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The GARD assay for assessment of chemical skin sensitizers

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Allergic contact dermatitis is a skin disease caused by an immunologic reaction to low molecular weight compounds, so called haptens. These substances are commonly present in products used by humans in daily life, such as in cosmetics and fragrances, as well as within chemical industry and in pharmaceuticals. The frequent usage of these compounds in different applications has led to increasing incidences of allergic contact dermatitis, which has become a substantial economic burden for society.

As a consequence, chemicals are routinely tested for their ability to induce skin sensitization, using animal models such as the murine Local Lymph Node Assay. However, recent legislations regulate the use of animal models within chemical testing. Thus, there is an urgent need for *in vitro* alternatives to replace these assays for safety assessment of chemicals.

Recently, we identified a signature of predictive genes, which are differentially regulated in the human myeloid cell-line MUTZ-3 when stimulated with sensitizing compounds compared to non-sensitizing compounds. Based on these findings, we have formulated a test strategy for assessment of sensitizing compounds, called Genomic Allergen Rapid Detection, GARD. In this paper, we present a detailed method description of how the assay should be performed.

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1. Introduction

Allergic contact dermatitis (ACD) is a type IV hypersensitivity reaction, mediated by effector CD8$^+$ and CD4$^+$ T cells (Fonacier et al., 2010). The disease is caused by low molecular weight (LMW) compounds, which act as haptens that form a functional allergen after binding to endogenous proteins present in skin. During the sensitization phase of ACD, the protein is taken up by dermal dendritic cells (DCs) that are present in the epidermis at the site of exposure. Consequently, DCs will mature and migrate to local lymph nodes, presenting fragments of the LMW complex on either MHC class I or II, depending on the route of antigen uptake (Friedmann, 2006). Provided that the DCs also become activated and signal using co-stimulatory molecules, as reviewed in (Martin et al., 2011), this antigen presentation will lead to differentiation of naïve T cells into specific effector and memory T cells. Upon re-exposure to the specific LMW compound, memory T cells elicit a rapid immune response, mainly by the release of the proinflammatory cytokines interferon (IFN) γ and tumor necrosis factor (TNF) α. LMW compounds able to induce ACD are termed skin sensitizers.

Chemicals with sensitizing properties are commonly found within chemical and pharmaceutical industry, and in products used in everyday life such as cosmetics and fragrances, which has led to increasing incidences of ACD, with prevalence rates of up to 18.6% in specific cohorts in Europe (Mortz et al., 2001; Nielsen et al., 2001), which corresponds approximately to 20% of all reported cases of contact dermatitis, with the remaining 80% being cases of immunologically non-specific irritant contact dermatitis (Fonacier et al., 2010). In addition, contact dermatitis, both irritant and allergic, accounts for 85–90% of all occupational skin diseases among the working population of the Western world (Friedmann, 2006), thereby causing a substantial economic burden for society. In order to minimize the use of sensitizing compounds, chemicals are routinely tested for their sensitizing potency. Such assays are today performed with animal models, preferably the murine local lymph node assay (LLNA) (Basketter et al., 2002). However, the REACH (Registration, Evaluation, and Authorization of Chemicals) regulation will have a huge impact on the number of animals required for testing. In addition, the 7th Amendment to the Cosmetics Directive (76/768/EEC) regulates the use of animals for testing cosmetic ingredients. Thus, there is an urgent need of alternative *in vitro* assays for assessment of sensitizers, which reflects clinical experience and that exhibits an improved reliability and accuracy.

Consequently, several groups are currently developing animal-free testing strategies, using a number of different approaches.

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In silico strategies based on quantitative structure–activity relationship (QSAR) has e.g. shown promising results (Golla et al., 2009; Gunturi et al., 2010). However, such *in silico* assays are likely troubled by the diversity among molecular structures of sensitizers, since very similar structures give dissimilar sensitization results (Natsch, 2010). Furthermore, *in chemico* strategies predict sensitization by measuring the peptide reactivity of compounds (Gerberick et al., 2004). Still, the most extensively explored strategy is *in vitro* cell based assays, among them the most frequent ones being *in vitro* models of DCs, due to their key function as initiators of the immune response leading to skin sensitization. Numerous cell systems and biomarkers have been suggested, such as measurement of CD86 in the U-937 cell line (Python et al., 2007), combined measurement of CD86 and CD54 in the THP-1 cell line (Ashikaga et al., 2006; Sakaguchi et al., 2006), or monitoring of the activity of transcription factors, such as nuclear factor-erythroid 2-related factor 2 (NRF2) in a reporter cell line (Emter et al., 2010). While these assays are functional and relevant, they are all limited in their readout.

Recently, we presented a predictive biomarker signature in the myeloid cell line MUTZ-3 (Johansson et al., 2011). We hypothesized that, given a good *in vitro* DC model is available, such cells could be explored for biomarkers for sensitization, due to their roles as decision-makers in the immunologic response to foreign substances. MUTZ-3 is a human acute myelomonocytic leukemia cell line, which mimics primary DCs in terms of transcriptional profile and their ability to induce specific T cell responses (Larsson et al., 2006; Masterson et al., 2002; Santegoets et al., 2006). Furthermore, proliferating MUTZ-3 express an immunologically relevant phenotype similar to immature primary DCs, with expression of CD1a, HLA-DR and CD54, as well as low expression of CD80 and CD86 (Johansson et al., 2011). Using a panel of reference chemicals, including 18 well-known sensitizers, 20 non-sensitizers and vehicle controls, we were indeed able to identify differentially regulated transcripts in MUTZ-3, depending on if the cells were exposed to a sensitizer or a non-sensitizer. The identified transcripts where found to be involved in immunologically relevant pathways, regulating recognition of foreign substances and leading to DC maturation. Thus, these biomarkers are potent predictors of different sensitizers. We have developed the usage of this biomarker signature into a novel assay for skin sensitization, called genomic allergen rapid detection, GARD. The assay is based on the measurement of these transcripts, collectively termed the GARD Prediction Signature, using a complete genome expression array. Classifications of unknown compounds as sensitizers or non-sensitizers are performed with a support vector machine (SVM) model, trained on the 38 reference chemicals used for GARD development. In this paper, we present a detailed method description for how to accurately predict skin sensitization, using GARD.

2. Materials and methods

2.1. MUTZ-3 growth and seeding of cells for stimulation

The human myeloid leukemia-derived cell line MUTZ-3 (DSMZ, Braunschweig, Germany) is maintained in α-MEM (Thermo Scientific Hyclone, Logan, UT) supplemented with 20% (volume/volume) fetal calf serum (Life Technologies, Carlsbad, CA) and 40 ng/ml rhGM-CSF (Bayer HealthCare Pharmaceuticals, Seattle, WA), as described (Johansson et al., 2011). A media change every 3–4 days is recommended, or when cell-density exceeds 500.000–600.000 cells/ml. Proliferating progenitor MUTZ-3 are used for the assay, with no further differentiation steps applied. During media exchange, cells should be counted and resuspended to 200.000 cells/ml. Working stocks of cultures should not be grown for more than 20 passages or 2 months after thawing. For chemical stimulation of cells, 1.8 ml MUTZ-3 is seeded in 24-well plates at a concentration of 222.222 cells/ml. The compound to be used for stimulation is added in a volume of 200 μl, diluting the cell density to 200.000 cells/ml during incubation.

2.2. Phenotypic analysis of MUTZ-3

Prior to any chemical stimulation, a qualitative phenotypic analysis of MUTZ-3 is performed to ensure that proliferating cells are in an immature stage. All cell surface staining and washing steps were performed in PBS containing 1% BSA (w/v). Cells were incubated with specific mouse monoclonal antibodies (mAbs) for 15 min at 4 °C. The following mAbs were used for flow cytometry: FITC-conjugated CD1a (DakoCytomation, Glostrup, Denmark), CD34, CD86, and HLA-DR (BD Biosciences, San Diego, CA), PE-conjugated CD14 (DakoCytomation), CD54 and CD80 (BD Biosciences). Mouse IgG1, conjugated to FITC or PE were used as isotype controls (BD Biosciences) and propidium iodide (PI) (BD Biosciences) was used to assess cell viability. FACSDiva software was used for data acquisition with FACSCanto II instrument (BD Bioscience). 10,000 events were acquired, gates were set based on light scatter properties to exclude debris and non-viable cells, and quadrants were set according to the signals from isotype controls. Further data analysis was performed, using FCS Express V3 (De Novo Software, Los Angeles, CA).

2.3. Chemical handling and assessment of cytotoxicity

All chemicals should be stored according to instructions from the supplier, in order to ensure stability of compounds. Chemicals should be dissolved in water when possible or DMSO for hydrophobic compounds. As many chemicals will have a toxic effect on the MUTZ-3 cells, this toxicity needs to be monitored. Some chemicals are poorly dissolved in cell media; therefore the maximum soluble concentration needs to be assessed as well. The chemical that is to be tested should be titrated to concentrations ranging from 1 μM to the maximum soluble concentration in cell media. For freely soluble compounds, 500 μM should be the upper end of the titration range. For cell stimulations, chemicals should be dissolved in its appropriate solvent as 1000× stocks of target in-well concentration, called stock A. A 10× stock, called stock B, is prepared by taking 10 μl of stock A to 990 μl of cell media. 200 μl of stock B is then added to the wells containing 1.8 ml seeded cells. For the samples dissolved in DMSO, the in-well concentration of DMSO will thus be 0.1%. Following incubation for 24 h at 37 °C and 5% CO_2, harvested cells are stained with PI and analyzed with a flow cytometer. The relative viability of cells stimulated with each concentration in the titration range are calculated as

$$\text{Relative vialbility} = \frac{\text{fraction of viable stimulated cells}}{\text{fraction of viable unstimulated cells}} \cdot 100$$

For toxic compounds, the concentration yielding 90% relative viability (Rv90) should be used for the GARD assay. For non-toxic compounds, a concentration of 500 μM should be used if possible. For non-toxic compounds that are insoluble at 500 μM in cell media, the highest soluble concentration should be used. Whichever of these three criteria is met, only one concentration will be used for the genomic assay. The concentration to be used for any given chemical is termed the 'GARD input concentration'.

2.4. Chemical exposure of cells for GARD

Once the testing concentration for chemicals to be assayed is established, the cells are stimulated again as described above, this

time only using GARD input concentration. All assessments of unknown compounds should be assayed in biological duplicates, performed at different time-points and different cell cultures. At each experiment, duplicate wells are used for each stimulation, providing two technical replicates for each biological replicate. Following 24 h incubation for 24 h at 37 °C and 5% CO_2, cells from one well are lysed in 1 ml TRIzol reagent (Life Technologies) and stored at −20 °C until RNA extraction. 200.000 cells/well is a large surplus of what is required for cDNA preparation (see below), but a second sample (technical replicate) is stored as backup. In parallel, a small sample of stimulated cells are taken for PI staining and analysis with flow cytometry, to ensure the intended viability of the cells is reached.

2.5. Preparation of cDNA and gene chip hybridization

RNA isolation from lysed cells is performed as described by the TRIzol supplier (Life Technologies). A minimum of 300 ng total RNA is required to perform preparation of cDNA. The preparation of labeled sense DNA is performed according to Affymetrix GeneChip™ whole transcript (WT) sense target labeling assay (100 ng Total RNA labeling protocol), using the recommended kits and controls (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of the Human Gene 1.0 ST arrays should be performed according to the manufacturer's protocol (Affymetrix).

2.6. Data acquisition and analysis

The microarray data should be normalized and quality checked with the RMA algorithm, using Affymetrix expression console (Affymetrix). At this point, data should be merged with existing training data created during GARD development (Johansson et al., 2011). The readout for the assay is the decision value output from a support vector machine (SVM) (Noble, 2006). SVMs are constructed in R (R Development Core Team, 2008), with the additional package e1071 (R package e1071). The SVM should be trained on the training data available, using only the 200 analytes in the GARD Prediction Signature (Johansson et al., 2011). The samples that are being assayed are then evaluated by the trained and frozen SVM, as a test set. The classification of a sample as a sensitizer or a non-sensitizer is based on the SVM prediction; a positive decision value means a sample is a sensitizer, and a negative decision value means a sample is a non-sensitizer. The SVM prediction is in this paper illustrated with a Sammon projection (Sammon, 1969) constructed in R, and with a principal component analysis (PCA) (Ringner, 2008) constructed in Qlucore Omics Explorer 2.1 (Qlucore AB, Lund, Sweden).

3. Results

3.1. GARD background and workflow

The complete workflow of the GARD assay is summarized in Fig. 1A. First, a qualitative phenotypic analysis of MUTZ-3 is performed to ensure that proliferating cells are in an immature stage. As MUTZ-3 is known to be a heterogeneous population of cells, variations in surface antigen expression does commonly occur. However, an example of a MUTZ-3 phenotype in unstimulated cells has been previously reported (Johansson et al., 2011). Furthermore, the viability of MUTZ-3 prior to stimulation should be assessed with PI staining. The viability criteria for accepting a cell culture for use in the assay is set to >85%. Instructions on how to gate cells for phenotypic quality control and viability analysis are provided in Fig. 1B and C, respectively.

The GARD input concentration of chemicals to be assayed is determined as described in the material & methods section. Following 24 h incubation, cells are harvested, RNA is isolated, cDNA is prepared and arrays are hybridized, washed and scanned as described. Once the array data is acquired, it should be merged with a training data set, which consists of measurements of all 38 reference chemicals run during assay development (Johansson et al., 2011). The data is normalized with Affymetrix's RMA algorithm. A data set consisting of both train data and any new samples that are to be assayed is now available for analysis.

At this point, an SVM is trained on the training data. The trained SVM is a model, or an equation, that describes the hyperplane that best separates sensitizers from non-sensitizers in the train data. This model can then be applied to predict any unknown samples, i.e. the test data, as either sensitizers or non-sensitizers. The trained data is shown in a 3D PCA plot based on the GARD Prediction Signature in Fig. 1D, with a hyperplane represented as a 2D plane. This illustrates the classifications performed by the SVM, visible and interpretable by the human eye, as unknown samples of a hypothetical test set (dark red) that group together with sensitizers of the training data (bright red) on one side of the hyperplane would be classified as sensitizers, while unknown samples that group together with non-sensitizers of the training data (green) on the other side of the hyperplane would be classified as non-sensitizers. The actual SVM output is displayed as prediction values, corresponding to the Euclidean distance between the sample to be classified and the hyperplane. Thus, the decision value for any given sample represents the position of the sample in comparison to the hyperplane. Consequently, a positive prediction value denotes a sensitizer, and a negative value denotes a non-sensitizer. In addition, potency of a predicted sensitizer will be determined by the absolute value of the decision value, i.e. the actual distance to the hyperplane. A large decision value corresponds to a strong sensitizer, while a small decision value corresponds to a weaker sensitizer.

3.2. An example of how to assess the sensitizing capacity of unknown chemicals

In this section, the assessment of two chemicals will be exemplified, step by step. We will study the two compounds 2-nitro-1,4-phenylendiamine, a strong sensitizer according to the LLNA, and methyl salicylate, a non-sensitizer. Both of these compounds were used for the development of GARD, but for the sake of this exercise, they will be removed from the available data set and treated as unknown samples. Triplicate samples are available of each stimulation, providing true biological replicates.

The two compounds are freely soluble in cell media to a concentration of 500 μM, if they are first dissolved in DMSO. Thus, when determining the GARD input concentration, 500 μM will be the high end of the titration range. Cell stimulations were performed as described, and harvested cells were stained with PI (Fig. 2A). The relative viability of cells stimulated with 2-nitro-1,4-phenylendiamine decreases with increasing stimuli concentration. The Rv90 value for this compound is identified at a concentration of 300 μM. In contrast, methyl salicylate does not have any cytotoxic effect on MUTZ-3, as the relative viability remains unchanged with increasing stimuli concentration. Thus, the GARD input concentrations for 2-nitro-1,4-phenylendiamine and methyl salicylate are 300 and 500 μM, respectively.

Once the GARD input concentration for all samples to be assayed are established, cell stimulations for 24 h are repeated. Cells are harvested, RNA is isolated, cDNA is prepared and arrays are hybridized as described. Both stimulations are performed in triplicate, independent experiments. Thereafter, the array data from the triplicate stimulations are normalized, together with available

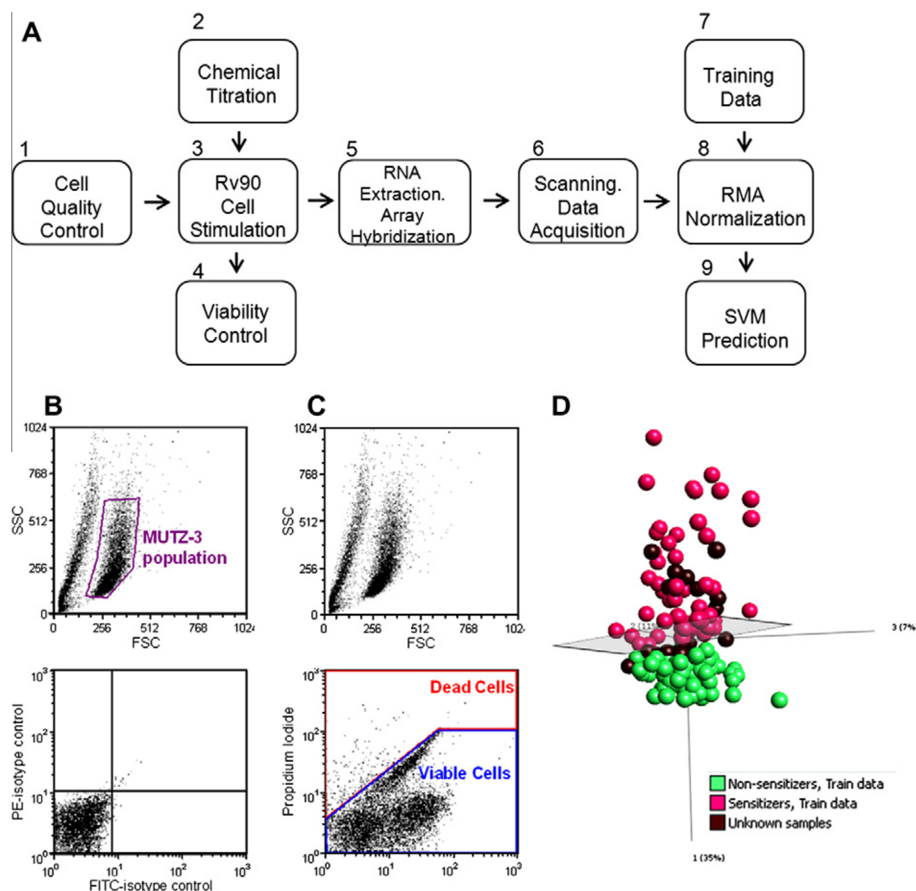

Fig. 1. GARD Workflow. (A) A schematic view of the different steps of sensitizer assessment using GARD. (1) MUTZ-3 cells are phenotyped, ensuring that unstimulated cells are in an immature stage. (2) The chemicals to be tested are titrated and investigated for cytotoxic effects. A GARD input concentration is established. (3) MUTZ-3 cells are stimulated with the chemicals, using the GARD input concentration. (4) A cell sample is stained with PI, ensuring that the desired relative viability is reached. (5) RNA is extracted, cDNA is synthesized and arrays are hybridized, according to standard protocols. (6) Following scanning of arrays, expression data is acquired. (7) The data is merged with available trained data. (8) The trained data and test data are normalized with an RMA normalization algorithm. (9) An SVM is trained and the SVM model is then used for predicting the test data. An output will be given for each sample in the form of a decision value. (B) Gating instructions for phenotypic analysis of MUTZ-3 cells, showing unstimulated cells stained with isotype controls. A gate is set in the FSC/SSC-plot to exclude dying cells and cell debris. Observing only gated cells, quadrants are set according to signals of isotype controls. (C) Gating instructions for viability analysis using propidium iodide. No gates are set in the FSC/SSC-plot. Dead cells are distinguished from live cells by their uptake of Propidium Iodide and fluorescence in the PE-channel. (D) SVM predictions, illustrated with a 3D PCA based on the 200 genes of GARD prediction signature. Unknown samples are plotted in the PCA without contributing to the principal components. The SVM hyperplane is in three dimensions illustrated with a 2D plane, from which decision values are calculated, serving as the basis for classification.

training data, with the RMA algorithm, In this case, the training data refer to the remaining 36 stimulations and vehicle controls used for the establishment of the GARD Prediction Signature (Johansson et al., 2011), a total of 131 arrays. At this point, the training data is used for training an SVM model. The model is then used to classify the test data, i.e. 2-nitro-1,4-phenylindiamine and methyl salicylate, as either sensitizer or non-sensitizer (Fig. 2B). The obtained decision values for this experiment are presented in Table 1.

3.3. Robustness, stability and reproducibility

The reproducibility of GARD was determined by assessing the correlation between the triplicate samples of each of the 38 reference chemicals used for assay development. RNA from these triplicate samples were collected at different days and on different batches of cells. Thus, biological variations in terms of cell cycle and growth rate are integrated in the assessment of reproducibility, as well as technical variation during RNA isolation, array hybridization and variation between array batches.

The variation in raw signal was assessed by studying Pearson's correlation coefficient (Table 2). The correlation coefficient is calculated by comparing data for the 200 genes in the GARD Prediction Signature, or for data derived from the complete array. For the GARD Prediction Signature, the correlation coefficient is 0.99 or above in 86% of all comparisons made. The lowest correlation between replicates is observed for penicillin G and *p*-phenylendiamine, with a coefficient of 0.97. When comparing replicates based on the full array, only Penicillin G has a coefficient below 0.99. Thus, the data is highly reproducible, with stable expression levels of the measured transcripts in technical and biological replicates.

The variation in decision, naturally dependent on the raw signal values will have an impact on how samples are classified. As can be seen in Fig. 2B, the replicates of both 2-nitro-1,4-phenylendiamine group closely together in a 2D Sammon projection, indicating a strong robustness and reproducibility of the assay. If triplicate samples of any one stimulation end up on both sides of the hyperplane, it should be regarded as a sensitizer. Indeed, while the cutoff of a sample being a sensitizer or a non-sensitizer is currently set to zero, this cutoff should and will be evaluated in connection with pre-validation of the assay. Furthermore, a sample being ambiguously classified by the SVM is likely a weak sensitizer, as the absolute value of the decision value may be correlated to the potency of the sensitizer; the further away from the cloud of negative samples

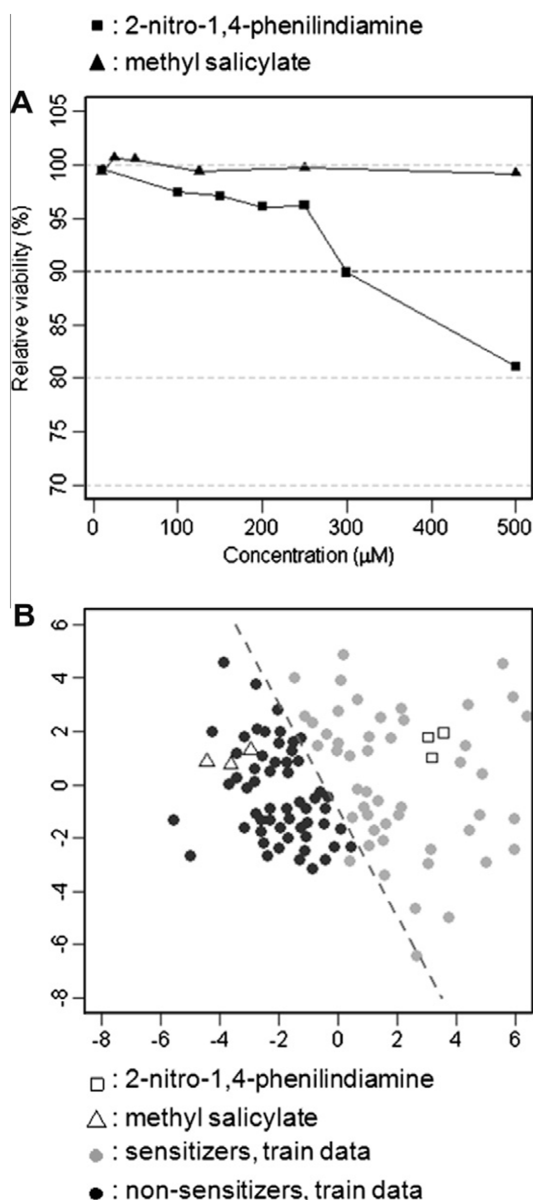

Fig. 2. Sensitizer assessment using GARD. Methyl salicylate and 2-nitro-1,4-phenilendiamine are used to exemplify how unknown samples are classified as either sensitizer or non-sensitizer. (A) The GARD input concentrations are determined by studying cytotoxic effects induced by the chemicals. Shown data are averages of duplicate samples. Data was obtained through propidium iodide staining and analysis with flow cytometry. No gates were set, and the reported viability is relative to unstimulated control. (B) Sammon projection in two dimensions of samples in the train set together with the two compounds to be classified. The hyperplane from which SVM decision values are measured is in this 2D representation illustrated with a line, exemplifying how classifications are made.

Table 1
SVM predictions of test data.

Sample	SVM prediction value	SVM classification
2-Nitro-1,4-phenilindiamine 1	6.3	Sensitizer
2-Nitro-1,4-phenilindiamine 2	5.1	Sensitizer
2-Nitro-1,4-phenilindiamine 3	5.4	Sensitizer
Methyl salicylate 1	−1.8	Non-sensitizer
Methyl salicylate 2	−1.8	Non-sensitizer
Methyl salicylate 3	−0.9	Non-sensitizer

a sensitizer is positioned, the higher its potency as a sensitizer, as discussed in (Johansson et al., 2011).

4. Discussion

Prediction of a compound's ability to induce skin sensitization is an important aspect of safety assessment of chemicals, and is currently performed with animal models, such as the murine LLNA. However, a number of factors, such as the REACH legislation and the 7th amendment to the Cosmetics Directive, make animal models unsuitable for assessment of sensitization. Furthermore, these assays are known to not correlate perfectly with clinical experience of human data. Indeed, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) reported the accuracy of the LLNA to be 72% (Haneke et al., 2001).

Genomic allergen rapid detection, GARD, is a novel assay for assessment of sensitization. It is based on a genomic readout, measuring 200 transcripts in the myeloid cell line MUTZ-3 following compound stimulation. The 200 transcripts, collectively called GARD Prediction Signature, participate in signaling pathways that are involved with recognition of foreign substances. A number of these pathways, such as nuclear factor-erythroid-related factor 2 (NRF2) mediated oxidative response, aryl hydrocarbon receptor (AHR) signaling and Toll-like receptor (TLR) signaling, are known to lead to transcription of cytoprotective enzymes and DC maturation (Johansson et al., 2011) as a response to xenobiotic challenges. Thus, GARD utilizes human MUTZ-3 as an *in vitro* DC model, taking advantage of its decision-making role in the immune response leading to skin sensitization for predicting sensitizing potency in unknown chemicals.

As a consequence of being an assay with a biomarker signature as readout, simultaneously monitoring a number of different cell events, GARD is well suited to detect positive compounds from a wide chemical space. The assay has been shown to be robust and highly reproducible, as well as accurate, with respect to the 38 reference compounds run so far. Although GARD has yet to go through a formal pre-validation, a thorough cross-validation has been performed during assay development, estimating the accuracy of the assay to >95% (Johansson et al., 2011). An internal study that will test the accuracy of GARD is currently being performed, using an additional panel of reference chemicals, including eight sensitizers and four non-sensitizers. In addition, 27 blinded samples have been made available to us from third parties, which will be assayed together with our internal validation panel. The results from these experiments are currently under analysis.

The great versatility that comes with analyzing the complete genome of cells allows for further development and broadening of the assay. Studies are currently being performed to evaluate GARD's applicability for respiratory sensitizers, both chemical haptens and proteins. Methods for assessment of respiratory sensitization are greatly underdeveloped, with no validated assay available to date (Verstraelen et al., 2008). However, efforts are being made to develop cell-based assays for sensitization of the respiratory tract, using both DC-like cell lines such as THP-1 (Verstraelen et al., 2009c), as well as epithelial cell lines such as BEAS-2B (Verstraelen et al., 2009b) and A549 (Verstraelen et al., 2009a). Furthermore, chemical reactivity assays are being explored within respiratory sensitization as well (Lalko et al., 2011). However, peptide reactivity has been shown to be a common feature for many sensitizers of both skin and respiratory tract, which would make it hard for such assays to discriminate between the two. In contrast, we envision GARD as being a single assay for both groups of sensitizers and this would be accomplished by having separate or overlapping Prediction Signatures for skin and respiratory sensitizers. The prerequisite for accomplishing such an assay is that stimulated MUTZ-3 possesses the informational content necessary for separating respiratory sensitizers from negative controls, i.e. that such a respiratory Prediction Signature can be identified. Indeed, we have recently identified a biomarker signature that

Table 2
Correlation between the three replicates of all reference compounds used for assay development. All values are calculated Pearson coefficients between two replicates.

Stimulation	GARD prediction signature			Full array		
	1 vs 2	1 vs 3	2 vs 3	1 vs 2	1 vs 3	2 vs 3
Non-sensitizers						
1-Butanol	0.99	0.99	1.00	0.99	0.99	1.00
4-Aminobenzoic acid	0.99	0.98	0.98	0.99	0.99	0.99
Benzaldehyde	1.00	1.00	1.00	1.00	1.00	1.00
Chlorobenzene	0.99	0.99	1.00	1.00	1.00	1.00
Diethyl phthalate	1.00	0.99	0.99	1.00	0.99	1.00
Dimethyl formamide	0.99	0.99	1.00	0.99	0.99	1.00
Ethyl vanillin	1.00	1.00	0.99	0.99	0.99	0.99
Glycerol	0.99	1.00	1.00	0.99	0.99	1.00
Isopropanol	0.99	0.99	0.99	0.99	0.99	0.99
Lactic acid	0.99	0.99	0.99	0.99	0.99	0.99
Methyl salicylate	0.99	0.99	0.99	0.99	0.99	0.99
Octanoic acid	1.00	0.99	0.99	1.00	1.00	1.00
Phenol	1.00	0.99	1.00	1.00	1.00	1.00
p-Hydroxybenzoic acid	0.99	0.99	0.99	0.99	0.99	0.99
Potassium permanganate[a]	1.00	–	–	1.00	–	–
Propylene glycol	1.00	1.00	1.00	1.00	1.00	1.00
Salicylic acid	1.00	0.99	0.99	1.00	0.99	1.00
Sodium dodecyl sulfate	0.99	0.99	0.99	1.00	0.99	0.99
Tween 80	0.99	0.99	0.99	1.00	0.99	0.99
Zinc sulfate	0.99	0.99	0.98	1.00	0.99	0.99
Sensitizers						
2,4-Dinitrocholorobenzene	0.99	0.99	0.99	0.99	0.99	0.99
2-Aminophenol	0.99	0.99	0.99	1.00	1.00	0.99
2-Hydroxyethyl acrylate	0.99	0.99	0.98	0.99	0.99	0.99
2-Mercabtobenzothiazole	1.00	0.99	0.99	1.00	0.99	0.99
2-Nitro-1,4-phenylendiamine	0.99	0.99	0.99	1.00	0.99	1.00
Cinnamic alcohol	0.99	0.99	0.98	0.99	0.99	0.99
Ethylendiamine	0.98	0.99	0.99	0.99	0.99	0.99
Eugenol	0.99	1.00	0.99	1.00	1.00	1.00
Formaldehyde	1.00	0.99	0.99	0.99	0.99	0.99
Geraniol	0.99	1.00	0.99	0.99	0.99	0.99
Glyoxal	0.99	1.00	0.99	0.99	1.00	1.00
Hexylcinnamic aldehyde	0.98	0.99	0.99	0.99	0.99	0.99
Isoeugenol	0.99	0.99	0.99	0.99	0.99	0.99
Kathon CG (MC/MCI)	0.99	0.99	0.99	1.00	1.00	1.00
Penicillin G	0.99	0.97	0.97	0.99	0.99	0.98
Potassium dichromate	1.00	0.99	0.99	0.99	0.99	0.99
p-Phenylendiamine	0.98	0.99	0.97	0.99	0.99	0.99
Resorcinol	1.00	1.00	1.00	1.00	1.00	1.00

[a] Only two replicates of potassium permanganate were used during assay development, due to a faulty array.

discriminates between respiratory sensitizers and non-sensitizing controls, with results currently being summarized in a manuscript.

While the analysis of the complete genome has been powerful during assay development and identification of the GARD prediction signature, the assay in its final form might benefit from a technological platform transfer to multiplex quantitative PCR or custom arrays. Such a platform transfer, along with the necessary reduction of prediction signature sizes, will be evaluated in connection to pre-validation.

The transferability of the assay remains to be tested although we foresee no immediate problems with the technology transfer. Maintenance of the MUTZ-3 cell line, chemical exposure and flow cytometric analysis are all steps easily transferred between laboratories, as demonstrated recently for the DC migration assay (Rees et al., 2011). The scripts used to perform SVM classifications are written for R, an open source program freely available for download (R Development Core Team, 2008).

In conclusion, GARD is a novel assay for assessment of sensitization. The powerful analysis of the full genome of MUTZ-3, or parts thereof, using so called Prediction Signatures, allows for a robust readout that may answer questions of unknown chemicals' ability to induce skin or respiratory sensitization, or both. The assay is simple to perform, with a majority of the laboratory steps being conducted according to standardized protocols provided by platform suppliers, thus constituting an attractive replacement for animal tests.

Conflict of interest

GARD signatures have been patented by the authors.

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