발 간 등 록 번 호
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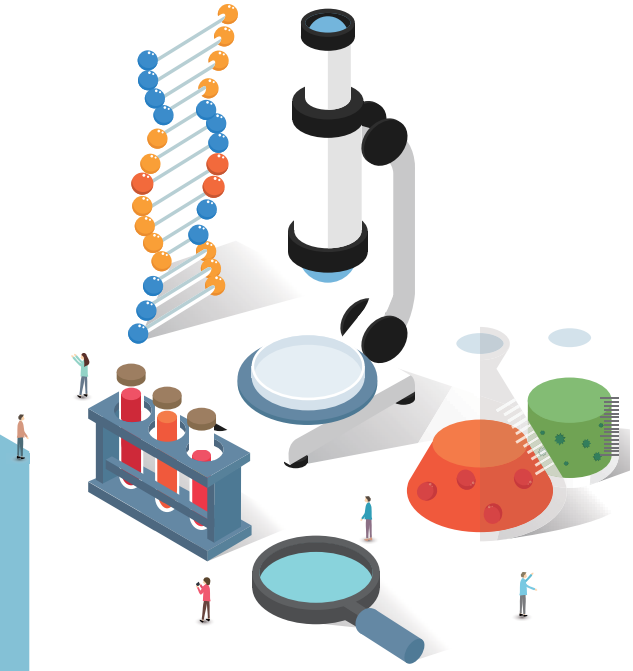
| 민원인 안내서 |

국민 안심이 기준입니다
YOUR SAFETY IS OUR STANDARD

화장품 등 피부감작성 동물대체시험법 가이드라인

국소림프절시험법:BrdU-ELISA

2021. 8.



식품의약품안전처
식품의약품안전평가원

지침서 · 안내서 제 · 개정 점검표

명칭	화장품 등 피부감작성 동물대체시험법(국소림프절시험법: BrdU-ELISA) 가이드라인 (민원인 안내서)

아래에 해당하는 사항에 체크하여 주시기 바랍니다.

등록대상 여부	□ 이미 등록된 지침서 · 안내서 중 동일 · 유사한 내용의 지침서 · 안내서가 있습니까?	□ 예 ■ 아니오
	☞ 상기 질문에 '예'라고 답하신 경우 기존의 지침서 · 안내서의 개정을 우선적으로 고려하시기 바랍니다. 그럼에도 불구하고 동 지침서 · 안내서의 제정이 필요한 경우 그 사유를 아래에 기재해 주시기 바랍니다. (사유 :)	
	□ 법령(법 · 시행령 · 시행규칙) 또는 행정규칙(고시 · 훈령 · 예규)의 내용을 단순 편집 또는 나열한 것입니까?	□ 예 ■ 아니오
	□ 단순한 사실을 대외적으로 알리는 공고의 내용입니까?	□ 예 ■ 아니오
	□ 1년 이내 한시적 적용 또는 일회성 지시 · 명령에 해당하는 내용입니까?	□ 예 ■ 아니오
	□ 외국 규정을 번역하거나 설명하는 내용입니까?	□ 예 ■ 아니오
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	□ 대내외적으로 법령 또는 고시 · 훈령 · 예규 등을 알기 쉽게 풀어서 설명하거나 특정한 사안에 대하여 식품의약품안전처의 입장을 기술하는 것입니까? (민원인용)	■ 예(☞안내서) □ 아니오
기타 확인사항	□ 상위 법령을 일탈하여 새로운 규제를 신설 · 강화하거나 민원인을 구속하는 내용이 있습니까?	□ 예 ■ 아니오
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상기 사항에 대하여 확인하였음.

2021년 8 월 31일

담당자 강남희

확 인(부서장) 김광진

이 안내서는 화장품 등 피부감작성 동물대체시험법(국소림프절시험법: BrdU-ELISA) 가이드라인에 대하여 알기 쉽게 설명하거나 식품의약품안전처의 입장을 기술한 것입니다.

본 안내서는 대외적으로 법적 효력을 가지는 것이 아니므로 본문의 기술방식('~하여야 한다' 등)에도 불구하고 민원인 여러분께서 반드시 준수하셔야 하는 사항이 아님을 알려드립니다. 또한, 본 안내서는 2021년 8월 현재의 과학적·기술적 사실 및 유효한 법규를 토대로 작성되었으므로 이후 최신 개정 법규 내용 및 구체적인 사실관계 등에 따라 달리 적용될 수 있음을 알려드립니다.

※ "민원인 안내서"란 대내외적으로 법령 또는 고시·훈령·예규 등을 알기 쉽게 풀어서 설명하거나 특정한 사안에 대하여 식품의약품안전처의 입장을 기술하는 것 (식품의약품안전처 지침서등의 관리에 관한 규정 제2조)

※ 본 안내서에 대한 의견이나 문의사항이 있을 경우 식품의약품안전평가원 독성평가연구부 특수독성과에 문의하시기 바랍니다.

전화번호: 043-719-5153, 5155
팩스번호: 043-719-5150

제·개정 이력

연번	제·개정번호	승인일자	주요내용
1	B1-2013-4-001	2013.3.	화장품 독성시험 동물대체시험법 가이드라인(Ⅳ) 제정
2	안내서-0751-01	2017.5.	「식약처 지침서등의 관리에 관한 규정」 개정에 따른 일괄 재분류 (규제개혁담당관실-3761호,2017.5.16)
3	안내서-1144-01	2021.8.	제목을 “화장품 등 피부감작성 동물대체시험법 (국소림프절시험법: BrdU-ELISA) 가이드라인”으로 수정, 내용 정비 및 OECD 가이드라인 영문본을 추가하여 개정

▸▸ Contents

Ⅰ 개 요

본 시험법은 피부감작성의 독성발현경로(AOP, Adverse Outcome Pathway) 중 네 번째 핵심 단계(key event)인 T-세포의 활성화와 증식을 평가하는 방법으로서 UN GHS 기준에 따라 시험물질의 피부감작성을 평가하는 국소림프절시험법(LLNA: BrdU-ELISA)이다.

본 시험법은 피부감작성 반응 중 유도기(induction phase)에 나타나는 반응을 측정하는 것으로 시험물질 적용 부위와 가까운 림프절 내에서 림프구의 증식 수준을 나타내는 5-Bromo-2-deoxyuridine(BrdU) 양을 ELISA 방법을 이용해 정량적으로 측정하여 피부감작물질을 구별한다.

LLNA: BrdU-ELISA 시험법은 기니픽 시험(TG 406)과 비교 시, 사용되는 동물의 수를 감소시킬 수 있으며 야기(challenge)에 의해 유도되는 피부 과민반응 유발이 필요하지 않고, 보조제(adjuvant)를 사용하지 않기 때문에 동물의 고통을 줄일 수 있는 장점이 있다.

Ⅱ 시험원리

LLNA: BrdU-ELISA 시험법의 기본 원리는 피부감작성 시험물질에 의한 적용 부위와 가까운 림프절 내에서 유발되는 림프구의 증식을 평가하는 것이다. 이러한 림프구의 증식은 시험물질 적용 후 이개 림프절(auricular lymph node)에서 증식된 세포의 증가를 나타내는 BrdU 양을 측정하여 평가한다. BrdU는 티미딘(thymidine)의 유사체로서 증식하는 세포의 DNA에 들어가 결합하며, BrdU의 결합(incorporation)은 과산화효소로 표지된 BrdU를 특이적으로 인지하는 항체를 사용하는 ELISA 방법으로 측정한다. 시험결과는 부형제대조군의 평균 증식에 대한 시험물질군의 평균 증식의 비율인 감작지수(Stimulation Index, SI)로 나타내며, 시험물질을 피부감작물질로 판정하기 위해서는 SI 지수가 1.6 이상($SI \geq 1.6$)이어야 한다.

Ⅲ 제한점 및 고려사항

시험을 수행하기 전 시험물질의 특성 및 화학 구조, 물리화학적 성질, 생체외(*in vitro*) 또는 생체내(*in vivo*) 독성시험 결과, 구조적으로 유사한 물질의 독성시험 결과 등 시험물질에 대해 모든 가능한 정보를 고려하여 LLNA: BrdU-ELISA 시험법이 해당 시험물질에 적합한지를 결정해야 한다.

특정 금속물질, 일부 계면활성제 유형의 물질(피부자극물질인 경우 빈번하게 위양성 결과가 나타남), 용해도가 매우 낮거나 불용성 물질, 잠재적 교란기능의 작용기를 포함하는 시험물질류(test chemical classes) 또는 단일 시험물질(substances) 등의 경우 LLNA 시험법 적용에는 제한이 있기 때문에 기니픽 시험(TG 406)이 필요할 수 있다.

본 시험법의 검증 데이터베이스에 따르면 LLNA: BrdU-ELISA 시험법에서 위양성 결과가 나온 비감작성 물질은 감작지수 1.6~1.9의 값을 나타냈다(예: 양성값 경계). 따라서 감작지수 값이 1.6~1.9일 때, 비감작성물질에서 양성값 경계 결과가 나올 수 있는 가능성을 고려해야 한다.

Ⅳ 시험방법

4.1 실험동물 및 시험물질 준비

실험동물은 CBA/JN 마우스 계통이 선호되며 임신 및 출산 경험이 없는 8~12주령의 건강한 암컷 마우스를 사용하고, 마우스의 체중은 평균 체중의 20%를 초과해서는 안 된다. 마우스는 실험실 환경에 순화를 위해 최소 5일간 케이지에 키우고 육안상 피부 병변이 없음을 확인한다.

고체시험물질은 마우스에 적용하기 전에 적절한 용매/부형제에 용해시키거나 현탁액을 만들고, 필요한 경우 이를 희석해야 한다. 액체시험물질은 원물질 그대로 사용하거나 또는 희석하여 사용한다. 의료기기에서 일반적으로 나타나는 불용성 시험물질들은 적절한 용매를 사용하여 용출 가능한 성분들이 모두 용출되도록 하는 과장용출법(exaggerated extraction)[1]으로 추출한다. 용매/부형제는 아세톤 : 올리브 오일(AOO, 4:1 v/v), N,N-디메틸포름아미드(N,N-dimethylformamide), 메틸에틸케톤(methyl ethyl ketone), 프로필렌글리콜(propylene glycol), 디메틸설폭사이드(dimethyl sulfoxide)가 권장되며 충분한 과학적 근거가 제시되는 경우 다른 용매/부형제도 사용할 수 있다.

시험물질의 농도는 보통 적절히 연속되는 3개의 농도를 선정한다(예: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% 등). 시험물질과 관련된 기존의 모든 독성정보(예: 급성 독성 및 피부 자극), 구조 및 물리화학적 정보(해당 정보가 없는 경우 구조적으로 유사한 시험물질의 정보) 등을 고려하여 투여용량을 설정해야 한다. 이와 같은 정보가 없는 경우 예비시험을 실시해야 한다.

음성대조물질은 시험물질을 용해한 용매/부형제를 사용하고(부형제대조군), 양성대조물질은 아세톤 : 올리브오일(acetone: olive oil, AOO, 4:1, v/v)에 희석한 25% 헥실시나믹알데히드(hexyl cinnamic aldehyde, HCA)와 25% 유제놀(eugenol)이 권장된다. 동시 양성대조군을 포함하는 것을 권장하지만 LLNA: BrdU-ELISA 시험을 정기적으로 수행하고(한 달에 최소 한 번 이상), 양성대조군의 재현성 있는 정확한 결과를 도출하는 능력을 보여주는 배경자료를 확립한 실험실의 경우, 양성대조군의 주기적인 시험(예: 6개월 이하 간격)도 가능하다(별첨1-12항 참조).

시험 시 마우스는 군당 최소 4마리를 사용하고, 매 시험마다 최소 3개 농도의 시험물질군, 부형제대조군 및 양성대조군을 포함한다.

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1) 과장용출법(exaggerated extraction): 모의 사용 용출법(simulated-use extraction)*에서 용출되는 양과 비교하여 더 많은 양의 화학적 조성물을 용출하기 위한 용출 방법.
* 제품의 사용방법을 모의한 용출법

4.2 예비시험

최고 투여용량을 결정하기 위한 정보가 없는 경우 본 시험법에서 적절한 투여용량을 결정하기 위해 예비시험을 수행해야 한다. 시험의 최대 투여용량은 액체 시험물질의 경우 100% 농도를 사용하며, 고체 시험물질 또는 현탁액의 경우 용해 가능한 최대 농도를 사용해야 한다.

예비시험은 LLNA: BrdU-ELISA 본시험과 동일한 조건에서 수행되며 단, 림프절 증식은 평가하지 않고 투여용량군당 한 마리 또는 두 마리의 마우스를 사용한다. 모든 마우스의 일반증상은 매일 관찰하고 체중은 시험 전 및 종료 전(6일차)에 측정하여 기록한다(표 1). 또한 각 마우스 양쪽 귀의 홍반을 관찰하여 표 2에 따라 점수화한다. 본시험의 최고 투여용량은 예비시험에서 사용된 농도 중 전신 독성이나 과도한 국소 피부 자극을 유발하지 않는 농도 중 두 번째로 높은 농도를 선택한다.

표 1. 예비시험 일정

	1일	2일	3일	4일	5일	6일
일반증상 관찰	O	O	O	O	O	O
체중 측정	O					O
귀두께 측정	O		O			O
홍반 평가		O	O	O	O	O
물질 도포	O	O	O			
안락사						O

표 2. 홍반에 따른 부여 점수

관찰 및 측정항목	점수
홍반이 없음	0
매우 가벼운 정도의 홍반(거의 인지하기 어려움)	1
명확히 나타나는 홍반	2
중등도 이상의 홍반	3
딱지가 생성되어 홍반 수준을 결정하기 어려운 심각한 홍반(빨개짐)	4

4.3 본시험

1일차에는 각 마우스의 체중, 귀두께 및 모든 일반증상을 관찰하여 기록한다. 시험물질 희석액 25 μL, 부형제 또는 양성대조물질(실험실 방침에 근거한 동시 또는 최근에 사용된 양성대조물질)을 양쪽 귀의 배면(귓등) 전체에 바른다. 2일차에는 1일차의 적용 절차를 반복하고, 일반증상 및 홍반을 관찰한다. 3일차에는 1일차의 적용 절차를 반복하고, 귀두께를 측정하며 일반증상 및 홍반을 관찰한다. 4일차에는 일반증상 및 홍반을 관찰하고, 아무것도 처리하지 않는다. 5일차에는 BrdU(10 mg/mL) 용액(마우스당 5 mg) 0.5 mL을 복강 내에 주사하고, 일반증상 및 홍반을 관찰한다. 6일차에는 각 마우스의 체중, 귀두께, 홍반 및 일반증상을 관찰하여 기록하며, 5일차에 투여한 BrdU 용액이 적용된 후 약 24시간이 경과하면 동물을 안락사 시킨다. 각 마우스에서 시험물질을 도포한 부위의 이개 림프절을 절개하고 인산완충용액(PBS)에 넣어 보관한다(표 3).

표 3. 본시험 일정

	1일	2일	3일	4일	5일	6일
일반증상 관찰	O	O	O	O	O	O
체중 측정	O					O
귀두께 측정	O		O			O
홍반증상 평가		O	O	O	O	O
물질 도포	O	O	O			
BrdU 용액 주사					O	
안락사						O

림프절 단일세포 현탁액은 200 마이크로 메쉬 스테인리스 스틸 거즈를 통한 부드러운 물리적인 분해 방법이나 또는 단일 세포 현탁액을 만들 수 있는 다른 기법 등을 이용하여 준비한다. 세포 현탁액을 anti-BrdU 항체와 반응시킨 후 마이크로 플레이트리더를 이용하여 370nm와 492nm(참조파장)에서 흡광도를 측정한다.

4.4 결과 평가

(1) BrdU 표지 지수(BrdU labelling index)는 다음과 같이 계산한다.

$$\text{BrdU 표지 지수} = (ABS_{em} - ABS\ blank_{em}) - (ABS_{ref} - ABS\ blank_{ref})$$

* ABS= 흡광도, em= 방출 파장, ref= 참조 파장

(2) 각 시험물질군의 결과는 평균 감작지수(SI)로 나타낸다. 감작지수는 각 시험물질군과 양성대조군 마우스의 평균 BrdU 표지 지수를 부형제대조군의 평균 BrdU 표지 지수로 나누어 계산한다.

$$\text{감작지수(SI)} = \frac{\text{시험물질군 또는 양성대조군의 평균 BrdU 표지 지수}}{\text{부형제대조군의 평균 BrdU 표지 지수}}$$

V 결과 판정

5.1 결과 판정

판정기준은 평균 감작지수가 1.6 이상(SI ≥ 1.6)일 때 결과를 양성으로 간주한다.

감작지수(SI)	판정
≥1.6	피부감작성
<1.6	비감작성

하지만 양성값 경계 결과(예: SI 값 1.6~1.9)에 대해서는 이러한 결과 값이 양성임을 확인하기 위해 SI 값과 함께 용량-반응 상관성 정도, 전신 독성 또는 과도한 자극의 증거, 통계적 유의성(해당되는 경우) 등의 추가 정보를 고려할 수 있다.

5.2 신뢰성 확인

양성대조군을 사용하여 재현성 있는 결과를 보여줌으로써 본 시험이 적절하게 수행되었는지 확인한다. 양성대조군은 SI가 14를 초과하는(>14) 과도한 피부자극 또는 전신독성을 나타내지 않는 농도에서 부형제대조군 대비 SI가 1.6 이상(≥1.6)으로 양성 반응을 나타내야 한다.

VI 시험결과 및 보고

데이터는 개별 마우스 BrdU 표지 지수 값, BrdU 표지 지수의 그룹 평균, 관련 오차 (예: 표준편차(SD) 또는 표준오차(SEM)), 용매/부형제대조군에 대한 각 투여군의 평균 SI를 나타내는 표 형식으로 요약한다. 시험결과보고서에는 다음의 내용을 포함하도록 한다.

시험물질

- 공급원, 로트 번호, 사용기한(가능한 경우), 시험 물질의 안정성(알려진 경우)
- 단일성분물질의 경우 화학물질 식별정보, 물리적 형태, 수용성, 추가적인 관련 물리화학적 특성
- 다성분물질, UVBCs 및 복합물질의 경우 구성성분의 화학물질 정보, 정량적 비율, 관련 물리화학적 특성 등 가능한 자세히 특성 규명
- 용매/부형제 및 대조군의 식별 데이터(예: CAS 번호, 순도 등)
- 부형제 선정의 타당성에 대한 근거

실험동물

- 마우스의 출처, 수와 주령, 사육 조건, 식이 정보, 미생물학적 상태(알려진 경우) 등

시험조건

- ELISA 키트의 공급원, 로트 번호, 제조자의 품질 보증/품질관리 데이터

- 시험물질 준비와 적용 등에 관한 세부사항
- 투여 용량 선정의 타당성에 대한 근거(수행된 경우, 예비시험에 대한 결과 포함)
- 사용된 부형제와 시험물질 농도, 적용된 시험물질의 총량
- 양성 또는 음성 판정 기준

신뢰성 확인

- 최근 실시한 신뢰성 확인 결과 요약(사용된 시험물질, 농도, 양성대조군, 부형제대조군 등)
- 실험실의 동시 또는 과거 양성대조군과 동시 부형제대조군 데이터

결과

- 도포 시작 시점 및 예정된 안락사 시 개별 마우스 체중, 각 처리군의 평균 및 관련 오차(예: SD, SEM)
- 독성 증상(도포 부위의 피부 자극 등) 및 발생 경과
- 각 처리군의 개별 마우스 BrdU 표지 지수, SI 값, 평균 및 관련 오차
- 용량-반응 상관성, 이상치(outliers) 분석 결과, 통계 분석(적절한 경우)

결과 토의

- 시험 결과, 용량-반응 상관성 분석, 통계분석에 대한 간단한 해설과 시험물질의 피부감작성 물질 분류에 대한 결론

별첨 1 번역본(OECD TG 442B)

국소림프절시험법: BrdU-ELISA
Local Lymph Node Assay: BrdU-ELISA

생체내(*in vivo*) 피부감작성: 국소림프절시험법: BrdU-ELISA

초기 고려사항, 적용가능성 및 제한점

1. LLNA: BrdU-ELISA 시험법은 검증 및 검토가 이루어지고 독립적인 국제 전문평가 위원단에 의해 일부 제한점을 가지면서 피부 감작성 및 비감작성 물질을 식별하는데 유용한 시험법으로 추천되었다(1)(2)(3).

2. LLNA: BrdU-ELISA 시험법은 특정 제한점을 가지면서 잠재적인 피부 감작성 시험물질을 식별하기 위한 변형된 비방사성 LLNA 시험법(a modified non-radioactive LLNA method)이다. 이는 생체내(*in vivo*) 시험이 필요한 모든 경우에 방사성 LLNA (TG 429) 또는 기니픽 시험(TG 406)(4)을 대신하여 LLNA: BrdU-ELISA 시험법을 사용해야 한다는 의미가 아니라, LLNA: BrdU-ELISA 시험법이 동일한 기능을 가지며 양성 및 음성 결과에 일반적으로 더 이상 추가적인 확인이 필요하지 않은 대체시험법으로 사용할 수 있는 것이다(1)(2). 시험을 수행하는 실험실은 시험 전 시험물질에 대해 모든 가능한 정보를 고려해야 한다. 이러한 정보에는 시험물질의 특성 및 화학 구조, 물리화학적 성질, 생체외(*in vitro*) 및 생체내(*in vivo*) 독성시험 결과, 구조적으로 유사한 물질의 독성시험 결과 등이 포함된다. LLNA: BrdU-ELISA 시험법이 해당 시험물질에 적합한지(일부 화학물질의 경우 LLNA: BrdU-ELISA 시험법을 사용할 수 없음[3항 참조])를 결정하는 것과 투여용량을 설정하는데에 이러한 정보를 참고해야 한다.

3. LLNA: BrdU-ELISA 시험법은 생체내 시험법으로서, 알레르기성 접촉 감작 활성(allergic contact sensitising activity)의 평가에 동물 사용을 배제할 수 없다. 따라서 적절한 *in vitro*, *in chemico*, *in silico* 시험법의 적용 가능 범위를 검토하여, 이에 따라 동물 시험 대신에 이러한 접근방식의 사용 가능성을 고려해야 한다. 하지만 다른 LLNA 시험법과 마찬가지로, LLNA: BrdU-ELISA 시험법은 기니픽 시험(TG 406)(4)과 비교 시, 알레르기성 접촉 감작 활성을 평가하는데 사용되는 동물의 수를 줄일 수 있다. 더욱이 LLNA: BrdU-ELISA 시험법은 TG 406 시험법과는 달리 야기(challenge)에 의해 유도되는 피부 과민반응 유발이 필요하지 않기 때문에 알레르기성 접촉 감작성 시험에서 동물의 사용 방식을 상당히 개선(통증 및 고통 경감)한다. 또한, LLNA: BrdU-ELISA 시험법은 기니픽 극대화 시험(4)처럼 보조제
(adjuvant)를 사용하지 않기 때문에 동물의 고통을 줄여준다. TG 406(4)에 비해 LLNA: BrdU-ELISA 시험법의 이점이 있음에도 불구하고, 일부 제한점으로 인해 TG 406 시험법이 필요한 경우가 있다(예: 특정 금속물질의 시험, 피부자극물질의 위양성 결과[일부 계면활성제 유형의 물질](5)(6), 시험물질의 용해도[거의 용해되지 않거나 불용성 물질 등]). 또한 잠재적 교란기능의 작용기를 포함하는 시험물질류(test chemical classes) 또는 단일 시험물질(substances)(예: 지방산 글루타메이트, 올레산, 올레산 에스터, 지방알코올 1, 지방알코올 2, 폴리아미노기능성 실록산(polyaminofunctional siloxane)(7))의 경우 기니픽 시험(TG 406(4))이 필요할 수 있다. LLNA 시험에 대해 확인된 다른 제한점(6)은 LLNA: BrdU-ELISA 시험법에도 적용하는 것을 권장한다(1). 이와 같이 확인된 제한점을 제외하면 시험물질의 특성이 LLNA: BrdU-ELISA 시험법의 정확도에 영향을 주지 않는 한, 모든 시험물질에 적용할 수 있다. 또한 감작지수(SI) 1.6~1.9 값이 얻어지는 경우(31~32항 참조) 양성 경계값 결과의 가능성을 고려해야 한다. 이는 SI ≥ 1.6(6항 참조) 기준을 사용하는 43개 물질의 검증 데이터베이스에 근거하고 있는데, LLNA: BrdU-ELISA 시험법은 모든 32개 LLNA 감작성 물질을 정확하게 식별했지만 비감작성 물질 11개 중 2개는 잘못 식별하였고, 그 물질들은 SI 1.6~1.9의 값을 나타냈다(예: 양성값 경계)(1). 하지만 SI 값을 설정하고 시험의 예측력을 계산하는데 동일한 데이터세트가 사용되었으므로 이 결과는 실제 예측력을 과대추정한 값일 수 있다.

4. 혼합물 또는 시험하기 어려운 화학물질(예: 불안정성) 및 본 가이드라인의 적용 범위에 명확하게 포함되지 않는 시험물질에 대해서는 시험 전에 시험이 과학적으로 의미 있는 결과를 도출하는지 우선적으로 검토해야 한다.

5. 용어정의는 부록 1에 수록되어 있다.

시험 원리

6. LLNA: BrdU-ELISA 시험법의 기본 원리는 감작성 시험물질이 적용 부위의 가까운 림프절 내에서 림프구 증식을 유발하는 것이다. 이러한 림프구의 증식은 적용하는 알레르기 유발 항원의 용량(dose) 및 감작능(potency)에 비례하기 때문에 감작성의 정량적 측정이 가능하다. 림프구 증식은 각 시험물질군과 부형제대조군의 평균 림프구 증식을 비교하여 측정한다. 부형제대조군의 평균 증식에 대한 시험물질군의 평균 증식의 비율(감작지수, Stimulation Index, SI)을 계산하며, 시험물질을 피부감작물질로 판정하기 위해서는 SI 지수가 1.6 이상(SI ≥ 1.6)이어야 한다. 본 가이드라인의 시험법은 물질 적용 부위의 이개 림프절(auricular lymph node)에서 증식된 세포수의 증가를 나타내는 BrdU 양의 측정을 기반으로 한다. BrdU는 티미딘(thymidine)의 유사체로서 증식하는 세포의 DNA에 들어가 결합한다. BrdU의 결합(incorporation)은 BrdU에 특이적인 과산화효소로 표지된 항체(antibody labelled with peroxidase)를 사용하는 ELISA 방법으로 측정한다. 이때, 과산화효소의 기질을 첨가하면 기질과 반응하여 특정 파장에서 정량 가능한 유색 결과물을 생성하고 이는 미량정량판 판독기(microtiter plate reader)를 통해 측정된다.

시험 설명

동물종 선택

7. 실험동물로는 마우스를 사용한다. LLNA: BrdU-ELISA 시험법의 검증 연구가 CBA/JN 계통으로 수행됨에 따라 해당 종이 선호되며(1)(3), 출산 및 임신 경험이 없는

암컷 마우스를 사용한다. 시험 시작 시 마우스는 8~12주령이어야 하며, 체중 편차는 최소로 하되 평균 체중의 20%를 초과해서는 안 된다. LLNA: BrdU-ELISA 시험법 결과에서 마우스의 계통이나 성별에 의한 차이가 없다는 것을 증명하는 데이터가 충분히 있는 경우 다른 계통이나 수컷을 대체하여 사용할 수 있다.

사육 및 사료조건

8. 마우스 개별 사육에 관한 과학적 근거가 별도로 제공되지 않는 한, 마우스는 그룹별(8)로 바닥이 단단한 케이지(9)에서 적절한 바닥재(substrate) 및 깔짚(10)(11)(12)(13)을 사용하여 사육해야 한다. 실험동물실의 온도는 22 ± 3℃로 유지되어야 하고, 상대습도는 최소한 30%이상 단, 가급적 70%를 넘지 않아야 하며, 실험동물실 청소 시를 제외하고 50~60%의 범위에 있어야 한다. 조명은 명/암 주기를 12시간 간격으로 설정한다. 사료는 일반적인 실험동물용 사료를 사용하며 음용수는 무제한 공급한다.

실험동물 준비

9. 마우스는 무작위로 선별하고 가급적 비침습적인 제모(14)(15)로 개체 식별을 표시하며 실험실 환경에 순화를 위해 투여 시작 전 최소 5일간 케이지에 키운다. 투여 시작 전 모든 마우스를 검사하여 육안상 피부 병변이 없음을 확인한다. 마우스는 모든 검사 과정 중 비혐오적인(non-aversive) 방법(예: cupping, tunnel handling)으로 다루어야 한다(16).

시험 용액의 조제

10. 고체시험물질은 마우스에 적용하기 전 적절한 용매/부형제에 용해시키거나 현탁액을 만들고, 필요한 경우 이를 희석해야 한다. 액체시험물질은 원물질 그대로 사용하거나 또는 희석하여 사용한다. 의료기기에서 일반적으로 나타나는 불용성

시험물질들은 적용하기 전에 적절한 용매를 사용하여 용출 가능한 성분들이 모두 용출되도록 하는 과장용출법(exaggerated extraction)을 사용해야 한다(35). 시험물질 보관에 대한 안정성 자료가 없는 경우 시험 당일에 조제 해야 한다.

신뢰성 확인

11. 양성대조군(PC)은 반응정도가 잘 알려진 감작성 물질의 적절하고 재현성 있는 민감도에 의해 본 시험이 적절하게 수행되었는지 증명하기 위해 사용한다. 실험실의 수행능력을 확인하고 실험실내 및 실험실간 재현성과 유사성(comparability)을 평가하기 위하여 동시 양성대조군을 포함할 것을 권고한다. 일부 규제 당국 또한 각 시험마다 양성대조군의 사용을 요구하기 때문에 LLNA: BrdU-ELISA 시험법을 수행하기 전에 관계 당국과 상의할 것을 권장한다. 따라서 양성대조군을 주기적으로 사용함에 따라 발생할 수 있는 추가적인 동물실험이 필요(규제당국의 요구사항을 충족하기 위해)하지 않도록 동시 양성대조군의 사용을 권장한다(12항 참조). 양성대조군은 감작지수가 부형제대조군 대비 1.6 이상(≥)으로 예상되는 노출 수준에서 LLNA: BrdU-ELISA 양성 반응을 나타내어야 한다. 양성대조물질의 용량은 과도한 피부자극 또는 전신독성은 야기하지 않고, 유도반응을 재현성 있고 과도하지 않게(예: SI 〉 14은 과한 것으로 간주됨) 유발하는 농도를 선택해야 한다. 선호하는 양성대조물질은 아세톤 : 올리브오일(acetone: olive oil, AOO, 4:1, v/v)에 희석한 25% 헥실시나믹알데히드(hexyl cinnamic aldehyde, HCA) (CAS 번호 101-86-0)와 25% 유제놀(eugenol) (CAS 번호 97-53-0)이다. 충분한 타당성이 제시되는 경우, 위의 기준에 부합하는 다른 양성대조물질을 사용할 수 있다.

12. 동시 양성대조군의 포함을 권장하는 반면, LLNA: BrdU-ELISA 시험을 정기적으로 수행하고(한 달에 최소 한 번 이상), 양성대조군 결과를 재현성 있고 정확하게 도출하는 능력을 입증하는 배경자료(historical PC database)를 확립한 실험실의 경우, 양성대조군의 주기적인 시험(예: 6개월 이하 간격)도 가능하다. 실험자가 적절한 기간 내에(1년 미만) 최소 10회의 독립적인 시험에서 양성대조군의 일관적인 양성 결과를 도출함으로써 LLNA: BrdU-ELISA 시험법의 숙련도를 보일 수 있다.

13. LLNA: BrdU-ELISA 시험 과정에 변경이 있는 경우(예: 숙련된 직원, 시험재료 및 시약, 시험장비, 실험동물 공급처 등의 변경) 동시 양성대조군을 반드시 포함해야 하며 변경사항은 시험보고서에 기록해야 한다. 이러한 시험 절차의 변경이 이전에 확립된 배경자료의 적절성에 어떠한 영향을 주는지 고려하여 양성대조군 결과의 일관성을 문서화 하기 위한 새로운 배경자료의 확립이 필요한지 결정해야 한다.

14. 시험자는 동시 양성대조군 시험 대신 주기적으로 시험하는 결정이 주기적 양성대조군 시험 사이에 생성된 음성 시험 결과(동시 양성대조군 없이)의 적합성(adequancy) 및 수용성(acceptability)에 영향을 준다는 것을 알아야 한다. 예를 들어, 주기적 양성대조군 시험에서 위음성 결과가 나왔다면, 주기적 양성대조군 시험결과가 나온 마지막 시험과 위음성 결과가 나온 시험 사이에 수행된 시험의 음성 결과에 대해서는 신뢰할 수 없다. 동시 양성대조군을 포함할 것인지 또는 주기적 양성대조군을 사용할 것인지를 결정할 때에는 이러한 영향을 신중하게 고려해야 한다. 동시 양성대조군에 사용되는 동물의 수를 줄이는 것이 과학적으로 타당하게 설명되고, 시험 기관이 배경자료를 근거하여 적은 수의 동물 사용이 가능하다는 것을 보여주는 경우, 동시 양성대조군의 동물 수의 감소를 고려해야 한다(17).

15. 양성대조물질의 용매는 일관된 반응을 유발하는 것으로 알려진 부형제(예: 아세톤 : 올리브오일, AOO, 4:1, v/v)를 사용해야 하지만, 규제 여건에 따라 비표준 부형제(임상적/화학적으로 관련 있는 제형)가 또한 필요할 수 있다(23). 만약 동시 양성대조군을 시험물질과는 다른 부형제를 사용하여 시험하는 경우 동시 양성대조군에 대한 부형제를 별도로 시험에 포함시켜야 한다.

16. 특정 화학물질류(specific chemical class) 또는 특정 반응 범위의 시험물질을 평가하는 경우에 있어서, 기준시험물질(benchmark test chemicals)을 사용하는 것은 본 시험법이 이런 종류의 시험물질의 피부감작능을 식별하는 데 적절하게 기능한다는 것을 입증하는데 유용할 수 있다. 적절한 기준시험물질은 다음의 특성을 가져야 한다.

- 시험물질군과 구조적 및 기능적 유사성
- 알려진 물리적/화학적 특성

- LLNA: BrdU-ELISA 시험으로 얻은 근거 자료(supporting data)
- 다른 동물 종이나 인체 시험으로 얻은 근거 자료(supporting data)

시험 방법

동물 수 및 투여용량

17. 투여용량군당 최소 4마리의 마우스를 사용하며, 최소 3개 농도의 시험물질군, 동시 부형제대조군 및 양성대조군(실험실 규정을 근거로, 11~15항에서 언급된 것을 고려하여 동시 또는 최근 사용된 양성대조군)을 준비한다. 특히, 양성대조군 시험을 가끔씩(on an intermittent basis) 수행하는 경우, 양성대조군을 여러 용량으로 시험하는 것을 고려해야 한다. 시험물질을 처리하지 않는 것을 제외하고, 대조군(control group) 마우스는 시험물질군 마우스와 동일한 방식으로 처리 및 취급해야 한다.

18. 투여용량 및 부형제 선택은 참고문헌 2와 27에 제시된 권고사항을 기반으로 한다. 3개 연속 투여용량의 경우, 보통 적절히 연속되는 농도를 선정한다(예: 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5% 등). 시험농도의 선정에는 충분한 과학적 근거가 뒷받침되어야 한다. 3개의 연속되는 농도 선정 시 가능하면 시험물질과 관련된 기존의 모든 독성정보(예: 급성 독성 및 피부 자극), 구조 및 물리화학적 정보(또는 구조적으로 관련된 시험물질)를 고려하여 전신 독성 또는 과도한 국소 피부 자극을 피하면서 가장 높은 농도로 노출을 최대화한다(19)(20). 이와 같은 정보가 없는 경우 사전 예비시험이 필요하다(21~24항 참조).

19. 부형제는 시험 결과에 영향을 주지 않아야 하며, 시험물질 적용에 적합한 용액/현탁액을 만들면서 가장 높은 농도를 얻기 위하여 용해도를 최대화 하는 것에 기반하여 선정해야 한다. 권장되는 부형제는 아세톤 : 올리브 오일(AOO, 4:1 v/v), N,N-디메틸포름아미드(N,N-dimethylformamide), 메틸에틸케톤(methyl ethyl ketone), 프로필렌글리콜(propylene glycol), 디메틸설폭사이드(dimethyl sulfoxide)(5)이며

충분한 과학적 근거가 제시되는 경우 다른 부형제를 사용할 수 있다. 특정 상황에서는 시판되는 형태의 시험물질 또는 임상적으로 적절한 용매를 추가적인 대조군으로 사용할 수 있다. 친수성 물질인 경우, 적절한 용해제(예: 1% Pluronic® L92)를 사용해서 시험물질 용액이 부형제에 혼합되어, 피부를 적시고 적용하는 즉시 흘러내리지 않도록 특별한 주의를 기울여야 한다. 따라서 완전히 수용성인 부형제는 피한다.

20. 개별 마우스 림프절 결과의 처리는 마우스 간의 변동성 평가와 시험물질군과 부형제대조군 간의 차이에 대한 통계적 비교가 가능하다(33항 참조). 또한, 양성대조군 동물 수의 감소 가능 여부에 대한 평가는 개별 마우스의 자료가 수집된 경우에만 가능하다(22). 뿐만 아니라 일부 국가의 규제 당국은 개별 마우스 데이터의 수집을 요구한다. 향후 규제 당국이 원래의 방식으로 수집한 결과(예: 집단 동물 데이터)를 나중에 다른 요구사항(예: 개별 동물 데이터)에 의해 고려하는 경우, 개별 동물 데이터를 수집하는 것이 추후 필요한 중복 시험을 피함으로써 동물 복지 측면에서 이점을 제공한다.

예비시험

21. 최고 투여용량을 결정하기 위한 정보가 없는 경우(18항 참조) LLNA: BrdU-ELISA 시험법에서 적절한 투여용량을 결정하기 위해 예비시험을 수행해야 한다. 예비시험의 목적은 전신 독성(24항 참조)이나 과도한 국소 피부자극(23항 참조)을 유발하는 농도에 대한 정보가 없는 경우 본시험의 최대 투여용량 선택에 대한 지침을 제공하는 것이다. 시험의 최대 투여용량은 액체 시험물질의 경우 100% 농도, 고체 시험물질 또는 현탁액의 경우 가능한 최대 농도이어야 한다.

22. 예비시험은 LLNA: BrdU-ELISA 본시험과 동일한 조건에서 수행되며 단, 림프절 증식은 평가하지 않고 투여용량군당 한 마리 또는 두 마리의 사용이 제안된다. 모든 마우스의 전신 독성 또는 적용 부위의 국소 자극 등 모든 일반증상을 매일 관찰한다. 체중은 시험 전 및 종료 전(6일 차)에 기록한다. 각 마우스 양쪽 귀의 홍반을 관찰하고, 표 1에 따라 점수화한다(20). 두께측정기(예: 디지털 마이크로미터 또는 피코크 다이얼

두께 측정기)를 사용하여 1일 차(적용 전), 3일 차(첫 번째 적용 약 48시간 후) 및 6일 차에 귀 두께를 측정한다. 또한 6일 차의 귀 두께는 마우스를 안락사 시킨 후 귀 부위를 이어펀치로 뚫어 무게를 측정하여 결정할 수 있다. 과도한 국소 자극은 홍반 점수 3 이상 또는 귀 두께가 25% 이상 증가한 경우를 말한다(21)(22). LLNA: BrdU-ELISA 본시험의 최고 투여용량은 예비시험에서 사용된 농도(18항 참조) 중 전신 독성이나 과도한 국소 피부 자극을 유발하지 않는 농도 중 두 번째로 높은 농도를 선택한다.

표 1: 홍반에 따른 부여 점수

관찰	점수
홍반이 없음	0
매우 가벼운 정도의 홍반(거의 인지하기 어려움)	1
명확히 나타나는 홍반	2
중등도 이상의 홍반	3
딱지가 생성되어 홍반 수준을 결정하기 어려운 심각한 홍반(빨개짐)	4

23. 귀 두께의 25% 증가(21)(22) 이외에도 부형제대조군 대비 시험물질군에서 귀 두께의 통계적으로 유의한 증가는 LLNA에서 자극을 확인하는 데 사용된다(22)(23)(24)(25)(26)(27)(28). 그러나 귀 두께의 증가가 25% 미만일 때 통계적으로 유의하게 나타날 수 있지만, 이는 특별하게 과도한 자극과 연관되지는 않는다(25)(26)(27)(28)(29).

24. 통합 평가의 일환으로 사용할 경우, 신경계 기능 변화(예: 입모, 운동실조, 떨림, 경련), 행동 변화(예: 공격성, 털 손질 행동의 변화, 활동 수준의 현저한 변화), 호흡 양상 변화(예: 호흡 곤란, 헐떡임, 수포음과 같이 호흡의 빈도 및 강도의 변화), 음수 및 섭취량 변화 등의 일반증상은 전신 독성(30)의 징후일 수 있으며 따라서 LLNA: BrdU-ELISA 본시험의 최대 투여용량을 시사할 수 있다. 또한 무기력 또는 무반응 증상, 통증과 괴로움, 경미하거나 일시적인 것 이상의 모든 일반증상, 1~6일차 5% 초과의 체중감소 및 치사율은 평가 시 고려되어야 한다. 빈사 상태의 마우스 또는 심한 통증과 고통을 보이는 마우스는 안락사 시킨다(31).

본시험 일정

25. 시험 일정은 다음과 같다.

- 1일차(Day 1):
  각 마우스의 체중 및 모든 일반증상을 관찰하여 기록한다. 시험물질 희석액 25 μL, 부형제 또는 양성대조물질(11~15항에서 고려된 것처럼 실험실 방침에 근거한 동시 또는 최근에 사용된 양성대조물질)을 각 귓등에 바른다.
- 2일차 및 3일차(Day 2, 3):
  1일차의 적용 절차를 반복한다.
- 4일차(Day 4):
  아무것도 처리하지 않는다.
- 5일차(Day 5):
  BrdU(10 mg/mL) 용액(마우스 당 5 mg) 0.5 mL을 복강 내에 주사한다.
- 6일차(Day 6):
  각 마우스의 체중 및 모든 일반증상을 기록한다. BrdU 용액을 투여하고 약 24시간 후, 동물을 안락사 시킨다. 각 마우스에서 시험물질을 도포한 부위의 이개 림프절을 절개하고 인산완충용액(PBS)에 넣어 보관한다. 림프절의 확인과 절개에 대한 도표 및 세부 사항은 참고문헌(17)을 참조한다. 본시험에서 국소 피부 반응의 관찰을 위해서는 귀 홍반의 점수화 또는 귀 두께 측정(두께 측정기를 사용하거나 해부 시 이어펀치 무게 측정)과 같은 추가적인 항목들이 시험 프로토콜에 포함될 수 있다.

세포 현탁액의 준비

26. 200 마이크로 메쉬 스테인리스 스틸 거즈를 통한 부드러운 물리적인 분해 또는 단일 세포 현탁액을 만들 수 있는 다른 기법(예: 일회용 플라스틱 막자를 사용하여 림프절을 으깨고 #70 나일론 메쉬로 거름)으로 각 마우스 양쪽에서 적출한 림프절의 단일 세포 현탁액을 준비한다. 이 시험에서는 림프절 세포 현탁액 준비 과정이

중요하므로 모든 실험자는 사전에 숙달된 기술을 갖추고 있어야 한다. 부형제대조군 마우스의 림프절은 크기가 작기 때문에 SI 값에 인위적인(artificial) 영향을 주지 않도록 세심한 작업이 필요하다. 각 경우에 림프절 세포 현탁액의 목표량(target volume)은 결정된 최적의 양(optimised volume)(약 15 mL)으로 맞춰야 한다. 최적의 양(optimised volume)은 부형제대조군의 평균 흡광도를 0.1~0.2 이내로 얻는 것에 기반한다.

세포증식 측정(림프구 DNA의 BrdU 함량 측정)

27. BrdU는 상용 키트(검증연구에서는 Roche Applied Science, Mannheim, Germany, 사용)를 사용하여 ELISA 방법으로 측정한다. 일치하는 결과를 주는 경우 다른 BrdU-ELISA 키트를 사용할 수 있다. 림프절 세포 현탁액 100 μL를 편평한 바닥을 가진 마이크로플레이트의 웰(well)에 넣는다(3개 반복시료). 림프절 세포를 변성 및 고정한 후 페록시다아제가 결합된 anti-BrdU 항체를 각 웰(well)에 넣어 반응을 유도한다. 이후 세척을 통해 결합되지 않은 anti-BrdU 항체를 제거하고 TMB(tetramethyl benzidine) 기질 용액을 넣고 발색체(chromogen)가 생성되도록 한다. 그리고 370 nm와 참조 파장 492 nm에서 흡광도를 측정한다. 모든 경우에 시험 조건을 최적화해야 한다(26항 참조).

관찰

일반증상 관찰

28. 각 마우스에서 적용 부위의 국소 자극이나 전신 독성에 대한 일반증상이 있는지를 최소 하루에 한 번 주의 깊게 관찰해야 한다. 모든 관찰 사항은 각 마우스 별로 체계적으로 기록하여 유지한다. 관찰(monitoring) 계획은 전신 독성 또는 과도한 국소 피부 자극, 안락사가 필요한 피부 부식을 즉시 식별할 수 있는 기준을 포함해야 한다(31).

체중

29. 25항에서 언급한 바와 같이, 각 마우스의 체중은 시험 시작 시점과 예정된 안락사 시 측정해야 한다.

결과의 계산

30. 각 시험물질군의 결과는 평균 감작지수(SI)로 나타낸다. 감작지수는 각 시험물질군과 양성대조군 마우스의 평균 BrdU 표지 지수를 부형제대조군의 평균 BrdU 표지 지수로 나누어 계산한다. 부형제대조군에 대한 감작지수의 평균은 “1”이 된다.

31. BrdU 표지 지수(BrdU labelling index)는 다음과 같이 정의된다.

32. BrdU 표지 지수 = $(ABS_{em} - ABS\ blank_{em}) - (ABS_{ref} - ABS\ blank_{ref})$

33. 여기서, em = 방출 파장, ref = 참조 파장

34. 판정기준은 평균 감작지수가 1.6 이상(SI ≥ 1.6)일 때 결과를 양성으로 간주한다(1). 하지만 경계값 결과(예: SI 값 1.6~1.9)를 양성으로 판정할 때에는 용량-반응 상관 정도, 통계적 유의성, 용매/부형제 및 양성대조물질 반응의 일관성을 또한 고려한다(5)(32)(33).

35. SI 1.6~1.9의 양성반응 경계값에 대해서 그러한 결과 값이 양성임을 확인하기 위해 SI 값과 함께 용량-반응 상관성, 전신 독성 또는 과도한 자극의 증거, 통계적 유의성(해당되는 경우) 등의 추가 정보를 고려할 수 있다(1). 또한 알려진 피부감작물질과 구조적인 관계 여부, 마우스에서의 과도한 피부자극 유발 여부, 관찰된 용량-반응 상관성의 유형 등을 포함하여 시험물질의 다양한 특성을 고려해야 한다. 이를 비롯한 기타 고려사항에 대해서는 참고문헌(34)에서 자세히 논의된다.

36. 개별 마우스의 데이터 수집은 데이터의 용량-반응 상관성 여부 및 상관 정도에 대한 통계적 분석을 가능하게 한다. 모든 통계적 평가는 적절하게 보정된 시험군 간의

비교뿐 아니라 용량-반응 상관성에 대한 평가를 포함할 수 있다(예: 투여군 대비 동시 부형제대조군 짝 비교(pair-wise comparison)). 통계 분석에는 용량-반응 상관성 평가를 위한 선형 회귀법(linear regression)이나 Williams's test와 짝 비교를 위한 Dunnett's test 등이 포함된다. 통계 분석의 적절한 방법을 정할 때 연구자는 데이터 변환이나 비모수 통계 분석을 필요로 하는 불균등한 분포의 가능성(possible inequalities of variances) 및 문제 등을 파악하고 있어야 한다. 어떤 경우라도, 특정 데이터 포인트("이상치(outliers)"라고도 함)를 넣거나 넣지 않는 것을 모두 포함하여 SI 계산 및 통계적 분석을 모두 수행해야 할 필요가 있다.

시험자료 및 보고

데이터

37. 데이터는 개별 마우스 BrdU 표지 지수 값, BrdU 표지 지수의 그룹 평균, 관련 오차(예: 표준편차(SD), 표준오차(SEM)), 용매/부형제대조군에 대한 각 투여군의 평균 SI를 나타내는 표 형식으로 요약한다.

시험 보고서

38. 시험 보고서에는 다음의 정보를 포함해야 한다.

시험물질

- 공급원, 로트 번호, 사용기한(가능한 경우)
- 시험 물질의 안정성(알려진 경우)

단일성분물질

- 물리적 형태, 수용성, 추가적인 관련 물리화학적 특성
- IUPAC 또는 CAS명, CAS 번호, SMILES 또는 InChI 코드, 구조식, 순도, 적절하고 실질적으로 제공 가능한 불순물의 화학물질 정보 등의 화학물질 식별정보

다성분물질, UVBCs, 복합물질

- 구성성분의 화학물질 정보, 정량적 비율, 관련 물리화학적 특성 등 가능한 자세히 특성 규명

대조군

- 식별 데이터(예: CAS 번호, 가능한 경우 출처, 순도, 알려진 불순물, 로트 번호)
- 물리적 성질 및 물리화학적 특성(예: 휘발성, 안정성, 용해도)

용매/부형제

- 식별 데이터(순도, 농도(해당되는 경우), 사용된 용량)
- 부형제 선정의 타당성에 대한 근거

실험동물

- CBA 마우스의 출처
- 마우스의 미생물학적 상태(알려진 경우)
- 마우스의 수와 주령
- 마우스의 출처, 사육 조건, 식이 정보 등

시험조건

- ELISA 키트의 공급원, 로트 번호, 제조자의 품질 보증/품질관리 데이터(항체 민감도, 특이도, 감지 허용치)
- 시험물질 준비와 적용에 관한 세부사항
- 투여 용량 선정의 타당성에 대한 근거(수행된 경우, 예비시험에 대한 결과 포함)
- 사용된 부형제와 시험물질 농도, 적용된 시험물질의 총량
- 사료와 음용수 품질에 관한 세부사항(식이 유형/공급원, 음용수원 등)
- 도포 및 샘플링 일정에 관한 세부사항
- 독성 측정 방법

- 양성 또는 음성 판정 기준
- 프로토콜 편차에 대한 세부사항 및 편차가 시험 설계와 결과에 어떻게 영향을 주었는지에 대한 설명

신뢰성 확인

- 최근 실시한 신뢰성 확인 결과 요약(사용된 시험물질, 농도, 양성대조군, 부형제대조군, 기준시험물질에 대한 정보 포함)
- 실험실의 동시 또는 과거 양성대조군과 동시 부형제대조군 데이터
- 동시 양성대조군이 포함되지 않은 경우, 가장 최근의 주기적 양성대조군에 대한 수행일자 및 보고서와 동시 양성대조군 미수행 근거가 되는 실험실의 양성대조군 배경자료의 세부사항이 기술된 보고서

결과

- 도포 시작 시점과 예정된 안락사 시 개별 마우스 체중 및 각 처리군의 평균과 관련 오차(예: SD, SEM)
- 각 마우스 도포 부위의 피부 자극을 포함한 독성 증상 및 발생 경과
- 각 처리군의 개별 마우스 BrdU 표지 지수 및 SI 값의 표
- 각 처리군 마우스의 BrdU 표지 지수 평균 및 관련 오차(예: SD, SEM)와 각 처리군의 이상치(outliers) 분석 결과
- SI 계산 결과 및 변동성에 대한 적절한 측정값(시험물질군 및 대조군의 마우스 간 변동성 고려)
- 용량-반응 상관성
- 통계 분석(적절한 경우)

결과 토의

- 결과, 용량-반응 분석, 통계분석(적절한 경우)에 대한 간단한 해설과 시험물질의 피부감작성 물질 분류에 대한 결론

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부록 1 - 용어 정의

정확도(Accuracy): 시험 결과와 허용된 참고치의 일치 정도. 시험 수행에 대한 평가 척도이고 상관성(relevance)의 한 측면이다. 정확하게 맞춘 시험결과의 비율을 의미하는 "일치성(concordance)"과 "정확도(Accuracy)"는 같은 의미로 쓰임

독성발현경로(Adverse Outcome Pathway, AOP): 분자 수준의 시작단계를 거쳐 체내 유해반응까지 표적 화학물질 또는 유사한 화학물질 그룹으로부터 일어나는 일련의 현상

기준 시험물질(Benchmark test chemical): 시험물질의 비교기준으로 사용되는 물질. 기준물질은 다음의 특성을 가져야 한다. (i) 일관성 있고 신뢰할 수 있는 공급원; (ii) 시험되는 물질과 구조적, 기능적 유사성; (iii) 알려진 물리/화학적 특성; (iv) 알려진 효과 입증 자료; (v) 원하는 반응의 범위 내 알려진 효력

위음성(False negative): 양성물질이 음성으로 판정되는 것

위양성(False positive): 음성물질이 양성으로 판정되는 것

유해성(Hazard): 물질이 생명체, 생태계 또는 특정 인구집단에 노출될 때, 유해영향을 야기할 가능성이 있는 물질 또는 상황(situation)의 본질적 특성

실험실 간 재현성(Inter-laboratory reproducibility): 다른 실험실에서 동일한 시험절차와 시험물질로 시험을 수행하였을 때 양적 또는 질적으로 유사한 결과를 생산할 수 있는지 측정하는 것으로서 시험법이 실험실간 전수될 수 있는지 여부를 나타내는 것

실험실 내 재현성(Intra-laboratory reproducibility): 동일한 실험실에서 자격을 갖춘 사람이 다른 시점에서 특별한 실험절차로 같은 결과를 생산할 수 있는 정도

혼합물질(Mixture): 서로 반응하지 않는 2가지 이상의 물질로 구성된 혼합물 또는 용액

단일성분물질(Mono-constituent substance): 정량적인 구성으로 정의되며, 하나의 주요성분이 적어도 80% (w/w) 이상인 물질

다성분물질(Multi-constituent substance): 두 가지 이상의 주요성분의 양이 ≥ 10% (w/w) 및 〈 80% (w/w)인 물질. 다성분 물질은 생산과정의 산물임. 혼합물과 다성분 물질의 차이는 혼합물은 두 가지 이상의 물질을 화학반응 없이 섞어서 얻고, 다성분 물질은 화학반응의 산물임

이상치(Outlier): 집단에서 무작위로 채취한 샘플이 다른 수치와 상당히 다른 값을 보이는 것

유사시험법평가기준(Performance Standards): 검증된 시험방법을 기반으로 기전적, 기능적으로 유사하게 제시된 시험방법의 비교 가능성을 평가하는 기준. 다음 사항을 포함함

(1) 필수적 시험 방법 구성요소;
(2) 검증된 시험방법의 허용 가능한 수행 검증을 위해 사용된 화학물질들 중에서 선택된 최소한의 기준 물질 리스트;
(3) 검증된 시험 방법에서 얻은 내용을 기반으로 정확성과 신뢰도의 비교 수준.

이는 제시된 시험 방법이 기준 화학물질의 미니멈리스트를 이용해 평가했을 때 증명되어야 함

숙련도 물질(Proficiency chemicals(substances)): 표준화된 시험 방법의 기술적인 능력을 증명하기 위해 실험실에서 사용되는 수행 기준에 포함된 기준물질. 이 물질의 선택 기준은 일반적으로 반응 범위를 대표한다는 것과 시중에서 구할 수 있는 것, 그리고 고품질의 유용한 시험자료가 있는 것

신뢰성보증(Quality assurance): 시험 수행과 독립된 개인이 실험실 시험 기준, 장비, 기록 보관 절차의 준수에 대한 관련 절차를 평가하는 것

참고 물질(Reference chemicals(substances)): 관심의 대상이 되는 종(species)이나 참고할 수 있는 *in vitro* 또는 *in vivo* 시험계(reference test system)에서의 반응이 이미 알려져 있고, 검증과정에서 선택되어 이용된 시험물질, 이 물질들은 시험법이 사용될 것으로 예상되는 시험물질 종류를 대표해야 하며, 시험물질이 일으킬 것으로 예상되는 반응의 모든 범위(강, 약, 음성)를 나타내야 한다. 검증절차의 단계, 시험법 및 시험목적에 따라 다른 참고물질 목록이 필요할 수도 있음

상관성(Relevance): 시험과 효과의 관련성 및 시험이 특정 목적에 의미 있고 유용한 지에 대한 설명. 상관성은 시험이 얼마나 생물학적 효과를 정확하게 측정하고 예측하는 지 나타내며, 상관성은 시험법의 정확도(일치성)를 내포함

신뢰도(Reliability): 동일한 시험방법에 따라 반복 시행하였을 때 동일 실험실과 다른 실험실에서 시험 결과를 재현할 수 있는 정도. 신뢰도는 실험실 내, 실험실 간 재현성(reproducibility)과 실험실 내 반복성(repeatability)으로 평가됨

재현성(Reproducibility): 동일한 방법으로 동일한 물질을 시험하였을 때 나온 결과의 일치

수신자 조작 특성(Receiver Operating Characteristic, ROC) 분석: 예측 모형에 대하여 최적의 판정 기준값(cut-off value)을 설정하기 위한 분석. 판정 기준값을 사용한 예측 모형을 통해, 시험물질이 양성 혹은 음성으로 분류될 수 있음. 판정 기준값의 모든 편차는 민감도(sensitivity) 및 특이도(specificity)의 변화에 따라 반대 반향으로 변하는 결과로 이어질 것임. ROC 분석은 대개 진단 시험에 대한 최적의 판정 기준값을 구하는 데 사용함

민감도(Sensitivity): 시험법으로 모든 양성/활성 화학물질이 정확하게 분류되는 비율. 민감도는 시험법의 범주 결정에 대한 정확도의 척도이며 시험법의 상관성을 평가하는 중요 고려사항

피부감작성(Skin sensitization): 면역학적 과정으로 감수성 있는 사람이 화학적 항원에 국소적으로 노출되었을 때 나타나며, 화학적 항원은 접촉성 감작성(contact sensitization)을 발병시킬 수 있는 피부 면역반응을 촉발함

특이도(Specificity): 시험법으로 모든 음성/비활성 화학물질이 정확하게 분류되는 비율. 특이도는 시험법의 범주 결정에 대한 정확도의 척도이며 시험법의 상관성을 평가하는 중요 고려사항

감작지수(Stimulation Index, SI): 시험물질의 피부감작 가능성을 평가하기 위해 산출된 값. 부형제 대조군과 시험물질 처리군의 증식 정도의 비율

물질(Substance): 생산과정을 통해 얻어지거나 또는 자연 상태로 얻어진 화학원소들(elements)과 이들로 이루어진 구성물질(compounds). 생산품의 안정성을 유지시키는데 필요한 첨가제와 생산과정에서 유래하는 불순물을 포함하지만, 해당물질의 안정성이나 조성의 변화에 영향을 주지 않고 분리될 수 있는 용매는 제외함

시험물질(Test chemical): 시험의 대상이 된 물질을 말함

UVCB: 알려지지 않은 물질이거나 가변적인 구조를 가지며, 복잡한 반응물이거나 생물학적인 재료

별첨 2 원문(OECD TG 442B)

OECD/OCDE **442B**

Adopted:
25June 2018

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Local lymph node assay: BRDU-ELISA or –FCM

GENERAL INTRODUCTION

1. A skin sensitiser refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (UN GHS) (1).

2. There is general agreement regarding the key biological events underlying skin sensitisation. The current knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an Adverse Outcome Pathway (AOP) (2), starting with the molecular initiating event through intermediate events to the adverse effect, namely allergic contact dermatitis. This AOP focuses on chemicals that react with thiol (i.e. cysteine) and primary amines (i.e. lysine) such as organic chemicals. In this instance, the molecular initiating event (i.e. the first key event) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The first key event can be addressed using the in chemico Direct Peptide Reactivity Assay (DPRA) TG 442C (3). The second key event in this AOP takes place in the keratinocytes and includes inflammatory responses as well as changes in gene expression associated with specific cell signalling pathways such as the antioxidant/electrophile response element (ARE)-dependent pathways. This key event can be addressed using the in vitro ARE-Nrf2 Luciferase Test Methods (KeratinoSensTM or LuSens) TG 442D (4). The third key event is the activation of dendritic cells (DC), typically assessed by expression of specific cell surface markers, chemokines and cytokines, and can be addressed using either the in vitro Human Cell Line Activation Test (h-CLAT), the in vitro U937 Cell Line Activation Test (U-SENS™) or the Interleukin-9 Reporter Gene assay (IL-8 Luc assay) as described in TG 442E (5). The fourth key event is T-cell proliferation, which is indirectly assessed in the in vivo murine Local Lymph Node Assays (LLNA) (6).

3. The first Test Guideline (TG) for the determination of skin sensitisation in the mouse, the Local Lymph Node Assay (LLNA; TG 429) was adopted in 2002, and has since then been revised (7). The details of the validation of the LLNA and a review of the associated work have been published (8) (9) (10) (11) (12) (13) (14) (15) (16). In the LLNA, radioisotopic thymidine or iodine is used to measure lymphocyte proliferation and therefore the assay has limited use in regions where the acquisition, use, or disposal of radioactivity is problematic.

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

4. This Test Guideline describes two non-radioactive modifications to the LLNA test method, which utilise non-radiolabelled 5-bromo-2-deoxyuridine (BrdU) (Chemical Abstracts Service [CAS] No 59-14-3) in an ELISA [Enzyme-Linked Immunosorbent Assay] - or FCM [Flow Cytometry Method]-based test system to measure lymphocyte proliferation:

- The Local Lymph Node Assay: BrdU-ELISA (Appendix IA), and
- The Local Lymph Node Assay: BrdU-FCM (Appendix IB).

5. Similar to the LLNA, the LLNA: BrdU-ELISA and the LLNA: BrdU-FCM study the induction phase of skin sensitisation and provide quantitative data suitable for dose-response assessment. Furthermore, an ability to detect skin sensitisers without the necessity for using a radiolabel for DNA eliminates the potential for occupational exposure to radioactivity and waste disposal issues. This in turn may allow for the increased use of mice to detect skin sensitisers, which could further reduce the use of guinea pigs to test for skin sensitisation potential (i.e. TG 406) (17).

6. This Test Guideline is designed for assessing skin sensitisation potential of chemicals in animals. TG 406 utilises guinea pig tests, notably the guinea pig maximisation test and the Buehler test (17). The LLNA (TG 429) (7) and the non-radioactive modifications, LLNA: BrdU-ELISA and FCM (TG 442 B) and LLNA: DA (TG 442 A) (18), all provide an advantage over the guinea pig tests in TG 406 (17) in terms of reduction and refinement of animal use.

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Annex I – Definitions

Accuracy: The closeness of agreement between test method results and accepted reference values. It is a measure of test method performance and one aspect of "relevance." The term is often used interchangeably with "concordance", to mean the proportion of correct outcomes of a test method (12).

AOP (Adverse Outcome Pathway): sequence of events from the chemical structure of a target chemical or group of similar chemicals through the molecular initiating event to an in vivo outcome of interest (2).

Benchmark test chemical: A sensitising or non-sensitising substance used as a standard for comparison to a test chemical. A benchmark chemical should have the following properties: (i) a consistent and reliable source(s); (ii) structural and functional similarity to the class of substances being tested; (iii) known physical/chemical characteristics; (iv) supporting data on known effects; and (v) known potency in the range of the desired response.

False negative: A test chemical incorrectly identified as negative or non-active by a test method, when in fact it is positive or active (12). The false negative rate is one indicator of the test method performance.

False positive: A test chemical incorrectly identified as positive or active by a test, when in fact it is negative or non-active (12). The false positive rate is one indicator of the test method performance.

Hazard: Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub) population is exposed to that agent.

Inter-laboratory reproducibility: A measure of the extent to which different qualified laboratories, using the same protocol and testing the same test chemical, can produce qualitatively and quantitatively similar results. Inter-laboratory reproducibility is determined during the pre-validation and validation processes, and indicates the extent to which a test can be successfully transferred between laboratories, also referred to as between-laboratory reproducibility (12).

Intra-laboratory reproducibility: A determination of the extent that qualified people within the same laboratory can successfully replicate results using a specific protocol at different times. Also referred to as within-laboratory reproducibility (12).

Mixture: A mixture or a solution composed of two or more substances in which they do not react.

Mono-constituent substance: A substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).

Multi-constituent substance: A substance, defined by its quantitative composition, in which more than one main constituent is present in a concentration ≥ 10% (w/w) and < 80% (w/w). A multi-constituent substance is the result of a manufacturing process. The difference between mixture and multi-constituent substance is that a mixture is obtained

by blending of two or more substances without chemical reaction. A multi-constituent substance is the result of a chemical reaction.

Outlier: An outlier is an observation that is markedly different from other values in a random sample from a population.

Performance standards: Standards, based on a validated test method, that provide a basis for evaluating the comparability of a proposed test method that is mechanistically and functionally similar. Included are (i) essential test method components; (ii) a minimum list of reference chemicals selected from among the chemicals used to demonstrate the acceptable performance of the validated test method; and (iii) the comparable levels of accuracy and reliability, based on what was obtained for the validated test method, that the proposed test method should demonstrate when evaluated using the minimum list of reference chemicals (12).

Proficiency chemicals (substances): A subset of the Reference Chemicals included in the Performance Standards that can be used by laboratories to demonstrate technical competence with a standardised test method. Selection criteria for these substances typically include that they represent the range of responses, are commercially available, and have high quality reference data available.

Quality assurance: A management process by which adherence to laboratory testing standards, requirements, and record keeping procedures, and the accuracy of data transfer, are assessed by individuals who are independent from those performing the testing.

Reference chemicals (substances): A set of chemicals to be used to demonstrate the ability of a new test method to meet the acceptability criteria demonstrated by the validated reference test method(s). These chemicals should be representative of the classes of chemicals for which the test method is expected to be used, and should represent the full range of responses that may be expected from the chemicals for which it may be used, from strong, to weak, to negative.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy (concordance) of a test method (12).

Reliability: Measures of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility and intra-laboratory repeatability (12).

Reproducibility: The agreement among results obtained from testing the same substance using the same test protocol (see reliability) (12).

Receiver operating Characteristic (ROC) analysis: An analysis to set an optimal cut-off value for the prediction model. The prediction models using cut-off values allow test chemical to be categorized as positive or negative. Any variation of the cut-off value will result in changes of the sensitivity and specificity, in opposite directions. ROC analysis is commonly used to obtain optimal cutoff values for diagnostic tests.

Sensitivity: The proportion of all positive / active chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results, and is an important consideration in assessing the relevance of a test method (12).

Skin sensitisation: An immunological process that results when a susceptible individual is exposed topically to an inducing chemical allergen, which provokes a cutaneous immune response that can lead to the development of contact sensitisation.

Specificity: The proportion of all negative / inactive chemicals that are correctly classified by the test method. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (12).

Stimulation Index (SI): A value calculated to assess the skin sensitisation potential of a test chemical that is the ratio of the proliferation in treated groups to that in the concurrent vehicle control group.

Substance: Chemical elements and their compounds in the natural state or obtained by any production process, including any additive necessary to preserve the stability of the product and any impurities deriving from the process used, but excluding any solvent which may be separated without affecting the stability of the substance or changing its composition (1).

Test chemical: The term "test chemical" is used to refer to what is being tested. It is not related to the applicability of the test methods to the testing of mono-constituent substances, multi-constituent substances and/or mixtures.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials.

OECD/OCDE **442B**

Adopted:
25June 2018

Appendix IA: In Vivo Skin Sensitisation: The Local Lymph Node Assay: BrdU-ELISA

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The LLNA: BrdU-ELISA has been validated and reviewed, and recommended by an international independent scientific peer review panel as considered useful for identifying skin sensitising and non-sensitising test chemicals, with certain limitations (1) (2) (3).

2. The LLNA: BrdU-ELISA is a modified non-radioactive LLNA method for identifying potential skin sensitising test chemicals, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-ELISA should be used in place of the radioactive LLNA (TG 429) or guinea pig tests (i.e. TG 406) (4), when the use of an in vivo method is deemed necessary, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation (1) (2). The testing laboratory should consider all available information on the test chemical prior to conducting the study. Such information will include the identity and chemical structure of the test chemical; its physicochemical properties; the results of any other in vitro or in vivo toxicity tests on the test chemical; and toxicological data on structurally related test chemicals. This information should be considered in order to determine whether the LLNA: BrdU-ELISA is appropriate for the test chemical (given the incompatibility of limited types of test chemicals with the LLNA: BrdU-ELISA [see paragraph 3]) and to aid in dose selection.

3. The LLNA: BrdU-ELISA is an in vivo method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitising activity. Therefore, consideration should be given to the applicability domain of suitable in vitro, in chemico and in silico methods and consequently, the possibility of using these approaches rather than testing on animals. Like other LLNA test methods, the LLNA: BrdU-ELISA has, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (4). Moreover, the LLNA: BrdU-ELISA offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing, since unlike TG 406, the LLNA: BrdU-ELISA does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-ELISA does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (4). Thus, the LLNA: BrdU-ELISA reduces animal distress. Despite the advantages of the LLNA: BrdU-ELISA over TG 406 (4), there are certain limitations applicable to the LLNA test, that may necessitate the use of TG 406 (e.g. the testing of certain metals, false positive findings with certain skin irritants [such as some surfactant-type substances] (5) (6), solubility of the test chemicals [such as rarely soluble or non-soluble substances]). In addition, test chemical classes or substances containing functional groups shown to act as potential confounders (e.g. fatty acid glutamate, oleic acid, oleic acid ester, fatty alcohol 1, fatty alcohol 2, polyaminofunctional siloxane (7)) may necessitate the use of guinea pig tests (i.e. TG 406 (4)). Other limitations that have been identified for the LLNA (6) have also been recommended to apply to the LLNA: BrdU-ELISA (1). Other than such identified limitations, the LLNA: BrdU-ELISA should

In accordance with the decision of the Council on a delegation of authority to amend Annex I of the decision of the council on the Mutual Acceptance of Data in the assessment of chemicals [C(2018)49], this Guideline was amended by the OECD's Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology by written procedure on 25 June 2018.

be applicable for testing any test chemicals unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: BrdU-ELISA. In addition, consideration should be given to the possibility of borderline positive results when Stimulation Index (SI) values between 1.6 and 1.9 are obtained (see paragraphs 31-32) in the LLNA: BrdU-ELISA. This is based on the validation database of 43 substances using an SI ≥ 1.6 (see paragraph 6) for which the LLNA: BrdU-ELISA correctly identified all 32 LLNA sensitisers, but incorrectly identified two of 11 LLNA non-sensitisers with SI values between 1.6 and 1.9 (i.e. borderline positive) (1). However, as the same dataset was used for setting the SI-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties.

4. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically.

5. Definitions are provided in the Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-ELISA is that sensitisers induce proliferation of lymphocytes in the lymph nodes draining the site of test chemical application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitisation. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control group (VC). The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the SI, is determined, and should be ≥1.6 before further evaluation of the test chemical as a potential skin sensitiser is warranted. The methods described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by ELISA, which utilises an antibody specific for BrdU that is also labelled with peroxidase. When the substrate is added, the peroxidase reacts with the substrate to produce a coloured product that is quantified at a specific absorbance using a microtiter plate reader.

DESCRIPTION OF THE ASSAY

Selection of animal species

7. The mouse is the species of choice for this test. Validation studies for the LLNA: BrdU-ELISA were conducted exclusively with the CBA/JN strain, which is therefore considered the preferred strain (1) (3). Young adult female mice, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Alternatively, other strains or males may be used when sufficient data are generated to demonstrate that significant strain and/or gender-specific differences in the LLNA: BrdU-ELISA response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (8) on solid-bottomed cages (9) with suitable substrate and nesting material (10) (11) (12) (13), unless adequate scientific rationale for alternative housing mice individually is provided. The temperature of the experimental animal room should be 22 ± 3°C. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

9. The animals are randomly selected, humanely marked to permit individual identification preferably by non-invasive hair clipping (14) (15), and kept in their cages for at least five days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions. During all examinations, the mice should be handled using non-aversive methods such as cupping or tunnel handling (16).

Preparation of dosing solutions

10. Solid test chemicals should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test chemicals may be applied neat or diluted prior to dosing. Insoluble chemicals, such as those generally seen in medical devices (35), should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to an ear of the mice. Test chemicals should be prepared daily unless stability data demonstrate the acceptability of storage.

Reliability check

11. Positive controls (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitising test chemical for which the magnitude of the response is well characterised. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-, and inter-laboratory reproducibility and comparability. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA: BrdU-ELISA. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLNA: BrdU-ELISA response at an exposure level expected to give an increase in the SI $\geq$ 1.6 over the VC group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (e.g. SI > 14 would be considered excessive). Preferred PC test chemicals are 25% hexyl cinnamic aldehyde (CAS No 101-86-0) and 25% eugenol (CAS No 97-53-0) in acetone: olive oil (4:1, v/v). There may be circumstances in which, given adequate justification, other PC test chemicals, meeting the above criteria, may be used.

12. While inclusion of a concurrent PC group is recommended, there may be situations in which periodic testing (i.e. at intervals $\leq$6 months) of the PC test chemical

may be adequate for laboratories that conduct the LLNA: BrdU-ELISA regularly (i.e. conduct the LLNA: BrdU-ELISA at a frequency of no less than once per month) and have an established historical PC database that demonstrates the laboratory's ability to obtain reproducible and accurate results with PCs. Adequate proficiency with the LLNA: BrdU-ELISA can be successfully demonstrated by generating consistent positive results with the PC in at least 10 independent tests conducted within a reasonable period of time (i.e. less than one year).

13. A concurrent PC group should always be included when there is a procedural change to the LLNA: BrdU-ELISA (e.g. change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the PC results.

14. Investigators should be aware that the decision to conduct a PC study on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false negative result is obtained in the periodic PC study, negative test chemical results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent PCs or to only conduct periodic PCs. Consideration should also be given to using fewer animals in the concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used (17).

15. Although the PC test chemical should be tested in the vehicle that is known to elicit a consistent response (e.g. acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary (18). If the concurrent PC test chemical is tested in a different vehicle than the test chemical, then a separate VC for the concurrent PC should be included.

16. In instances where test chemicals of a specific chemical class or range of responses are being evaluated, benchmark test chemicals may also be useful to demonstrate that the test method is functioning properly for detecting the skin sensitisation potential of these types of test chemicals. Appropriate benchmark test chemicals should have the following properties:

- structural and functional similarity to the class of the test chemical being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: BrdU-ELISA;
- supporting data from other animal models and/or from humans.

TEST PROCEDURE

Number of animals and dose levels

17. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test chemical, plus a concurrent VC group treated only with the

vehicle for the test chemical, and a PC group (concurrent or recent, based on laboratory policy in considering paragraphs 11-15). Testing multiple doses of the PC should be considered especially when testing the PC on an intermittent basis. Except for absence of treatment with the test chemical, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

18. Dose and vehicle selection should be based on the recommendations given in the references 2 and 27. Three consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g. acute toxicity and dermal irritation) and structural and physicochemical information on the test chemical of interest (and/or structurally related test chemicals) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation (19) (20). In the absence of such information, an initial pre-screen test may be necessary (see paragraphs 21-24).

19. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test chemical. Recommended vehicles are acetone: olive oil (4:1 v/v), N,N-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (5) but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test chemical is marketed as an additional control. Particular care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (e.g. 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

20. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test chemical and VC group measurements (see paragraph 33). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (18). Further, some national regulatory authorities require the collection of individual animal data. Regular collection of individual animal data provides an animal welfare advantage by avoiding duplicate testing that would be necessary if the test chemical results originally collected in one manner (e.g. via pooled animal data) were to be considered later by regulatory authorities with other requirements (e.g. individual animal data).

Pre-screen test

21. In the absence of information to determine the highest dose to be tested (see paragraph 18), a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-ELISA. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-ELISA study, where information on the concentration that induces systemic toxicity (see paragraph 24) and/or excessive local skin irritation (see paragraph 23) is not available. The maximum dose level tested should be a concentration of 100% of the test chemical for liquids or the maximum possible concentration for solids or suspensions.

22. The pre-screen test is conducted under conditions identical to the main LLNA: BrdU-ELISA study, except there is no assessment of lymph node proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema and scored using Table 1 (20). Ear thickness measurements are taken using a thickness gauge (e.g. digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6. Additionally, on Day 6, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score ≥3 and/or ear thickness of ≥25% on any day of measurement (21) (22). The highest dose selected for the main LLNA: BrdU-ELISA study will be the next lower dose in the pre-screen concentration series (see paragraph 18) that does not induce systemic toxicity and/or excessive local skin irritation.

Table 1. Erythema Scores

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

23. In addition to a 25% increase in ear thickness (21) (22), a statistically significant increase in ear thickness in the treated mice compared to solvent/vehicle control mice has also been used to identify irritants in the LLNA (22) (23) (24) (25) (26) (27) (28). However, while statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (25) (26) (27) (28) (29).

24. The following clinical observations may indicate systemic toxicity (30) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-ELISA: changes in nervous system function (e.g. pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (e.g. aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (i.e. changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 6 and mortality should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (31).

Main study experimental schedule

25. The experimental schedule of the assays is as follows:

- Day 1:

  Individually identify and record the weight of each animal and any clinical observation. Apply 25 µL of the appropriate dilution of the test chemical, the vehicle alone, or the PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15), to the dorsum of each ear.

- Days 2 and 3:

  Repeat the application procedure carried out on Day 1.

- Day 4:

  No treatment.

- Day 5:

  Inject 0.5 mL (5 mg/mouse) of BrdU (10 mg/mL) solution intra-peritoneally.

- Day 6:

  Record the weight of each animal and any clinical observation. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline (PBS) for each animal. Details and diagrams of the lymph node identification and dissection can be found in reference (17). To further monitor the local skin response in the main study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included into the study protocol.

Preparation of cell suspensions

26. From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension (e.g. use of a disposable plastic pestle to crush the lymph nodes followed by passage through a #70 nylon mesh). The procedure for preparing the LNC suspension is critical in this assay and therefore every operator should establish the skill in advance. Further, the lymph nodes in VC animals are small, so careful operation is important to avoid any artificial effects on SI values. In each case, the target volume of the LNC suspension should be adjusted to a determined optimised volume (approximately 15 mL). The optimised volume is based on achieving a mean absorbance of the VC group within 0.1-0.2.

Determination of cellular proliferation (measurement of BrdU content in DNA of lymphocytes)

27. BrdU is measured by ELISA using a commercial kit (e.g. in the validation study the Roche Applied Science, Mannheim, Germany, was used). Other BrdU ELISA kits may be used if they provide consistent results. Briefly, 100 µL of the LNC suspension is added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the LNC, peroxidase-conjugated anti-BrdU antibody is added to each well and allowed to react. Subsequently, the anti-BrdU antibody is removed by washing and the substrate solution is then added and allowed to produce chromogen. Absorbance

at 370 nm with a reference wavelength of 492 nm is then measured. In all cases, assay test conditions should be optimised (see paragraph 26).

OBSERVATIONS

Clinical observations

28. Each mouse should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with records being maintained for each mouse. Monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity, excessive local skin irritation, or corrosion of skin for euthanasia (31).

Body weights

29. As stated in paragraph 25, individual animal body weights should be measured at the start of the test and at the scheduled humane kill.

CALCULATION OF RESULTS

30. Results for each treatment group are expressed as the mean SI. The SI is derived by dividing the mean BrdU labelling index/mouse within each test chemical group and the PC group by the mean BrdU labelling index for the solvent/VC group. The average SI for the VCs is then one.

31. The BrdU labelling index is defined as:

32. BrdU labelling index = (ABSem – ABS blankem) – (ABSref – ABS blankref)

33. Where; em = emission wavelength; and ref = reference wavelength.

34. The decision process regards a result as positive when SI $\geq$ 1.6 (1). However, the strength of the dose-response relationship, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result (i.e. SI value between 1.6 and 1.9) is declared positive (5) (32) (33).

35. For a borderline positive response between an SI of 1.6 and 1.9, users may want to consider additional information such as dose-response relationship, evidence of systemic toxicity or excessive irritation, and where appropriate, statistical significance together with SI values to confirm that such results are positives (1). Consideration should also be given to various properties of the test chemical, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation in the mouse, and the nature of the dose-response observed. These and other considerations are discussed in detail elsewhere (34).

36. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (e.g. pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, e.g. linear regression or Williams's test to assess dose-response trends, and Dunnett's test for pair-wise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical

analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

DATA AND REPORTING

Data

37. Data should be summarised in tabular form showing the individual animal BrdU labelling index values, the group mean BrdU labelling index/animal, its associated error term (e.g. SD, SEM), and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

Test report

38. The test report should contain the following information:

Test chemical

- source, lot number, limit date for use, if available;
- stability of the test chemical, if known;

Mono-constituent substance

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Controls

- identification data (e.g. CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (e.g. volatility, stability, solubility);

Solvent/vehicle

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle;

Test animals

- source of CBA mice;
- microbiological status of the animals, when known;

- number and age of animals;
- source of animals, housing conditions, diet, etc.;

Test conditions

- source, lot number, and manufacturer's quality assurance/quality control data (antibody sensitivity and specificity and the limit of detection) for the ELISA kit;
- details of test chemical preparation and application;
- justification for dose selection (including results from pre-screen test, if conducted);
- vehicle and test chemical concentrations used, and total amount of test chemical applied;
- details of food and water quality (including diet type/source, water source);
- details of treatment and sampling schedules;
- methods for measurement of toxicity;
- criteria for considering studies as positive or negative;
- details of any protocol deviations and an explanation on how the deviation affects the study design and results;

Reliability check

- a summary of results of latest reliability check, including information on test chemical, concentration, PC, VC and benchmark test chemical used, as appropriate;
- concurrent and/or historical PC and concurrent VC data for testing laboratory;
- if a concurrent PC was not included, the date and laboratory report for the most recent periodic PC and a report detailing the historical PC data for the laboratory justifying the basis for not conducting a concurrent PC;

Results

- individual weights of mice at start of dosing and at scheduled humane kill; as well as mean and associated error term (e.g. SD, SEM) for each treatment group;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal;
- a table of individual mouse BrdU labelling indices and SI values for each treatment group;
- mean and associated error term (e.g. SD, SEM) for BrdU labelling index/mouse for each treatment group and the results of outlier analysis for each treatment group;
- calculated SI and an appropriate measure of variability that takes into account the inter-animal variability in both the test chemical and control groups;
- dose-response relationship;
- statistical analyses, where appropriate;

Discussion of results:

- a brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test chemical should be considered a skin sensitiser.

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Appendix IB: In Vivo Skin Sensitisation: The Local Lymph Node Assay: BrdU-FCM

INITIAL CONSIDERATIONS, APPLICABILITY AND LIMITATIONS

1. The LLNA: BrdU-FCM has been validated and recommended, following an international independent scientific peer review, as useful for identifying skin sensitising and non-sensitising test chemicals, with certain limitations (1) (2) (3) (4). The validation study for the LLNA: BrdU-FCM was performed in compliance with the performance standards (PS) for assessment of proposed similar or modified LLNA test methods for skin sensitisation in Annex 1 of the OECD Guideline for the testing of chemicals, Skin sensitisation: Local lymph node assay (TG 429).

2. The LLNA: BrdU-FCM is a modified non-radioactive LLNA method for identifying potential skin sensitising test chemicals, with specific limitations. This does not necessarily imply that in all instances the LLNA: BrdU-FCM should be used in place of the radioactive LLNA (TG 429) or guinea pig tests (i.e. TG 406) (5), when the use of an in vivo method is deemed necessary, but rather that the assay is of equal merit and may be employed as an alternative in which positive and negative results generally no longer require further confirmation (1) (2). The testing laboratory should consider all available information on the test chemical prior to conducting the study. Such information will include the identity and chemical structure of the test chemical; its physicochemical properties; the results of any other in vitro or in vivo toxicity tests on the test chemical; and toxicological data on structurally related test chemicals. This information should be considered in order to determine whether the LLNA: BrdU-FCM is appropriate for the test chemical (given the incompatibility of limited types of test chemicals with the LLNA: BrdU-FCM [see paragraph 3]) and to aid in dose selection.

3. The LLNA: BrdU-FCM is an *in vivo* method and, as a consequence, will not eliminate the use of animals in the assessment of allergic contact sensitising activity. Therefore, consideration should be given to the applicability domain of suitable *in vitro*, *in chemico* and *in silico* methods and consequently, the possibility of using these approaches rather than testing on animals. Like other LLNA test methods, the LLNA: BrdU-FCM has, however, the potential to reduce the animal use for this purpose when compared to the guinea pig tests (TG 406) (5). Moreover, the LLNA: BrdU-FCM offers a substantial refinement of the way in which animals are used for allergic contact sensitisation testing, since unlike TG 406, the LLNA: BrdU-FCM does not require that challenge-induced dermal hypersensitivity reactions be elicited. Furthermore, the LLNA: BrdU-FCM does not require the use of an adjuvant, as is the case for the guinea pig maximisation test (5). Thus, the LLNA: BrdU-FCM reduces animal distress. Despite the advantages of the LLNA: BrdU-FCM over TG 406 (5), there are certain limitations applicable to the LLNA test, that may necessitate the use of TG 406 (*e.g.* the testing of certain metals, false positive findings with certain skin irritants [such as some surfactant-type substances] (6) (7), solubility of the test chemicals [such as practically insoluble or insoluble substances]). In addition, test chemical classes or substances containing functional groups shown to act as potential confounders (e.g. fatty acid glutamate, oleic acid, oleic acid ester, fatty alcohol 1, fatty alcohol 2, polyaminofunctional siloxane (8)) may necessitate the use of guinea pig tests (i.e. TG 406 (5)). Other limitations that have been identified for the LLNA (7) have also been recommended to apply to the LLNA:

BrdU-FCM (1). Other than such identified limitations, the LLNA: BrdU-FCM should be applicable for testing any test chemicals unless there are properties associated with these substances that may interfere with the accuracy of the LLNA: BrdU-FCM. According to the validation study, the LLNA: BrdU-FCM correctly identified 20 among the 22 reference substances listed in the TG 429 PS on the basis of the LLNA results (1). One moderate skin sensitiser, 2-mercaptobenzothiazole, and one weak skin sensitiser, methyl methacrylate for which the other LLNA variants have limitation in prediction, were misclassified in the LLNA: BrdU-FCM (1) (2) (9). However, as the same dataset was used for setting the Stimulation Index (SI)-values and calculating the predictive properties of the test, the stated results may be an over-estimation of the real predictive properties.

4. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

5. Definitions are provided in the Annex 1 of the General Introduction.

PRINCIPLE OF THE TEST

6. The basic principle underlying the LLNA: BrdU-FCM is that sensitisers induce proliferation of lymphocytes in the lymph nodes draining the site of test chemical application. This proliferation is proportional to the dose and to the potency of the applied allergen and provides a simple means of obtaining a quantitative measurement of sensitisation. Proliferation is measured by comparing the mean proliferation in each test group to the mean proliferation in the vehicle treated control group (VC). The ratio of the mean proliferation in each treated group to that in the concurrent VC group, termed the SI, is determined, and should be $\geq$2.7 before further evaluation of the test chemical as a potential skin sensitiser is warranted. The methods described here are based on the use of measuring BrdU content to indicate an increased number of proliferating cells in the draining auricular lymph nodes. BrdU is an analogue of thymidine and is similarly incorporated into the DNA of proliferating cells. The incorporation of BrdU is measured by FCM, which utilises an antibody specific for BrdU that is also labelled with fluorescein isothiocyanate (FITC). The FCM quantifies the number of BrdU-positive viable cells using a flow cytometer, which is widely employed in analysing lymphocyte population.

DESCRIPTION OF THE ASSAY

Selection of animal species

7. The mouse is the species of choice for this test. Validation studies for the LLNA: BrdU-FCM were conducted exclusively with the BALB/c strain, which is therefore considered the preferred strain (1) (2). The CBA/J strain can also be used in the LLNA: BrdU-FCM. CBA/J strain responses are highly correlated with and more sensitive than BALB/c strain responses (2) (10) (11) (12). However, different cut-off SI values may have to be adopted for each strain to maximize sensitivity after Receiver Operating Characteristic (ROC) analysis. Young adult female mice, which are nulliparous and non-pregnant, are used. At the start of the study, animals should be between 8-12 weeks old, and the weight variation of the animals should be minimal and not exceed 20% of the mean weight. Alternatively, other strains or males may be used when sufficient data are

generated to demonstrate that significant strain and/or gender-specific differences in the LLNA: BrdU-FCM response do not exist.

Housing and feeding conditions

8. Mice should be group-housed (13) on solid-bottomed cages (34) with suitable substrate and nesting material (35) (36) (37) (38), unless adequate scientific rationale for alternative housing mice individually is provided. The temperature of the experimental animal room should be 22 ± 3°C. Although the relative humidity should be at least 30% and preferably not exceed 70%, other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

9. The animals are randomly selected, humanely marked to permit individual identification preferably by non-invasive hair clipping (39) (40), and kept in their cages for at least five days prior to the start of dosing to allow for acclimatisation to the laboratory conditions. Prior to the start of treatment all animals are examined to ensure that they have no observable skin lesions. During all examinations, the mice should be handled using non-aversive methods such as cupping or tunnel handling (41).

Preparation of dosing solutions

10. Solid test chemicals should be dissolved or suspended in solvents/vehicles and diluted, if appropriate, prior to application to an ear of the mice. Liquid test chemicals may be applied neat or diluted prior to dosing. Insoluble chemicals, such as those generally seen in medical devices (33), should be subjected to an exaggerated extraction in an appropriate solvent to reveal all extractable constituents for testing prior to application to an ear of the mice. Test chemicals should be prepared daily unless stability data demonstrate the acceptability of storage.

Reliability check

11. Positive controls (PC) are used to demonstrate appropriate performance of the assay by responding with adequate and reproducible sensitivity to a sensitising test chemical for which the magnitude of the response is well characterised. Inclusion of a concurrent PC is recommended because it demonstrates competency of the laboratory to successfully conduct each assay and allows for an assessment of intra-, and inter-laboratory reproducibility and comparability. Some regulatory authorities also require a PC for each study and therefore users are encouraged to consult the relevant authorities prior to conducting the LLNA: BrdU-FCM. Accordingly, the routine use of a concurrent PC is encouraged to avoid the need for additional animal testing to meet such requirements that might arise from the use of a periodic PC (see paragraph 12). The PC should produce a positive LLNA: BrdU-FCM response at an exposure level expected to give an increase in the SI $\geq$ 2.7 over the VC group. The PC dose should be chosen such that it does not cause excessive skin irritation or systemic toxicity and the induction is reproducible but not excessive (e.g. SI > 27 would be considered excessive). Preferred PC test chemicals are 25% hexyl cinnamic aldehyde (CAS No 101-86-0) and 25% eugenol (CAS No 97-53-0) in acetone: olive oil (4:1, v/v). There may be circumstances

in which, given adequate justification, other PC test chemicals, meeting the above criteria, may be used.

12. While inclusion of a concurrent PC group is recommended, there may be situations in which periodic testing (i.e. at intervals ≤ 6 months) of the PC test chemical may be adequate for laboratories that conduct the LLNA: BrdU-FCM regularly (i.e. conduct the LLNA: BrdU-FCM at a frequency of no less than once per month) and have an established historical PC database that demonstrates the laboratory's ability to obtain reproducible and accurate results with PCs. Adequate proficiency with the LLNA: BrdU-FCM can be successfully demonstrated by generating consistent positive results with the PC in at least 10 independent tests conducted within a reasonable period of time (i.e. less than one year).

13. A concurrent PC group should always be included when there is a procedural change to the LLNA: BrdU-FCM (e.g. change in trained personnel, change in test method materials and/or reagents, change in test method equipment, change in source of test animals), and such changes should be documented in laboratory reports. Consideration should be given to the impact of these changes on the adequacy of the previously established historical database in determining the necessity for establishing a new historical database to document consistency in the PC results.

14. Investigators should be aware that the decision to conduct a PC study on a periodic basis instead of concurrently has ramifications on the adequacy and acceptability of negative study results generated without a concurrent PC during the interval between each periodic PC study. For example, if a false negative result is obtained in the periodic PC study, negative test chemical results obtained in the interval between the last acceptable periodic PC study and the unacceptable periodic PC study may be questioned. Implications of these outcomes should be carefully considered when determining whether to include concurrent PCs or to only conduct periodic PCs. Consideration should also be given to using fewer animals in the concurrent PC group when this is scientifically justified and if the laboratory demonstrates, based on laboratory-specific historical data, that fewer mice can be used (14).

15. Although the PC test chemical should be tested in the vehicle that is known to elicit a consistent response (e.g. acetone: olive oil; 4:1, v/v), there may be certain regulatory situations in which testing in a non-standard vehicle (clinically/chemically relevant formulation) will also be necessary (15). If the concurrent PC test chemical is tested in a different vehicle than the test chemical, then a separate VC for the concurrent PC should be included.

16. In instances where test chemicals of a specific chemical class or range of responses are being evaluated, benchmark test chemicals may also be useful to demonstrate that the test method is functioning properly for detecting the skin sensitisation potential of these types of test chemicals. Appropriate benchmark test chemicals should have the following properties:

- structural and functional similarity to the class of the test chemical being tested;
- known physical/chemical characteristics;
- supporting data from the LLNA: BrdU-FCM;
- supporting data from other animal models and/or from humans.

TEST PROCEDURE

Number of animals and dose levels

17. A minimum of four animals is used per dose group, with a minimum of three concentrations of the test chemical, plus a concurrent VC group treated only with the vehicle for the test chemical, and a PC group (concurrent or recent, based on laboratory policy in considering paragraphs 11-15). Testing multiple doses of the PC should be considered especially when testing the PC on an intermittent basis. Except for absence of treatment with the test chemical, animals in the control groups should be handled and treated in a manner identical to that of animals in the treatment groups.

18. Dose and vehicle selection should be based on the recommendations given in the references 2 and 19. Three consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1%, 0.5%, etc. Adequate scientific rationale should accompany the selection of the concentration series used. All existing toxicological information (e.g. acute toxicity and dermal irritation) and structural and physicochemical information on the test chemical of interest (and/or structurally related test chemicals) should be considered, where available, in selecting the three consecutive concentrations so that the highest concentration maximises exposure while avoiding systemic toxicity and/or excessive local skin irritation (16) (17). In the absence of such information, an initial pre-screen test may be necessary (see paragraphs 21-24).

19. The vehicle should not interfere with or bias the test result and should be selected on the basis of maximising the solubility in order to obtain the highest concentration achievable while producing a solution/suspension suitable for application of the test chemical. Recommended vehicles are acetone: olive oil (4:1 v/v), *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol, and dimethyl sulphoxide (6) but others may be used if sufficient scientific rationale is provided. In certain situations it may be necessary to use a clinically relevant solvent or the commercial formulation in which the test chemical is marketed as an additional control. Particular care should be taken to ensure that hydrophilic substances are incorporated into a vehicle system, which wets the skin and does not immediately run off, by incorporation of appropriate solubilisers (e.g. 1% Pluronic® L92). Thus, wholly aqueous vehicles are to be avoided.

20. The processing of lymph nodes from individual mice allows for the assessment of inter-animal variability and a statistical comparison of the difference between test chemical and VC group measurements (see paragraph 33). In addition, evaluating the possibility of reducing the number of mice in the PC group is only feasible when individual animal data are collected (14). Further, some national regulatory authorities require the collection of individual animal data. Regular collection of individual animal data provides an animal welfare advantage by avoiding duplicate testing that would be necessary if the test chemical results originally collected in one manner (e.g. via pooled animal data) were to be considered later by regulatory authorities with other requirements (e.g. individual animal data).

Pre-screen test

21. In the absence of information to determine the highest dose to be tested (see paragraph 18), a pre-screen test should be performed in order to define the appropriate dose level to test in the LLNA: BrdU-FCM. The purpose of the pre-screen test is to provide guidance for selecting the maximum dose level to use in the main LLNA: BrdU-

FCM study, where information on the concentration that induces systemic toxicity (see paragraph 24) and/or excessive local skin irritation (see paragraph 23) is not available. The maximum dose level tested should be a concentration of 100% of the test chemical for liquids or the maximum possible concentration for solids or suspensions.

22. The pre-screen test is conducted under conditions identical to the main LLNA: BrdU-FCM study, except there is no assessment of lymph node proliferation and fewer animals per dose group can be used. One or two animals per dose group are suggested. All mice will be observed daily for any clinical signs of systemic toxicity or local irritation at the application site. Body weights are recorded pre-test and prior to termination (Day 6). Both ears of each mouse are observed for erythema and scored using Table 1 (17). Ear thickness measurements are taken using a thickness gauge (*e.g.* digital micrometer or Peacock Dial thickness gauge) on Day 1 (pre-dose), Day 3 (approximately 48 hours after the first dose), and Day 6. Additionally, on Day 6, ear thickness could be determined by ear punch weight determinations, which should be performed after the animals are humanely killed. Excessive local irritation is indicated by an erythema score ≥3 and/or ear thickness of ≥ 25% on any day of measurement (18) (19). The highest dose selected for the main LLNA: BrdU-FCM study will be the next lower dose in the pre-screen concentration series (see paragraph 18) that does not induce systemic toxicity and/or excessive local skin irritation.

Table 1. Erythema Scores

Observation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation preventing grading of erythema	4

23. In addition to a 25% increase in ear thickness (18) (19), a statistically significant increase in ear thickness in the treated mice compared to solvent/vehicle control mice has also been used to identify irritants in the LLNA (19) (20) (21) (22) (23) (24) (25). However, while statistically significant increases can occur when ear thickness is less than 25%, they have not been associated specifically with excessive irritation (22) (23) (24) (25) (26).

24. The following clinical observations may indicate systemic toxicity (27) when used as part of an integrated assessment and therefore may indicate the maximum dose level to use in the main LLNA: BrdU-FCM: changes in nervous system function (*e.g.* pilo-erection, ataxia, tremors, and convulsions); changes in behaviour (*e.g.* aggressiveness, change in grooming activity, marked change in activity level); changes in respiratory patterns (i.e. changes in frequency and intensity of breathing such as dyspnea, gasping, and rales), and changes in food and water consumption. In addition, signs of lethargy and/or unresponsiveness and any clinical signs of more than slight or momentary pain and distress, or a >5% reduction in body weight from Day 1 to Day 6 and mortality

should be considered in the evaluation. Moribund animals or animals showing signs of severe pain and distress should be humanely killed (28).

Main study experimental schedule

25. The experimental schedule of the assays is as follows:

- Day 1:
  - Individually identify and record the weight of each animal and any clinical observation. Apply 25 µL of the appropriate dilution of the test chemical, the vehicle alone, or the PC (concurrent or recent, based on laboratory policy in considering paragraphs 11-15), to the dorsum of each ear.
- Days 2 and 3:
  - Repeat the application procedure carried out on Day 1.
- Day 4:
  - No treatment.
- Day 5:
  - Inject 0.1 mL (2 mg/mouse) of BrdU (20 mg/mL) solution intra-peritoneally.
- Day 6:
  - Record the weight of each animal and any clinical observation. Approximately 24 hours (24 h) after BrdU injection, humanely kill the animals. Excise the draining auricular lymph nodes from each mouse ear and process separately in phosphate buffered saline (PBS) for each animal. Details and diagrams of the lymph node identification and dissection can be found in reference (14). To further monitor the local skin response in the main study, additional parameters such as scoring of ear erythema or ear thickness measurements (obtained either by using a thickness gauge, or ear punch weight determinations at necropsy) may be included into the study protocol.

Preparation of cell suspensions

26. From each mouse, a single-cell suspension of lymph node cells (LNC) excised bilaterally is prepared by gentle mechanical disaggregation through 200 micron-mesh stainless steel gauze or another acceptable technique for generating a single-cell suspension (*e.g.* use of a disposable plastic pestle to crush the lymph nodes followed by passage through a #70 nylon mesh). The procedure for preparing the LNC suspension is critical in this assay and therefore every operator should establish the skill in advance. Further, the lymph nodes in VC animals are small, so careful operation is important to avoid any artificial effects on SI values. The LNC are harvested with an appropriate volume of cold PBS (*e.g.* 2 mL) and, if necessary, the LNC suspension can be diluted (*e.g.* 1/10 dilution). The number of LNC should be counted and then 1.5×10^6 LNC are needed for the next step.

Determination of cellular proliferation (measurement of BrdU-positive lymphocytes)

27. BrdU-positive lymphocytes are counted through the FCM using a commercially available kit (*e.g.* in the validation study the BD Pharmingen, Franklin Lakes, NJ, USA, was used). Other anti-BrdU antibody kits may be used if they provide consistent results. Briefly, the LNC suspension (1.5×10^6) is washed once with PBS by centrifugation and then re-suspended. Cells are permeabilised with the buffer supplied with the kit and then treated with DNase. After washing, FITC-conjugated anti-BrdU antibody is added and after another wash, 7-aminoactinomycin D (7-AAD) solution is added. The number of BrdU-positive cells within the viable 7-AAD-expressing cell population (10^4 cells) is counted with a flow cytometer.

OBSERVATIONS

Clinical observations

28. Each mouse should be carefully observed at least once daily for any clinical signs, either of local irritation at the application site or of systemic toxicity. All observations are systematically recorded with records being maintained for each mouse. Monitoring plans should include criteria to promptly identify those mice exhibiting systemic toxicity, excessive local skin irritation, or corrosion of skin for euthanasia (28).

Body weights

29. As stated in paragraph 25, individual animal body weights should be measured at the start of the test and at the scheduled humane kill.

CALCULATION OF RESULTS

30. Results for each treatment group are expressed as the mean SI. The SI for the LLNA: BrdU-FCM is derived by dividing the number of BrdU-positive LNCs/mouse of test chemical group or the PC group by the mean number of BrdU-positive LNCs in the solvent/VC group. The average SI for the VCs is then one.

The number of BrdU-positive LNCs is defined as (See Appendix IB-Annex 1 paragraph 7):
Number of BrdU-positive LNCs = % of BrdU-positive cells (% of Q2[1]) × number of LNCs

31. The decision process regards a result as positive when SI $\geq$ 2.7 (1) (2) (10). However, the strength of the dose-response relationship, the statistical significance and the consistency of the solvent/vehicle and PC responses may also be used when determining whether a borderline result is declared positive (6) (29) (30).

32. If it is necessary to clarify the results obtained, consideration should also be given to various properties of the test chemical, including whether it has a structural relationship to known skin sensitisers, whether it causes excessive skin irritation in the

[1] The gated percentage data (Q2 region %) from 'Quadrant Statistics' in the flow cytometer analysis.

mouse, and the nature of the dose-response observed. These and other considerations are discussed in detail elsewhere (31).

33. Collecting data at the level of the individual mouse will enable a statistical analysis for presence and degree of dose-response relationship in the data. Any statistical assessment could include an evaluation of the dose-response relationship as well as suitably adjusted comparisons of test groups (*e.g.* pair-wise dosed group versus concurrent solvent/vehicle control comparisons). Statistical analyses may include, e.g. linear regression or Williams's test to assess dose-response trends, and Dunnett's test for pair-wise comparisons. In choosing an appropriate method of statistical analysis, the investigator should maintain an awareness of possible inequalities of variances and other related problems that may necessitate a data transformation or a non-parametric statistical analysis. In any case, the investigator may need to carry out SI calculations and statistical analyses with and without certain data points (sometimes called "outliers").

DATA AND REPORTING

Data

34. Data should be summarised in tabular form showing the number of BrdU-positive LNCs for the individual animal, the group mean number of BrdU-positive LNCs/animal, or, its associated error term (*e.g.* SD, SEM), and the mean SI for each dose group compared against the concurrent solvent/vehicle control group.

Test report

35. The test report should contain the following information:

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical, if known;

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Controls:

- identification data (*e.g.* CAS number, if available; source; purity; known impurities; lot number);
- physical nature and physicochemical properties (*e.g.* volatility, stability, solubility);

Solvent/vehicle:

- identification data (purity; concentration, where appropriate; volume used);
- justification for choice of vehicle;

Test animals:

- source of BALB/c mice or CBA mice;
- microbiological status of the animals, when known;
- number and age of animals;
- source of animals, housing conditions, diet, etc.;

Test conditions:

- source, lot number, and manufacturer's quality assurance/quality control data (antibody sensitivity and specificity and the limit of detection) for the FCM kit;
- details of test chemical preparation and application;
- justification for dose selection (including results from pre-screen test, if conducted);
- vehicle and test chemical concentrations used, and total amount of test chemical applied;
- details of food and water quality (including diet type/source, water source);
- details of treatment and sampling schedules;
- methods for measurement of toxicity;
- criteria for considering studies as positive or negative;
- details of any protocol deviations and an explanation on how the deviation affects the study design and results;

Reliability check:

- a summary of results of latest reliability check, including information on test chemical, concentration, PC, VC and benchmark test chemical used, as appropriate;
- concurrent and/or historical PC and concurrent VC data for testing laboratory;
- if a concurrent PC was not included, the date and laboratory report for the most recent periodic PC and a report detailing the historical PC data for the laboratory justifying the basis for not conducting a concurrent PC;

Results:

- individual weights of mice at start of dosing and at scheduled humane kill; as well as mean and associated error term (e.g. SD, SEM) for each treatment group;
- time course of onset and signs of toxicity, including dermal irritation at site of administration, if any, for each animal;

- a table of number of BrdU-positive LNCs, and SI values of individual mouse for each treatment group;
- mean and associated error term (e.g. SD, SEM) for number of BrdU-positive LNCs/mouse for each treatment group and the results of outlier analysis for each treatment group;
- calculated SI and an appropriate measure of variability that takes into account the inter-animal variability in both the test chemical and control groups;
- dose-response relationship;
- statistical analyses, where appropriate;

Discussion of results:

- a brief commentary on the results, the dose-response analysis, and statistical analyses, where appropriate, with a conclusion as to whether the test chemical should be considered a skin sensitiser.

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APPENDIX IB - ANNEX I: MEASUREMENT OF BrdU-POSITIVE LNCs WITH FLOW CYTOMETRY

This method is based on the LLNA: BrdU-FCM protocol, which was used for the KoCVAM- coordinated validation study (1). It is recommended that this protocol is used when implementing and using the LLNA: BrdU-FCM in the laboratory.

Preparation prior to measurement

1. To measure incorporated BrdU, the following samples should be prepared prior to the measurement.

- Blank sample (n=1): LNCs from the mouse not injected with BrdU.
- Non-treatment sample (n=1): LNCs from the mouse not treated with any substances, but received a BrdU injection.
- Vehicle control-treatment sample (n≥4): LNCs from the mouse treated with the vehicle control and received a BrdU injection.
- Test chemical-treatment sample (n≥4, a minimum of three concentrations): LNCs from the mouse treated with test chemicals and received a BrdU injection.
- Positive control-treatment sample (n≥4): LNCs from the mouse treated with the positive control and received a BrdU injection.

Analysis of flow cytometric results

2. A flow cytometer should be calibrated using appropriate tools (e.g. 'BD FACSComp' for FACSCaliburTM or 'Beckman coulter FlowCheck' for Cytomics FC500) prior to testing or regularly.

Forward scatter-side scatter (FSC-SSC) graph

1) Both the X axis (FSC) and Y axis (SSC) should be on a linear scale.

2) Set up a zone (gate) with a flock of viable lymph nodes at its centre in the FSC-SSC graph.

3) Outline the gate such that it has at least 10,000 cells.

7-AAD-BrdU graph

1) The X axis (7-AAD, FL3) should be on a linear scale, whereas the Y (BrdU, FL1) axis should be a log scale (Figure 1).

* Compensation should be set using unstained, only BrdU-stained, only 7-AAD stained samples, and double stained with both anti-BrdU and 7-AAD at the time of beginning this assay. The compensation can be saved for future use.

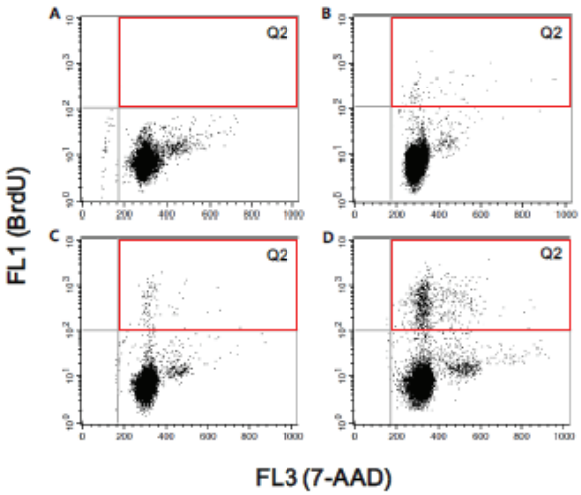
Set up Q2 following the steps below

1) Using the blank sample, set up Q2 (upper right) where no cells are present (Figure 1A).

2) Using the non-treatment sample, set up Q2 so that % BrdU-positive cells are about 1% of all cells (Figure 1B).

3) The Q2 region percentage indicates the proportion of FITC conjugated anti-BrdU-Antibody positive live lymphocyte in 10,000 LNCs.

Figure 1. Flow cytometry configuration for the calculation of % of BrdU-positive cells (% of Q2)



Note: A, blank sample; B, non-treatment sample; C, vehicle control-treatment sample; D, test chemical or positive control-treatment sample

Count of % BrdU-positive cells

3. Perform flow cytometric operation for the vehicle control-treatment samples (Figure 1C), the test chemical-treatment samples and the positive control-treatment samples (Figure 1D). Obtain the gated percentage data (Q2 region %) from 'Quadrant Statistics' for each sample.

Calculation of the SI and the EC2.7

4. The number of BrdU-positive LNCs in the LNs of the vehicle control-treatment group is obtained by multiplying the number of LNCs in the LNs by the ratio of cells expressing BrdU in 10,000 LNCs (obtained by flow cytometry). The number of BrdU-positive LNCs in the LNs of the test chemical-treatment group is obtained by the method described above. Individual SIs are calculated by dividing the number of BrdU-positive LNCs/mouse in the test chemical-treatment group by the mean number of BrdU-positive LNCs in the vehicle control-treatment group. The mean SI of each test chemical group is calculated based on individual SIs.

$$\text{Stimulation Index (SI)} = \frac{\text{Number of BrdU-positive LNCs/mouse exposed to a test chemical}}{\text{Mean number of BrdU-positive LNCs in the vehicle control group}}$$

5. For the positive results, the EC2.7 value, i.e. an estimated concentration showing 2.7 of SI, could be calculated by linear regression method using the following equation.

$$Y\ (SI) = aX(\text{concentration}) + b \ \rightarrow \ EC2.7 = (2.7-b)/a$$

* Parameters a (slope) and b (y-intercept) can be derived using linear least squares method.

Other estimation methods (e.g. linear interpolation or extrapolation formulas) could be utilized to calculate EC2.7 value (32).

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