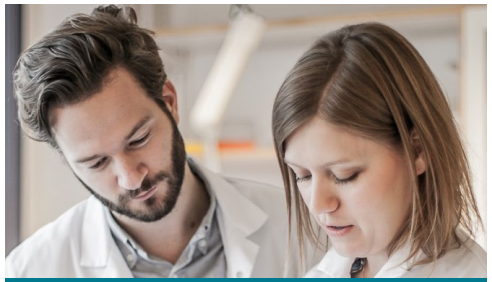
How are the *in vitro* GARD™ assays used in real life?

Cases and applications for testing of skin and respiratory sensitizers

Andy Forreryd, PhD

About **SenzaGen**

- Founded in 2010
- Spin-out from Lund University after over 10 years research
- Highly multidisciplinary team: 20+ employees today



- Business model: global industrial and CRO partnerships
- Own laboratory, continuous development of the technology

Our lead product, GARD™ stands for Genomic Allergen Rapid Detection and is a state-of-the art test platform for assessment of chemical sensitizers

Launched assays:

- GARD™skin/GARD™potency for skin sensitization
- GARD™air for respiratory sensitization
- GARD ™skin Medical Device for skin sensitization assessment of medical devices

Items tested include:

- Active pharmaceutical ingredients
- Cosmetic ingredients
- Industrial chemicals
- Agrochemicals
- Medical device materials
- UVCBs, Pre-/pro haptens

- Since Sept. 2017, SenzaGen AB's shares have been traded on Nasdaq First North, Stockholm (SENZA)
- Partners in USA, EU, China, Korea

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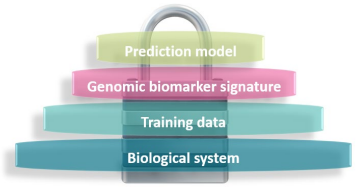
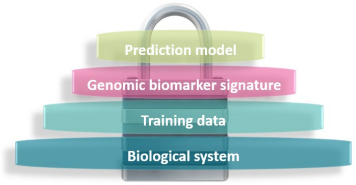
The **GARD platform** – Currently available assays



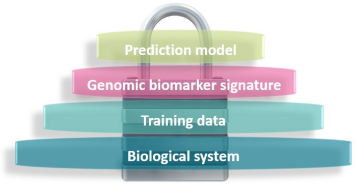
GARD™skin (200 genes)

To identify the skin sensitization hazard of chemicals



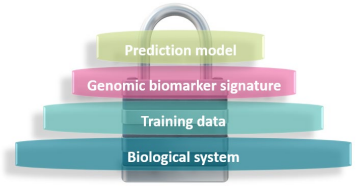
GARD™skin Medical Device (200 genes)

To identify the skin sensitization hazard of medical devices



GARD™potency (51 genes)

Skin sensitization potency classification according to GHS/CLP



GARD™air (28 genes)

To identify the respiratory sensitization hazard of chemicals

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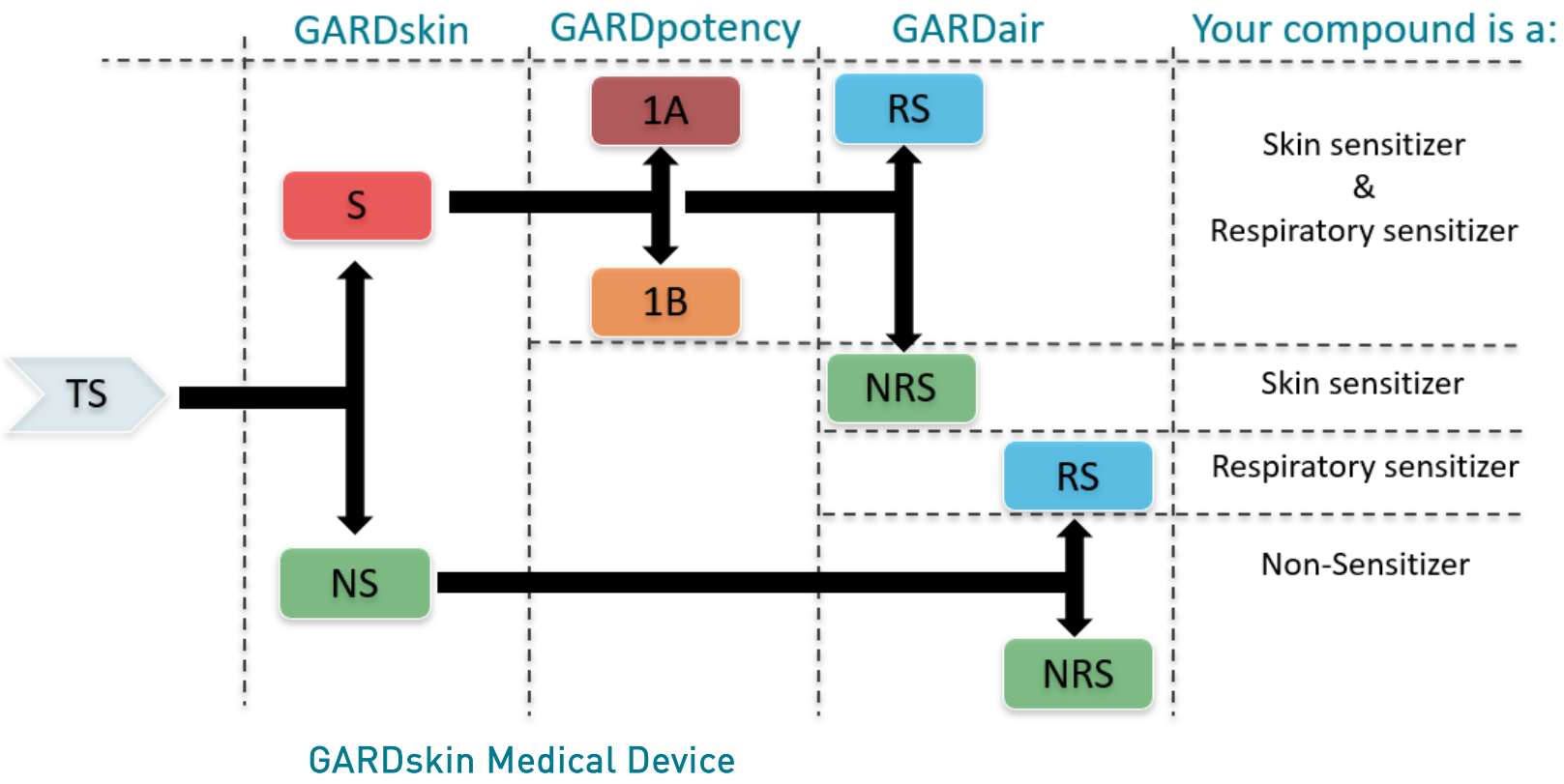
The GARD platform – One testing platform for many answers

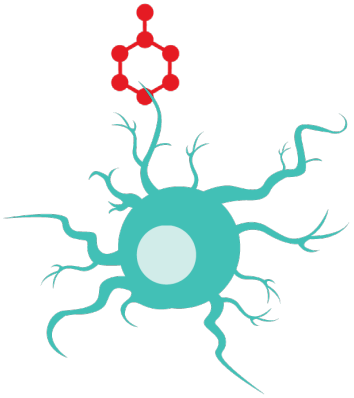


The GARD technology platform

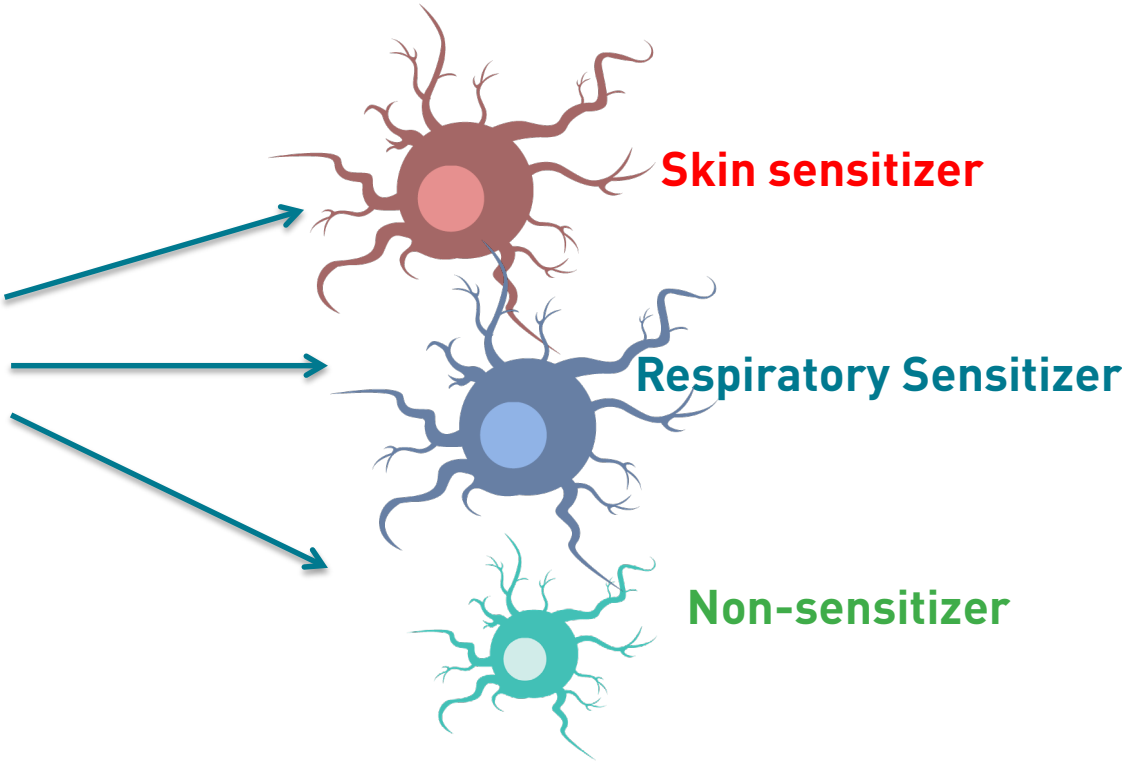
Human relevant cells in combination with Genomics and machine learning

The GARD platform – how it works

SenzaCells: a human dendritic-like cell-line



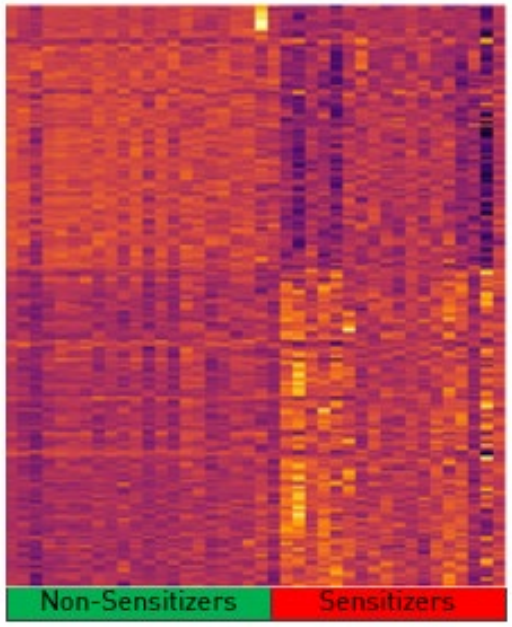
Cellular responses



The GARD platform – how it works

Cellular response is monitored using biomarker signatures – **Not only a single biomarker**

GARDskin prediction signature
200 genes



Recognition of foreign substances
e.g. **TLR**s, **RXR**, **AHR**

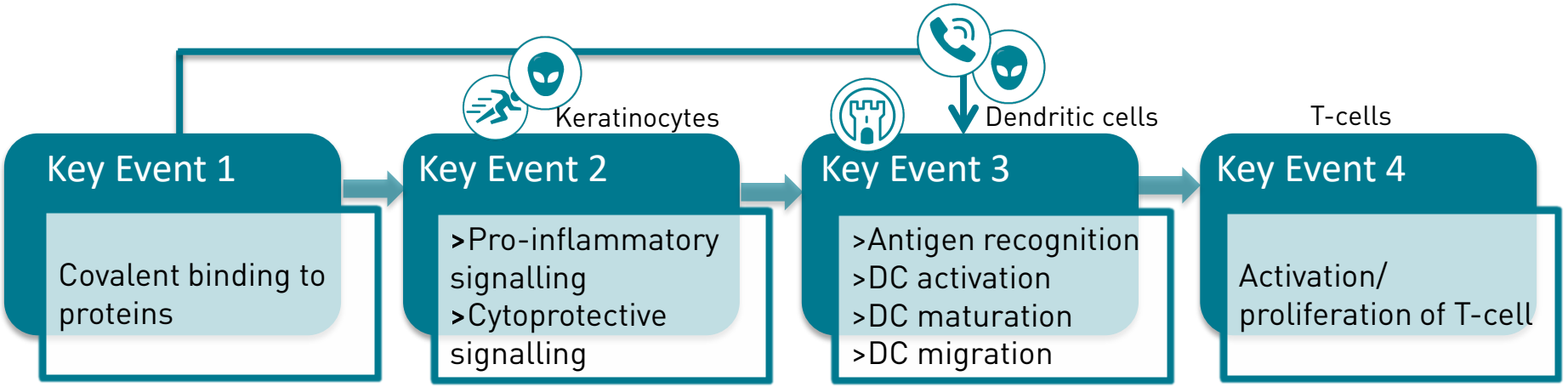
Immunological self-defence mechanisms
e.g. **CD80**, **CD86**

Cellular stress responses
e.g. **NRF2**-pathway

Communication
e.g. chemotaxis receptors

BMC Genomics
Johansson *et al. BMC Genomics* 2011, **12**:399
http://www.biomedcentral.com/1471-2164/12/399

The GARD platform – how it works



Captures events downstream of KE1

Metabolic activity & identifies pre/pro haptens
ALDH
NAT-1
CYP - Cytochrome p-450

> Keap1-Nrf2-ARE pathway & AHR signalling
NQO1
HMOX1
Thioredoxin reductase I

> Pro-Inflammatory cytokines mediating e.g. TNFα, INFγ, IL-8
FAS
MAP2KI
COX20

> Inflammasome
NLRP
PSTPIP1

> DC migration & maturation
CD86
MAPK- activation
PKA- and GPCR- mediated signalling

> Antigen recognition & Innate immune activation
TLR-4
TLR-6
RXRA – retinoic X receptor
NLRP
PSTPIP1

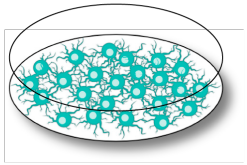
> Self-defence mechanisms
C3a/C5a-activation pathways

Covers the 3 Key steps for T-cell activation:

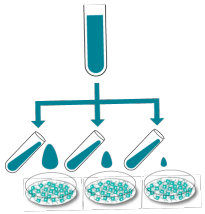
Antigen presentation
Co-stimulation
Cytokine secretion

How to **GARD** your product - in **6 Steps**

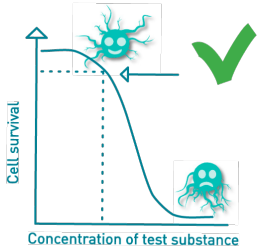
1 GARD Input Finder



Grown SenzaCells

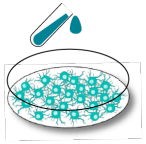


Add different concentrations of the test substance to the cells

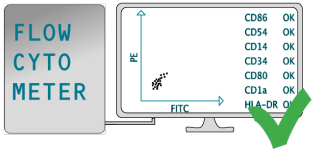


Determine the concentration of the test substance where the cells react and 90% survive

2 GARD Main Stimulation

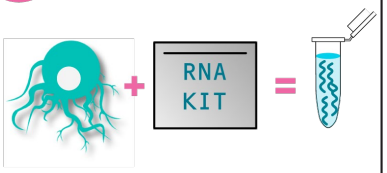


Take test substance at determined concentration and add to fresh batch of cells



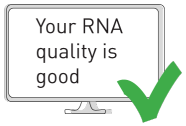
Quality control of the cells

3 RNA extraction

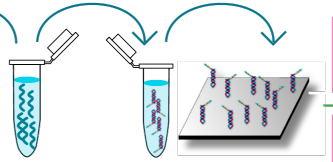


Extract RNA from the cells

4 Gene expression profiling



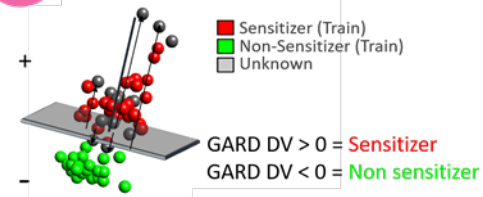
Check the RNA quality



Mix the isolated RNA with reporter probes and load onto a cassette

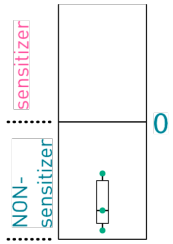
Analyze the probe cassette to quantify the RNA

5 GARD data analysis application



Upload the results to the GDAA web app. One press of the button and the algorithm crunches the data

6 Results



Dear customer,

We have determined that your test substance is a non-sensitizer.

Kindly,

The results are yours!

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GARDskin – Performance data

Data set	Sensitivity	Specificity	Accuracy	Reference
Validation of the GARD assay	**93% (51/55)**	**96% (24/25)**	**94% (75/80)**	**Johansson, 2019**
Accumulated performance	92% (134/145)	81% (50/62)	**89% (184/207)**	-

TOXICOLOGICAL SCIENCES, 170(2), 2019, 374–381

doi: 10.1093/toxsci/kfz108
Advance Access Publication Date: May 17, 2019
Research Article

Validation of the GARD™skin Assay for Assessment of Chemical Skin Sensitizers: Ring Trial Results of Predictive Performance and Reproducibility

Henrik Johansson,[1] Robin Gradin,[1] Angelica Johansson,[1] Els Adriaens,[2] Amber Edwards,[3] Veronika Zuckerstätter,[4] Anders Jerre,[1] Florence Burleson,[3] Helge Gehrke,[4] and Erwin L Roggen[5]

Within Laboratory Reproducibility:

SenzaGen	82%
Burleson	83%
Eurofins	89%

Between Laboratory Reproducibility:

Concordance: 92%

GARDskin & GARDpotency – REACH registration

"The REACH Regulation allows the use of non-adopted in vitro methods in case they meet the EURL ECVAM criteria for entering pre-validation. **For the GARD assay this criteria is met**, as it is currently being validated."

"The current REACH information requirements require that three KEs are examined and **GARD assay can be used to assess the KE 3**."

ECHA
EUROPEAN CHEMICALS AGENCY

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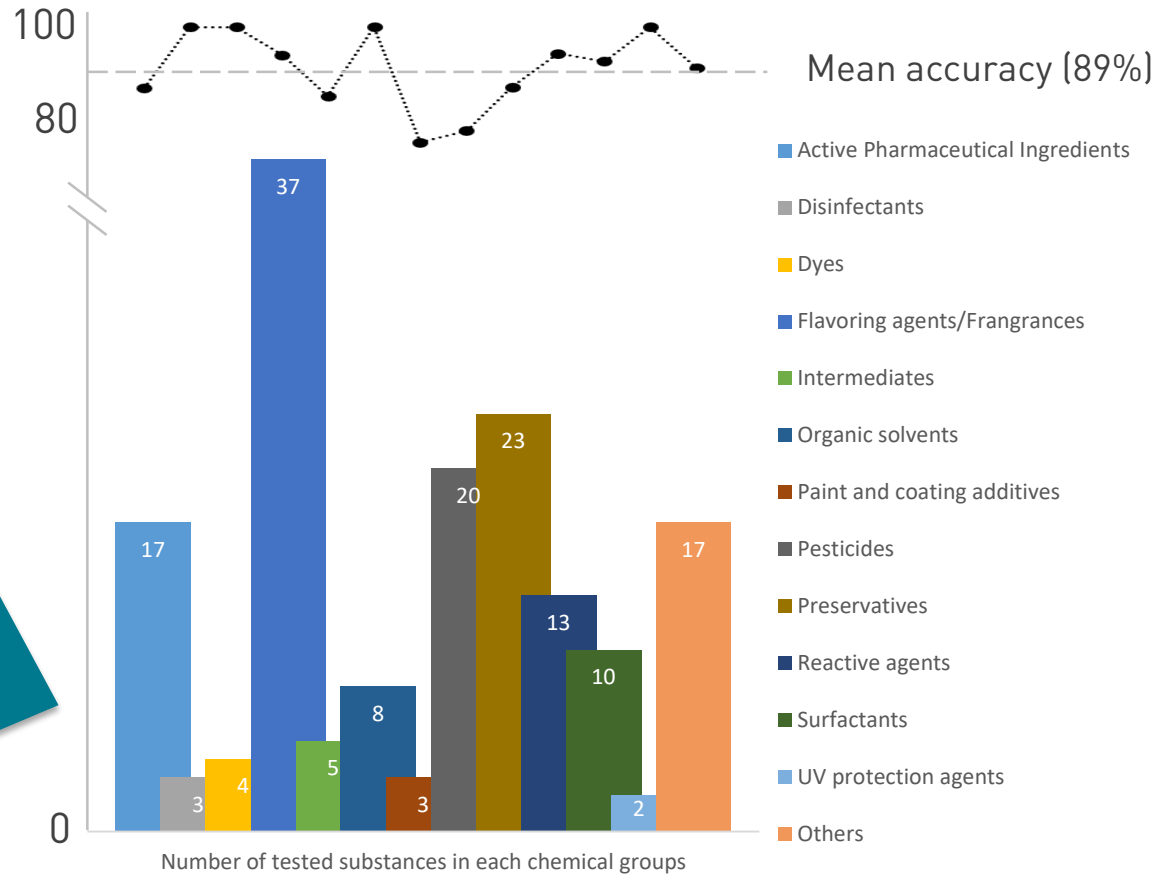
GARDskin – Broad applicability domain

The standard assay protocol is applicable for small molecules in general: from cosmetics ingredients to various industrial chemicals, including pre- and pro-haptens

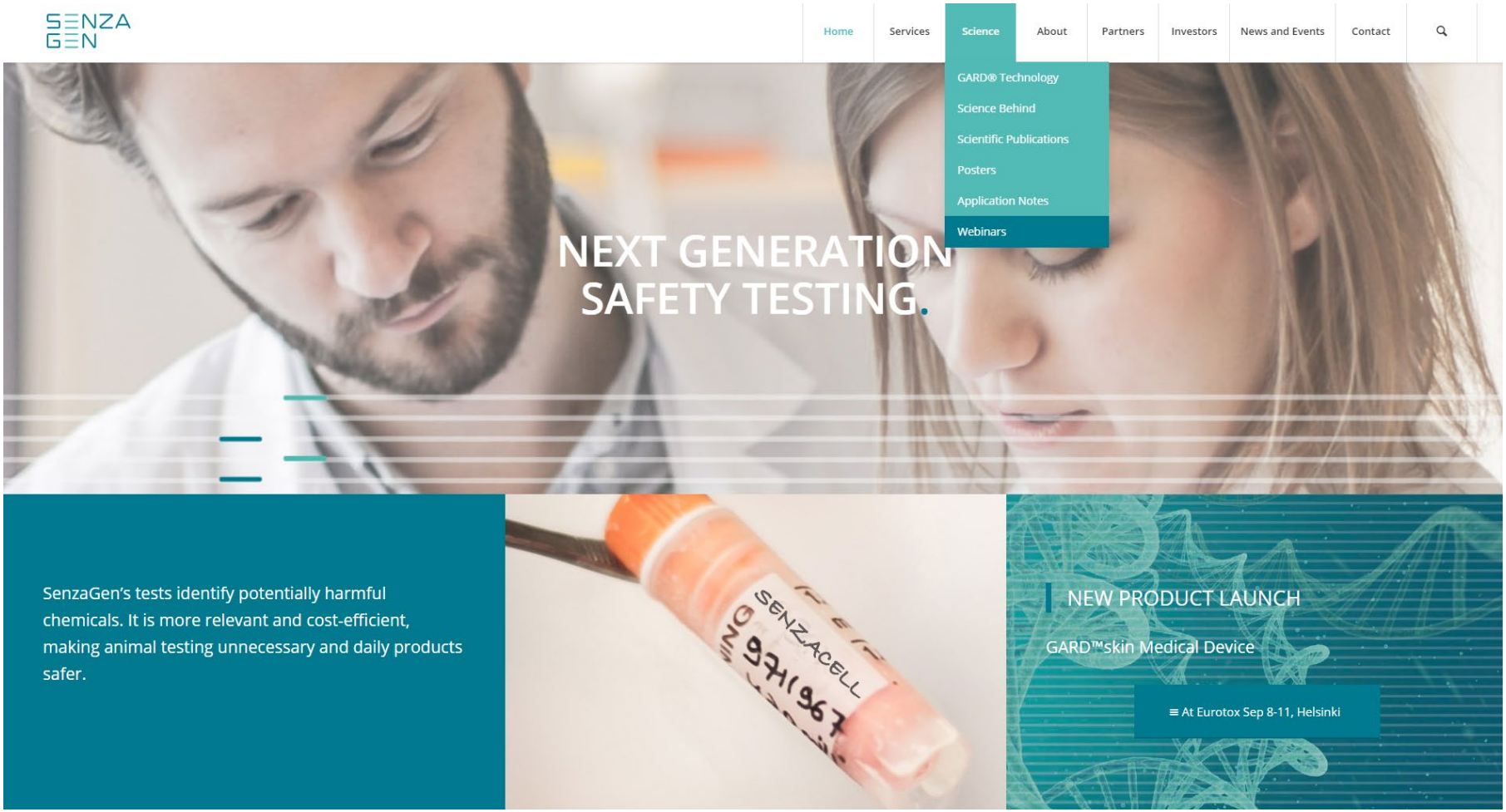
SenzaGen and its partners also offer lab services for the explorative assessment of 'difficult-to-test' substances:

- UVCBs
- Natural extracts and mixtures
- Pesticides
- Pharmaceutical ingredients (drug candidates)
- ...and more



Source: SenzaGen in-house studies, excluding data from customer projects

More info at: **www.senzagen.com**



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Case study I – GARDskin for "difficult to test" substances

Why substances can be difficult to test:

Pre/Pro haptens: Activation may be needed to create the allergen.

SenzaCells:
Aldehyde dehydrogenases ***(ALDH)***
Cytochrome p-450 ***(CYP)***
N-acetyltransferase 1 ***(NAT-1)***

Solubility: High log $P_{o/w}$ value or other properties leading to low solubility in aqueous media.

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Case study I – GARDskin for "difficult to test" substances

Compound	Pre/pro hapten	High logP	DPRA (TG442C)	ARE-NRF2 (TG 442D)	h-CLAT (TG442E)	GARD (TGP 4.106)
2-Aminophenol	YES	-	S	S	S	S[1]
2-nitro-1,4-Phenylendiamine	YES	-	S	S	S	S[1]
Aniline	YES	-	NS	NS	S	S[1]
Cinnamic alcohol	YES	-	S	S	S	S[1]
Ethylene diamine	YES	-	NS	S	S	NS[1]
Eugenol	YES	-	S	NS	S	S[1]
Geraniol	YES	-	NS	S	S	S[1]
Hydroquinone	YES	-	S	S	S	S[2]
Isoeugenol	YES	-	S	S	NS	S[1]
Linalool	YES	-	NS	NS	S	S[1]
p-Phenylenediamine	YES	-	S	S	S	S[1]
Propyl gallate	YES	-	S	S	S	S[1]
Resorcinol	YES	-	NS	NS	S	S[1]
Farnesol	YES	-	NS	S	S	S[1]
Abietic acid	YES	YES (6.5)	S	S	NS	S[1]
Chlorpromazine	YES	YES (5.4)	NA	NS	S	S[1]
Lauryl gallate	YES	YES (6.2)	S	S	S	S[3]
Amylcinnamyl alcohol	YES	YES (4.4)	S	NS	NS	S[1]
Limonene	YES	YES (4.6)	NS	NS	S	S[1]
Benzoyl peroxide	-	YES (3.5)	NS	NS	S	S[3]
Hexylcinnamic aldehyde	-	YES (4.8)	S	NS	NS	S[1]
Isopropyl myristate	-	YES (8.5)	NS	NS	S	NS[4]
propyl paraben*[1]	-	YES (3.4)	NS	S	S	S[1]
Tocopherol	-	YES (6.9)	NS	S	NS	S[1]
Accuracy			61%	58%	71%	92%

References:

[1] Johansson et al. 2017
[2] Forreryd et al. 2016
[3] Zeller et al. 2017
[4] Johansson et al. 2019

*Basketter
Human potency Class 5

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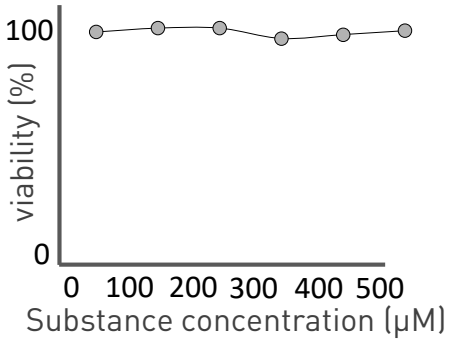
Case study II – Testing of UVCBs

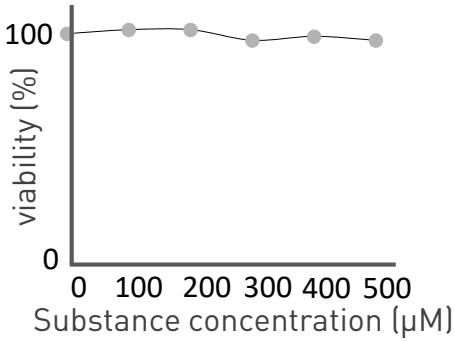
UVCBs: Unknown or Variable composition, Complex reaction products and Biological materials

The challenges: Complexity and uncertainty of compositions; Hydrophobic, often has solubility issues for water-based test systems

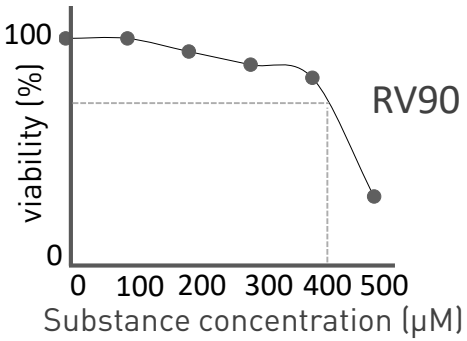
Vehicle: DMSO

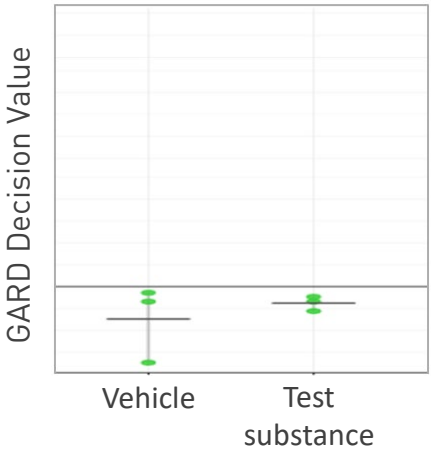


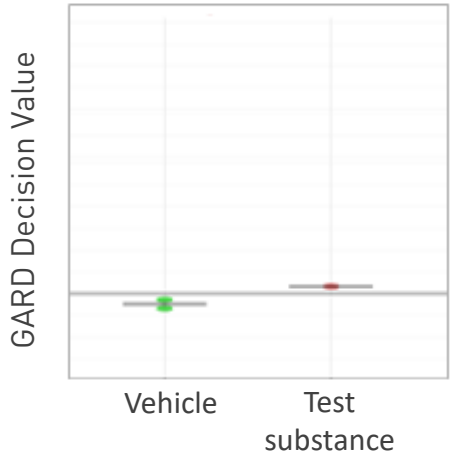
Vehicle: H_2O

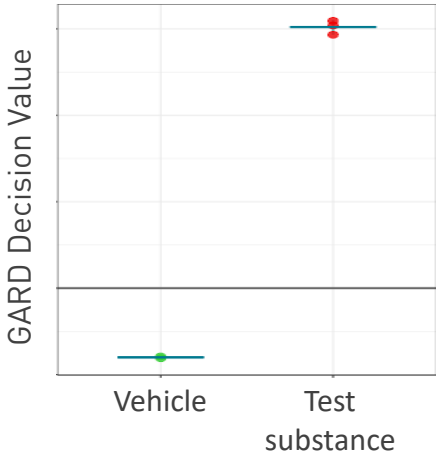


Vehicle: DMF/Glycerol









Available solvent: Acetone, Chlorobenzene, DMF, DMSO, Isopropanol, Ethanol, Glycerol, Hexane, Olive oil and sesame oil.

Case study II – Testing of UVCBs

Materials & Methods

Test item	GARDskin
7 UVCBs with known average MW	Additional solubility tests performed to select suitable solvents

Results in comparison with existing *in vivo* data

Test items	Existing data	Existing classification	GARDskin
1	LLNA, Buehler	S, 1B	S
2	LLNA	NS	S
3	LLNA, Buehler	S, 1B	S
4	LLNA	S, 1B	S
5	Buehler, HRIPT	NS	NS
6	LLNA, Buehler, HRIPT	S, 1B	S
7	Buehler	S, 1B	S

Conclusion

GARDskin:
good applicability potential for UVCBs, consistent with *in vivo* data.

The assessment of UVCBs need to be handled case by case.

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Case study III– Testing of natural mixtures

Henna: natural dye from Lawsonia inermis.

Henna-based hair colouring products: often considered safer alternatives to synthetic hair dyes containing known skin sensitizers such as p-phenylenediamine (PPD). However, skin allergic reactions to henna products have been reported.

Materials & Methods

Pre study	Main study
GARDskin assessment of 10 commonly used hair dye ingredients	GARDskin assessment of 10 henna-based products (powder mixtures)
	HPLC examination of the presence of PPD in the products

*The images are from the internet and only serve as examples of commercially available henna products

This study is a collaboration between Federal University of Goiás, Brazil and Lund University, Sweden. For more details, please see the original publication (de Ávila et al. Contact Dermatitis, 2019)

Case study III– Testing of **natural mixtures**

Table 1.GARDskin prediction results for commonly used hair dye ingredients

Test materials	Human classification	Animal classification	mDPRA	IL-18	USENS™	GARD™ skin
Reference controls						
Dimethyl sulfoxide	NS	NS	NS	NS	NS	NS
Glycerol	NS	NS	NS	NS	NS	NS
Sodium dodecyl sulfate	NS	S	S	NS	S	NS
DNCB	S	S	S	S	S	S
Eugenol	S	S	S	S	S	S
2-Hydroxyethyl acrylate	S	S	-	-	-	S
Hair dye ingredients						
1,4-Diaminoanthraquinone	S	S	S	S	S	S
2-Amino-3-hydroxypyridine	S	NS	S	S	S	S
Lawsone	NA	Equivocal	S	NS	S	S
5-Amino-*o*-cresol	S	S	S	NS	S	S
Hydroquinone	S	S	S	S	S	S
p-Phenylenediamine	S	S	S	S	S	S
Resorcinol	S	S	S	S	S	S
Disperse orange 3	S	S	S	S	S	S
Basic red 51	NA	NS	S	S	S	S
Pyrogallol	S	NS	S	S	S	S
Concordance *vs*. Human data			91.7%	92.3%	92.3%	100%
Concordance *vs*. Animal data		78.5%	76.9%	64.3%	78.6%	73.3%

For more details of the study, please see the original publication (de Ávila et al. Contact Dermatitis, 2019)

Case study III– Testing of natural mixtures

Table 2. GARDskin prediction results for 10 commercial henna-based products

Henna Product	PPD (label)	PPD	mDPRA	IL-18	USENS™	GARDskin™
1		1.091 ± 0.028	S	S	S	S
2	+	2.970 ± 0.046	S	S	S	S
3		0.030 ± 0.001	S	S	NS	S
4		0.032 ± 0.006	S	S	NS	S
5		4.321 ± 0.028	S	S	S	S
6		1.020 ± 0.100	S	S	S	S
7		0.577 ± 0.015	S	NS	S	S
8	+	2.541 ± 0.057	S	NS	S	S
9		0.760 ± 0.017	S	S	S	S
10		3.354 ± 0.163	S	S	S	S

Conclusion

GARDskin show high concordance to human data for testing of **hair dye ingredients**

GARDskin is applicable for **henna-based hair colouring products (powder mixtures).**

For more details of the study, please see the original publication (de Ávila et al. Contact Dermatitis, 2019)

Case study IV– Testing of oil-based mixtures

The Customer: a leading supplier of natural based functional ingredients/ raw materials for cosmetic and personal care applications

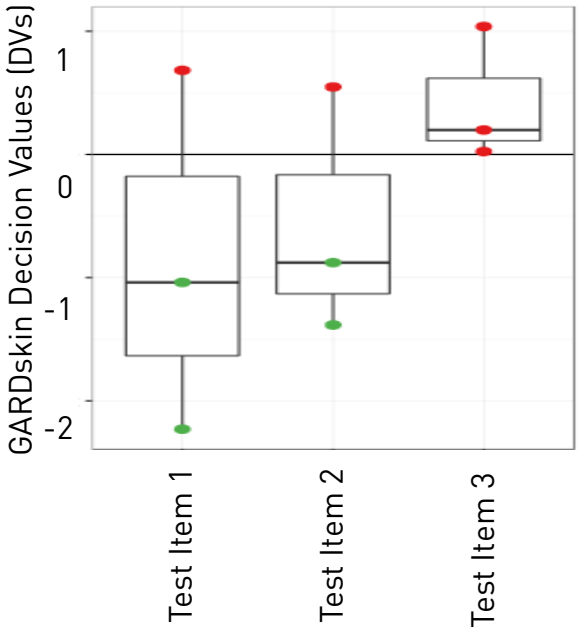
The problem: An oil-based mixture with **unpleasant and characteristic smell**

- Used as raw materials sold to cosmetic and personal care companies
- The oil-based mixture contains **chemical x** as contaminant, which is the suspect for the unpleasant smell.
- Distillation to get rid of contaminants of **chemical x**

Case study IV– Testing of oil-based mixtures

Methods & Results (GARDskin)

Test item 1	Test item 2	Test item 3
Pure **chemical x**	Oil mixture containing 1200 ppm **chemical x**	Distilled oil mixture containing 10 ppm **chemical x**
Non sensitizer	Non sensitizer	Sensitizer



Conclusion

This case is a good example of how GARDskin can be used for assessment of oil-based mixtures and essential oils.

Case study V– Testing **Medical Devices** for skin sensitization hazard

***In vitro* skin sensitization testing in the Biological Evaluation of Medical Devices according to ISO 10993-12:2012**

- Use polar and non-polar extraction vehicles according to ISO 10993-12:2012 (saline, sesame oil, super refined olive oil and cell culture media)
- Classify leachables in extracts of medical devices as either skin sensitizers or non-sensitizers
- 200 genomic biomarkers in GARDskin Predictive Signature (GPS)

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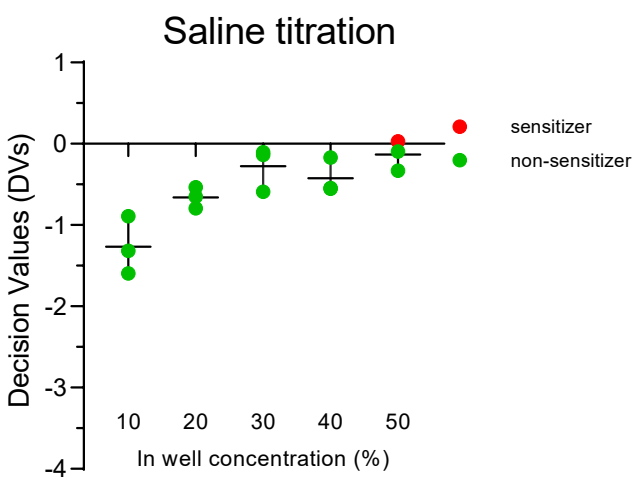
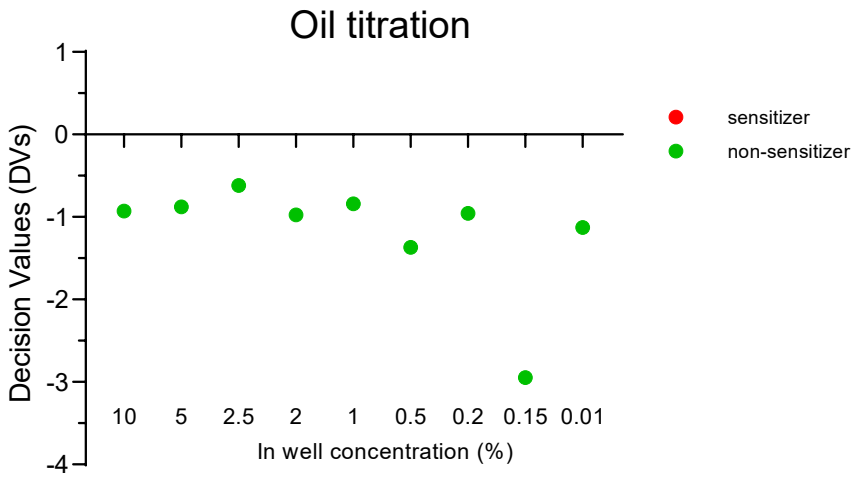
Case study V– Testing **Medical Devices** for skin sensitization hazard

Extraction steps of solid material follow the international standards for safety assessment of **Medical Device** (**ISO 10993-12**).





Case study V– Testing **Medical Devices** for skin sensitization hazard

Table 1. Summary of the results from the in-house validation of GARD®skin Medical Device compared with LLNA (as listed in the CE STTF database) and Human potency classification (HP) for the chemicals (Basketter et al. 2014) .

Test material	Chemical	Sensitizing potential		GARD®skin Medical Device Prediction		
		LLNA	HP	Saline	Olive oil	Sesame oil
Silicone	None	N/A	N/A	Non-sensitizer	Non-sensitizer	Non-sensitizer
	2-aminophenol	Strong	Cat 2	Sensitizer	Sensitizer	Sensitizer
	Cinnamic aldehyde	Moderate	Cat 2	Sensitizer	Sensitizer	Sensitizer
	Propyl gallate	Strong	Cat 2	Sensitizer	Sensitizer	Sensitizer
	Phenyl benzoate	Weak	Cat 3	Sensitizer	Sensitizer	Sensitizer
TPU	None	N/A	N/A	Non-sensitizer	Non-sensitizer	Not tested
	Propyl gallate	Strong	Cat 2	Sensitizer	Sensitizer	Not tested
	Phenyl benzoate	Weak	Cat 3	Sensitizer	Sensitizer	Not tested
Silicone tube	-	N/A	N/A	Non-sensitizer	Non-sensitizer	Non-sensitizer
TPU tube	-	N/A	N/A	Non-sensitizer	Non-sensitizer	Non-sensitizer
PVC tube	-	N/A	N/A	Non-sensitizer	Non-sensitizer	Non-sensitizer
Vehicle control	-	Neg		Non-sensitizer	Non-sensitizer	Non-sensitizer
Positive control	p-Phenylenediamine	Pos		Sensitizer	Sensitizer	Sensitizer

Case study VI – Safety assessment of e-liquids

Materials & Methods

In this customer study, **GARDskin**, **GARDpotency** and **GARDair** were used to predict and compare the skin and respiratory sensitisation potential of **three experimental** and **two commercial e-liquids**. To our knowledge this is the first published study assessing e-liquids using in vitro assays.

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The use of Genomic Allergen Rapid Detection (GARD) assays to predict the respiratory and skin sensitising potential of e-liquids

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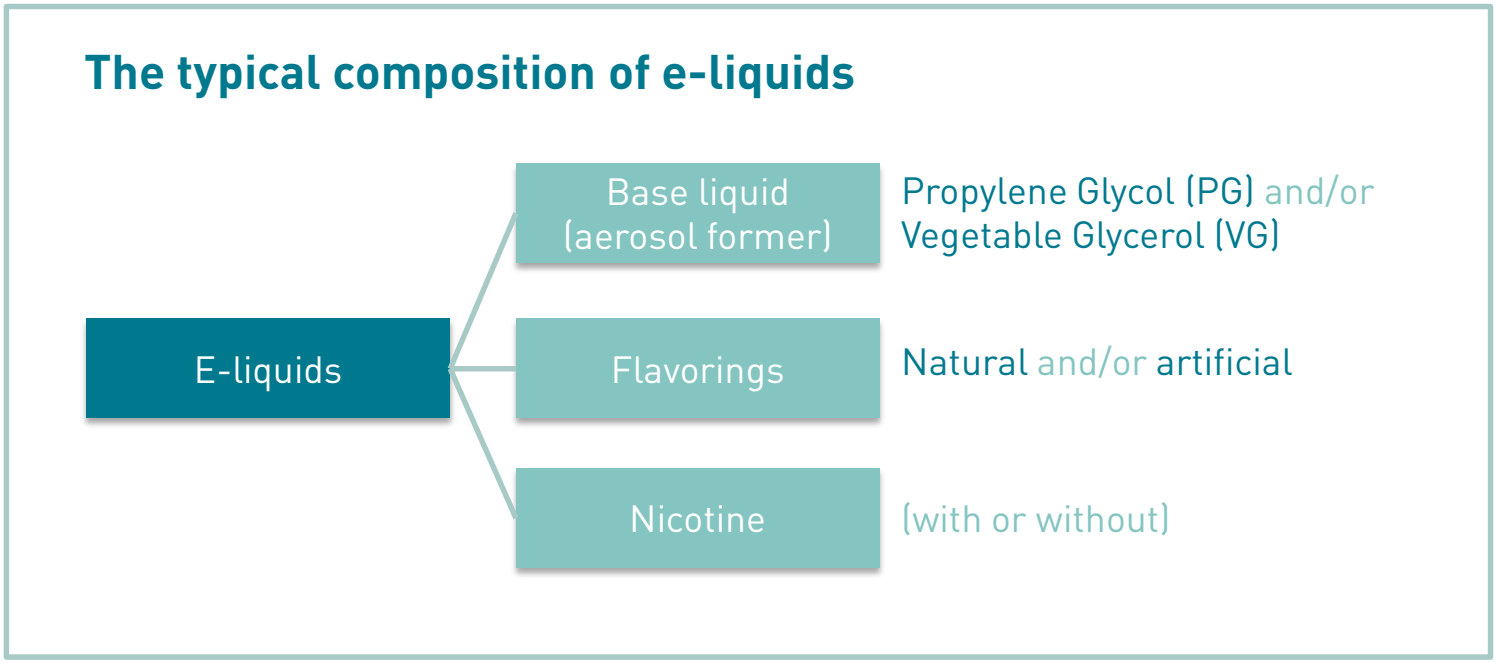
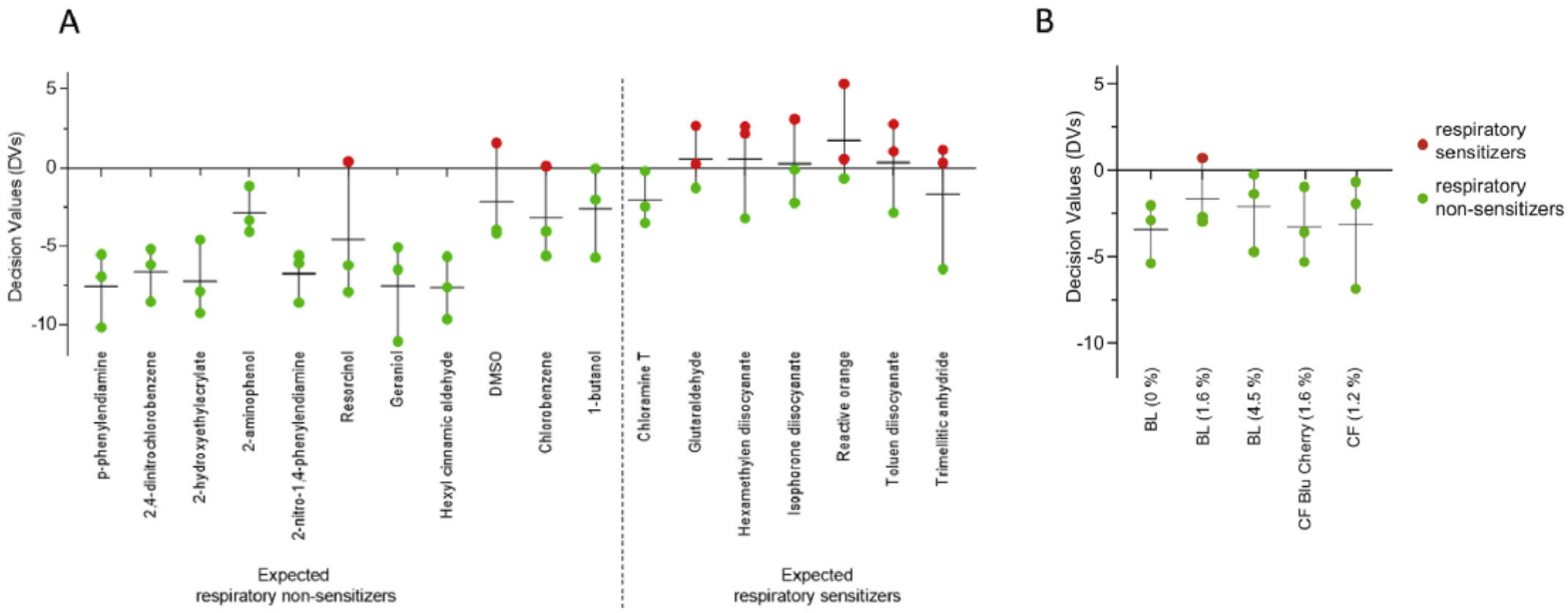
Case study VI – Safety assessment of e-liquids



E-liquids are usually **complex mixtures**, especially when they include natural flavourings

Case study VI – Safety assessment of e-liquids



GARDair prediction

A controls: 5 out of 7 accurately classified as respiratory sensitizers, no false positives. Sensitivity 71%, Specificity 100%, Overall accuracy 89%

B test substances: None of the base liquids nor commercial e-liquids were classified as respiratory sensitizers.

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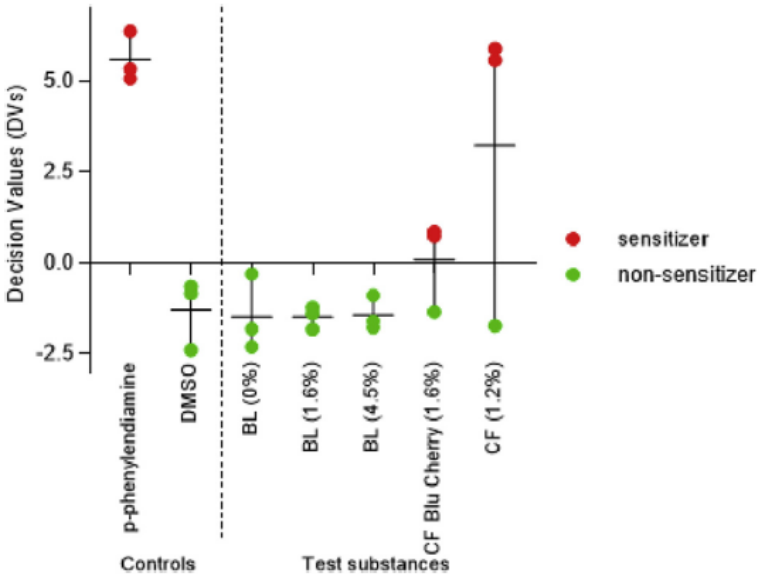
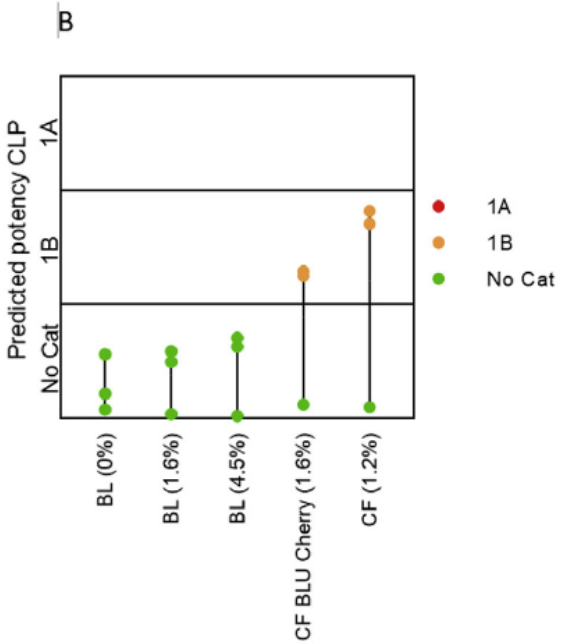
Case study VI – Safety assessment of e-liquids





GARDskin prediction :

2 commercially available flavoured e-liquids were predicted as skin sensitizers

GARDpotency prediction :

Test substances: 2 commercially available flavoured e-liquids were further classified as weak skin sensitizers (1B)

Case study VI – Safety assessment of e-liquids

Composition of test materials. PG: Propylene glycol; VG: Vegetable glycerol; BL: base liquid; CF: commercially available flavoured e-liquid.

Test material	Content [w/w %]			
	PG	VG	Nicotine	Other substances (e.g. water, flavourings)
BL 0%[a]	50	50	–	–
BL 1.6%[a]	49.2	49.2	1.6	–
BL 4.5%[a]	47.75	47.75	4.5	–
CF Blu Cherry 1.6%[a]	40.4	53.4	1.6	4.6
CF 1.2%[a]	66.5	18.8	1.1	13.6

[a] % refer to % nicotine content.

Test substance classification with the GARD assay for respiratory sensitisers, GARDskin and GARDpotency assays.

	Respiratory assay	GARDskin	GARDpotency
BL 0%	Non-Sensitiser	Non-Sensitiser	No Cat
BL 1.6%	Non-Sensitiser	Non-Sensitiser	No Cat
BL 4.5%	Non-Sensitiser	Non-Sensitiser	No Cat
CF Blu Cherry 1.6%	Non-Sensitiser	Sensitiser	1 B
CF 1.2%	Non-Sensitiser	Sensitiser	1 B

Conclusion

The GARD platform offers an integrated test strategy for assessment of skin and respiratory sensitization potential of **complex mixtures** such as e-liquids.

The assays are potentially useful for product development and hazard identification of other types of complex formulations.

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The **GARD platform –** How can I get my **substances tested?**

Contact us:
We help to design the testing strategy for your specific substances.

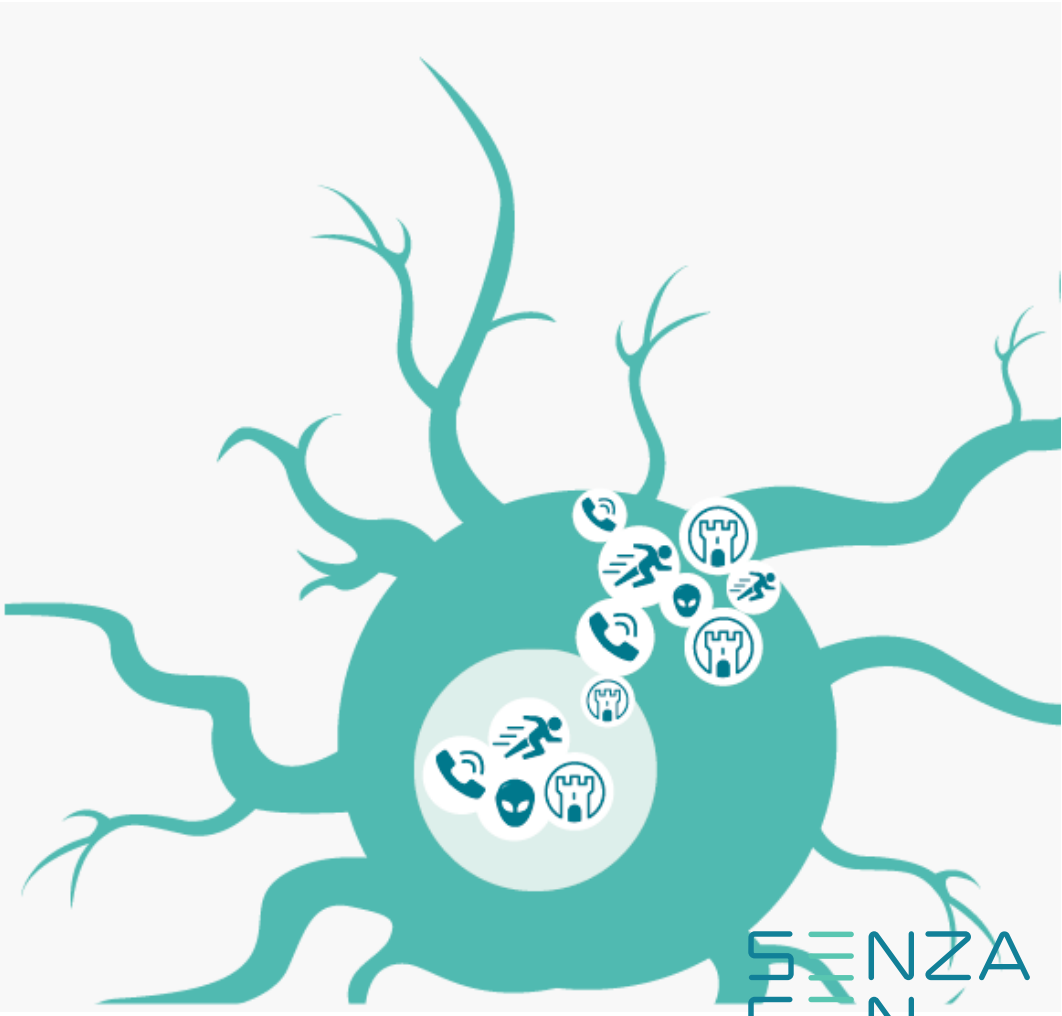
Select assay(s):
GARDskin, GARDpotency, GARDair, GARDskin Medical Device

Test Substance Questionnaire:
Do you have information on vehicles? If not, we evaluate it for you.

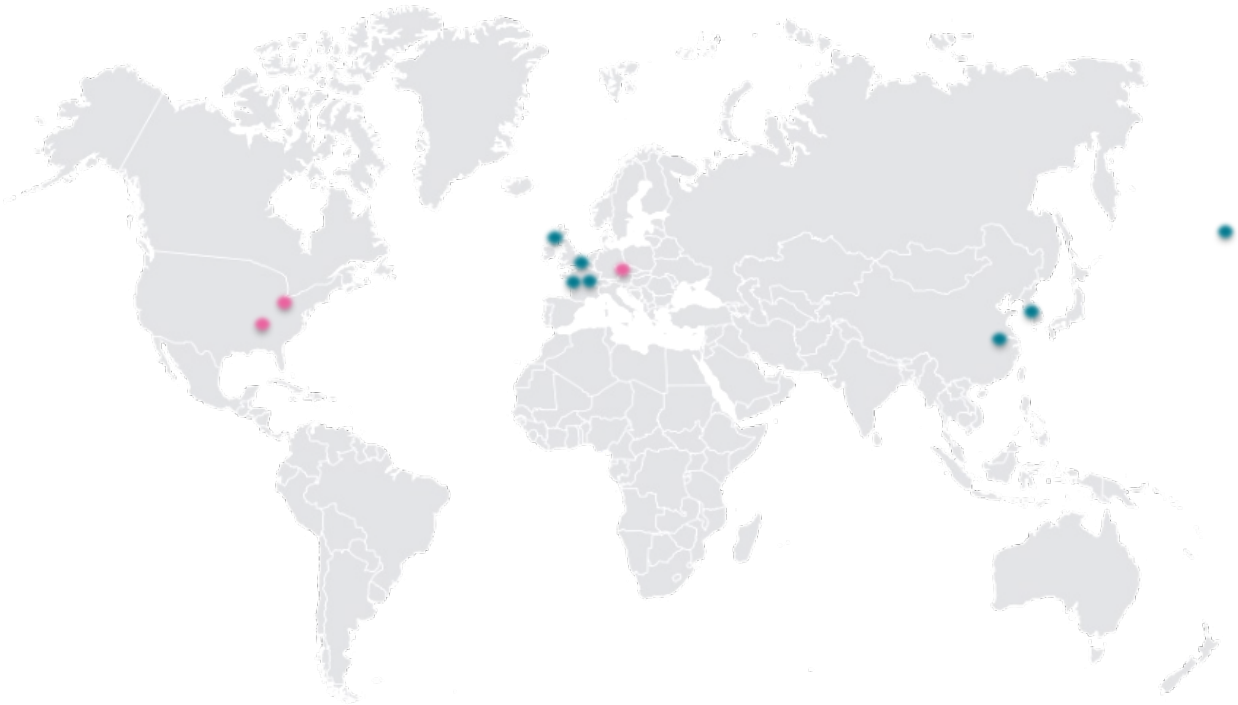
Turnaround time:
4-6 weeks.

Sample requirements:
0.5 g (solids) or 1 ml (liquids). Can be adapted to lower amounts.

Your preferred testing site:
Select where you like to have your testing done.



The **GARD platform – Where** can I get my **testing done?**



- **Licence Labs*:**
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  Eurofins BPT
  MB Research Laboratories

- **Distributors*:**
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  Eurosafe
  Guangzhou CHN-ALT Biotech Co., Ltd
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  XCellR8

* In alphabetic order

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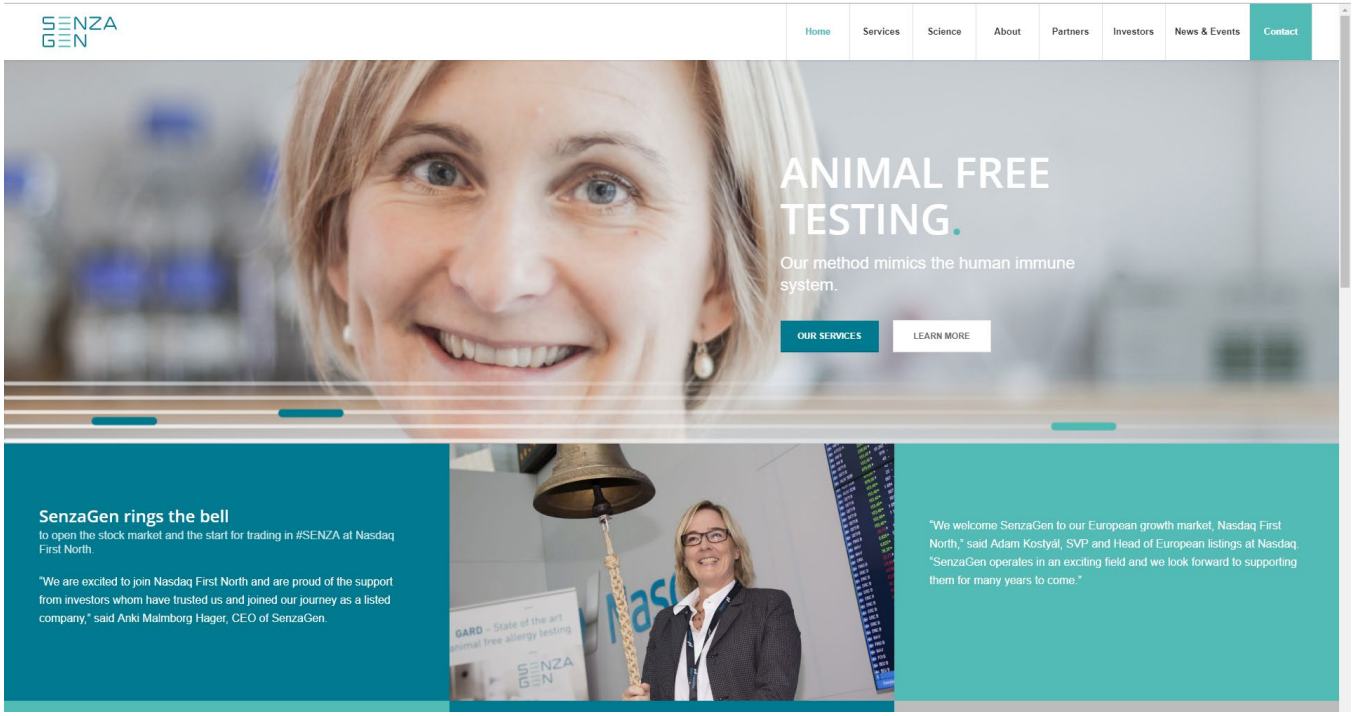
Information and contacts

www.senzagen.com



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