Report on epiCS® Skin Irritation Test method Validation Study - final testing and submission

Introduction

The "ESAC Opinion on the validation study of the epiCS® test method based on the EURL ECVAM/OECD Performance Standards for in vitro skin irritation testing using Reconstructed human Epidermis (RhE)" (Ref. Ares (2014)4229618 - 16/12/2014) dated 17.11.2014 was sent to CellSystems by EURL ECVAM on 16.12.2015 (Annex 1).

ESAC recommends to additionally test the "remaining 14 and 13 RCs in the two non-naïve laboratories in order to arrive at a complete data set for all 20 RCs, generated under identical conditions of SOP implementation" (Annex 1,2).

ESAC asked to "report the newly generated data (13 and 14 RCs from the two naïve laboratories) together with the data set from 2013 (i.e. 20 RCS from ACS, 6 and 7 RCs in IIVS and Harlan using our EURL ECVAM reporting sheet" (Annex 2). The EURL ECVAM reporting sheet is Annex 3.

"ESAC strongly suggested that the same laboratories are used for this third phase testing." (Annex 2).

We hereby report the final testing and results as recommended by ESAC.

Excel spread sheets that were also used in the phase of "additional testing" in 2013 were used by the participating laboratories for collecting data.

We exactly followed this recommendation for the final testing. Reporting of data to EURL ECVAM is done with the EURL ECVAM reporting sheet provided by EURL ECVAM (Claudius Griesinger and Bertrand Desprez, latest update 26.10.2015).

The testing procedure and the SOP for this final testing were identical to the "additional testing" in 2013.

1. Participants of the study

The same non-naïve test laboratories that carried out the testing in 2013 carried out the final testing in 2015.

1.1 Test laboratories

Harlan, Roßdorf, Germany - testing was carried out by Christine Behle-Wagner.

Institute for In Vitro Sciences (IIVS) - testing was carried out by Nicole Barnes and Nathan Wilt.

1.2 Training of test laboratories

The laboratories had used the epiCS SIT method in 2013 and did not use it until the beginning of the final testing in 2015. Due to this long period of not using the method, we decided to re-train the laboratories in order to make sure, that they carry out the epiCS SIT method as in 2013 and follow strictly the SOP. This training was divided into two parts:

I) Going step by step through the SOP to make sure the SOP was well followed, especially the steps of tissue rinsing and the post-incubation part. Here, it was essential, that one 6-well plate was used to incubate tissues that were exposed to one chemical only.

II) Testing a set of chemicals as a pre-test

Chemicals (non coded): potassium hydroxide 5%, heptanal, Isopropanol hexylsalicylate, 1-bromohexane

These chemicals were shipped from CellSystems to Harlan, and from CAAT-Europe to IIVS - concurrently with the blinded RC.

During the pre-test at IIVS, it was discovered that potassium hydroxide (KOH) was shipped as solid substance - 100% KOH as pellets and not as KOH, 5% aqueous solution. Communication with CAAT-Europe revealed that solid 100% KOH was sent as Reference Chemical (RC) to IIVS and to Harlan. For correction CAAT-Europe replaced 100% KOH at IIVS and at Harlan by KOH, 5% aqueous solution, the correct RC.

Concurrently the coded RC 1-bromohexane was replaced by 1-bromohexane with a different code. Both RC where newly coded to keep blinding for all chemicals.

Due to the testing schedule IIVS carried out all tests with correct RC, whereas Harlan carried out the first trial before detection of this error, so only from trial 2 onwards the correct RC were tested. (see 2.2.1.1)

1.3 Coding and distribution of reference chemicals

Center for Alternatives to Animal Testing (CAAT)-Europe, Konstanz, Germany - Mardas Daneshian

Aliquoting, coding and sending of the RC was done by CAAT-Europe.

After finalization of the testing in the laboratories, the RC were decoded by CAAT-Europe.

2. Course of the study

2.1 Blinding and distribution of substances

The listed RC were coded by CAAT-Europe and sent to IIVS and Harlan:

			Harlan		IIVS	
No.	**RC**	**CAS number**	**CAAT code**	**Intern. code**	**CAAT code**	**Intern. code**
1	methyl stearate	112-61-8	A531	C1	E936	15AD68
2	di-n-propyl disulphide	629-19-6	A420	C2	E862	15AD69
3	hexyl salicylate	6259-76-3	A365	C3	E451	15AD70
4	heptanal	111-71-7	A674	C4	E922	15AD71
5	diethyl phthalate	84-66-2	A932	C5	E153	15AD72
6	1-bromohexane	111-25-1	A852 (A243)	C6	E273 (E853)	15AD73
7	naphthalene acetic acid	86-87-3	A197	C7	E642	15AD74
8	isopropanol	67-63-0	A255	C8	E843	15AD75
9	1-decanol	112-30-1	-	-	E358	15AD76
10	potassium hydroxide (5% aq)	1310-58-3	A795 (A188)	C9	E512 (E133)	15AD77
11	2-chloromethyl-4-methoxy-3,5-dimethlypryridine HCl	86604-75-3	A436	C10	E234	15AD78
12	cinnamaldehyde	104-55-2	A268	C11	E543	15AD79
13	2-methyl-3-tert-butylthiophenol	7340-90-1	A519	C12	E680	15AD80
14	tetrachlorethylene	127-18-4	A631	C13	E415	15AD81

Intern. = internal code; Harlan and IIVS applied this additional internal coding to RC

RC = reference chemical

code in brackets "()" = code of replacing RC.

2.2 Testing phase

Testing was carried out using the same SOP as for the RC testing in 2013.

Data collection was done with the same EXCEL sheets that were used in 2013.

2.2.1 Harlan

In all trials, the values for the positive controls (PC) and negative controls (NC) were within the acceptance range, i.e. the mean OD_{570} of the NC tissues was ≥ 1.0 and did not exceed 2.8. The mean viability of PC expressed as % of the negative control was ≤ 20 % in all trials.

2.2.1.1 Trial 1

This trial was carried out in week 19 with epiCS lot: 100-AE0816-1;

NC (OD = 1.549) and PC (mean viability = 1.9%) were within the acceptance range.

All runs, except for the run for chemical di-n-propyl disulphide (code A420), were valid.

Remark:

After having carried out the first trial it was discovered that potassium hydroxide (KOH) was not tested as 5% aqueous solution but as a solid substance - 100% KOH (see 1.2). The correction measure was: 100% KOH (code A795) and 1-bromohexane (code A852) were replaced with 5% KOH (new code A188) and 1-bromohexane (new code A243) in a blinded way. This exchange of two coded substances secured that all substances remained blinded during the testing phase.

Retesting:

Di-n-propyl disulphide (code A420) had to be retested.

Although 5% KOH was not tested in the first trial, and 1-bromohexane was already tested in the first trial, we decided to allow 3 test runs for 5% KOH (new code A188) and additional 3 test runs for 1-bromohexane with the new code (new code A243). Only the first three test runs of 1-bromohexane (irrespective of chemical coding) were transferred to the EURL ECVAM reporting sheet.

The values for testing of 100% KOH were not transferred into the EURL ECVAM reporting sheet. The respective cells in the sheet were kept blank.

2.2.1.2 Trial 2

This trial was carried out in week 21 with epiCS lot: 100-AE0844-1;

NC (OD = 1.796) and PC (mean viability = 0.5 %) were within the acceptance range.
All runs were valid.

Substances 5% KOH (A188) and 1-bromohexane (A243) were tested for the first time with this new code. Hence, it was the first run for code A188 and A243 but the second run for the RC 1-bromohexane.

Retesting:

none

2.2.1.3 Trial 3

This trial was carried out in week 22 with epiCS lot: 100-AE0858-1.

NC (OD = 1.588) and PC (mean viability = 1.5%) were within the acceptance range.

All runs - except for naphthalene acetic acid (code A197) - were valid.

Retesting:

Naphthalene acetic acid (code A197) had to be retested.

2.2.1.4 First retesting trial and third run for 5% KOH and 1-bromohexane as code A243.

This trial was carried out in week 25 with epiCS lot: 100-AE1040-2;
NC (OD = 1.826) and PC (mean viability = 1.7%) were within the acceptance range.

For this trial, the following chemicals were identified to be retested caused by results in trial 1 and 3:

Di-n-propyl disulphide (code A420) - one invalid run in trial 1.

Naphthalene acetic acid (code A197) - one invalid run in trial 3.

For 5% KOH (code A188) and 1-bromohexane (code A243) - these runs were the third runs of the replaced RC.

All runs were valid.

Only the first three runs of 1-bromohexane were reported in the EURL ECVAM reporting sheet for data analysis.

2.2.2 IIVS

Before trial 1 was carried out it was discovered that instead of KOH, 5 % aqueous solution, KOH 100% was shipped to IIVS (see 1.2). As correction the solid KOH (code E512) from the blinded RC and another blinded liquid RC (1-bromohexane, code E273) were excluded from testing. A differently coded 5% KOH solution (new code E133) and the differently coded 1-bromohexane (new code E853) were sent to IIVS. This exchange of two coded substances secured that all substances remained blinded during the testing phase.

2.2.2.1 Trial 1

Carried out in week 28 with epiCS lot 100- AE1220-1.

NC (OD = 1.838) and PC (1.9% mean viability) were within the acceptance range.

All runs were valid except for the run of 1-decanol (code E358) with SD=18.65%.

Retesting:

1-decanol (code E358) had to be retested.

2.2.2.2 Trial 2 (non valid)

Carried out in week 29 with epiCS lot 100-AE1234-1.

NC (OD = 1.903) was within the acceptance range, but PC (39% mean viability) was not.

For the PC SD was 61.20 %.

Therefore this was a non valid trial.

Thus, data were not transferred into the EURL ECVAM reporting sheet.

2.2.2.3 Trial 3 (second valid trial)

Carried out in week 30 with epiCS lot 100-AE1250-1.

NC (OD=1.764) and PC (4.5% mean viability) were within the acceptance range.

KOH 5% (code E133) showed SD=33.52% and therefore this test run was invalid.

Retesting:

KOH 5% (Code E133) had to be retested

2.2.2.4 Trial 4 (third valid trial)

Carried out in week 33 with epiCS lot 100-AE1444-2.

NC (OD=1.916) and PC (0.6 % mean viability) were within the acceptance range.

All runs were valid.

Retesting:

none

2.2.2.5 Trial 5 (non valid retesting)

Carried out in week 36 with epiCS lot 100-AE1608-1.

Bacterial / fungal contamination was observed.

PC showed 36,5 % viability and was above the acceptance criterion ≤ 20 %.

Therefore, this trial was invalid and not reported in the EURL ECVAM reporting sheet for data analysis.

2.2.2.6 Trial 6 (first valid retesting)

Carried out in week 37 with epiCS lot 100-AE-1624-1

NC (OD=2.034) and PC (0.8 % mean viability) were within the acceptance range.

Run for 1-decanol (code E358) was invalid (SD=58.14 %). This resulted in a second retesting run.

Run for KOH 5% (code E133) was valid.

Retesting:

1-decanol had to be retested

2.2.2.7 Trial 7 (second valid retesting)

Carried out in week 41 with epiCS lot 100-AE-1818-1

NC (OD=2.103) and PC (1.8 % mean viability) were within the acceptance range.

Run for 1-decanol (code E358, internal code 15AD76) was valid.

3. Decoding of chemicals

After finalization of the trials and the retesting, the chemicals were decoded by CAAT-Europe. Harlan and IIVS received the decoding tables from CAAT-Europe. Both laboratories checked if any peculiarities had appeared.

Harlan and IIVS did not observe any peculiarities, which allowed finalization of the testing phase.

4. Data analysis

For data analysis, the values from the data collecting spread sheets provided by Harlan and IIVS were transferred into the EURL ECVAM reporting sheet.

The EURL ECVAM reporting sheet contains data generated during final testing as well as the data set from 2013 (i.e. 20 RCs from ACS, 6 and 7 RCs from IIVS and Harlan).

The reporting sheet shows that all criteria of the Performance Standards were met.

5. Abbreviations

PC	positive control
NC	negative control
OD	optical density
SD	standard deviation
CAAT	Center for Alternatives to Animal Testing
IIVS	Institute for In Vitro Sciences
RC	reference chemical
KOH	potassium hydroxide
SOP	standard operating procedure

6. Annexes

1. ESAC opinion
2. EURL ECVAM letter: ESAC Peer review on the revised full submission on the EST1000 SIT/epiCS® SIT for skin irritation testing (ref. TM2009-09)
3. EURL ECVAM reporting sheet