Quality assurance in NAMs (cell viability, cell source, positive controls) and limitations for materials with limited solubility – How are these currently addressed?

IDEA meeting on NAM

10.12.2019, Andreas Natsch

Agenda

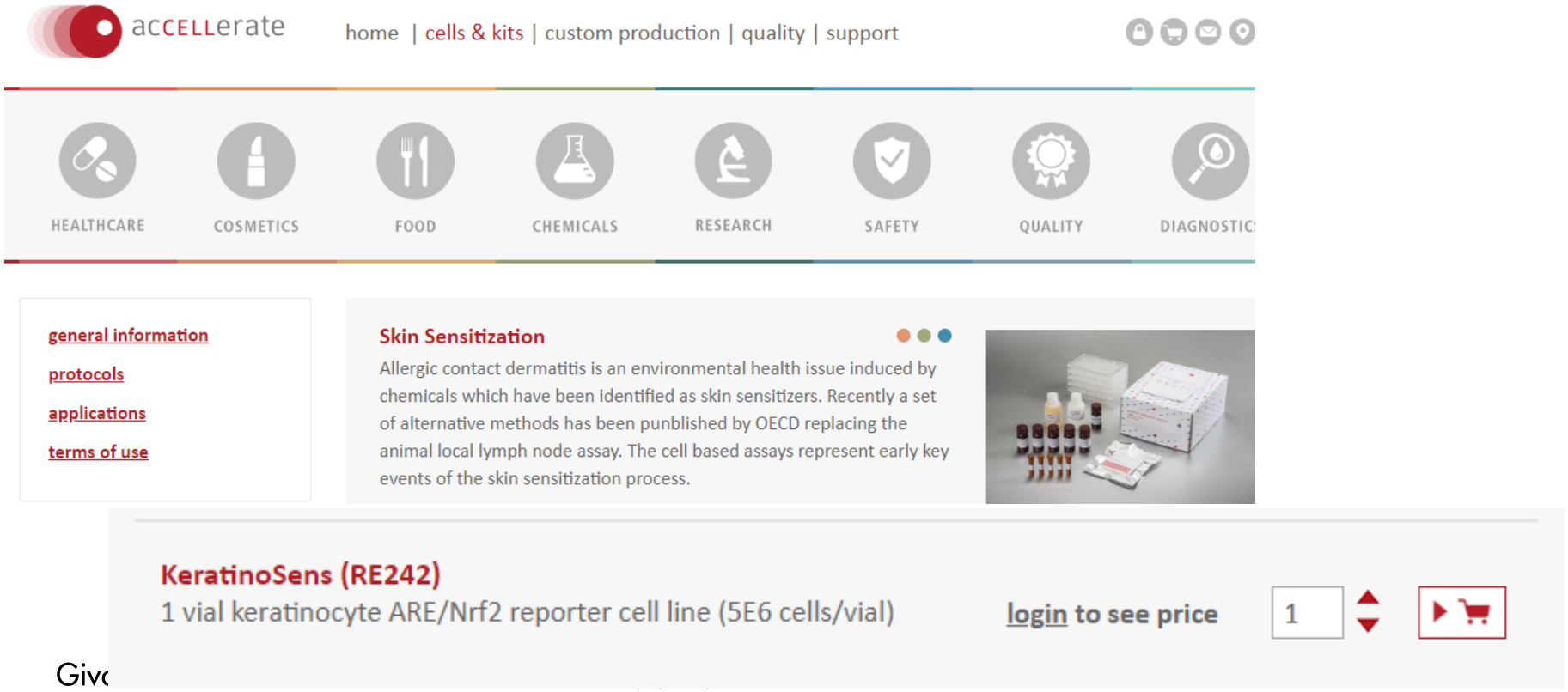
1. **Cell source**
   1. KeratinoSens and stability over time
   2. hClat

2. **Positive controls and proficiency chemicals**
   1. KeratinoSens positive controls and proficiency chemicals over time
   2. DPRA proficiency chemicals and positive controls over time
   3. kDPRA reproducibility

3. **Limitations for materials with limited solubility**
   1. Testing above saturation in DPRA
   2. Testing above saturation in KeratinoSens

Cell source for KeratinoSens®

- Intitially, cells were shipped to all labs from Givaudan directly
- Every batch was tested on 7 proficiency chemicals by Givaudan
- Now cells are sold by Accelerate: they produced one very large batch – this batch was validated by Givaudan on the same 7 proficiency chemicals



Cell source for hClat

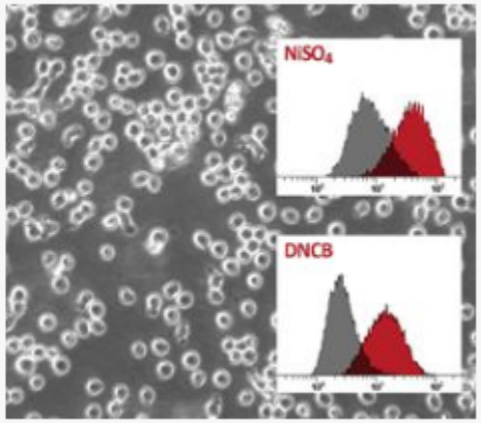
- Unlike KeratinoSens, which is a transgenic cell line, hClat is performed with standard THP-1 cells
- Can be obtained form any cell vendor
- Accelerate also provides tested 'assay ready cells'
- No details known about extend of internal validation done, but response by DNCB and Nickel verified

instaCELL skin sensitization assay kit I (h-CLAT) (SF210)
- THP-1 assay ready cells (4 vial)
- reagents, buffers and antibodies to perform 1x dose finding and 3x h-CLAT according to DB-ALM protocol N° 158

login to see price 1

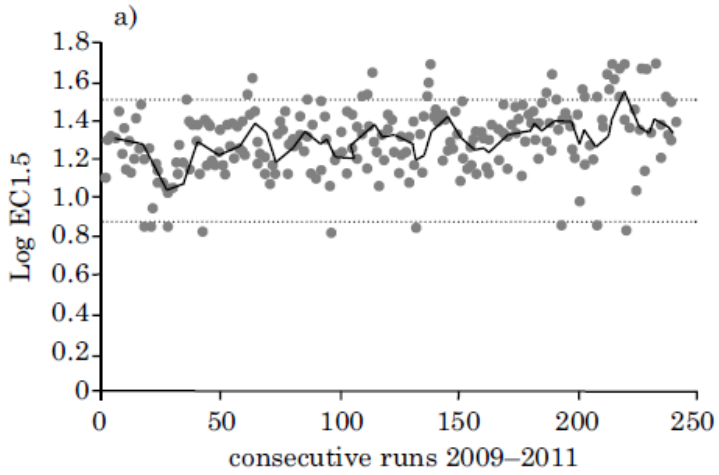
h-CLAT the human Cell Line Activation Test and U-Sens using THP-1 or U937 cells which respond to skin sensitizes with the expression of CD54 and CD86 (OECD 442E). The instaCELL skin sensitization kit I contain all required reagents including assay ready THP-1 cells, which can be used for h-CLAT without prior cultivation. The cells recover at high viability and reproducibly discriminate between sensitizers and non-sensitizers.

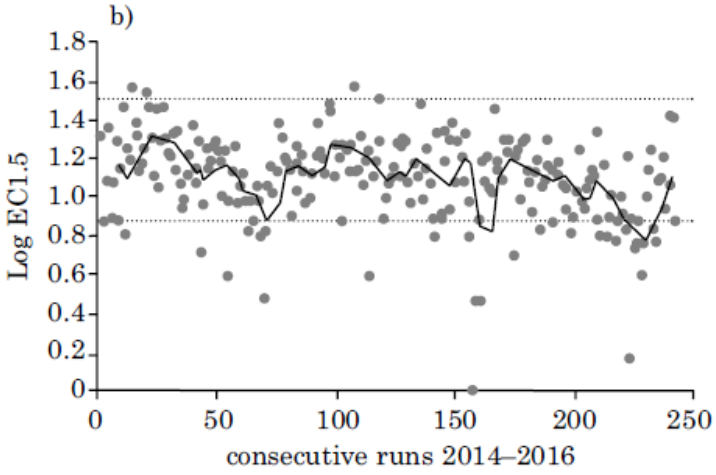
The keratinocyte cell lines KeratinoSens® and LuSens have been developed by Givaudan and BASF and express Luciferase reporter upon activation of oxidative stress pathways (OECD 442D). The cell lines are provided by acCELLerate.

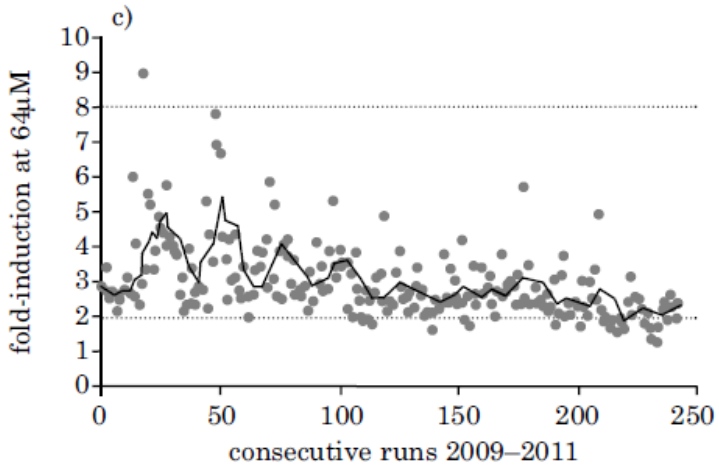


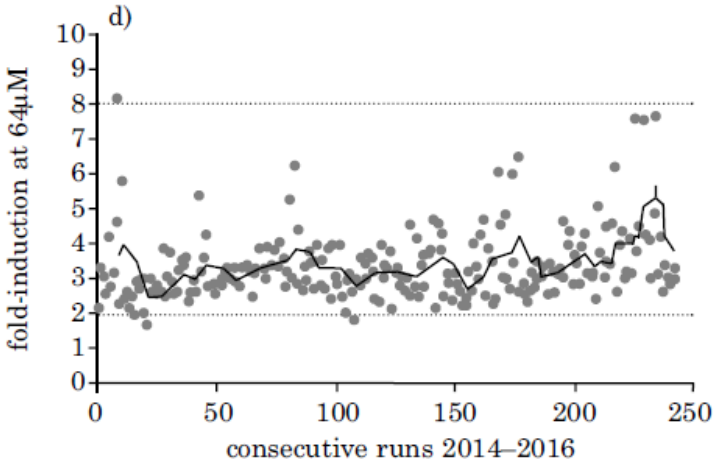
KeratinoSens positive controls over time

- Cinnamic aldehyde needs to be rated positive -> Always fullfilled
- EC 1.5 should be in range 7 – 30 µM
- SI at 64 µM 2 - 8 fold









KeratinoSens proficiency chemicals over time

- Every batch sent to external labs was tested on seven chemicals also tested in the validation study
- In general high stability of qualitative **AND quantitative data**
- **Guarantees that labs receive cells fully compatible with the test as validated**

Table 2: Reproducibility and stability of the KeratinoSens assay over time — Luciferase determinations

	QC batch 2016[a]	QC batch 2014[a]	QC batch 2013[a]	Givaudan historical 2009[b]	Median ring study 2009[c]	Range ring study 2009
Cinnamic alcohol	94.64	107.60	57.86	104.1/123.5	108.1	86.0–131.0
Cinnamic aldehyde	19.65	9.32	12.79	14.3/16.1	9.1	6.0–14.8
p-phenylenediamine	10.69	6.24	6.00	9.9/5.0	8.7	6.0–10.1
Ethylenglycol dimethacrylate	70.92	36.33	41.44	81.5/56.5	46.7	10.6–112.2
DNCB	1.99	2.14	2.60	3.3/2.5	2.3	1.4–3.3
Sulphanilamide	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Lactic acid	n.i.	n.i.	n.i..	n.i.	n.i.	n.i.

The values shown correspond to the EC1.5 value (the concentration, in µM, that induces luciferase activity 1.5-fold, up to a concentration of 1000µM).

[a]*Verification of quality control (QC) cell batches used for assay transfer;*

[b]*the first value is the Givaudan value in the ring study (2009–2010), the second value is the historical Givaudan value prior to the ring study (2009);*

n.i. = no induction over threshold of 1.5.

DPRA proficiency chemicals

- DPRA also requires testing of 10 proficiency chemicals
- Test in house to set up test, with exception of Farnesal, all very nicely fulfilled the range

Table 4: Tests on proficiency chemicals for demonstrating technical proficiency with the Direct Peptide Reactivity Assay: Cysteine Peptide.

Proficiency chemicals	CASRN	Historical Range[3] of % Cys-peptide depletion	Historical Average % Cys-peptide depletion	Measured average % Cys-peptide depletion	Measured Standard deviation Cys-peptide depletion	Criteria passed
2,4-Dinitrochlorobenzene	97-00-7	90-100	99.5	100.0	0.0	Ok
Oxazolone	15646-46-5	60-80	74.1	60.8	0.2	Ok
Formaldehyde	50-00-0	30-60	47.3	49.2	1.6	Ok
Benzylideneacetone	122-57-6	80-100	92.2	94.6	0.3	Ok
Farnesal	19317-11-4	15-55	34.0	74.8	3.5	not ok
2,3-Butanedione	431-03-8	60-100	77.8	82.3	1.7	Ok
1-Butanol	71-36-3	0-7	0.7	1.1	0.7	Ok
6-Methylcoumarin	92-48-8	0-7	1.5	2.7	1.8	Ok
Lactic Acid	50-21-5	0-7	1.4	1.0	1.5	Ok
4-Methoxyacetophenone	100-06-1	0-7	1.3	0.7	1.2	Ok

[1]The in vivo hazard classification (and potency classification) is based on LLNA data.

[2] A DPRA prediction should be considered in the framework of an IATA and in accordance with the provisions of paragraphs 9 and 11.

[3]Ranges determined on the basis of at least 10 depletion values generated by 6 independent laboratories.

G

DPRA proficiency chemicals

- Also for Lys-peptide: Very close to historical average

Table 5: Tests on proficiency chemicals for demonstrating technical proficiency with the Direct Peptide Reactivity Assay: Lysteine Peptide

Proficiency chemicals	CASRN	Historical Range[3] of % Lys peptide depletion	Historical Average % Lys peptide depletion	Measured average % Lys peptide depletion	Measured Standard deviation Lys peptide depletion	Criteria passed
2,4-Dinitrochlorobenzene	97-00-7	15-45	25.8	24.0	3.0	Ok
Oxazolone	15646-46-5	10-55	42.2	54.9	3.3	Ok
Formaldehyde	50-00-0	0-24	5.2	1.9	0.8	Ok
Benzylideneacetone	122-57-6	0-7	1.6	4.8	1.4	Ok
Farnesal	19317-11-4	0-25	6.7	8.4	0.9	Ok
2,3-Butanedione	431-03-8	10-45	26.5	34.0	0.6	Ok
1-Butanol	71-36-3	0-5.5	0.2	0.4	0.4	Ok
6-Methylcoumarin	92-48-8	0-5.5	0.9	0.6	0.9	Ok
Lactic Acid	50-21-5	0-5.5	0.3	0.2	0.3	Ok
4-Methoxyacetophenone	100-06-1	0-5.5	0.8	0.5	0.1	Ok

DPRA: Stability over time

- PC is always tested in each run
- Very high stability over time

Table C1: Data on repeated testing of positive control at testing facility

	% depletion	Standard deviation
Historical range Cysteine peptide according to DBALM protocol	***60.8-100***	
Experiment 1 Cysteine peptide	77.2	0.7
Experiment 2 Cysteine peptide	78.9	0.2
Experiment 3 Cysteine peptide	78.1	0.8
Experiment 4 Cysteine peptide	90.0	0.7
Experiment 5 Cysteine peptide	78.8	0.6
Experiment 6 Cysteine peptide	65.9	0.7
Experiment 7 Cysteine peptide	75.9	1.1
Experiment 8 Cysteine peptide	64.4	1.3
Experiment 9 Cysteine peptide	67.2	0.5
Experiment 10 Cysteine peptide	66.1	1.1
Experiment 11 Cysteine peptide (this study)	68.4	0.3
Historical range Lysine peptide according to DBALM protocol	***40.2-69.4***	
Experiment 1 Lysine peptide	64.2	0.9
Experiment 2 Lysine peptide	66.0	0.4
Experiment 3 Lysine peptide	62.8	1.0
Experiment 4 Lysine peptide	57.2	0.3
Experiment 5 Lysine peptide	50.7	2.8
Experiment 6 Lysine peptide	50.0	3.2
Experiment 7 Lysine peptide	43.5	1.7
Experiment 8 Lysine peptide	52.0	18.9*
Experiment 9 Lysine peptide	53.6	2.0
Experiment 10 Lysine peptide (this study)	57.7	2.2

*co-elution in one sample

kDPRA quality assurance

- K DPRA measures kinetic rate at 90 min in each run for quality assurance
- Very high reproducibility in Phase I and Phase II of the validation study

Table 1. Reproducibility of positive control: log $k_{90\ min}$ values [$M^{-1}s^{-1}$]

	Average	SD	Min	Max
All labs Phase II	**-1.58**	**0.04**	**-1.64**	**-1.53**
Lab A	-1.53	0.07	-1.66	-1.43
Lab B	-1.60	0.04	-1.68	-1.53
Lab E	-1.62	0.08	-1.75	-1.48
Lab F	-1.64	0.07	-1.75	-1.52
Lab D	-1.60	0.08	-1.75	-1.48
Lab C	-1.54	0.07	-1.66	-1.37
Lab G	-1.56	0.04	-1.64	-1.47
All labs Phase I [1)]	*-1.60*	*0.08*	*-1.73*	*-1.38*

[1)] *Shown for comparison*

kDPRA reproducibility

- Just an example of 12 chemicals tested in the validation for interlaboratory reproducibility

Figure 1. Log k_{max} values from inter-laboratory testing, Chemicals 13-24. For laboratory 5 – 8, the average from repeated testing is plotted. Shown are the 7 individual lab results (circles), the interquartile range box (blue) and the average (horizontal line).

Quality assurance in LLNA

- OECD 429:”Adequate proficiency with the LLNA 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)”
- Only positive control tested at one concentration (and sometimes only with pooled animals, i.e. no statistics)

- Thus in *in vitro* tests, much more quality assurance is built into the guidelines
  - PC run everytime – including dose-response data and statistically evaluated data
  - Proficiency chemicals run before laboratory is considered proficient, includes not only yes/no but also a target range

  - **Nothing to blame with the LLNA - This higher data requirement is of course due to the fact that it is much easier to do in vitro testing than adding many animal test control groups for ethical and logistic reasons !!!**

Limitations for materials with limited solubility

- Limited solubility in the currently validated *in vitro* assays (442c,442d and 442e) is frequently discussed
- All these assays use solution of the chemicals in a solvent, which is then added to the aqueous test medium
- Top-test concentrations are often above saturation level
- Thus a saturated solution is formed – but part of the chemical will drop out of the solution as small oil droplets /emulsion /precipitate
- The dissolved concentration is different from the nominal test concentration

- For Keratinosense and hClat, some, but overall few of the false-negatives can be attributed to such limited solubility
- However, also for many chemicals precipitating at top-doses, still correct identification of a positive sensitization potential has been reported

- This has recently been studied in more detail for DPRA and kDPRA ….

Precipitation in DPRA

- At the test concentration (5 mM) in the DPRA, precipitation was observed for 16 of 82 test chemicals under the Cys-peptide conditions, i.e. at the top concentration of the kDPRA by Yamamoto *et al.*
  - Precipitation indicates that the test chemical concentration is above saturation and that actually a lower concentration of the chemical is available for reaction.
  - In case the molecule reacts with the peptide, one would expect that more chemicals will re-dissolve and be able to participate in the reaction, but the molar ratio test chemical to peptide will not be as expected and not constant
- In the study of Yamamoto *et al.*, however, this observation had no effect on the prediction accuracy
- **Actually for the chemicals for which precipitation was observed, there were five false-negatives with the DPRA and seven false negatives with the ADRA, the latter being conducted at non-precipitating concentrations**.

Precipitation in kDPRA

- Also for chemicals precipitating in DPRA according Yamamoto, strong reactivity and GHS1A attribution was found in kDPRA

Chemicals for which Yamamoto observed precipitation under Cys-conditions in DPRA	**CAS**	**LLNA EC3 con-solidated**	**Consoli-dated GHS LLNA**	**Consoli-dated GHS Human**	**log k_{max}**	**Potency Classif-ication reviseed threshold**
Benzoyl peroxide	94-36-0	0.06	1A	1B	0.741614	1A
CMI	26172-55-4	0.009	1A	1A	0.596191	1A
TCS	*1154-59-2*	*0.04*	*1A*		*(-0.458) Autoflu-orescence*	*1A*
DNCB	97-00-7	0.06	1A	1A	-0.55653	1A
Lauryl gallate	1166-52-5	0.3	1A	1A	-0.97626	1A
methyl-2-nonynoate	111-80-8	2.5	1B	1A	-1.66122	1A
Phenylacetaldehyde	122-78-1	4.7	1B	1A	-2.36271	1B/NC
2-phenyl-propionaldehyde	93-53-8	6.3	1B	1B	-2.67551	1B/NC
palmitoyl chloride	112-67-4	8.8	1B		-3.38468	1B/NC
Farnesal	4602-84-0	4.8	1B	1B	-3.40789	1B/NC
Cyclamen aldehyde	103-95-7	22.3	1B		-3.53135	1B/NC
IPM	110-27-0	44	1B	NC	not reactive	1B/NC

Conclusions

- For NAM, many more questions are being asked as compared to traditional animal testing
  - For example nobody asked in the LLNA, when a chemical is tested in Acetone:olive oil 4:1, what the solution state of a chemicals in the olive oil layer on the skin actually is after acetone has evaporated.
  - Dissolution state also may affect dose entering the skin…..
- Due to the medium-throughput nature of the *in vitro* assays, more data on quality assurance is being produced (positive controls, proficiency chemicals)
- For KeratinoSens and DPRA we have very good understanding of variability of the *quantitative* data
- Less data published on hClat – but quantitative data correlate well to KeratinoSens and also appear to be reproducible

Thank you

Contact