

# The Research Institute for Fragrance Materials' human repeated insult patch test protocol

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## Abstract

With implementation of the dermal sensitization QRA approach for fragrance ingredients, IFRA/RIFM are recommending use of the RIFM standard human repeated insult patch test (HRIPT) protocol for generation of confirmatory human data for the induction of dermal sensitization in a normal human population. Details of this standard HRIPT protocol are provided in this paper. The study protocol consists of two phases—Induction and Challenge. In the Induction phase, patches treated with fragrance ingredients in 75% diethyl phthalate/25% ethanol are applied to backs of volunteers for 24 h. Following patch removal there is a 24-h rest period and volunteers are patched again at the same site. This procedure is repeated to achieve 9 applications over a 3-week period. There is an approximate 2-week rest period followed by a Challenge phase of a single 24-h patch application of test article applied to a naïve site on the back. Skin reactions at the naïve site observed at Challenge may be suggestive of dermal sensitization, and a Rechallenge is performed to confirm the nature of the reactivity. This study is designed to confirm the No-Observed-Effect-Level for induction of dermal sensitization in a normal human population.

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## 1. Introduction

Historical human data either from human repeated insult patch tests (HRIPT) or human maximization tests are available for raw materials found in consumer products and a variety of those products. This is certainly true for fragrance ingredients.

The HRIPT is a version of the modified Draize procedure (Draize, 1959; Draize et al., 1944; Marzulli and Maibach, 1976) and is a patch test that is now used to confirm the No-Observed-Effect-Level (NOEL) for the induction of dermal sensitization in a normal human population. The protocol described here has been in use by the Research Institute for Fragrance Materials, Inc. (RIFM) for the last twenty years for the testing of individual fragrance materials.

RIFM has a historical database that contains more than 1000 HRIPTs and greater than 1200 human maximization tests conducted on individual fragrance ingredients. This includes more than 200 HRIPTs that have been conducted by RIFM using the same (RIFM standard) protocol. In addition, the RIFM database contains a significant and increasing number of murine local lymph node assays (LLNA) that can be used in combination with confirmatory human dermal sensitization data in a weight of evidence approach for establishing the No Expected Sensitization Induction Level (NESIL) that is used in a dermal sensitization Quantitative Risk Assessment (QRA) approach (Api et al., 2008).

The objective of the HRIPT protocol is to confirm, in healthy human volunteers, the dermal sensitization NOEL that has been obtained from dermal sensitization quantitative structure–activity relationships, animal pre-clinical data, and historical human data. This protocol is not used to determine hazard. The test is not used as a predictive

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method nor is it used on substances with unknown dermal sensitization potential. It is a test to confirm the lack of dermal sensitization at an exposure level which was identified as a NOEL in an animal model and/or historical human data. This methodology can also be used to assess skin irritation through repeated patching during the Induction phase.

The test articles are evaluated for the induction of dermal sensitization and irritation by repeated applications to the skin of healthy human volunteers. A review of the HRIPT design and the critical factors that can affect the induction of dermal sensitization can be found in McNamee (2008).

These HRIPT data add an important aspect to the overall evaluation of dermal sensitization, based on a weight of evidence approach, for a fragrance ingredient when conducting a QRA (Api et al., 2008). In fact, currently the HRIPT is the primary way of confirming in humans a predicted dermal sensitization NOEL from animal testing. With implementation of the QRA approach, the International Fragrance Association (IFRA) and RIFM are recommending the use of the RIFM standard HRIPT protocol for generation of confirmatory human data for use in QRA.

## 2. Protocol

### 2.1. Test subjects

A sufficient number of subjects (approximately 130), male and female between the ages of 18 and 70, are to be empanelled so that approximately 100 complete the study. The subjects are informed of the nature of the test, including possible adverse reactions. Written informed consent is obtained. Additionally, the subjects must be considered dependable and able to read, understand, and follow instructions. Prior to test initiation, each subject is to complete a medical history form. The subjects should not exhibit any physical or dermatological condition which would preclude application of the test articles. The subjects must fit all of the inclusion and exclusion criteria listed in Table 1.

### 2.2. Test articles

The amount of test article applied should be expressed as the quantity of chemical per unit area of the skin,  $\mu\text{g}/\text{cm}^2$ , and as a percentage. A vehicle that contains ethanol (EtOH) must be used to dilute the test article. The preferred vehicle of RIFM is 75% diethyl phthalate (DEP)/25% EtOH. In addition to the test article, the test subject should also be patched with the vehicle control and a saline control. It has been well documented that the vehicle in which an allergen is presented to the skin has an effect on its skin-sensitizing potency (Kligman, 1966; Lalko et al., 2004; Stotts and Ely, 1977). The DEP and EtOH combination in use by RIFM was selected because the majority of fragrance materials are soluble in DEP and EtOH, it is representative of the matrix often used in commercial products, and the 3:1 DEP/EtOH ratio was selected since on rare occasions increasing levels of EtOH can induce sensitization in humans (Stotts and Ely, 1977).

### 2.3. Experimental design

#### 2.3.1. Induction phase

The quantity of test article applied per test patch is 0.3 ml or 0.3 g. The test articles are dispensed onto 25 mm Hilltop Chamber patches (Hill Top Research, Miami, OH) and the patches are applied to normal skin between the left scapula and the spinal mid-line. Hill Top Chamber

patches are composed of a flexible molded plastic chamber with a double rim that fits close to the skin and the chamber is lined with a nonwoven Webril pad. The patch is held in place with semioclusive tape. Fresh patches of test article are prepared daily. Following application of test article to the patch, the patch must be applied to the skin a minimum of 15 min after preparation but not longer than 40 min after preparation. Sample preparation and volatilization times should be documented.

Test article patch applications to the same patch site are generally made on Monday, Wednesday, and Friday for three consecutive weeks. The test subjects are instructed to return to the Testing Facility on Tuesday and Thursday (approximately 24 h after patch application) for supervised removal of patches. Test subjects are instructed to remove patches that were applied on Friday approximately 24 h after patch application. Twenty-four-hour rest periods follow Tuesday and Thursday removals and 48-h rest periods follow each Saturday removal. The patch sites are scored by a trained evaluator just prior to the next patch application according to the Draize scoring system (Draize et al., 1944) modified by Phillips et al. (1972) and further modified by McNamee (2008, Table 2). This procedure is repeated until nine Induction applications of the test article are made.

Test subjects are free to withdraw from the study at any time or they may be disqualified by the Testing Facility if in the opinion of the Principal Investigator clinical observations indicate it would be unwise for them to continue. A minimum of nine Induction patches are required in order to satisfactorily complete the Induction phase of the study. Test subjects are permitted a maximum of one missed scheduled patch application during the Induction phase of the study which must be made up during the Induction phase of the study. Test subjects will be discontinued by the Testing Facility if they miss more than one Induction patch application and the corresponding make-up patch application. Test subjects are permitted to miss one regularly scheduled supervised patch removal, and they are instructed to remove the patches themselves at home approximately 24 h following application. Test subjects will be discontinued by the Testing Facility if they miss more than one supervised patch removal. Discontinued subject data are reported up to the point of discontinuation, but their data are not used in the results, discussion, or conclusion sections of the final report.

The patch sites are scored by a trained evaluator according to the criteria referenced above (Table 2). Accompanying edema (swelling) or other dermal sequelae is recorded and described as mild, moderate, or severe. If a test subject develops a positive reaction of a 2-level (moderate) or greater during the Induction phase, the patch will then be applied to an adjacent naïve (previously unpatched) site for the next application. If a 2-level or greater reaction occurs at this naïve site, no further Induction applications are made. Reactive subjects are to be subsequently patched with the test article at a naïve test site during the Challenge phase of the study with the rest of the panel, unless in the opinions of the Investigators it would be unwise for them to do so (due to very strong reactions).

#### 2.3.2. Challenge phase

Ten to fourteen days after application of the last Induction patch, a single Challenge patch is applied to a naïve site of normal skin on the contralateral side of the back. Test subjects are instructed to return to the Testing Facility 24 h later for removal of the patch by the trained evaluator. The Challenge site is scored 24, 48, and 72 h after application by a trained evaluator, with both a clinical dermatologist and the study monitor present at the 72-h reading. The evaluators and clinical dermatologist must not know the identity of the test articles, vehicle or negative controls. Test subjects are asked to report any delayed reactions that might occur after the final Challenge patch reading. Test subjects who miss the Challenge patch application are discontinued from the study.

#### 2.3.3. Rechallenge

Test subjects who exhibit skin reactivity suggestive but not clearly indicative of induced allergic contact dermatitis during the Challenge phase of the study are requested to participate in a Rechallenge procedure following a four week rest period. The Rechallenge procedure consists of

Table 1  
Inclusion and exclusion criteria

*Inclusion criteria*

1. Males or females, age 18 to 70 years of age and in good general health (not more than 20% of the panel should be greater than 65 years of age)
2. Individuals of any skin type or race provided their degree of skin pigmentation does not significantly interfere with evaluations
3. Individuals free of any systemic or dermatological disorder including known allergies to skin care products or topical drugs, or, other medical conditions which, in the opinion of the investigator, might interfere with the conduct of the study, interpretations of the results, or increase the risk of adverse reactions
4. Individuals able to read, understand, and provide written informed consent
5. Individuals who are believed to be dependable, who agree to complete the course of the study, and comply with instructions

*Exclusion criteria*

1. Women who are self-reported pregnant, nursing or planning a pregnancy
2. Individuals with a history of any dermatological disease or condition, including but not limited to active atopic dermatitis, psoriasis, eczema, active seasonal allergies or skin cancer within the past 6 months
3. Individuals with abnormal skin pigmentation at the test sites which might interfere with subsequent evaluations of dermal responsiveness
4. Individuals taking medications which might interfere with the test results, including any regimen of steroidal/non-steroidal anti-inflammatory drugs, antihistamines or immunosuppressive drugs
5. Individuals who have applied any type of topical anti-inflammatory medication to the test sites within two weeks prior to enrollment
6. Individuals with any other skin condition that would interfere with the conduct of the study
7. Individuals who have undergone a bilateral mastectomy with lymph node removal, a unilateral mastectomy with lymph node removal within the last year, or a bilateral axillary lymph node removal
8. Individuals with a history of immune deficiency or auto-immune disease
9. Individuals who are currently receiving allergy injections, have received allergy injections within one week prior to enrollment, or expect to begin receiving allergy injections during the study
10. Individuals treated for malignancy within 6 months prior to enrollment
11. Individuals who are currently under treatment for asthma or diabetes
12. Current enrollment in any other research study or participation in a patch test study within 30 days prior to the start of this study
13. Individuals who have ever participated on a patch test study with a cologne or a perfume
14. Individuals who are known tape/adhesive reactors

Table 2  
HRIPT scoring scale

Erythema scale

This scale is used only for grading degree of erythema (redness). A score on this scale will be assigned following every application of a test material

- 0 No visible erythema
- 1 Mild erythema (faint pink to definite pink)
- 2 Moderate erythema (definite redness)
- 3 Severe erythema (very intense redness)

Designations for elevated responses

Edema, papules, vesicles, and bullae, if present, are graded as independent responses

- E Edema—definite swelling
- P Papules—many small, red, solid elevations; surface of reaction has granular feeling
- V Vesicles—small circumscribed elevations having translucent surfaces so that fluid is visible (blister-like); vesicles are no larger than 0.5 cm in diameter
- B Bullae—vesicles with a diameter >0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site

Other responses/recording characteristics

- S Spreading—evidence of the reaction beyond the pad area (does not include obvious signs of leakage of test material away from pad)
- W Weeping—evidence of release of fluid from a vesicular or bullous reaction
- A Marked reaction to adhesive (patch relocated)
- X Succeeding patch not applied and succeeding grade is for residual reaction
- L Patch lost (came off) during first 12 h

both an occlusive and semi-occlusive 24-h back patches of the test article, vehicle control, and saline control to naïve sites with readings of the patch test sites at 24, 48, 72, and 96 h post-patch applications. If the reactivity is still inconclusive, the test subject undergoes a use test, in which an open application of the test article to the antecubital fossa of either the left or right arm is repeated 3 times per day for 5 days, with the first application performed at the Testing Facility.

### 3. Discussion

The results of the HRIPT protocol are presented as the number of induced sensitization reactions observed out of the total number of volunteers who completed the study. The results of the individual patch sites are presented in tabular format.

The induction of dermal sensitization is determined by enhancement of the skin reaction observed at Challenge greater than that observed during Induction. Low grade reactions observed during Induction but which are not observed at Challenge are considered to be irritant in nature. If a volunteer has an erythematous and edematous reaction during the early part of the Induction phase that is confirmed at Challenge, the subject is considered to be presensitized and the reaction is not considered having been induced during the Induction phase.

If a test subject reacted during the earliest part of the Induction phase and returns to confirm the reactivity at Challenge, the subject is considered to have exhibited

reactions indicative of presensitization to the test material. This subject is considered to have completed that study but is not included in the number of induced sensitization reactions.

An erythematous and edematous reaction to the test article (and none to the vehicle or negative control) during Challenge that is confirmed at Rechallenge is indicative of induction of dermal sensitization. If no dermal sensitization is induced, then the concentration of test article tested is determined to be a NOEL for the induction of dermal sensitization. Since the HRIPT is used to confirm a NOEL, it will determine the negative or the lack of a sensitization reaction. As such, 'a' NOEL for the induction of sensitization may be determined, not necessarily 'the' NOEL, since the method is not used to define a Lowest-Observed-Effect-Level (LOEL) or NOEL on the dose response curve. The NOEL may be determined if there are historical data in humans which provide a known LOEL.

To ensure an adequate safety evaluation, approximately 100 subjects must complete the entire HRIPT protocol. Henderson and Riley (1945) investigated statistical calculations of patch tests adapted for the detection and evaluation of chemical agents. If no reactions were observed in a group of 100 test subjects, then the rate of positive reactions in a larger population is not likely to exceed 2.9%, based on a confidence level of 95%, under identical conditions (Henderson and Riley, 1945). The likely maximum rate of 2.9% positive reactions is often misinterpreted to mean that there would be an expected rate of 2.9% in the marketplace. The test conditions in the HRIPT are not identical to real life scenarios. To increase the sensitivity of the test whilst using such numbers of subjects, if appropriate one generally tests a higher concentration of test material and usually more exaggerated exposure conditions than would actually be encountered in intended and foreseeable use situations among the general population. Other factors that further increase the sensitivity and reliability of the test, in some HRIPT protocols, are exaggerated through possible minor skin irritation of a test material, use of occluded patches, and vehicle effects from the test conditions (Basketter et al., 2006; McNamee et al., 2008).

The induction of human dermal sensitization from the HRIPT is rare. Hall (2006) estimated the rate of dermal sensitization induction to be 0.09% of volunteers in tests on cosmetic products. In addition, Hall (2006) identified there has been no evidence of adverse sequelae from these tests.

## Conflict of Interest

Valerie T. Politano is an employee of the Research Institute for Fragrance Materials an independent research institute supported by the manufacturers of fragrances and consumer products containing fragrances.

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