KC constitutively express high IL-18 mRNA levels, which could

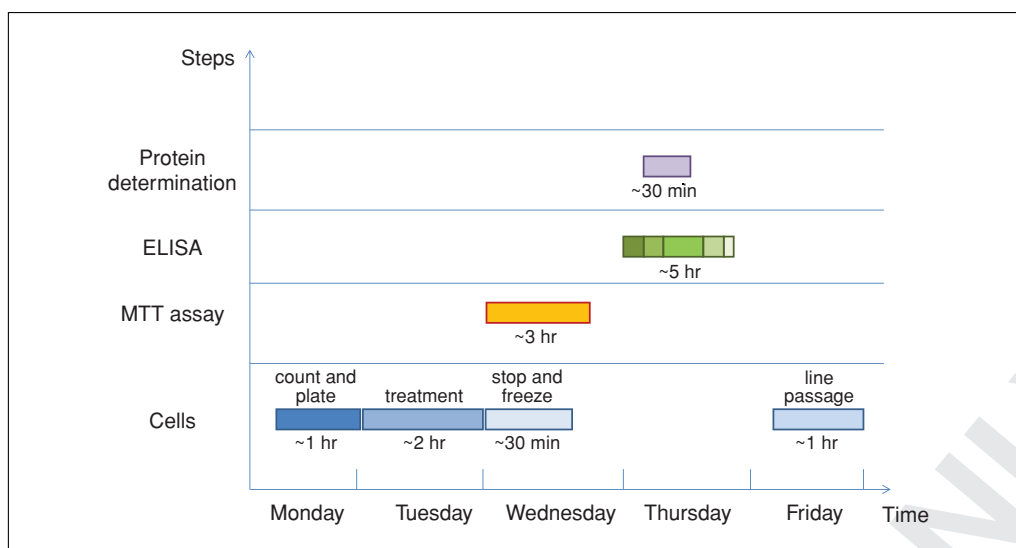


**Figure 20.8.2** Time consideration for the protocols. Counting and plating of the cells can be done on Monday; treatment can be completed on Tuesday and line passage on Friday (about 4 hr in a week). MTT can be done on Wednesday (about 3 hr) and ELISA on Thursday (about 5 hr plus half an hour for protein determination).

not be modulated further following treatment with contact allergens as assessed by real-time PCR. The latest finding is consistent with data obtained by Naik et al. (1999) in primary human KC treated with DNCB.

Cultures of adult human KC are readily available, even commercially, but differences in metabolism and response to stimuli can exist between cultures derived from different donors (Berghard et al., 1990); therefore, established lines of human KC represent a good alternative. Similar results are also obtained using both the NCTC cell line and primary human KC, confirming the relevance of the proposed model and the possibility to use a different source of KC (Corsini et al., 2009).

The NCTC cell line not only compares well with other in vitro models such as reconstructed epidermis and primary cultured KC, but have even better metabolic properties. The characterization of phase I and phase II metabolism expressed in these cells gives sustained evidence for their suitability to in vitro toxicology studies (Gelardi et al., 2001).

In regards to relationship with potency, there is a general trend for IL-18 induction at lower concentrations for extreme/strong sensitizers whereas higher concentrations are needed in the case of weak sensitizers. However, the Pearson correlation between in vitro EC1.2 ( $\mu\text{g/ml}$ ) and in vivo LLNA EC3 (%) has a correlation coefficient ( $r$ ) = 0.4787, with a  $p$  value of 0.0711 (considered not quite significant).

To summarize, we propose the NCTC assay as a screening, replacement, reduction test for hazard identification and general classification and labeling of skin sensitizers (R43 or no label). Our data suggest that IL-18 production by NCTC cells may represent a promising in vitro model for the screening of potential contact allergens. The test may also be complementary to the LLNA, allowing for a rapid and easy discrimination of contact sensitizers from respiratory allergens. Results were reproducible, and the method could be transferred to other laboratories, suggesting the potential use of the test in immunotoxicity testing strategies. The assay is currently under prevalidation in a two-tiered approach project (*Prevalidation of a novel tiered approach to determine the skin sensitizing capacity and potency of chemicals*) sponsored by the Dutch association ZonMw. It is, however, important to stress that due to the complexity of hypersensitivity reactions and the chemical diversity of allergens, a more realistic approach should consider several markers of activation and different cell types, such as dendritic-like cells activation.

### Time Considerations

See Figure 20.8.2 for information about the duration of tasks for this method.

### Literature Cited

??Andersen, 1993

Antonopoulos, C., Cumberbatch, M., Mee, J.B., Dearman, R.J., Wei, X.Q., Liew, F.Y., Kimber,

- I., and Groves, R.W. 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. *J. Leukoc. Biol.* 83:361–367.
- Berghard, A., Gradin, K., and Toftgard, R. 1990. Serum and extracellular calcium modulate induction of cytochrome P-450IA1 in human keratinocytes. *J. Biol. Chem.* 265:21086–21090.
- ??Cavani et al., 2007
- Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L., and Marinovich, M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* 23:789–796.
- Cumberbatch, M., Dearman, R.J., Antonopoulos, C., Groves, R.W., and Kimber, I. 2001. Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor- $\alpha$ - and IL-1 $\beta$ -dependent mechanism. *Immunology* 102:323–330.
- De Groot, D.M. 1988. Comparison of methods for the estimation of the thickness of ultrathin tissue sections. *J. Microsc.* 151:23–42.
- Galbiati, V., Mitjans, M., Lucchi, L., Viviani, B., Galli, C.L., Marinovich, M., and Corsini, E. 2011. Further development of the NCTC 2544 IL-18 assay to identify in vitro contact allergens. *Toxicology In Vitro* 25:724–732.
- Gelardi, A., Morini, F., Dusatti, F., Penco, S., and Ferro, M. 2001. Induction by xenobiotics of phase I and phase II enzyme activities in the human keratinocyte cell line NCTC 2544. *Toxicol. In Vitro* 15:701–711.
- ??Gober and Gaspari, 2008
- Hartwig, C., Tschernig, T., Mazzega, M., Braun, A., and Neumann, D. 2008. Endogenous IL-18 in experimentally induced asthma affects cytokine serum levels but is irrelevant for clinical symptoms. *Cytokine* 42:298–305.
- Kimber, I. and Basketter, D.A. 1992. The murine local lymph node assay: a commentary on collaborative studies and new directions. *Food Chem. Toxicol.* 30:165–169.
- Magnusson, B. and Kligman, A.M. 1969. The identification of contact allergens by animal assay. The guinea pig maximization test. *J. Invest. Dermatol.* 52:268–276.
- Naik, S.M., Cannon, G., Burbach, G.J., Singh, S.R., Swerlick, R.A., Wilcox, J.N., Ansel, J.C., and Caughman, S.W. 1999. Human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene. *J. Invest. Dermatol.* 113:766–772.
- Nethercott, J.R. and Holness, D.L. 1989. Occupational allergic contact dermatitis. *Clin. Rev. Allergy* 7:399–415.
- Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., et al. 1995. Cloning of a new cytokine that induces IFN- $\gamma$  production by T cells. *Nature* 378:88–91.
- Picardo, M., Zompetta, C., De Luca, C., Cristaudo, A., Cannistraci, C., Faggioni, A., and Santucci, B. 1990. Nickel-keratinocyte interaction: A possible role in sensitization. *Br. J. Dermatol.* 122:729–735.
- Remaut, K. and Thune, P. 1992. Occurrence of cosmetic allergy. *Tidsskr Nor Lægeforen* 112:1275–1277.
- Ryan, C.A., Kimber, I., Basketter, D.A., Pallardy, M., Gildea, L.A., and Gerberick, G.F. 2007. Dendritic cells and skin sensitization: Biological roles and uses in hazard identification. *Toxicol. Appl. Pharmacol.* 221:384–394.
- Teunis, M., Corsini, E., Smits, M., Madsen, C.B., Eltze, T., Ezendam, J., Galbiati, V., Gremmer, E., Krul, C., Landin, A., Landsiedel, R., Pieters, R., Rasmussen, T.F., Reinders, J., Roggen, E., Spiekstra, S., and Gibbs, S. 2012. Transfer of a two-tiered keratinocyte assay: IL-18 production by NCTC2544 to determine the skin sensitizing capacity and epidermal equivalent assay to determine sensitizer potency. *Toxicology In Vitro* In press.
- Vandebriel, R.J., Van Och, F.M., and van Loveren, H. 2005. In vitro assessment of sensitizing activity of low molecular weight compounds. *Toxicol. Appl. Pharmacol.* 207:142–148.
- ??Wahlberg, 1996