

Validation of the GARD™ skin assay for assessment of chemical skin sensitizers

– ring trial results of predictive performance and reproducibility.

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Abstract

Proactive identification of chemicals with skin sensitizing properties is a key toxicological endpoint within chemical safety assessment, as required by legislation for registration of chemicals. In order to meet demands of increased animal welfare and facilitate increased testing efficiency also in non-regulatory settings, considerable efforts have been made to develop non-animal approaches to replace current animal testing.

Genomic Allergen Rapid Detection (GARD™) is a state-of-the-art technology platform, the most advanced application of which is the assay for assessment of skin sensitizing chemicals, GARD™skin. The methodology is based on a dendritic cell (DC)-like cell line, thus mimicking the mechanistic events leading to initiation and modulation of downstream immunological responses. Induced transcriptional changes are measured following exposure to test chemicals, providing a detailed evaluation of cell activation. These changes are associated with the immunological decision-making role of DCs *in vivo* and include among other phenotypic modifications, up-regulation of co-stimulatory molecules, induction of cellular and oxidative stress pathways and xenobiotic responses and provide a holistic readout of substance-induced DC activation.

Here, results from an inter-laboratory ring trial of GARD™skin, conducted in compliance with OECD guidance documents and comprising a blinded chemical test set of 28 chemicals, are summarized. The assay was found to be transferable to naïve laboratories, with an inter-laboratory reproducibility of 92.0%. The within-laboratory reproducibility ranged between 82.1-88.9%, while the cumulative predictive accuracy across the three laboratories was 93.8%.

It was concluded that GARD™skin is a robust and reliable method for the identification of skin sensitizing chemicals and suitable for stand-alone use or as a constituent of integrated testing. These data form the basis for the regulatory validation of GARD™skin.

Keywords: GARD™, GARD™skin, *in vitro*, sensitization, chemical sensitizers.

Introduction

Skin sensitization resulting in allergic contact dermatitis (ACD) is a common occupational and environmental health problem. Topical exposure to chemical allergens triggers an adaptive immune response that causes immunological priming resulting in the acquisition of skin sensitization. The development of sensitization is dependent upon the stimulation of T lymphocyte responses. If the sensitized subject is exposed again to the same chemical allergen, a heightened response will be triggered resulting in Allergic Contact Dermatitis (ACD).

There is general agreement regarding the key biological events underlying skin sensitization, which have been continuously and extensively reviewed (Adler, Basketter et al. 2011, Kimber, Basketter et al. 2011, Martin, Esser et al. 2011, Ainscough, Frank Gerberick et al. 2013, Martin 2015). The existing knowledge of the chemical and biological mechanisms associated with skin sensitization has been summarized in the form of an Adverse Outcome Pathway (AOP) (OECD 2014). The AOP describes the sensitization process by the definition of four key events (KE). In order, KE:s include; (1) the covalent binding of a sensitizing chemical to endogenous proteins, a process also referred to as haptenization, (2) the induction of inflammatory responses in keratinocytes, (3) the activation of dendritic cells (DC), allowing for cell migration, antigen presentation and attenuation of downstream immunological pathways, thus bridging the innate and specific immunity of the host, and (4) the priming and subsequent proliferation of specific T-cells.

Assessment of skin sensitizing activity of chemicals initially required the use of laboratory animals. The classical methods based on guinea-pigs, the Guinea Pig Maximization Test (GPMT) (Magnusson and Kligman 1969) and the Buehler Occluded Patch Test (Test Guideline

(TG) 406) (OECD 1992), study both the induction and elicitation phases of skin sensitization. A murine test, the Local Lymph Node Assay (LLNA, TG 429) (OECD 2010) has gained acceptance as gold standard, since it provide advantages over the guinea pig tests in terms of animal welfare and predictive performance, while also having the ability to evaluate the relative potency of skin sensitizing chemicals, measured as a function of the lowest concentration of chemical required to trigger a certain level of T cell activation. However, driven by legislation (EU 1976, EC 2006), public opinion and economic interests, non-animal alternatives have become mandatory in predictive toxicology in general, and for skin sensitization testing in particular.

Recently, mechanistically-based *in chemico* and *in vitro* test methods have been described and validated. These methods include the Direct Peptide Reactivity Assay (DPRA) (Gerberick, Vassallo et al. 2004), the Keratinosens™ assay (Natsch 2010), and the h-CLAT assay (Ashikaga, Yoshida et al. 2006), addressing the first, second and third key events of the AOP, respectively. These methods are currently available as OECD test guidelines TG 442C (OECD 2015), TG 442D, which also include the LuSens assay (OECD 2018) and TG 442E, which also include the U-SENS and IL-8 Luc assays (OECD 2018). In addition, a TG is currently being drafted for the Sens-IS assay (Cottrez, Boitel et al. 2015).

However, information generated with the above-mentioned methods are not regarded as being sufficiently predictive for use as stand-alone tests for the identification of skin sensitization hazards and the current view is that more than one KE has to be monitored for safe hazard assessment. Rather, Defined Approaches (DA) in the context of Integrated Approaches to Testing and Assessment (IATA) are proposed as currently being the best paradigm for non-

animal testing (Hartung, Luechtefeld et al. 2013, Casati, Aschberger et al. 2018). Furthermore, none of the methods presently embraced by OECD guidelines are considered to allow potency-associated subclassification of sensitizers into categories 1A and 1B as defined by UN GHS (UN 2009). For these reasons, the need for complementary *in vitro* assays to assist in these predictions remains unfulfilled, and the room for improvement in terms of predictive accuracy in next-generation methodologies remains substantial.

The Genomic Allergen Rapid Detection (GARD™) assay for assessment of chemical skin sensitizers (GARD™skin), was conceived by whole genome analysis of human dendritic cell (DC)-like cells following chemical exposure. A predictive genomic biomarker signature, comprising genes associated with e.g. xenobiotic recognition, antigen presentation and co-stimulation and induction of cellular and oxidative stress pathways, was established (Johansson, Lindstedt et al. 2011). The methodology has since been transferred from a whole genome technological platform to the Nanostring nCounter system (Geiss, Bumgarner et al. 2008), a resource-effective gene expression readout system well suited for streamlined standard operating procedures (SOP) and widespread implementation (Forreryd, Johansson et al. 2014, Forreryd, Zeller et al. 2016). Furthermore, the GARD™skin assay has been shown, in a series of publications and industry-sponsored collaborations, to provide high levels of predictive accuracy (Johansson, Rydnert et al. 2014, Forreryd, Zeller et al. 2016, Johansson, Gradin et al. 2017).

Here, we describe the validation study of GARD™skin, based on an inter-laboratory ring trial, and report performance parameters of the method, including transferability, reproducibility and

predictive capacity. This publication constitutes a summary of the validation report that has been submitted to ECVAM and is currently in review of validating bodies.

Material and Methods

Participating organizations and structure of the study

The study was initiated by SenzaGen AB (Lund, Sweden), which acted as lead laboratory throughout the validation process. 3RsMC (Lyngby, Denmark) acted as validation manager, and formed a validation management group (VMG) with Adriaens Consulting (Aalter, Belgium) and Triskelion (Zeist, Netherlands). The overall purpose of the VMG was to define, guide, facilitate and evaluate the validation process, while ensuring compliance with the EURL-ECVAM modular approach to validation (Hartung, Bremer et al. 2004) and with the OECD Guidance documents on the validation and international acceptance of new or updated test methods for hazard assessment, as defined in OECD Guidance Documents (GD) No 1 and 34 (OECD 2005, OECD 2009). Two naïve laboratories, Burleson Research Technologies (BRT, Morrisville, NC, US), and Eurofins BioPharma Product Testing Munich GmbH (Eurofins, Planegg, Germany), were recruited. In addition, the two naïve laboratories were assisted in application of the technical readout system by Covance Genomics Laboratory (Redmond, WA, US), and KIGene (Karolinska University Hospital, Stockholm, Sweden), respectively.

The study was composed of a training phase, a transfer phase and a validation phase. During the training phase, naïve laboratories were given hands-on training in all aspects of the GARD™_{skin} SOP. Initial training was followed by an opportunity to provide feedback on the SOP, which was incorporated in a revised document. Following training, the transfer phase was performed using a set of non-blinded chemicals, giving the naïve laboratories the opportunity to demonstrate proficiency in all aspects of the protocols. Once the validation phase commenced, the revised SOP was locked for any further changes, all communication between the lead and naïve laboratories ceased and each participating laboratory individually assessed a larger set of blinded chemicals. Each laboratory performed three independent experiments, in

which all blinded test chemicals were assayed. The coding of test chemicals was unique for each participating laboratory, and for every independent experiment within each laboratory. All data were reported to the VMG, which evaluated and summarized the results which form the basis of this report. The true identities of the test chemicals were not decoded until after all data had been generated and submitted to the VMG.

Test chemicals

The set of chemicals used during the transfer phase is listed in Table 1. Chemicals used during training and transfer phases were chosen by the lead laboratory. For the validation phase, all chemical selection was done by the VMG, without any knowledge or influence from the participating laboratories. Chemical selection was based on predefined criteria relating to chemical reactivity, sensitizing potency diversity and solubility diversity, and the goal to minimize inclusion of chemicals previously assayed in GARD™skin, while maintaining a diverse and balanced chemical subset with enough data availability from clinical, *in vivo* and *in vitro* sources. The chemicals selected for inclusion in the blinded validation phase are presented in Table 2. All chemicals used during training, transfer and validation phases were purchased from Sigma Aldrich (Saint Louis, MI, USA) and in the case of the validation phase, distributed and randomly coded by the VMG.

GARD™ protocols

All protocols associated with the GARD™skin assay have been previously published (Johansson, Albrekt et al. 2013, Forreryd, Zeller et al. 2016) and summarized in SOP:s following the standard recommended format of the EURL ECVAM Database Service On Alternative Methods To Animal Experimentation (DB-ALM), with appropriate incremental

version control. Following feedback from training and transfer phases and inclusion of clarifying revisions, the updated GARD™ assay SOP v.05.01 was finalized and distributed prior to the commencement of the blinded validation phase. Hence, the GARD™ assay SOP v.05.01 was used as the sole guidance document available to participating laboratories during the validation phase. The GARD™ assay SOP v.05.01 is attached in its entirety to this publication as supplementary material S1 (Dryad Depository, doi:10.5061/dryad.sc65030).

In short, cultivated SenzaCells (ATCC Depository PTA-123875), are exposed *in vitro* to (the) test chemical(s) of interest for 24h. Following dose-response measurements of induced cell toxicity, an appropriate and test chemical specific input concentration is defined at non- to low-toxic levels. Genetic material (i.e. mRNA) is harvested from cells exposed to the appropriate input concentration of (the) test chemical(s) in three biological replicates, and the transcriptional levels of the GARD™ skin prediction signature (Johansson, Lindstedt et al. 2011) are quantified using the Nanostring nCounter system. The data is analyzed by a Support Vector Machine (SVM) (Cortes and Vapnik 1995), which has been appropriately trained on samples generated during technology platform transfer, consisting of expressional profiles generated by the reference panel of chemicals, as described (Forreryd, Zeller et al. 2016). Final prediction calls are derived from the mean SVM decision value (GARD™ DV) generated by the biological triplicate samples; Any test chemical inducing a positive (≥ 0) mean GARD™ DV is classified as a skin sensitizer. Consequently, any test chemical inducing a negative (< 0) mean GARD™ DV is classified as a non-sensitizer.

Statistical analysis

After data submission, the within-laboratory reproducibility (WLR), between-laboratory reproducibility (BLR) and predictive performance was calculated. WLR was assessed based on concordance between classifications in the three repeated experiments in each laboratory. BLR was assessed based on concordance between the majority classification of each chemical in the three different laboratories. Predictive performance was assessed by comparing the prediction results with the classification based on *in vivo* reference data. Therefore, 2x2 contingency tables (S versus NS) were constructed and sensitivity (probability of predicting S given the reference classification is S), specificity (probability of predicting NS given the reference classification is NS), and accuracy were calculated. In addition, the performance was evaluated based on the positive predictive value (PPV, proportion of positive classifications that are truly positive) and the negative predictive value (NPV, proportion of negative classifications that are truly negative). It was agreed by the VMG, prior to the commencement of the validation phase, to only consider data from valid experiments when assessing WLR, BLR and predictive performance, while documenting failed runs to report their occurrence and their underlying cause. All statistical analysis and strategic decisions relating to statistical analysis were done by the VMG, without the knowledge or influence of participating laboratories.

Results

Training and transfer

The method transfer was initiated by sharing of the SOP, web-based dissemination and discussions and material transfers. During the training phase, naïve laboratories were given the opportunity of on-site hands on training for a duration of four days, after which continued training pursued with reduced guidance. Naïve laboratories were given a chance to provide feedback on the SOP, and revisions were introduced into a finalized document (supplementary material S1). Using the updated SOP, naïve laboratories demonstrated their proficiency in the method by assaying and accurately classifying the transfer phase chemical set (Table 1, data not shown), estimating the transferability of GARD™skin to 100%.

GARD™ predictions of validation phase test chemicals

The results from the blind validation phase are summarized in Figure 1, presented as generated GARD™ DVs for each tested compound, across three independent experiments in the three laboratories. A complete classification table, as defined by the results presented in Figure 1, is attached to this publication as supplementary material S2, together with all calculations performed by the VMG of WLR, BLR and predictive capacity, as summarized below.

Each compound within each experiment across the different laboratories generated a classification (sensitizer (S) / non-sensitizer (NS)) or a missing value (N/A). From a total of 252 unique assessments, 12 missing values were generated. Missing values originated from solubility issues (dextran, all three experiments at Eurofins and BRT), incompatibility with flow cytometry-based viability assessments due to autofluorescence (cital, all three experiments at BRT), or non-compliance with cell viability quality control criteria defined by the SOP (2-

bromo-2-gluatarnitrile and 4-(methylamino)phenol sulphate, in two and one experiment at BRT, respectively). Based on this data, the failure rate was estimated to 4.7%. Missing values were excluded when estimating the prediction model performance, as agreed by the VMG in advance of the validation study.

Within laboratory reproducibility

WLR calculations were based on concordance between experiments. The result of a test chemical was considered concordant if three experiments generate the same outcome, irrespective of the true class of the test chemical. Based on the available data, WLR was estimated to 82.1%, 88.9% and 83.3%, for SenzaGen, Eurofins and BRT, respectively.

Between laboratory reproducibility

BLR calculations were based on concordance between each laboratory's majority outcome from three experiments. The result of a test chemical was considered concordant if all laboratories generate the same outcome, irrespective of the true class of a test chemical. Based on available data, BLR was estimated to 92.0%.

Discordant prediction results between the laboratories were observed for benzyl benzoate (false negative, SenzaGen) and vanillin (false positive, SenzaGen). While binary classifications indicate dissimilarities, it is noticed that generated signals (GARD DV) are highly reproducible (Figure 1). As such, no apparent technical variation could be attributed to observed discrepancies.

Predictive capacity

Predictive capacity calculations were based on concordance between each laboratory's majority outcome from three experiments and the true class of each test chemical. Contingency tables are presented in Table 3. The predictive accuracy was estimated to 89.3%, 96.3% and 96.0%, for SenzaGen, Eurofins and BRT, respectively. Taken together, the cumulative accuracy was estimated to 93.8%. Similarly, the method's cumulative sensitivity and specificity was estimated to 92.7% and 96%, respectively. The PPV and NPV for each laboratory was 94.4% and 80.0% (SenzaGen), 100.0% and 88.9% (Eurofins) and 100.0% and 88.9% (BRT).

Predictive capacity (Historical data)

In order to increase the confidence in reported data and to support estimated figures of predictive capacity, the GARD™skin validation report was complemented with historical data, generated by the lead laboratory and analyzed using the GARD™ assay SOP v.05.01. The results are attached to this publication as Supplementary Material S3. In short, the predictive performance was summarized as follows; accuracy, 95.8%; sensitivity, 100.0%; specificity, 92.3%; PPV, 91.7%; NPV, 100.0% (one false positive, n=24). Of important note, the obtained results were identical to those previously published (Forreryd et al., 2016).

Discussion

Predictive toxicology is a field that seeks to identify hazards associated with chemical exposure. Traditionally, predictive toxicology has been conducted primarily using animal experimentation. However, due to legislation, public opinion, concern for human environmental health and economic interests, there is not only a desire, but also a demand that toxicologists move away from animal tests and replace these with novel assays based on state-of-the-art technologies.

Decades of intensive research have resulted in a good understanding of the key biological mechanisms associated with and required for skin sensitization induction and progression. This knowledge has been used for the development of the OECD AOP for skin sensitization.

A variety of non-animal test methods that seek to reflect the various KEs of skin sensitization are available as OECD TGs. Furthermore, several testing strategies have emerged that combine the data from two or more of these test methods in the context of integrated approaches to testing and assessment (IATA) for skin sensitization (OECD 2016), which in their practical implementation are referred to as integrated testing strategies (ITS) or defined approaches (DA). Despite these developments there is more to be done in the development of novel non-animal tests for hazard identification. Furthermore, a key remaining challenge is the accurate assessment of the skin sensitizing potency of contact allergens using *in vitro* test methods.

GARD™skin is an assay for assessment of skin sensitizers, based on *in vitro* chemical exposure of DC-like cells. The assay interrogates high informational content gene expression data and

provides machine-learning assisted classifications based on pattern recognition, by utilizing a training data set and a predictive genomic biomarker signature, both interconnectedly defined during assay development. The aim has been to provide a more holistic examination of the biological changes that are associated with exposure to contact allergens than can be expected from methods that are based upon the exclusive use of a single endpoint.

In the present study, we report the results from an inter-laboratory ring trial. The study was conducted by strict adherence to OECD guidance documents and aims to provide the basis for a formal regulatory validation of the GARD™skin assay.

In summary, GARD™skin was found to be transferrable from the lead laboratory to two naïve laboratories, both of which were able to reproduce the expected outcomes from a limited set of chemicals used for training and demonstration of proficiency. Following the closure of a subsequent blind validation phase, comprising a larger set of chemicals across three independent experiments, GARD™skin was found to be reproducible, with a WLR ranging between 82.1-88.9%, and an inter-laboratory concordance of 92.0%. Lastly, GARD™skin was found to exhibit predictive accuracies ranging between 89.3-96.0%, with a cumulative accuracy of 93.8%.

Examining the discrepancies between the laboratories, valid predictions could not be consistently obtained for three of the substances. Dextran were deemed inapplicable by two of the laboratories due to solubility issues. Because ocular inspection is used to determine if compounds are dissolvable in vehicle solutions, results will be affected by the laboratory personals subjective judgement, which is likely the reason for this obtained difference. Citral

was another substance that was not consistently analyzed by all laboratories. In one of the laboratories, the substance was deemed inapplicable in all three experiments due to difficulties in determining suitable exposure concentration caused by autofluorescence during the flow-cytometry analysis. Autofluorescence was also reported by the other two laboratories but no definitive explanation could be identified for the conflicting outcomes. Finally, though all laboratories reported challenges when analyzing 2-bromo-2-glutaronitrile due to steep response curves during the viability assessment, only one laboratory failed to identify a suitable exposure concentration within the acceptable time-frame. It is possible that improved strategies for approaching difficult chemicals could further increase the consistency between laboratories.

The prediction performance attained by the GARDTM skin assay in this study should be regarded as comparable and competitive to the results produced by currently validated methods. Considering an excerpt of historical predictions on the set of herein studied substances, currently validated assays reached accuracies of 81% (n=26), 96% (n=27) and 89% (n=27) for DPRA, KeratinosensTM and h-CLAT respectively (Hoffmann, Kleinstreuer et al. 2018). Notable discrepancies compared to expected outcomes include 3-dimethylaminopropylamine (false negative, DPRA), ethylene diamine (false negative, GARDTM skin), isoeugenol (false negative, h-CLAT), benzyl benzoate (false negative, DPRA and h-CLAT), eugenol (false negative, KeratinosensTM), kanamycin (false positive, DPRA) and salicylic acid (false positive, DPRA and h-CLAT). While a review of historical data is preferably performed in a large chemical space in conjunction with a mechanistic discussion of reasonable explanations, such meta-analysis of historical data is outside of the scope of this article. However, ambitious efforts towards this end have recently been made (Roberts 2018).

Results obtained in this study closely mirror previous estimations of its predictive accuracy (Johansson, Rydnert et al. 2014, Forreryd, Zeller et al. 2016, Johansson, Gradin et al. 2017), which, in comparison with the current state of the art, must be considered consistently high and previously unsurpassed. Recently, a third-party evaluation conducted by the Cosmetics Europe explored the predictive capacity of several methods in a coherent chemical test set (n=128) (Hoffmann, Kleinstreuer et al. 2018). Predictive accuracies of included *in vitro* methods, including the three adopted TGs, ranged between 73.4-78.6% when predicting human hazard.

Originally, the loss of the holistic approach provided by animal models was believed to result in alternative methods with lower predictive performances. Therefore, testing strategies incorporating multiple alternative methods and other physicochemical properties of test substances, representative of the known mechanisms required for the induction of sensitization, were proposed (Jowsey, Basketter et al. 2006). The initially suggested methods were also designed to provide frameworks for ranking substances by their relative potency, which is still lacking in non-animal alternatives. Since the early proposals of ITS:s, the concept has been widely developed and multiple strategies employing different alternative test methods have been presented both for hazard identification and for hazard characterization (Ezendam, Braakhuis et al. 2016).

Nonetheless, though the development of non-animal alternatives has resulted in test methods achieving predictive performances in level with or above those attained by animal models, there is still a lack of confidence in their performance. This is particularly due to the belief that most alternative methods still only monitor a single mechanism or key event required for the induction of skin sensitization. Therefore, none of the currently validated test methods have been recommended for stand-alone use, and integration of test results is still advocated. In this context it is important to note that the study conducted by Cosmetics Europe also included an evaluation of several proposed DA:s (Kleinstreuer, Hoffmann et al. 2018). In the same chemical

test set as used to evaluate individual assays (n=128), proposed DA accuracies ranged between 75.6-85.0%.

These recent estimations of both individual predictive capacities and those generated by DA:s are consistent with historical data from previous compilations (Natsch, Ryan et al. 2013, Urbisch, Mehling et al. 2015). Furthermore, a meta-analysis of historical data, studying coherent chemical subsets, shows that ITS:s primarily based on a majority vote prediction model fail to increase the predictive capacity to and beyond the levels of the otherwise best performing assay (Roberts 2018). We have previously argued that the benefits of ITS:s and DA:s appear to be consistently overestimated in literature, among regulatory bodies and in the scientific community (Johansson and Gradin 2017). Evidence indicate that this is true both in terms of added value in respect to predictive capacity, as readily available data shows, but also in terms of the notion that accurate safety assessment must rely on a complete mechanistic monitoring of all KE:s of the AOP. Such information is in no way forwarded to the prediction model, which in the most advocated cases are solely constituted by a majority vote classification. Taken together, we encourage the field to further question the current paradigm in predictive toxicology. Naturally, a final recommendation on the regulatory adoption of GARD™_{skin} will be subject to a thorough peer-review of the submitted validation report by regulatory bodies within the frames of the current paradigm. However, a critical review of the possibility of relying on stand-alone tests for hazard assessment in the future, if a sufficiently predictive assay is readily available, is welcomed and encouraged.

It is appropriate to emphasize the unique technology features, which support the high observed predictive accuracy of GARD™skin; The data-driven assay development, the high informational content readout, and the machine-learning assisted prediction model.

Firstly, the genomic biomarkers utilized as predictors were identified by a data-driven approach, as opposed to the hypothesis-driven designs utilized in previously validated methods. In this context, the biomarker selection and prediction model design were not driven by *a priori* knowledge, e.g. an assumption that expression-levels of certain disease-associated genes or proteins would change following chemical exposure. Rather, genome-wide data generated by a reference panel of chemicals were interrogated to learn of actual measurable effects, in the context of the specified cellular system. While certain molecules previously described as relevant, e.g. CD86 and signaling entities controlled by the NRF2-pathway, were indeed identified, a data-driven and high-dimensional analysis allowed for a holistic view of induced transcriptional effects. As a direct consequence, this also relates to the second unique feature of GARD™skin, which is the high informational content. Generating high-dimensional data allows for models that generalize well. In context, this is an acknowledgement to the fact that sensitizers are indeed quite heterogeneous and may induce unique responses depending on a variety of factors (Albrekt, Johansson et al. 2014). Thus, while not all genes may be affected in the same way by all chemicals, when studied collectively, the GARD™skin prediction signature allows for a distinct separation between sensitizers and non-sensitizers, while assays relying on few biomarkers would fail to accurately classify certain test chemicals whenever the measured biomarker(s) fail to respond as hypothesized. The third unique feature of GARD™skin relates to the prediction model, which is based on state-of-the-art machine learning technology and pattern recognition. While these techniques are today ubiquitously utilized within almost every aspect of modern technology and innovation, including pure *in silico* applications for chemical

risk assessment, to the best of our knowledge, it is still only utilized by GARD™skin in the context of *in vitro* hazard assessments. These types of techniques allow for a streamlined, scientifically robust and statistically correct methodology for interpretation of high-dimensional data, which would otherwise be prone to overfitting.

In conclusion, investigations reported here summarizes the results generated in an inter-laboratory ring trial of GARD™skin. It was established that GARD™skin is transferable, reproducible and highly accurate.

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Tables

Table 1. Transfer phase chemical set.

Chemical name	CAS#	True group
2,4-dinitrochlorobenzene	97-00-7	sensitizer
resorcinol	108-46-3	sensitizer
geraniol	106-24-1	sensitizer
1-butanol	71-36-3	non-sensitizer
chlorobenzene	108-90-7	non-sensitizer

Table 2. Validation phase chemical set.

Chemical name	CAS#	True group
4-nitrobenzyl bromide	100-11-8	sensitizer
2-bromo-2-glutaronitrile	35691-65-7	sensitizer
cinnamal	104-55-2	sensitizer
formaldehyde	50-00-0	sensitizer
lauryl gallate	1166-52-5	sensitizer
4-(methylamino)phenol sulphate	55-55-0	sensitizer
methylisothiazolinone	2682-20-4	sensitizer
propyl gallate	121-79-9	sensitizer
tolouene diamine sulphate	615-50-9	sensitizer
diethyl maleate	141-05-9	sensitizer
3-dimethylaminopropylamine	109-55-7	sensitizer
ethylene diamine	107-15-3	sensitizer
isoeugenol	97-54-1	sensitizer
2-mercaptobenzothiazole	149-30-4	sensitizer
benzyl benzoate	120-51-4	sensitizer
cinnamyl alcohol	104-54-1	sensitizer
citral	5392-40-5	sensitizer
ethylene glycol dimethacrylate	97-90-5	sensitizer
eugenol	97-53-0	sensitizer
dextran	9004-54-0	non-sensitizer
glycerol	56-81-5	non-sensitizer
hexane	110-54-3	non-sensitizer
isopropanol	67-63-0	non-sensitizer
kanamycin	70560-51-9	non-sensitizer
lactic acid	50-21-5	non-sensitizer
propylene glycol	57-55-6	non-sensitizer
salicylic acid	69-72-7	non-sensitizer
vanillin	121-33-5	non-sensitizer

Table 3. Contingency tables.

True group	SenzaGen (19+9)		Eurofins (19+8)		BRT (17+8)		Cumulative (55+25)	
	S	NS	S	NS	S	NS	S	NS
S	17	2	18	1	16	1	51	4
NS	1	8	0	8	0	8	1	24
Accuracy (%)	89.3		96.3		96.0		93.8	
Sensitivity (%)	89.5		94.7		94.1		92.7	
Specificity (%)	88.9		100.0		100.0		96.0	

Figure legends

Figure 1. Summarized results generated in the ring trial, presented as mean decision values for each test chemical across three independent experiments and three independent laboratories. Error bars represent standard deviation in biological triplicate samples.

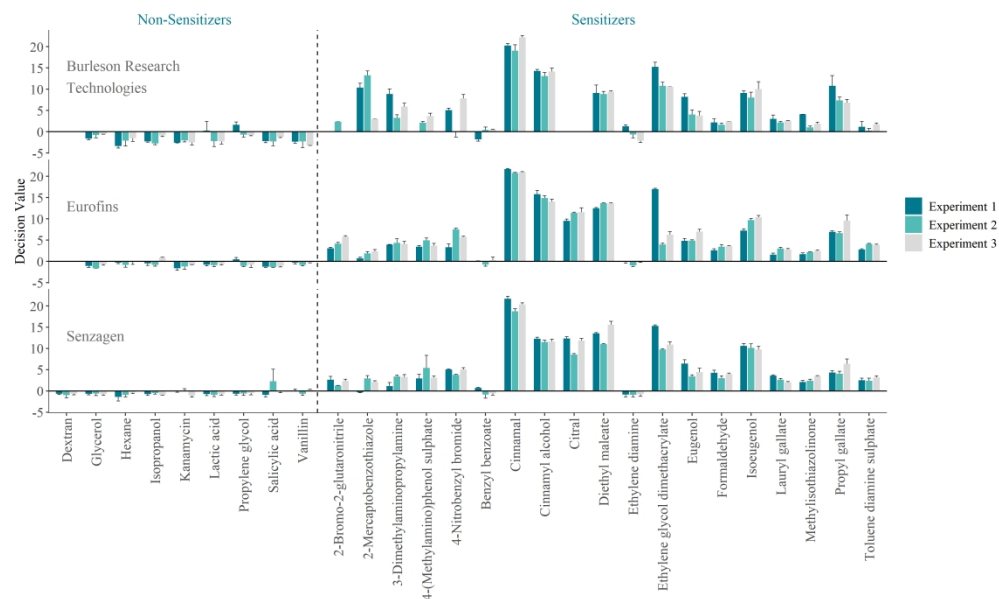


Figure 1

330x203mm (300 x 300 DPI)



DB-ALM Protocol

GARD – Genomic Allergen Rapid Detection (GARDskin)

PART A: PROTOCOL INTRODUCTION

PAGE 3

PART B: TECHNICAL DESCRIPTION

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Part A. Protocol Introduction

Protocol Name:

GARD assay v.05.01

Abstract

Genomic Allergen Rapid Detection (GARD) is an *in vitro* assay designed to predict the ability of chemical substances to induce skin sensitization based on the analysis of the relative expression levels of a biomarker signature of 196 genes using a human myeloid leukemia cell line called SenzaCell.

Résumé

GARD is based on chemical stimulation of a human myeloid leukemia cell line (SenzaCell), acting as an *in vitro* model of human Dendritic Cells (DCs). The readout of the assay is a transcriptional quantification of the genomic predictors, collectively termed the GARD Prediction Signature (GPS), using Nanostring nCounter technology. Substances are predicted as either Sensitizers or Non-sensitizers by a Support Vector Machine (SVM) model, i.e. a machine learning method that is trained using data collected from cell stimulations with known chemicals.

GARD is intended to be used as either a stand-alone assay, or as part of an integrated testing strategy (ITS), for the assessment of chemical skin sensitizers.

Experimental Description

Biological Endpoint and Endpoint Measurement:

Skin sensitization, giving rise to the clinical symptoms known as Allergic Contact Dermatitis, has been mechanistically defined in the OECD Adverse Outcome Pathways (AOP). The biological endpoint of GARD is categorized as:

AOP Key event 3: Activation of DCs

The endpoint measurement of GARD is the quantification of transcriptional levels of 196 genomic predictors, known as the GPS, using Nanostring nCounter technology.

Endpoint Value:

The Endpoint Value of each GARD measurement is a derived decision value (DV) from an SVM model.

Experimental System:

The experimental (biological) system of GARD is a human myeloid leukemia cell line called SenzaCell. The cells are available under a license agreement upon request.

Discussion

Ethical issues

The test is based on a human cell line established with the donor's consent. Thus, neither human nor animal tissues are required to perform the test. Fetal bovine serum (FBS) is used in the cell medium. Transition into using FBS-free, defined medium is possible, but not currently prioritized.

Special equipment needed

Table 1 indicates the required fixed equipment. For handling of chemical substances, any standard laboratory with appropriate equipment is sufficient. For maintenance of cell culture and cell stimulations, a cell culture laboratory designed for such purposes is recommended. For handling of RNA, any laboratory with RNase free workspace is sufficient. The readout of the assay is based on measurements made with a Nanostring nCounter system. In the case of a GARD assay transfer, a CRO or core facility for Nanostring services should be prioritized. Alternatively, cell stimulations could be performed in-house, with a subsequent shipment of RNA samples to a CRO providing Nanostring services for further analysis.

Amount of training required

The GARD assay includes safe chemical handling, cell culturing free of antibiotics, flow cytometry analysis and RNA isolation. Laboratory personnel with experience of these methods will be able to perform the GARD assay with the help of this protocol. Nevertheless, a short training period may accelerate method transfer. For the Nanostring analysis, a CRO or core facility accustomed to Nanostring equipment should be contracted.

Duration of the test

It is recommended that GARD is run in so-called campaigns, assaying up to 1-30 test substances simultaneously. One such campaign is performed within one-three months.

Potential of the protocol to be adapted for high-throughput testing

In its current form, the maximum throughput of GARD is reached at the rate given above. However, it should be noted that the limiting factor is cell growth and cell stimulation procedures, rather than end-point measurements. For one campaign of 1-30 test substances one laboratory personnel is sufficient to cover the workflow. However, with appropriate parallelisation of campaigns and with a team of technicians the throughput is substantially increased.

Costs

GARD is currently provided as a service by SenzaGen AB at the price of 5000 EUR per test substance.

Advantages and limitations

The advantage of GARD compared to other assays for prediction of chemical skin sensitizers is its predictive performance, where GARD has been used to assess the sensitizing capacity of >100 chemicals, with an average predictive accuracy of ~90%. This superior predictive performance is likely due to the sensitive readout, being based on a biomarker signature, rather than a single biomarker. Furthermore, the use of a multivariate readout also enables mechanistic-based classifications of test substances. Indeed, models for prediction of sensitizing potency have been established, although omitted from this protocol, which details the binary classifications of sensitizers. Finally, using a global readout allows for the simultaneous assessment of multiple biological endpoints by applying the same test system but with different biomarker prediction signatures. Prediction models for chemical respiratory sensitizers have been demonstrated, and yet another for protein respiratory sensitizers is in development. However, the discussion of such assays is outside the scope of this protocol. Limitations of the assay are mainly associated with the advanced equipment required, accompanied by high dimensional data analysis. However, all data analysis steps of transcriptional data has been automated and standardized using a web application specifically developed for GARD data analysis.

Status

As of March 2017, GARD should be considered a functional, scientifically validated, but not yet regulatory accepted assay. Further developments relating to the technical platform is ongoing by the test method developers.

In Development:

Current development of the assay related to the following areas:

- Applicability/Functionality domain; Chemical respiratory sensitizers
- Applicability/Functionality domain; Protein sensitizers
- Applicability/Functionality domain; Complex formulations and mixtures
- Potency assessment

None of the listed development areas concern or have any impact on this protocol, regarding binary classification of chemicals as skin sensitizers or non-skin sensitizers.

Known Laboratory Use:

As of January 2015, GARD has only been performed in the developing laboratory. In its current form, GARD is owned and performed by SenzaGen AB as a service for industry, collaborative partners and other interested stakeholders.

SenzaGen AB has established service agreements with customers representing pharmaceutical, cosmetic, chemical and biotech industry.

Participation in Evaluation Study:

GARD has previously been involved in two evaluation studies. The first evaluation study commenced in 2012 in collaboration with the Cosmetics Europe Skin Tolerance Task Force, and included the blind predictions of 10 substances. The positive outcome of the study prompted the inclusion of GARD in an extended second phase of the Cosmetic Europe evaluation, which included the blind predictions of an additional 55 substances. The study was concluded in late 2015 (Johansson et al. 2017).

GARD has also been evaluated by an industrial collaborative partner, using blinded samples of relevance for the cosmetic industry. The positive outcome of this study led to prolonged collaboration efforts aiming to determine GARD's ability to predict sensitizing potency.

Participation in Validation Study:

In addition to the evaluation studies mentioned above, GARD has been pre-validated in-house, in a proof-of-concept study demonstrating the functionality of the assay. Furthermore, GARD is currently involved in ring trial, forming the basis for a complete ECVAM submission for formal validation.

Regulatory Accepted:

GARD has not been regulatory accepted. A TST form was submitted to EURL ECVAM in January 2015, and a ring trial is currently being conducted, as of June 2016.

Proprietary and/or Confidentiality Issues

The IP rights of the GPS and any assay utilizing the GPS, in its entirety or parts thereof, are owned by SenzaGen AB.

Abbreviations and Definitions

AOP – Adverse Outcome Pathways

B - Benchmark

BSA – Bovine Serum Albumin

CASI – Campaign and Sample Information

csv file – comma-separated values file

DB-ALM – DataBase service on Alternative Methods to animal experimentation

DC – Dendritic Cell

DMSO – Dimethyl Sulfoxide

DV – Decision Value

FBS – Fetal Bovine Serum

GARD – Genomic Allergen Rapid Detection

GDA – GARD Data Analysis Application

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

GPS – GARD Prediction Signature

ITS – Integrated Testing Strategy

mAbs – Monoclonal antibodies

PBS – Phosphate Buffered Saline

PMCO – Prediction Model Cut-off

RCC – Reporter Code Count

RLF – Reporter Library File

Rv90 – Concentration Inducing 90% Relative viability

SVM – Support Vector Machine

TS – Test substance

WCB – Working Cell bank

Last Update: 2017-04-03

Part B. Technical Description

Protocol Details

Protocol Name: GARD assay v.05.01

Last Update: 2017-04-03

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Health and Safety Issues

The human myeloid leukemia cell line (SenzaCell) is a cell line of Biosafety level I. As such, no extraordinary safety issues are considered necessary, beyond those considered common for sterile work with mammalian cell lines in laboratories dedicated for such purposes.

The greatest health and safety issues associated with GARD are those related to the chemical substances that are to be tested, including the positive control chemical p-Phenylenediamine (PPD) which is sensitizing, acute toxic and an environmental hazard. When supplied, always read the SDS of each chemical substance and follow the precautions stated for each substance. In case of an unknown coded test substance consider it as a sensitizer and highly toxic compound and wear maximum protection.

DMSO is used as a solvent. DMSO easily penetrates the skin and therefore test substances dissolved in DMSO may be quickly absorbed.

The TRIzol™ reagent is corrosive and carcinogenic and should be handled according to instructions provided by the supplier.

Propidium iodide is a known toxic and irritant compound which should be used with care.

General precautions:

Always wear protective clothing and gloves and work in a fume hood when handling chemical substances and the TRIzol™ reagent. Wear protective glasses and breathing mask when handling the original stocks (powder or liquid) of the chemical substances, and preferably also disposable arm cuffs to avoid contact with the chemical substances.

SDS Information:

p-Phenylenediamine (CAS 106-50-3) and DMSO (CAS D5879), read SDS at www.sigmaaldrich.com.

TRIzol™ reagent (Cat# 15596026), read SDS at www.thermofisher.com

Propidium Iodide (Cat# 556463), read SDS at www.bdbioscience.com

GARD assay overview

Figure 1 describes the workflow of the GARD assay, starting with routine cell culturing of SenzaCells (provided by SenzaGen AB). In the GARD assay, cell stimulations of test substances are occurring for two reasons: for *GARD Input Finder* and for the *GARD Main Stimulation*.

To find the GARD Input concentration, every test substance is screened for cytotoxic effects. During this step, cells are exposed to a range of concentrations of the test substance, originating from a serial dilution.

Once the GARD Input concentration of each test substance is found, cells are exposed yet again to the test substance with the concentration identified. This step is repeated three times, using three different batches of cells, to achieve three biological replicate samples.

Thus, every GARD assessment of a test substance is based on three replicate *GARD Main Stimulations*. Depending on the number of test substances to be assayed, the three Main Stimulations can either be run in parallel or sequentially. The endpoint measurements of GARD, i.e. the quantification of the GPS mRNA transcripts, is performed on total RNA purified from cells in the Main Stimulation. The quantification is performed using the Nanostring instrument and the result is analysed with the GARD Data Analysis Application (GDAA) and each test substance can be binary predicted as **Sensitizer** or **Non-sensitizer**.

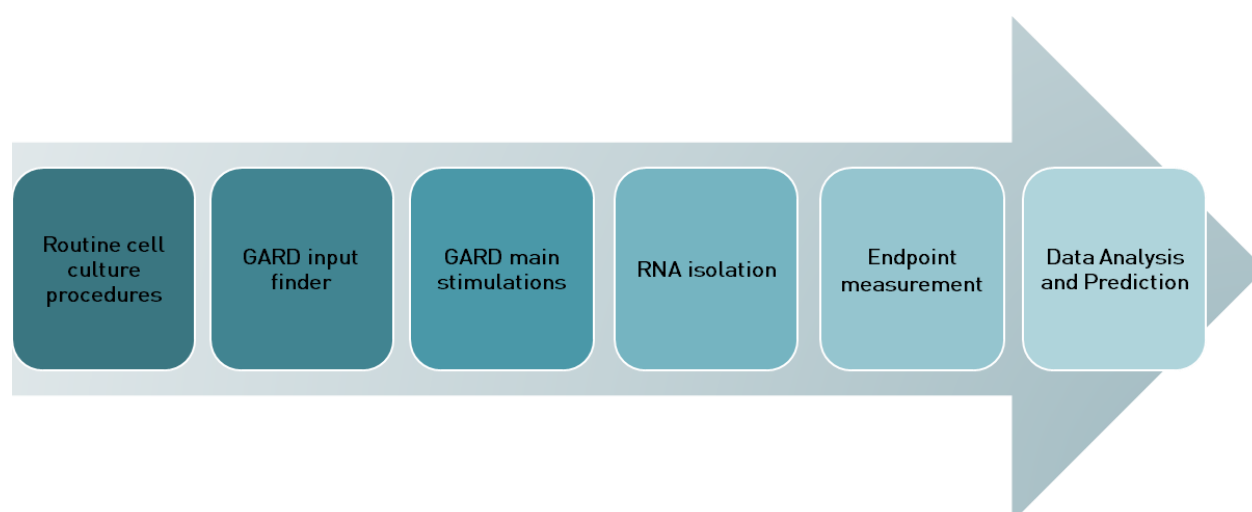


Figure 1. Overview of the GARD assay workflow.

Materials and Preparations

CELL OR EXPERIMENTAL SYSTEM

The human myeloid leukemia cell line SenzaCell is provided by SenzaGen AB and sent to the CRO on dry ice. The provided vial should be stored in liquid nitrogen. The vial is defined as a Master Cell Bank (MCB) stock and should be expanded into multiple (≥ 40) vials and frozen in liquid nitrogen as a homogenous Working Cell Bank (WCB) (see Annex 5 SG SOP 08 Establishing a Cell Bank).

EQUIPMENT

Table 1. Fixed Equipment

Equipment	Manufacturer*
Sterile (LAF) hood for cell culture work, Class II	-
Fume hood for handling of chemicals	-
Laboratory grade scale, capacity of weighing a minimum of 10 mg with reproducibility	-
Vessels for long-term storage of cells in liquid nitrogen	-
2 separate CO ₂ incubators (one for cell line culturing and one for chemical stimulation)	-
Benchtop centrifuge, swing-out rotor, 4°C	-
Centrifuge adapters for 5/15/50 ml tubes (adapter for 96 well plate if plates are used in flow cytometry analysis)	-
Freezer (-18 to -22°C)	-
Ultra low freezer (-70 to -80°C)	-
Refrigerator (2-8°C)	-
Flow cytometer (minimum equipped with a blue laser, f ex FACSVerse)	BD
Microcentrifuge for 1.5 ml micro tubes	-
Minicentrifuge for 0.2 ml tubes	-
BioAnalyzer 2100	Agilent
nCounter Prepstation	Nanostring
nCounter Digital Analyzer	Nanostring
Thermal cycler	-
Pipette controller (e.g. Pipetboy)	-
Pipettes 0.1-1000 µl	-
(Good to have: Vacuum system for preparation of flow cytometry samples)	-

*Listed are the equipment used at the laboratory of the test method developers. Equipment for which a specific manufacturer is not listed, the source of the equipment is considered arbitrary. The flow cytometer, thermocycler and BioAnalyzer could be exchanged for an **equivalent instrument**, whereas the Nanostring platform cannot be exchanged.

Table 2. Consumables

Product	Company*	Catalog Number
TC Flask, 162 cm ²	Corning	3151
TC Flask, 75 cm ²	Corning	430641U
TC Flask, 25 cm ²	Corning	430372
Centrifuge tube, 15 ml	Corning	430791
Centrifuge tube, 50 ml	Corning	430829
12-well plate	Corning	3512
24-well plate	Corning	3524
Stripettes, 10 ml	Corning	4101
Stripettes, 25 ml	Corning	4251
Sterile and RNase-free filter tips 0.1-1000 µl	-	-
Cryogenic Vial	Corning	430488
Sample tubes for Flow cytometry	Corning	352052
(Deep 96 well plate if used for flow cytometry)	-	-
0.2 µm sterile filter	-	-
1.5 ml micro tubes	-	-
RNase-free 1.5 ml micro tubes	Axygen	311-09-051
RNase-free 0.2 ml tubes	Sarstedt	72.991.002
Nitrile gloves, thickness 0.14 mm	Shieldskin	67625

*Listed are the consumables used at the laboratory of the test method developers. The exchange of any of these articles for an **equivalent product** should not interfere with the protocols and/or results, but needs to be assessed to ensure equivalence, especially the cell culture plastics.

Table 3. Medium, Serum, Buffers, Reagents and Chemicals

Product	Company*	Catalog Number
<i>Cell Medium</i>		
MEM/Alpha Modification with L-glut, Ribo-& Deoxyribo	Thermo Scientific	SH30265.01
FBS**	(to be assessed)	
rhGM-CSF (Premium grade. Purity >97%, endotoxin level <0.1 EU/μg cytokine, and activity of ≥5x10 ⁶ IU/mg)	Miltenyi Biotec	130-093-868
<i>Buffers & Solvents</i>		
D-PBS, HyClone	GE Healthcare	SH30028.02
BSA, Cohn fraction V	-	-
TRIzol	Ambion	15596018
Ethanol, 95-100%, Undenatured	Solveco	1015
<i>Antibodies*** & Staining</i>		
Mouse anti-human CD86-FITC	BD Pharmingen	555657
Mouse anti-human HLA-DR-FITC	BD	347400
Mouse anti-human CD34-FITC	BD	555821
Mouse anti-human CD1a-FITC	Dako	F7141
Mouse anti-human CD54-PE	BD Pharmingen	555511
Mouse anti-human CD14-PE	Dako	R0864
Mouse anti-human CD80-PE	BD	340294
Mouse polyclonal anti-IgG1-FITC	BD Pharmingen	555748
Mouse polyclonal anti-IgG1-PE	BD Pharmingen	555749
Propidium Iodide, 50 μg/ml	BD Pharmingen	556463
Trypan Blue Solution, 0.4%	Thermo Scientific	15250061
<i>Reagents & Kits</i>		
Direct-zol RNA MiniPrep	Zymo Research	R2052
RNA 6000 Nano Kit	Agilent	5067-1511
nCounter master kit	Nanostring	NAA-AKIT-###
GPS200_v.2 codeset	Nanostring	(Contact Nanostring)
<i>Chemicals</i>		
	CAS no	Catalog no****
p-Phenylenediamine (PPD)	106-50-3	695106
DMSO 100%	67-68-5	D5879

*Listed is the medium and reagents used at the laboratory of the test method developers. The exchange of any of these articles for an **equivalent product** should not interfere with the protocols and/or results, with the exception of the Nanostring products, which are specifically required in this protocol.

**Each lot of FBS needs to be assessed according to Annex 6 SG SOP 09 Assessment of Fetal Bovine Serum.

***Each lot of mAbs needs to be titrated to determine antibody concentration giving saturation.

**** Catalog numbers at Sigma-Aldrich for guidance of e.g. purity of each chemical.

PREPARATIONS

Media and Endpoint Assay Solutions

Cell medium

The cell medium for the SenzaCells is prepared by supplementing 500 ml MEM/Alpha with 125 ml FBS (final FBS concentration: 20%). FBS-supplemented media is referred to as *semi-complete medium* and is stored at 2-8°C for maximum 30 days. Aliquoted GM-CSF (150 μg/ml), long-term storage at -20°C±5°C or below (short-term storage at 4°C, maximum 1 week), is added freshly to the medium at every cell split and change of medium, 0.26 μl per 1 ml of cell suspension (final GM-CSF concentration: 40 ng/ml. See Table 3 for GM-CSF purity, endotoxin level and activity). Medium supplemented with both GM-CSF and FBS is referred to as *complete medium*.

Medium for freezing cells

The SenzaCells is frozen and stored in liquid nitrogen in complete medium supplemented with 10% DMSO.

Flow cytometry Wash buffer

For all washing and staining steps and carrier vehicle for flow cytometry, use PBS with 0.5-1% (w/w) BSA. Sterilize by filtration using a 0.2 µm filter. The prepared Wash buffer is stored at 2-8°C for 30 days.

Handling of Test substances

Chemicals that are to be tested for sensitization in the GARD assay are referred to as “test substances”. Test substances should be stored according to instructions from the supplier, in order to ensure its stability. Weighing of the substances can be performed prior to the day of cell stimulation if stored correctly and the stability of the substance can be ensured. Dissolved test substances should be prepared fresh on the day of cell stimulation. Test substances should be dissolved as 1000x stocks of target in-well concentration, in this document referred to as a Stock A concentration. Solubility of test substances should be ensured by a visual inspection of the solution. Extensive vortex and heat ($37^{\circ}\text{C} \pm 5^{\circ}\text{C}$) can be applied to achieve complete dissolution, see section *Solvent selection* below for assessment of solubility and solvent.

To prepare the Stock A concentration of a *solid* test substance, calculate the weight (see Note below about minimum weight of the scale) needed for 500 μL of Stock A according to Equation 1. The test substance is weighed into a pre-tared 1.5 ml micro tube. Recalculate the exact volume of solvent needed to reach the c_T , according to Equation 1.

$$v = \frac{m \cdot p \cdot 0.01}{M \cdot c_T} \quad (\text{Equation 1})$$

Where

V is the volume to be added in L
m is the exact weight added to the tube in g
M is the molecular weight of the test substance in g/mol
p is the purity of the test substance in %
 c_T is the desired target concentration in mol/L

To prepare the Stock A concentration of a *liquid* test substance, use Equation 2 to calculate a dilution factor and calculate the volume of the test substance and solvent needed for 500 μL of Stock A. Dilute the stock by the dilution factor into a 1.5 ml micro tube in the appropriate solvent.

$$df = \frac{c_S}{c_T} \quad (\text{Equation 2})$$

Where

df is the dilution factor
 c_S is the concentration of the stock in mol/L
 c_T is the desired target concentration in mol/L

Note:

- The target concentration (c_T) in Equation 1 and 2 is 1000x the desired in-well concentration.
- Take into consideration the minimum weight of the scale, preferably a five-decimal laboratory scale with a capability of weighing 10 mg with reproducibility.
- If the molecular weight is not available, use best available knowledge to approximate molecular weight of the test substance.
- If the purity of the substance is not available, use best available knowledge to approximate the purity of the test substance.
- If the molar concentration of a liquid test substance is not given by the customer, calculate the molar concentration using the molecular weight, density and purity of the test substance.
- If the density of a liquid test substance is not available, weigh the test substance.
- If the substance is too viscous for pipetting, weigh the test substance.

Positive and Negative Control(s)

For each GARD assay campaign, three benchmark controls are analysed in each of the three replicate *GARD Main Stimulations*. The controls are listed in Table 4, with relevant information for the laboratory work.

Note:

- The positive control chemical should be replaced once a year to ensure the properties of the chemical.

Table 4. List of Benchmark controls

Substance ID	Benchmark control	S/NS*	Solvent	GARD Input concentration (µM)	Relative/Absolute viability at GARD Input
pos ctrl	p-Phenylenediamine (PPD)	S	DMSO	75	84.5%-95.4% (Relative)
neg ctrl	DMSO	NS	-	0.1% (v/v)	≥95.5% (Relative)
unstim ctrl	Unstimulated cells (Medium)	NS	-	-	≥84.5% (Absolute)

*S/NS means Sensitizer and Non-sensitizer, respectively.

The purposes of the benchmark controls are twofold: Firstly, the unstimulated cells are used to normalize the data by removing confounding effects introduced by e.g. different batches. Secondly, the positive control (PPD) and the negative control (DMSO) must be accurately classified as Sensitizer and Non-sensitizer, respectively, for a GARD assay to be accepted as valid, see section *Acceptance Criteria*.

Flow cytometer instrument setup

A fluorescence compensation of the flow cytometer should be performed according to the specific instrument used when performing the GARD assay for the first time or following service of the instrument. This ensures that the fluorescence detected in a particular detector derives from the fluorochrome (PE or FITC) that is being measured. The compensation should preferably be performed using the SenzaCells, provided by SenzaGen AB, single stained with the antibodies HLA-DR-FITC and CD54-PE. For all flow cytometry analysis, the flow rate is set to 60-120 µl/min.

Campaign and Sample Information (CASI) document

As discussed previously in this protocol, it is recommended that the GARD assay is carried out in campaigns, assaying 1-30 test substances at a time. Such a campaign is carried out over a period of time in a series of laboratory experiments and data analysis.

Every GARD campaign should be accompanied by a Campaign and Sample information document (Annex 4 Campaign and Sample Info (CASI).xlsx, hereafter referred to as CASI). The CASI document records essential results as the campaign progresses and help the operators keep track of included test substances. It is also used to record key annotations that will be used in the final data analysis.

Once the study design of the present GARD campaign is finalized, create a campaign-specific CASI document based on the example given in the CASI document. This example campaign includes the assessment of 9 test substances. Along with the 3 benchmark controls, it gives a total of 36 unique RNA samples. Each of these RNA samples are designated a unique **Sample ID** corresponding to a row in the "Sample Information" sheet in the CASI document and will be analysed in the endpoint measurement. This protocol will indicate when data should be recorded into the CASI document, and how it should be used during final data analysis.

Recording of data:

For each test substance in the campaign, fill in the CASI document (sheet "Sample Information"):

- Substance ID (name of the test substance or a simplified code of your choice (avoid special characters) used throughout the campaign to identify the substance. For the Benchmark controls use "pos ctrl", "neg ctrl" and "unstim ctrl")
- Test substance chemical name or code from customer
- Source (i.e. customer name), optional

Method

ROUTINE CELL CULTURE PROCEDURES

All cell work should be performed under sterile conditions free of antibiotics; work in a laboratory designed for growth of mammalian cells, use LAF-workbenches and sterile plastics. All centrifugations are performed at 300-315xg, 5 min, 2-8°C. All incubations are performed at 37°C±1°C and 5%±0.5% CO₂. Working stocks of cultures should not be grown for more than 16 passages (~ 2 months) after thawing. A cell passage is defined in this document as each time the cell culture is counted and split, independently of how the cells has grown i.e. its doubling time (see below sections *Thawing of cells* for details about cell passage numbering, and *Cell seeding for test substance stimulation* for details about the range of cell passages used in cell stimulation).

For cell maintenance, grow cells in cell culture flasks. For volumes up to 10 ml, use TC Flask 25 cm². For volumes of 10-40 ml, use TC Flask 75 cm². For volumes of 40-90 ml, use TC Flask 162 cm². Note that for large cultures, more than one TC 162 cm² may be required.

Thawing of cells

The SenzaCells are stored in liquid nitrogen (liquid phase), 7 million cells /ml complete medium supplemented with 10% v/v DMSO.

- Thaw the cells by submerging the bottom half of a frozen vial in 37°C±1°C water bath.
- Add 10 ml semi-complete medium to a 15 ml tube and transfer the thawed cells to the tube. Centrifuge cells at 300-315xg, 5 min, 2-8°C.
- Remove supernatant by decantation. Resuspend cell pellet in 5 ml semi-complete medium. Add 0.26 µl GM-CSF (150 µg/ml) per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete.
- Move cells to a small cell culture flask (TC Flask 25 cm²). Incubate at 37°C±1°C and 5%±0.5% CO₂ (i.e. cell passage number P0).
- The next day, transfer the cell culture from the cell culture flask to a 50 ml tube. Centrifuge at 300-315xg, 5 min, 2-8°C.
- Remove supernatant by decantation. Resuspend to 1 ml semi-complete medium.
- Count the cells. Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2 x 10⁵ cells /ml.
- Add 0.26 µl GM-CSF (150 µg/ml) per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete. Incubate in cell culture flasks at 37°C±1°C and 5%±0.5% CO₂ (i.e. cell passage number P1).

Recording of data:

For each thawed cell batch, record into the CASI document (sheet "Cell maintenance log"):

- Identity of the working cell bank
- Date of thawing
- Purpose of the cell batch
- At each cell passage, record date, cell count and number of cells that are saved and/or used for the specific purpose

Maintenance of cells

Every 3-4 days the cells are counted and split to 2×10^5 cells /ml in fresh medium. The cell split is preferably performed on Mondays and Thursdays to coincide with cell stimulations (see *Cell seeding for test substance stimulation*).

- To split the cells, transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315xg, 5 min, 2-8°C.
- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells. Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2×10^5 cells /ml.
- Add 0.26 µl GM-CSF (150 µg/ml) per 1 ml of cell suspension to the cell culture. The cell culture media is now referred to as complete. Incubate in cell culture flasks at 37°C±1°C and 5%±0.5% CO₂.

Freezing of cells

- To freeze the cells, transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge cells at 300-315xg, 5 min, 2-8°C.
- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells. Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 14 million cells /ml.
- Add 0.26 µl GM-CSF (150 µg/ml) per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete.
- Transfer 0.5 ml of cell suspension to cryogenic vials (marked with cell bank identity, i.e. name of cells, date of freezing, type of cell bank stock, e.g. WCB).
- Prepare a separate solution of complete medium supplemented with 20% v/v DMSO.
- Add 0.5 ml of DMSO-supplemented complete medium to each of the cell-containing cryogenic vials.
- Immediately after adding the DMSO-supplemented complete medium, freeze the cells slowly in a temperature-controlled manner and the next day, vials are submerged into liquid nitrogen for long-time storage.

Preparing Flow cytometry samples

All washing steps are performed in Wash buffer. All centrifugations are performed at 300-315xg, 5 min, 2-8°C. All incubations are performed in dark at 2-8°C. Each lot of mAbs needs to be titrated to determine antibody concentration giving saturation.

Note:

- Removal of supernatant during preparation of flow cytometry samples is done by aspiration, e.g. by pipetting or by using a vacuum system, **not** by decantation.

Phenotypic Quality Control

The same day as performing a chemical stimulation, the cells are quality controlled by a phenotypic analysis. This is done to ensure cells are maintained in an inactivated state and to detect phenotypic drift.

Count cells and prepare 6 flow cytometry samples, with 2×10^5 cells in each sample.

- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315xg, 5 min, 2-8°C.
- Remove the supernatant by aspiration and repeat the washing step. Resuspend in 50 µl Wash buffer.
- Stain cells as indicated in Table 5 by adding titrated mAbs to each sample.

Table 5. Antibodies* and viability stain used in the Phenotypic Quality Control.

Sample 1	Isotype FITC (2 µl)	Isotype PE (2 µl)
Sample 2	CD86-FITC (2 µl)	CD54-PE (2 µl)
Sample 3	HLA-DR-FITC (0.5 µl)	CD80-PE (2 µl)
Sample 4	CD34-FITC (3 µl)	CD14-PE (1 µl)
Sample 5	CD1a-FITC (1 µl)	
Sample 6	Propidium Iodide (PI) (1 µl)	

*The indicated volumes of antibodies are used as guidance since each lot of antibody needs to be titrated.

- Incubate in dark at 2-8°C for ~15 min.
- Wash the cells once by adding ~1 ml Wash buffer and centrifuge at 300-315xg, 5 min, 2-8°C.
- Resuspend in 200 µl Wash buffer.

Analyze the samples on a flow cytometer according to manufacturer's instructions and set the flow rate to 60-120 µl/min. Record 10 000 events and analyze using the gating instructions below.

Analysis of Cell population

Exclude dead cells and cell debris by setting the "Cells" gate in the FSC/SSC scatter plot using Sample 1 (Isotype control), see Figure 2. Apply the "Cells" gate on Sample 2-6.

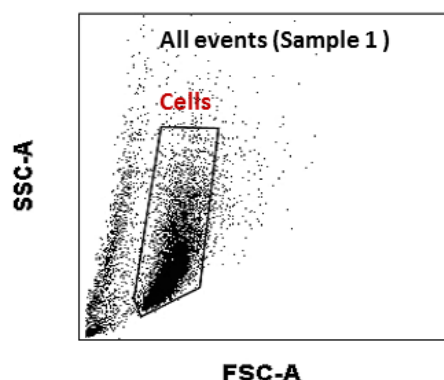


Figure 2. Instructions for setting the gate for the SenzaCell population.

Analysis of Phenotypic Quality Control markers

Show the "Cells" population in a PE/FITC scatter plot. Set quadrants for PE and FITC positive cells using Sample 1 (isotype controls) as Figure 3A. Apply the preset quadrants for PE and FITC positive cells in a PE/FITC scatter plot showing the "Cells" population of Sample 2-5 (mAb stained). Calculate and record the fraction of PE and FITC positive cells for each phenotypic marker, see example of Sample 4 below in Figure 3B, and compare with the accepted range in Table 6.

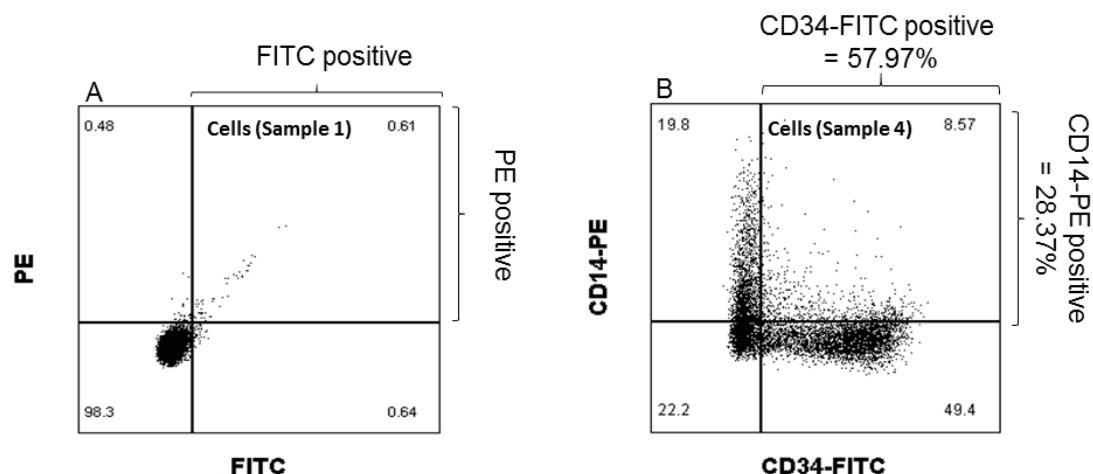


Figure 3. Instructions for setting the quadrants for PE and FITC positive cells (A). Apply the preset gate and quadrants to record the fraction of positive cells for each phenotypic marker (B).

Analysis of Absolute viability (PI negative cells)

Set the gate for “Absolute viability”, in the PE/FITC scatter plot showing “All events” on Sample 1 (Figure 4A). Apply the preset “Absolute viability” gate on the Sample 6 (PI stained), as in Figure 4B. Record the fraction of “Absolute viability” in % (PI negative cells) from Sample 6 and compare with the accepted range in Table 6.

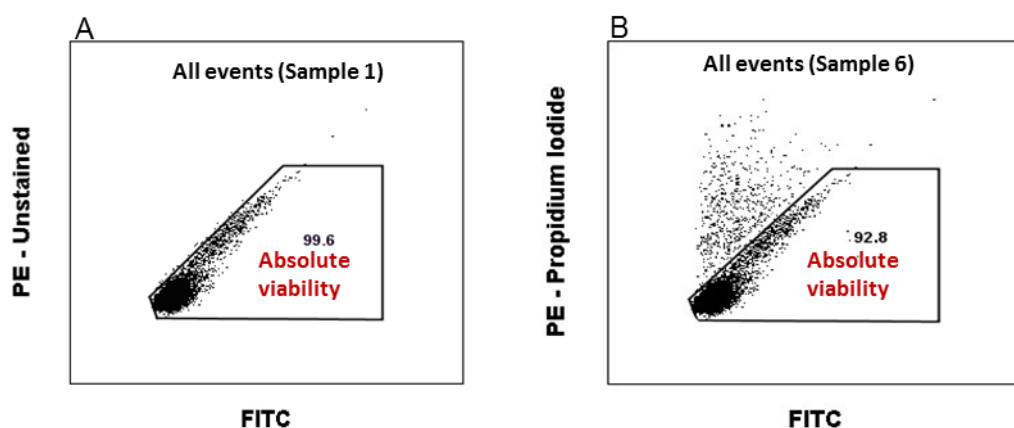


Figure 4. Instructions for setting the gate for “Absolute viability” (A). Apply the gate “Absolute viability” on Sample 6 (B).

Phenotypic Quality Control criteria

The accepted range of phenotypic biomarker expression (Table 6) is based on observations made in the developing laboratory during assay development. Variations within these ranges are to be considered normal. However, if any one biomarker is out of the specified ranges, it is recommended that the cell batch is discarded and not used for further stimulations.

Table 6. Accepted range for phenotypic markers of the SenzaCells.

Phenotypic biomarker	Accepted range of positive cells (%)*
CD86	10-40
CD54	+
HLA-DR	+
CD80	<10
CD34	+
CD14	+
CD1a	+
Phenotypic biomarker	Accepted range of negative cells (%)
Absolute viability (PI negative cells)	≥84.5

*A "+" in Table 6 indicates the presence of positive cells. An entirely positive cell population is not required. A representative example of a typical SenzaCell phenotype is presented in Figure 2 in Annex 2 SenzaCell Phenotype. Note that SenzaCells is known to be heterogenous, and deviations from given examples are expected.

Recording of data:

Record into the CASI document (sheet "Phenotypic Quality Control"):

- Identity of the working cell bank
- Date of thawing
- Purpose of the cell batch
- Passage number
- Date of phenotypic quality control
- The positive fraction of cells (%) for each phenotypic marker.
- Absolute viability (%) (PI negative fraction)

Cell seeding for test substance stimulation

Cells are seeded for stimulation directly following a cell split, i.e. all test substance stimulations are to be scheduled to coincide with routine cell culture maintenance. This has been shown by the GARD assay developers to be an important factor. The cell stimulations are initiated when a stable cell culture is established i.e. when at least a duplication of the cells between cell passages is seen, and depending on the purpose of the cell stimulation, at specific cell passage ranges:

- For *GARD Input Finder*, cells at passage number **P4-P16** are used.
- For *GARD Main Stimulation*, cells at passage number **P6-P12** are used.

TEST MATERIAL EXPOSURE PROCEDURES

GARD Input Finder

The GARD Input concentration should be established for each test substance. It is recommended to establish the GARD Input concentration for up to 10 test substances in each stimulation experiment. See Figure 5 for a schematic example of cell stimulation with two test substances and the benchmark controls unstimulated cells (unstim ctrl) and DMSO (neg ctrl).

Solvent Selection

To determine the GARD Input concentration for a test substance, cell stimulations are performed using in-well concentrations ranging from 500 μM to 1 μM (see Figure 5). Test substances should be dissolved in DMSO or water in 1.5 ml micro tubes as 1000x stocks of target in-well concentrations, in this document referred to as Stock A concentrations, to achieve a maximum in-well solvent concentration of 0.1%. For details about weighing and calculation, see section *Handling of Test substances*. For selection of solvent follow the decision tree below:

1. Prepare a stock A of 500 μl with a C_T of 500 mM (i.e. the highest Stock A concentration) in **100% DMSO** according to the equations in the section *Handling of Test substances*. Vortex well. Carefully apply heat ($37^\circ\text{C}\pm 5^\circ\text{C}$) if necessary to achieve complete dissolution. **If soluble**, use DMSO as solvent and continue to section *Serial dilution*.
2. **If not soluble in DMSO at 500 mM**, store the tube and continue to step 3.
3. Prepare a stock A of 500 μl with a C_T of 500 mM (i.e. the highest Stock A concentration) in **sterile water** according to the equations in the section *Handling of Test substances*. Vortex well. Carefully apply heat ($37^\circ\text{C}\pm 5^\circ\text{C}$) if necessary to achieve complete dissolution. **If soluble**, use water as solvent and continue to section *Serial dilution*.
4. **If not soluble in DMSO or water at 500 mM**, identify the highest possible soluble concentration in either solvent and use that as a starting point in the dilution range, section *Serial dilution*.

Note:

- Make sure to secure the lids before heating and vortexing the test substance.
- If a test substance is provided in limited amount the volumes of the Stock A dilution range can be scaled down.
- Take into consideration the minimum weight of the scale. If the test substance has a low molecular weight a pre-dilution or a higher Stock A volume (maximum 1.5 ml) might be necessary to weigh an appropriate amount of the substance (minimum weighed amount 10 mg).
- A test substance with solubility issues should be used from the highest possible concentration, down to 1 mM in the dilution range.

Serial dilution

- Perform a serial dilution down to 1 mM in the chosen solvent to get the full range of Stock A concentrations (see Figure 5). Vortex well between each dilution step.
- From Stock A, prepare the full range of Stock B concentrations by adding 10 μl of Stock A to 990 μl of semi-complete medium (100x dilution). Vortex Stock B well. If the substance is poorly soluble in semi-complete medium (typically identified through observation of precipitation in Stock B), the highest soluble concentration in semi-complete medium is used as the highest in the dilution range.
- In addition, prepare a Stock B concentration of DMSO (neg ctrl) in semi-complete medium to achieve the in-well concentration of 0.1% DMSO.

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315xg for 5 min at $2-8^\circ\text{C}$.

- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells. Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2.22×10^5 cells /ml (final cell concentration in wells after addition of test substance will be 2×10^5 cells /ml). Add 0.26 μ l GM-CSF (150 μ g/ml) per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete.
- Use 24-well plates and seed **1.8 ml** of cell suspension into the number of wells needed (see Figure 5).

Cell stimulation

For each cell stimulation, independently of number of test substances, include two wells of unstimulated cells (unstim ctrl) and one well with DMSO (neg ctrl) (Figure 5).

- Test substances: add 200 μ l of Stock B to the 1.8 ml cell suspension seeded in 24-well plates for the entire dilution range of each test substance. Mix well by carefully pipetting up and down. Final cell concentration in wells is 2×10^5 cells /ml.
- Negative control: add 200 μ l of DMSO Stock B to the 1.8 ml cell suspension, ending up with the in-well concentration of 0.1%.
- Unstimulated control: add 200 μ l of semi-complete medium to the 1.8 ml cell suspension, to achieve an in-well cell concentration and total volume equivalent to test substance treated samples.
- Incubate for 24 h (± 0.5 h) at $37^\circ\text{C} \pm 1^\circ\text{C}$ and $5\% \pm 0.5\%$ CO_2 .

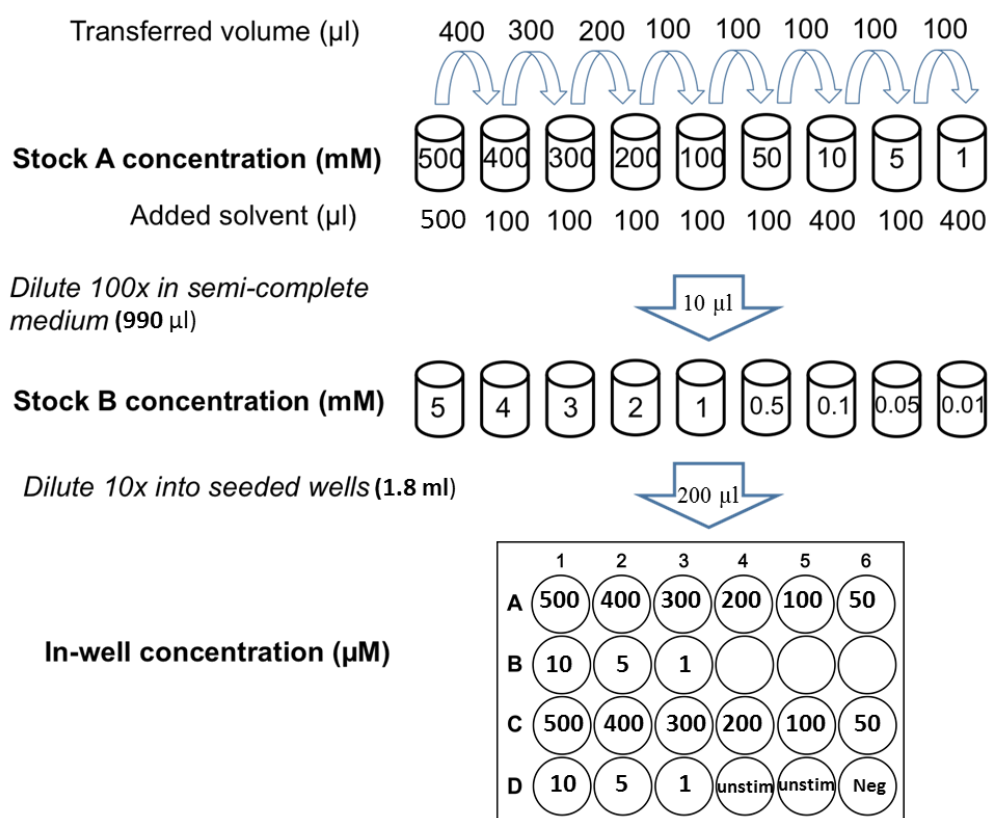


Figure 5. Schematic description of preparation of dilution series for *GARD Input Finder*.

After 24 h (± 0.5 h) incubation, harvest and prepare:

- duplicate flow cytometry samples of each test substance stimulation for the whole dilution range
- duplicate flow cytometry samples of DMSO (neg ctrl)
- four flow cytometry samples of unstimulated cells (unstim ctrl)

Sample preparation

- Mix the wells by carefully pipetting up and down and split each well into duplicate samples. **Note:** Harvest one plate at a time and put the cell samples in 2-8°C before continuing with washing.
- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315xg, 5 min, 2-8°C.
- Remove the supernatant by aspiration and repeat the washing step.
- Prepare a staining solution (enough for 50 µl for each flow cytometry sample) of Wash buffer and Propidium Iodide, 50:1.
- Resuspend each sample in 50 µl of the staining solution. **Note:** Leave 2 (out of 4) tubes with unstimulated cells unstained, resuspend them in 50 µl Wash buffer.
- Incubate in dark at 2-8°C for ~10 min.
- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315xg, 5 min, 2-8°C. Resuspend in 200 µl Wash buffer.
- Analyze the samples on a flow cytometer according to manufacturer's instructions and set the flow rate to 60-120 µl/min. Record 10 000 events and analyze using the gating instructions below.

Analysis of Cell population

Exclude dead cells and cell debris by setting the "Cells" gate in the FSC/SSC scatter plot using the unstimulated unstained sample, see Figure 6. Apply the "Cells" gate on all PI stained samples and record fraction of "Cells".

Note:

- The "Cells" population is not used for further analysis but used to keep track of the placement of the cell population in the FCS/SSC scatter plot, see Annex 1 *Cell population* for a common pitfall. A low "Cells" population can give a false percentage "Absolute viability".

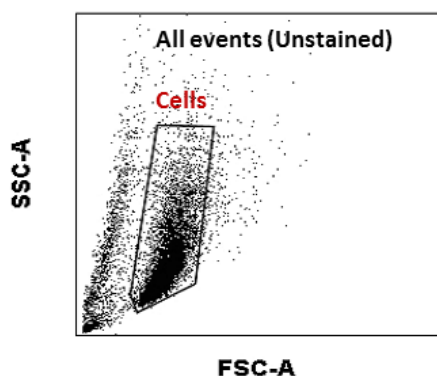


Figure 6. Instructions for setting the gate for the SenzaCells.

Analysis of Absolute viability (PI negative)

Use the unstimulated unstained sample to set the gate for "Absolute viability", in the PE/FITC scatter plot showing "All events" (Figure 7A). Apply the preset "Absolute viability" gate on all PI stained samples as in Figure 7B. Record the fraction of "Absolute viability" in % (PI negative cells).

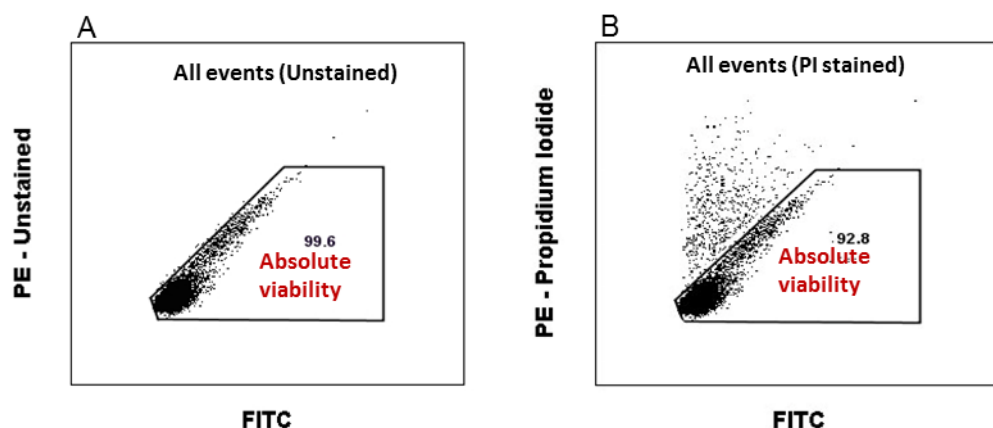


Figure 7. Instructions for setting the gate for “Absolute viability” (A). Apply the gate “Absolute viability” on PI stained samples (B).

Once the fraction of Absolute viability in % for the entire dilution range of a test substance has been recorded, the Relative viability for each sample is calculated according to Equation 3. For each concentration of the dilution range, calculate the mean value of the duplicate samples.

$$Rv = \frac{V_S}{V_C} \cdot 100 \quad (\text{Equation 3})$$

Where

Rv is the **Relative** viability of the sample in %

V_S is the **Absolute** viability of the sample in %

V_C is the mean **Absolute** viability of the two unstimulated PI stained control samples in %

Note:

- The benchmark controls should pass the Relative/Absolute viability acceptance criteria, see Table 4, unstim ctrl: **Absolute** viability ≥84.5% and neg ctrl: **Relative** viability ≥95.5%.

The **GARD Input concentration** of a test substance is decided according to the following decision tree:

- Test substances that induce cytotoxicity should be used for *GARD Main Stimulation* at the concentration that induces 90% Relative viability (Rv90), where an acceptance criterion for each sample is a Relative viability of 84.5%-95.4%. If multiple concentrations fulfill the acceptance criterion, the concentration that yields the Relative viability **closest to** 90% is chosen as the Rv90 value.
- Test substances that are not cytotoxic (Relative viability ≥95.5%) are used for *GARD Main Stimulation* at 500 μM (in-well concentration).
- Test substances that are not cytotoxic and not soluble to 500 μM (in-well concentration) are used at the highest soluble in-well concentration, see section *Solvent selection and Serial dilution* above.

Note:

- If the Relative viability decreases from ≥95.5% to <84.5% between two data points within the dilution range, additional experiments with a larger number of data points within the critical range is needed. These experiments are carried out according to the same protocol, but with a different Stock A c_T concentration. Interpolation between data points is not recommended, as linearity cannot be assumed.

Recording of data:

For each test substance, record into the CASI document (sheet "Sample Information"):

- Date of experiment/experiments
- Selected solvent
- Maximum concentration of the dilution range
- GARD Input concentration (μM)
- Relative (or Absolute for unstim ctrl) viability at GARD Input concentration

GARD Main Stimulation

Once the GARD Input concentration for each test substance is established, *GARD Main Stimulation* should be repeated three times, using three cell batches, on all test substances (TS) and benchmark controls to achieve three biological replicate samples (see Table 4 for details of the benchmark controls).

In Figure 8 a schematic example of a stimulation experiment with 8 TS and 3 benchmark controls is visualized, including two wells of the unstimulated controls.

Preparation of Test substances and Benchmark controls

- Prepare 500 μl of Stock A with a c_T of 1000x the GARD Input concentration of each substance (i.e. test substances and positive control, see Table 4) in the appropriate solvent as established in section *Solvent Selection* and according to the equations in the section *Handling of Test substances*. Vortex well. Carefully apply heat ($37^\circ\text{C} \pm 5^\circ\text{C}$) if necessary to achieve complete dissolution.
- Prepare the Stock B concentration by adding 10 μl of Stock A to 990 μl of semi-complete cell medium. Vortex Stock B well.
- In addition, prepare a Stock B concentration (100x dilution) of DMSO in semi-complete medium to achieve the in-well concentration of 0.1% DMSO (neg ctrl).

Note:

- Take into consideration the minimum weight of the scale. If a low Stock A concentration are to be prepared a pre-dilution or a higher Stock A volume (maximum 1.5 ml) might be necessary to weigh an appropriate amount of the substance (minimum weighed amount 10 mg).
- Take into consideration the solubility of the test substance and the strategy to achieve the GARD Input concentration (section *Solvent selection* above).

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315xg for 5 min in $2-8^\circ\text{C}$.
- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells. Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2.22×10^5 cells /ml (final cell concentration in wells after addition of substance will be 2×10^5 cells /ml). Add 0.26 μl GM-CSF (150 $\mu\text{g}/\text{ml}$) per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete.
- Use 12-well plates and seed **3.6 ml** of cell suspension into the number of wells needed for test substances and benchmark controls (see Figure 8).

Cell stimulations

For each cell stimulation experiment, include two wells of unstimulated cells (unstim ctrl).

- Add **400 μl** of Stock B to the 3.6 ml cell suspension seeded in 12-well plates. Mix well by carefully pipetting up and down. Final cell concentration in wells is 2×10^5 cells /ml.

- For the unstimulated cells, add **400 µl** of semi-complete medium to the 3.6 ml cell suspension, to achieve an in-well cell concentration and total volume equivalent to test substance treated samples.
- Incubate for 24 h (± 0.5 h) at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $5\% \pm 0.5\%$ CO_2 .

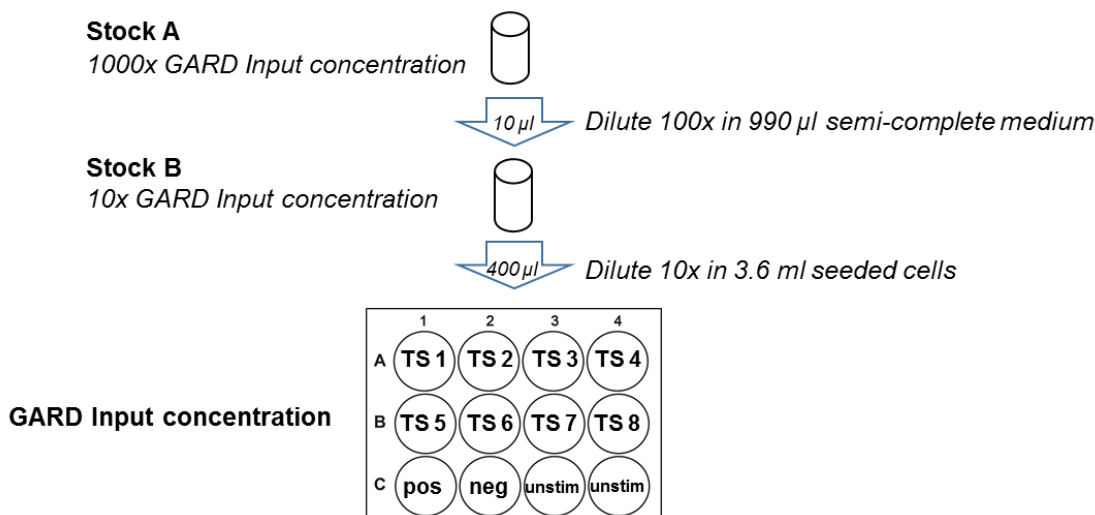


Figure 8. Example of preparation of a *GARD Main Stimulation*.

After 24 h (± 0.5 h) incubation, harvest and prepare for each substance stimulation (i.e. test substances and benchmark controls):

- triplicate TRIzol samples
- duplicate flow cytometry samples. For the unstimulated cells, harvest additional duplicate flow cytometry samples from the second well.

Harvest one plate at a time and store the samples at $2-8^{\circ}\text{C}$.

Sample preparation

- Take one plate at a time and mix cell culture in each well by pipetting up and down and harvest 3 x 1 ml from each substance, into 3 RNase-free 1.5 ml micro tubes (for TRIzol samples).
- Store the tubes at $2-8^{\circ}\text{C}$ and harvest the flow cytometry samples from the same plate by splitting the remaining cell suspension (< 1 ml) into two flow cytometry samples. Store the flow cytometry samples at $2-8^{\circ}\text{C}$.
- Harvest all samples from all plates before continuing.
- Centrifuge TRIzol samples at $300-315\times g$ for 5 min at $2-8^{\circ}\text{C}$. Remove supernatant carefully by aspiration. **Note:** aspirate only up to 12 tubes at a time, to avoid long-term contact with air and degradation of the RNA, before adding the TRIzol.
- Quickly add 300 µl of TRIzol reagent to each cell pellet. Homogenize cells by vortexing the samples for 10-20 sec. Homogenized samples can be stored short-term in RT for maximum 1 hour and long-term at $-20^{\circ}\text{C} \pm 5^{\circ}\text{C}$ (stable for one month) or $-80^{\circ}\text{C} \pm 10^{\circ}\text{C}$ (stable for one year).
- Wash the flow cytometry samples by adding ~1 ml Wash buffer and centrifuge at $300-315\times g$, 5 min, $2-8^{\circ}\text{C}$.
- Remove the supernatant by aspiration and repeat the washing step.
- Prepare a staining solution (enough for 50 µl for each flow cytometry sample) of Wash buffer and Propidium Iodide, 50:1.
- Resuspend each sample in 50 µl of the staining solution. **Note:** leave 2 (out of 4) tubes with unstimulated cells unstained, resuspend in 50 µl Wash buffer.
- Incubate in dark at $2-8^{\circ}\text{C}$ for ~10 min.

- Wash the cells by adding approximately 1 ml Wash buffer and centrifuge at 300-315xg, 5 min, 2-8°C. Resuspend in 200 µl Wash buffer.
- Analyze the samples on a flow cytometer according to manufacturer's instructions and set the flow rate to 60-120 µl/min. Record 10 000 events. Analyze in the same way as in *GARD Input Finder* (Figure 6, Figure 7 and Equation 3) and calculate the mean Relative viability for each substance using Equation 3.

The purpose of the PI stained samples is **Quality Control of the Relative viability** to ensure that test substances show the same Relative viability as in the *GARD Input Finder* and that benchmark controls show the same Relative (or Absolute for unstim ctrl) viability as described in Table 4.

Relative viability Quality Control criteria:

- Substances that are expected to induce cytotoxicity have a **Relative** viability of 84.5%-95.4%.
- Substances that are not expected to induce cytotoxicity have a **Relative** viability of $\geq 95.5\%$.
- The unstimulated control should have an **Absolute** viability of $\geq 84.5\%$.

If a test substance or benchmark control don't pass the Quality Control, see below procedures and Annex 3 for visualization of the procedures.

Procedures for failed Relative viability Quality Control

- If the Quality Control criteria is not reached for the **unstimulated control** (Absolute viability $< 84.5\%$), a new *GARD Main Stimulation* should be performed with all test substances and benchmark controls using a different cell batch, discarding the samples from the failed stimulation.
- If the Quality Control criteria for the Relative viability is not reached for ≥ 1 **test substance** because of an **incorrect GARD Input concentration**, the test substance is re-analyzed to find the correct GARD Input concentration. A Stock A of the test substance should be prepared in duplicate and cells stimulated with the correct GARD Input concentration in duplicate in the following *GARD Main Stimulation* to achieve a total of triplicate RNA samples for endpoint measurements.
- If the Quality Control criteria for the Relative viability is not reached for ≥ 1 **substance** (i.e. **test substances or pos/neg control**) because of a **technical error** in the 1st or 2nd *GARD Main Stimulation*, a Stock A of the test substance should be prepared in duplicate and cells stimulated in duplicate in the following *GARD Main Stimulation* to achieve a total of triplicate RNA samples for endpoint measurements. If this occurs in the 3rd *GARD Main Stimulation*, for: **1)** a *test substance*, a 4th *GARD Main Stimulation* should be performed (using the same cell batch at later passage), with failed test substances including all benchmark controls, discarding the failed samples from the 3rd repetition, **2)** the *pos or neg control*, only duplicate RNA samples are analyzed from the 1st and 2nd *GARD Main Stimulation* for endpoint measurement.

Recording of data:

In the CASI document (sheet "Sample Information"), copy the filled-in information in column *Test substance* and *GARD Input Finder* and paste in duplicates to be able to record in total three *GARD Main Stimulations* for each substance (see example in the CASI document).

For each test substance and benchmark control, record:

- *GARD Main stimulation* number
- Date of cell stimulation
- Relative (or Absolute for unstim ctrl) viability (%)
- If the Relative viability QC is passed

Part result:

For *each* test substance and benchmark control, 3 TRIzol replicate samples with passed Relative viability Quality Control are generated from each of the *GARD Main Stimulations* (i.e. 9 TRIzol samples from *each* test substance and benchmark control). Only one TRIzol sample from each of the *GARD Main Stimulations* will be used for RNA-isolation and further analyzed using the Nanostring platform. The additional replicates are stored as backup samples due to the possibility of having insufficient RNA concentration or RNA quality in only one TRIzol sample.

RNA isolation

The RNA isolation is recommended to be performed with maximum 24 TRIzol samples at a time and can be performed without randomization of the samples.

Prior to RNA isolation, the TRIzol samples for the endpoint measurement should be assigned a unique Sample ID (avoid extensive use of special characters for the Sample ID) in the CASI document under sheet "Sample Information" (three Sample ID's for the three TRIzol samples from each test substance and benchmark control) in order to keep track of the RNA samples isolated and QC assessed. After QC of RNA, a Sample ID can be discarded due to failure of QC (see below section *RNA quantification and quality control*). RNA from the replicate TRIzol samples is isolated and a new Sample ID is generated.

Recording of data:

In the CASI document use the list in sheet "Sample key for RNA isolation" as sample identification when isolating RNA.

Total RNA, including mRNA, is isolated from the TRIzol samples using a commercially available kit and reagents. Direct-zol RNA MiniPrep, Zymo Research, Cat.No R2052 is used by the assay developers and is recommended. RNase free tubes and ethanol (95-100%) are not provided in the kit.

- Thaw TRIzol samples on ice.

Prepare the buffers and follow the protocol in the instruction manual included in the recommended kit, but with the following adjustments:

- A DNase I treatment should not be performed.
- After the centrifugation with RNA Wash buffer, discard the flow-through of the RNA Wash Buffer (re-use the collection tube) and perform an *additional* 1 min centrifugation (10,000-16,000xg) to avoid RNA Wash Buffer residues in the eluate.
- Elute RNA by adding 25 μ l DNase/RNase free water directly to the column matrix and centrifuge at 16,000xg for 30 seconds. To increase RNA yield, it is recommended to perform a double elution by loading the eluted RNA once again on the same column for a second centrifugation.
- The eluted RNA can be used immediately or stored at -80°C $\pm$ 10°C.

It is recommended that a small aliquot (2 μ l) is stored separately or used immediately, for quantification and quality control purposes, according to instructions below.

RNA quantification and quality control

Assess the RNA from each sample using an Agilent Bioanalyzer, or an equivalent instrument. Follow protocols provided by the supplier.

Recording of data:

For each Sample ID, record into the CASI document (sheet "Sample Information):

- RNA isolation date
- Bioanalyzer run date
- RNA concentration
- RNA Integrity Number (RIN)

RNA Quality Control criteria

- The RNA concentration of a qualified sample should be ≥ 20 ng/ μ l
- The RNA Integrity Number (RIN) for a qualified sample should be ≥ 8.0

If the sample don't pass the quality control, *pool* the additional TRIzol replicates and isolate the RNA described above using *one* Zymo-Spin Column. The pooled TRIzol samples isolated for RNA is identified using a new Sample ID.

Recording of data:

For the Sample ID's *passing* the RNA Quality Control, record into the CASI document (sheet "Sample Information") the volume of sample and volume of vehicle required to produce a 10 μ l sample with an RNA concentration of 20 ng/ μ l.

Part result:

For each test substance and benchmark control, three RNA samples each identified with a Sample ID and passing the RNA Quality Control is generated.

The preparation of the RNA sample of the correct concentration for a Nanostring Hybridization assay is performed immediately prior to the hybridization (see protocols below).

ENDPOINT MEASUREMENT

The endpoint measurement of GARD is the mRNA quantification of the GPS, using the Nanostring nCounter system. A custom "CodeSet" (i.e. a set of oligonucleotide probes representing the genes of the GPS) has been developed by SenzaGen and Nanostring. To place an order for a batch of the GPS CodeSet, please contact SenzaGen AB first, and then place the order at Nanostring. The Nanostring analysis is performed with 12 RNA samples at a time (one cartridge and CodeSet) and can be performed without randomization of the samples.

Preparing a Cartridge Definition File for Nanostring analysis (is prepared by the Nanostring facility)

A Cartridge Definition File (CDF) needs to be created prior to any Nanostring run. It is used to map the reads from each lane in the cartridge to sample specific attributes. The easiest way to create a new CDF is to modify the existing "template.cdf". This is done by copying template.cdf and renaming it to a suitable name, such as run1.cdf. The file is then opened using a text editor such as Notepad.

The structure of the file needs to be kept consistent when altering the values, not introducing or removing any commas (,). The header tag contains information that is shared for all samples in the CDF, where only the CartridgeID should be changed to an appropriate identifier e.g. Run1. The sample tag contains one row for each lane in the cartridge with the columns; "LaneID", "SampleID", "Owner", "Comments", "Date", "FovCount" and "GeneRLF". The "LaneID's" are predefined as numbers between 1 and 12, representing the lanes on the cartridge, and should not be changed. The column "SampleID" should be changed so that each sample's Sample ID (as defined in the CASI document under sheet "Sample Information") is placed in the row where the LaneID matches the lane on the cartridge where the sample is placed. For example, if sample EN-001 is placed in the first lane on the cartridge, the "SampleID" in the first row of the file is changed to EN-001. The columns "Owner" and "Comments" can be left blank. "Date" is changed so that it corresponds to the date of the Nanostring run. "FovCount" is left as default (555) and "GeneRLF" is changed to the name of the Reporter Library File (RLF) that is used for the analysis.

The modifications are saved (without changing the file format) and the CDF is ready to be uploaded to the Nanostring Digital Analyzer.

Note:

- The unique Sample ID in the CASI document and in the RCC files must be identical.
- The Nanostring system has limited capability to recognize and use special characters. The available special characters are: @ ^ + - # % () ~ &, however avoid extensive use of special characters.

Setting up a Nanostring Hybridization assay

All hybridization reactions use a total RNA input of 100 ng. According to the protocol below, the sample is added to the reaction in a volume of 5 µl. Thus, all samples are to be diluted to a concentration of 20 ng/µl.

General Probe Handling warning: During setup of the assay, do not vortex or pipette vigorously to mix as it may shear the Reporter CodeSet. Mixing should be done by flicking or inverting the tubes. If using a microcentrifuge to spin down tubes, do not spin any faster than 1000 RCF for more than 30 seconds. Note that that a minicentrifuge usually has a max RCF of 2000xg, therefore it should be used with care. Do not “pulse” to spin because that will cause the centrifuge to go to maximum speed and may spin the CodeSet out of solution.

- Heat the thermocycler to 65°C, with a lid temperature of 70°C. If a time program is required, set it to infinity.
- Remove RNA samples, Reporter CodeSet and Capture CodeSet from storage at -80°C. Thaw and store RNA samples on ice. Flick and spin down. Thaw the CodeSets at room temperature and store on ice. Spin down.
- Dilute all samples to a concentration of 20 ng/µl using RNase-free water (provided in the Direct-zol RNA MiniPrep kit). Use RNase-free 0.2 mL tubes. Label each tube with Sample ID. Mix by flicking and inversion and spin down with a minicentrifuge.
- Prepare a master mix by adding 70 µl of the hybridization buffer (provided in the Nanostring master kit) to the Reporter CodeSet. Carefully mix by flicking and inversion and spin down with a minicentrifuge.
- Cut a 12-strip of hybridization-tubes (provided in the Nanostring master kit) in half, in order to fit them into a minicentrifuge. Note that the hybridization-tube strip has an orientation from 1-12, shown by the indent after the 1st and 8th position. Mark the tubes with Sample ID.
- Distribute 8 µl of master mix to each hybridization tube.
- Add 5 µl of RNA sample to each hybridization tube. Carefully mix by flicking and inversion and spin down with a minicentrifuge.
- Add 2 µl of the Capture CodeSet to each hybridization tube. Close the tubes with plastic lids and carefully mix by flicking and inversion and spin down with a minicentrifuge.
- Place the hybridization tubes in the thermocycler and incubate for 24h (±0.5 h).

Note:

- Program the cycler using 15 µl volume, at temperatures stated above. Do not set the thermal cycler to ramp down to 4°C at the end of the run.
- After thawing, inspect the tube of Reporter CodeSet to make sure no colored precipitate is present. If you see a colored precipitate, heat the entire tube to 75°C for ~10 min and cool at room temperature before using.

Setting up a Nanostring Prep Station Run

1. Remove the cartridge from storage at -20°C and let equilibrate to RT before the seal is broken.
2. Remove 2 reagent plates from storage at 4°C and let equilibrate to RT. Centrifuge the reagent plates 2000xg for 2 min, remove plastic lids before installing in the prep station.
3. Chose "Start processing" on the screen and select the high sensitivity mode.
4. Install all components into the Prep Station according to instructions on-screen. All required plastic material is provided in the Nanostring master kit "prep pack" (stored at RT).
5. Place reagent plates, pipette tips with black piercers to the bottom right, two empty hybridization tubes, holders for pipette tips, an empty cartridge (carefully close the holder without disrupting the positions of the electrodes) as described on-screen.
6. After 24h (± 0.5 h) hybridization, remove the hybridization tubes from the thermocycler and spin down with a minicentrifuge. Lift the metal lid and place them in the position highlighted on-screen. Orient the tubes so that sample #1 is positioned to the left and position #12 is positioned to the right. Remove plastic lid and close the metal lid.
7. Initiate the run by following on-screen instructions. Once the Prep Station protocol is finished, after approximately 3 hours, carefully remove the cartridge and place it on a lab-tissue, seal the cartridge with provided tape.
8. Discard used material in the Prep Station.

Note:

- If reagents or plastics are not inserted correctly the Prep Station will inform on the screen. Check before leaving the station.

Analysis with Digital Analyzer

1. Transfer the cartridge to the Digital Analyzer (make sure that the cartridge lays parallel in its position), chose "Add Cartridge" and follow instructions on screen (select 555 mode).
2. Upload the created CDF-file (specific for the Nanostring run) and the RLF file (specific for the batch of CodeSet) in the Digital Analyzer.
3. Follow the on-screen instructions to start quantify probes, the analysis takes 5 hours and the instrument can be running overnight. If the Digital Analyzer is quantifying another cartridge, press pause and follow the instructions to put in the new cartridge.
4. When the Digital Analyzer is finished in the morning, download the RCC files to a USB and save the cartridge in the original bag, seal with tape, mark date and run number and place in 2-8°C.

Recording of data:

For each Sample ID analyzed by Nanostring, record into the CASI document (sheet "Sample Information"):

- Nanostring Run date
- Nanostring Run (cartridge ID)
- Nanostring lane

Part result:

For each Sample ID analyzed by Nanostring, a Nanostring raw data file (RCC-file) is generated.

Nanostring Quality Control

Each RNA sample analyzed in the Nanostring will automatically be quality controlled with internal controls. This is notified during the section *Predictions using the GDAA* as described below and failed samples are automatically excluded before further analysis.

Data Analysis

The Nanostring raw data files are imported and analyzed on any desktop computer, using GARD Data Analysis Application (GDAA) accessible through a web browser. A username and password is required to access GDAA on the web. First time users will need to contact a SenzaGen representative (see contact persons above) in order to obtain the required login details. A RLF file corresponding to the specific batch of Nanostring CodeSet (and the resulting RCC files) should also be used during the Data Analysis and it will be obtained from SenzaGen or the Nanostring facility.

After scanning of Nanostring cartridges, data is downloaded from the nCounter Digital Analyzer as ZIP files containing *Reporter Code Count (RCC) files*, which contain digital counts of each of the CodeSet probes representing the GPS. One ZIP file is created for each Nanostring run and one RCC-file is generated for each analyzed Sample ID.

In the GDAA, a normalization algorithm further adjusts the test set to remove batch effects between the test set and the training set. This adjustment is performed in PCA space and uses the unstimulated samples in the data set. A support vector machine (SVM) is then built with an existing training dataset, which is used to generate decision values (DV) for each new sample. The mean DV of a test substance is used to classify the substance. The control substances are used to validate the experiment, where correct classifications of both control substances are required for an accepted campaign.

Predictions using the GDAA

1. To classify the raw data files with the GDAA, an annotation file in “.csv” format must first be created that maps each Sample ID’s counts to necessary descriptions. Create an annotation file by opening a new workbook in Excel. From the CASI document, copy the first 2 columns starting from row 5 (Sample ID and Substance ID) and paste into A1 of the new Excel workbook → Save as “Annotation_file_[campaign name].csv”, i.e. use CSV (Comma delimited). Ensure that the Substance ID of the benchmarks; PPD, DMSO and unstimulated cells are correctly annotated as “pos ctrl”, “neg ctrl” and “unstim ctrl” respectively.

Note:

- The unique Sample ID in the annotation file and in the RCC files (which is in turn defined by the Cartridge Definition File) must be identical. See Table 7 below for an example of a compatible layout for an annotation file. Make sure that the delimiters in the .csv files are commas and not any other symbol.

Table 7. Example of an annotation file compatible with the functions used in the data analysis.

	A	B
1	Sample ID	Substance ID
2	EN-001	TestSubstance 1
3	EN-002	TestSubstance 2
4	EN-003	TestSubstance 3
5	EN-011	pos ctrl
6	EN-012	neg ctrl

The GDAA accepts either the compressed ZIP-files or the extracted RCC-files to be uploaded and analyzed. Make sure that the ZIP/RCC-files are all stored in a unique project

folder. To further facilitate the analysis, the associated RLF-file and the annotation-file can be placed in the same folder.

2. Open an internet browser and login to the GDAA using the web address: <https://senzagen.shinyapps.io/GDAA/>
3. Ensure that "PCA" is selected under "Select Calibration Method".
4. Upload all of the ZIP/RCC-files in the "Zip or RCC files" browser, the annotation file in the "Annotation file" browser and the RLF-file in the "RLF file" browser.
5. Press the "Process..." button that appears when the files have been uploaded. The raw counts are now extracted, quality controlled and normalized. Make sure that the expected number of unstimulated control samples (≥ 3) were used in the normalization, as described in the grey text area that appears once the processing is complete. The Benchmark samples PPD and DMSO are classified and the results are displayed in a prediction plot. Any warnings that were encountered during the data processing are displayed below the "Upload Data" section. This area will also contain information about samples that failed the Nanostring Quality Control. Samples failing the Nanostring Quality Control are automatically removed from the analysis.

Recording of data:

Copy the prediction plot of the benchmark chemicals by right click on the plot → copy, and paste into the CASI document (sheet "Data analysis") by Paste special → choose Bitmap.

6. In the GDAA, press "Test new samples". A prediction plot of the binary predictions will be displayed and a table of the decision values will be created. Sample annotations for a point in the plot can be viewed by hovering the mouse pointer over it. Download the results by pressing "Download" and inspect the file by opening it in Excel. The new Excel workbook contains "Substance ID", "Sample ID", "Decision Values", "Mean DVs" and "Prediction" (column A-E) for each Substance ID analyzed.

Recording of data:

- Copy columns A-E and paste into the CASI document (sheet "Data analysis")

Part result:

Following complete analysis, each substance analyzed by Nanostring will now have a mean DV.

Acceptance Criteria

Test Substance Acceptance Criteria:

1. Each test substance should be soluble in either DMSO or water.
2. Each test substance should have three replicate samples with passed **Relative** viability Quality Control.
3. Each test substance should have $\geq$ two replicate samples with passed RNA Quality Control.
4. Each test substance should have $\geq$ two replicate samples with passed Nanostring Quality Control

If these criteria cannot be met for a test substance, the GARD assay needs to be repeated for the specific test substance (including benchmark controls).

GARD Campaign Acceptance Criteria:

1. Each stimulation (i.e. *GARD Input Finder* or *GARD Main Stimulation*) should be performed with SenzaCells that have passed the Phenotypic Quality Control.
2. The unstim ctrl should have $\geq$ three replicate samples with passed **Absolute** viability, RNA and Nanostring Quality Control.
3. Each pos/neg control should have $\geq$ two replicate samples with passed **Relative** viability, RNA and Nanostring Quality Control.
4. The positive (PPD) and negative (DMSO) benchmark controls should be accurately classified as Sensitizer and Non-sensitizer, respectively.

If these criteria cannot be met, the GARD assay needs to be repeated for all test substances and benchmark controls.

Prediction Model

For binary predictions, the prediction model is defined as:

“If the mean DV of biological replicate samples is ≥ 0 , the substance is classified as a **Sensitizer**. If the mean DV of biological replicate samples is < 0 , the substance is classified as a **Non-sensitizer**”.

Recording of data:

- For each substance summarize the result into the CASI document (sheet “Data report”).

Annexes

- Annex 1. Cell population
- Annex 2. SenzaCell Phenotype
- Annex 3. Procedures for failed Relative viability Quality Control
- Annex 4. Campaign and Sample Info (CASI).xlsx
- Annex 5. SG SOP 08 Establishing a Cell Bank
- Annex 6. SG SOP 09 Assessment of Fetal Bovine Serum

Annex 1. Cell population

During analysis of the viable cell population it is important to keep track of the “Cells” population in the FCS/SSC scatter plot and the diagonal displacement of the “Absolute viability” population in the PE/FITC scatter plot. Figure 1 is visualizing the FCS/SSC scatter plot and PE/FITC scatter plot for cells stimulated with 400 μ M (A) and 100 μ M (B), respectively. A low fraction of “Cells” can give a false percentage of Absolute viability, see Figure 1A.

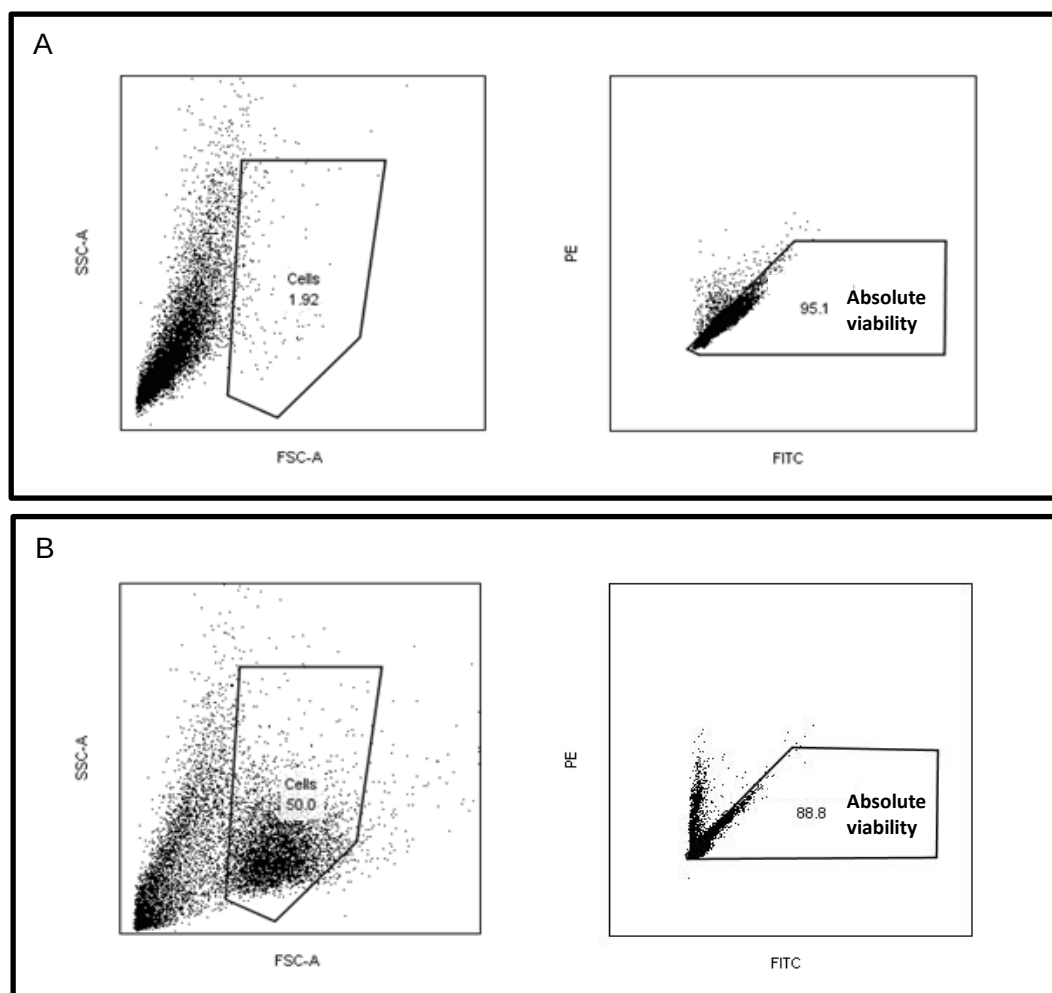


Figure 1. FCS/SSC and PE/FITC scatter plots of cells stimulated with a test substance at 400 μ M (A) and at 100 μ M (B).

Annex 2. SenzaCell Phenotype

An example of the SenzaCell phenotype is visualized in Figure 2.

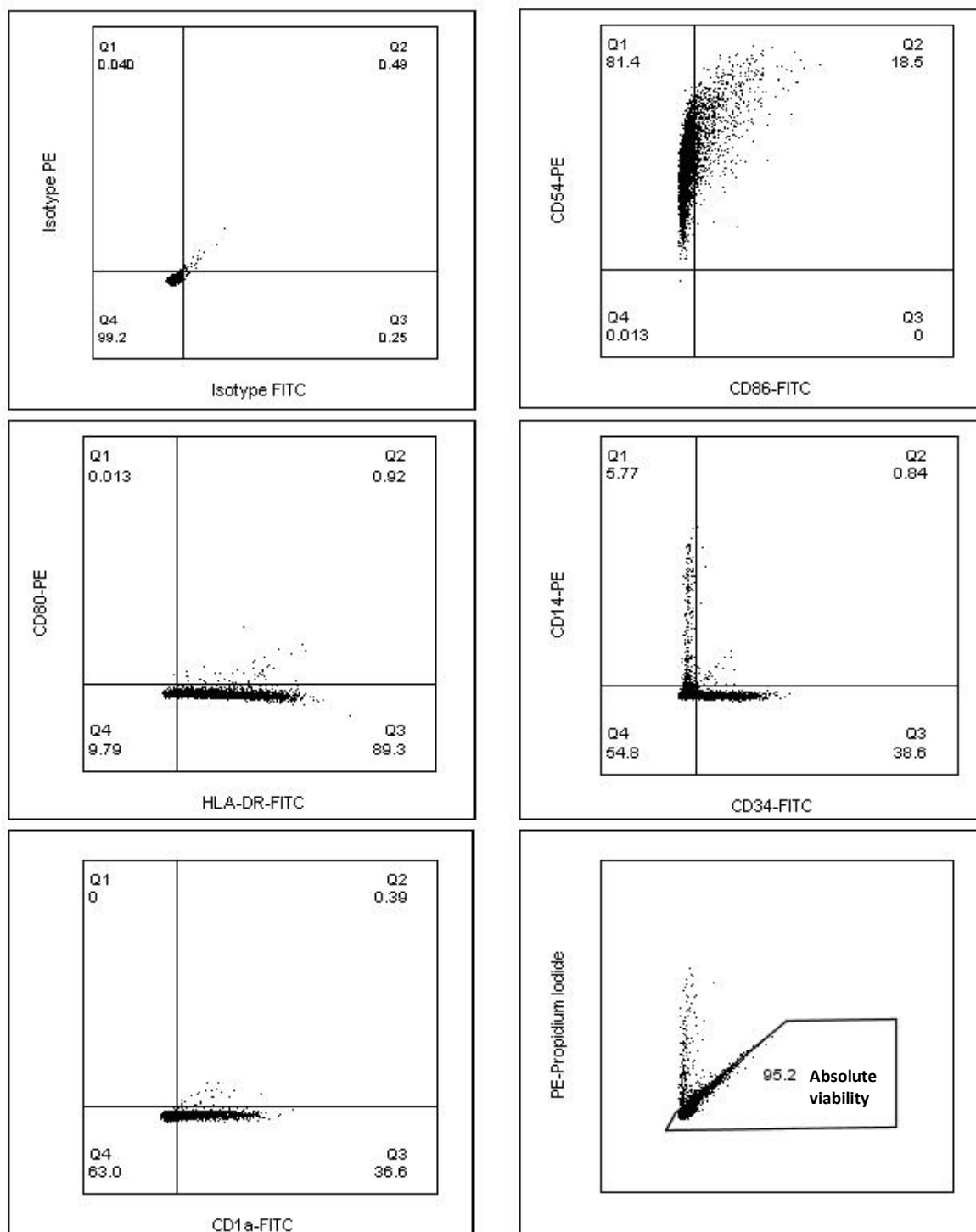


Figure 2. PE/FITC scatter plots of mAb stained SenzaCells for phenotypic control.

Annex 3. Procedures for failed Relative viability Quality Control

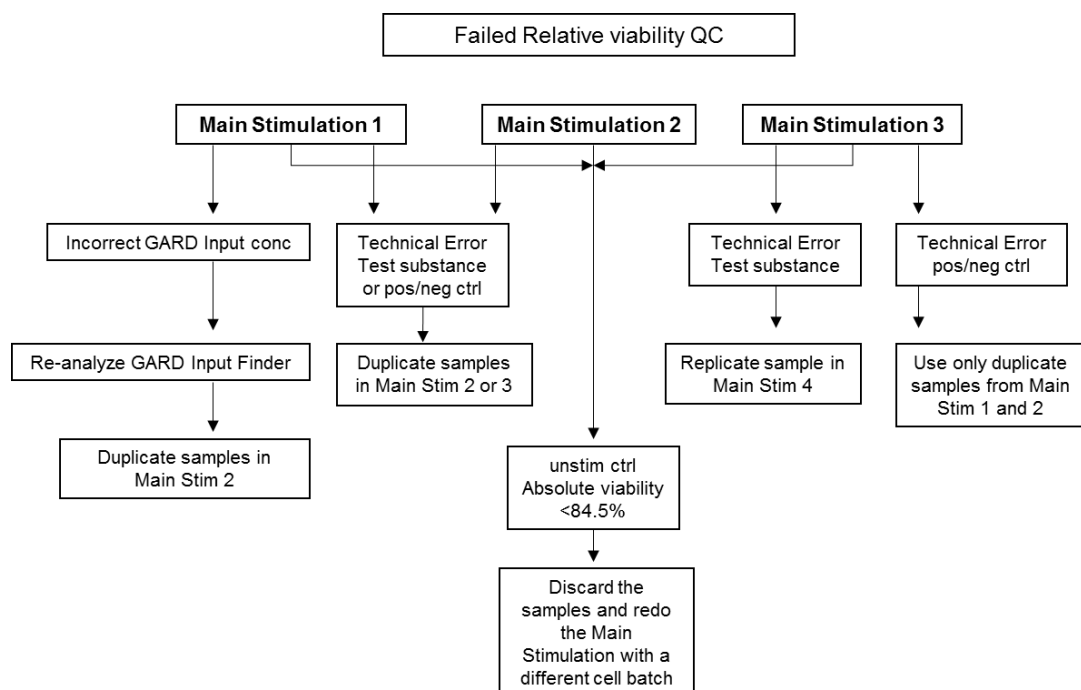


Figure 3. Visualization of the procedure in case of failed Relative viability Quality Control of a substance in *GARD Main Stimulations*.

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(PCT/GB2011/052082)

GARDpotency (ANALYTICAL METHODS AND ARRAYS FOR USE IN THE SAME)
(PCT/EP2016/056465)

SenzaCell (PCT/GB1616232.3)