STUDY REPORT

Assessment of Dermal and Respiratory Sensitization Potential of Phthalic Anhydride and Phthalic Acid Using the GARD™skin and GARD™air Assays

Study Number

NIEHSO 20220911-Phthalates

Testing Facility
Burleson Research Technologies, Inc. (BRT)
120 First Flight Lane
Morrisville, NC 27560

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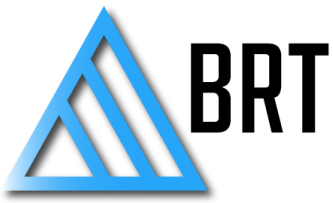

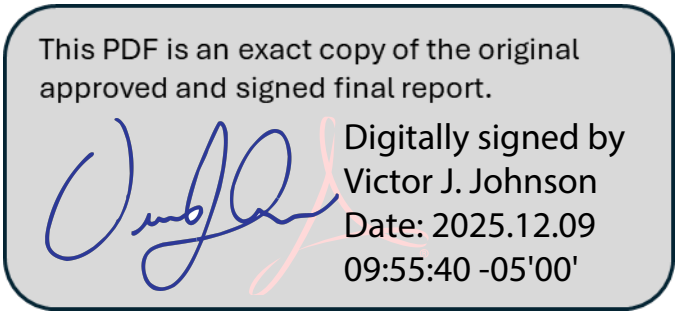

Study Identification

STUDY TITLE	Assessment of Dermal and Respiratory Sensitization Potential of Phthalic Anhydride and Phthalic Acid Using the GARD™skin and GARD™air Assays
STUDY NUMBER	NIEHSO 20220911-Phthalates
SPONSOR	National Institute of Environmental Health Sciences (NIEHS) Division of Translational Toxicology 530 Davis Drive Durham, NC 27713
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STUDY DIRECTOR:

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Study Schedule

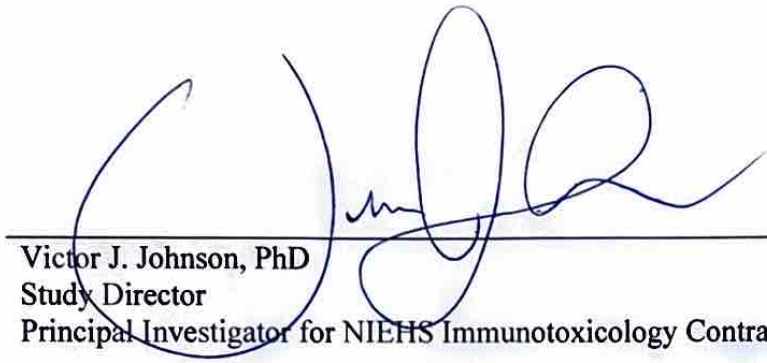
Study Initiation:	23 Feb 2023
Analytical Completion	07 Oct 2025
Study Completion	Date the Study Director signed the final report

Compliance – Non-GLP Study

Although this study was performed as indicated in the study protocol and applicable BRT standard operating procedures (SOPs), it is investigational in nature and does not conform to good laboratory practice (GLP) standards.

Approvals

Study Director Approval:



08 Dec 2025

Victor J. Johnson, PhD
Study Director
Principal Investigator for NIEHS Immunotoxicology Contract

Date

BRT Management Approval:

Florence G. Burleson, ~~PhD~~
Executive Vice President

08 Dec 2025
Date

Report Review:

Gary R. Burleson, PhD
President

08 Dec 2025
Date

Table of Contents:

Purpose and Summary

The purpose of this study was to use the GARD™skin and GARD™air assays to evaluate the sensitization potential of phthalic anhydride and phthalic acid to cause dermal and/or respiratory sensitization. These test compounds were nominated by the United States Environmental Protection Agency (USEPA) as part of a larger group of chemical nominations from NIEHS federal agency partners. These methods evaluate dendritic cell (DC) activation, the third key event of the skin sensitization adverse outcome pathway (AOP), which is also directly applicable to the pathogenesis of respiratory sensitization. The potential of compounds to activate DCs was assessed using a subclone of the human myeloid leukemia cell line MUTZ-3 called SenzaCell as a DC surrogate. Changes in gene expression were measured in SenzaCells following exposure to test compounds using Nanostring technology following RNA hybridization with code sets specific for dermal or respiratory sensitizers. Sensitization predictions were based on a Support Vector Machine (SVM) model that was developed using a machine learning algorithm with data collected from cell exposures to compounds with known sensitization potential. Phthalic anhydride was classified as a sensitizer in the GARDskin and GARDair assays indicating that it is a dermal and respiratory sensitizer. Phthalic acid was negative in both assays indicating that it is not a dermal or respiratory sensitizer.

Test and Control Materials

Phthalic anhydride and phthalic acid were nominated for testing by the USEPA. BRT was responsible for the procurement of test compounds. Table 1 provides compound identification and solvent, based on solubility testing.

Table 1: Test compounds identification, supplier, and solvent.

Test Compound ID	CAS RN®	Supplier	Lot Number	Purity	Solvent
Phthalic anhydride	85-44-9	Sigma-Aldrich	MKCK6719	99.0%	DMSO
Phthalic acid	88-99-3	Sigma-Aldrich	WXBF3775V	99.5%	DMSO

The negative control for both assays was 0.2% DMSO (solvent for both test compounds) and the positive control for GARDskin was 75 µM p-Phenylenediamine and for GARDair was 250 µM Reactive Black 5.

Unused Test Material

Test compound accountability was maintained by BRT. Prior to signing the final report, residual test compounds were discarded by BRT according to health and safety policies, SOPs, and work practices.

Health and Safety

All compounds were considered as potential sensitizing agents and handled with extreme care. Lab coats, nitrile gloves, sleeve guards, and safety glasses were always worn when handling the neat compounds. Neat compounds and stocks were prepared in a laboratory fume hood according

to the procedure outlined in the Health and Safety memo filed with the study records. These procedures were determined to be appropriate by a certified industrial hygienist. No spills or accidental exposure occurred during the conduct of this study.

Test System

SenzaCells

Activation of DCs by test compounds was evaluated using SenzaCells, a subclone of the human myeloid leukemia cell line MUTZ-3. Changes in gene expression were measured for validated panels of genes for dermal and respiratory sensitization, following 24 hours of exposure to determine if DCs were activated. Gene copies were quantified with Nanostring technology.

Methodology

GARDskin (OECD TG 442E; GARD™skin and GARD™potency assay protocol v.1.0) and GARDair (GARDair assay protocol v.1.2) assays were performed according to the SOPs provided by SenzaGen (method developer) and applicable BRT SOPs.

Prior to conducting an input finder experiment or main stimulation experiment, the cells were evaluated for phenotypic quality using flow cytometry. This was done at the time of plating the cells for the assay and served to demonstrate that the SenzaCells did not have an activated phenotype at the time of plating. The activation marker antibodies and acceptance criteria are provided in Table 2.

Table 2: Phenotypic quality acceptance criteria

Phenotypic biomarker	**Accepted range of positive cells (%)**
CD86	10-40
CD54	>0
HLA-DR	>0
CD80	<10
CD34	>0
CD14	>0
CD1a	>0
Phenotypic biomarker	**Accepted range of negative cells (%)**
Absolute viability (PI negative cells)	≥84.5

Briefly, test compounds were dissolved in DMSO as 1000x stock concentrations of 500 mM. Each test compound was evaluated in one or more input finder experiments to determine the appropriate concentration for the main runs based on viability following treatment. For the input finder, SenzaCells ($2x10^5$ cells/mL in 4 mL volume in 12-well plates) were exposed to each test compound at a range of concentrations (9 concentrations starting at 500 μM or maximum soluble concentration to a minimum of 1 μM; single well per concentration) for 24 hours and cytotoxicity was measured by flow cytometry using propidium iodide staining as a marker of dead cells. Results from the input finder were used to set an appropriate concentration [i.e.,

concentration that is closest to 90% relative viability (acceptance range is 84.5% to 95.4% relative viability) for cytotoxic test compounds; 500 μM or maximum soluble concentration for non-cytotoxic compounds] for main stimulation experiments (see Table 2 for test concentrations used in main stimulation experiments). In three independent main experiments (performed in parallel or sequentially; single well per test compound for each main stimulation experiment), SenzaCells ($2x10^5$ cells/mL in 4 mL volume in 12-well plates) were exposed to the appropriately adjusted test compound concentrations for 24 ± 0.5 hours following which, relative viability was assessed by flow cytometry, cells were lysed and RNA stabilized using TRIzol. The TRIzol samples were stored at ≤ -70°C until RNA was extracted using a Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. RNA samples were stored at ≤ -70°C until analyzed for gene expression using Nanostring technology and the GARDair50 CodeSet (Nanostring Technologies). The negative control for both assays was 0.2% DMSO (solvent for both test compounds) and the positive control for GARDskin was 75 μM p-Phenylenediamine and for GARDair was 250 μM Reactive Black 5.

SenzaCell viability for each treatment and control was based on the ability of live cells to exclude propidium iodide while dead cells allow the dye to complex with DNA resulting in fluorescence that is measured using flow cytometry. Viability acceptance criteria for input finder and main run stimulations were updated by the method developer to reflect those for GARDskin per OECD guidance document TG 442E. Table 3 provides the viability acceptance criteria.

Table 3: Viability acceptance criteria

Test chemical or control	Acceptance criteria[1]
Unstimulated control	Absolute viability of ≥84.5%
Negative control	Relative viability ≥95.5%
Positive control	Relative viability 84.5% - 95.4%
Test chemical with expected cytotoxicity	Relative viability 84.5% - 95.4%
Test chemical assayed at 500 mM or highest soluble concentration with no expected cytotoxicity	Relative viability ≥84.5%

[1]Listed acceptance criteria for unstimulated and negative controls apply to both cytotoxicity assessment experiments (input finders) and main stimulation experiments, while criteria for the positive control and test chemical are only applicable to main stimulation experiments.

Nanostring Analysis

RNA transcripts were measured to determine changes in gene expression using Nanostring technology. Total RNA was quantified, and quality was determined using a Qubit 4 fluorometer (ThermoFisher Scientific) and a total of 100 ng of RNA was used as sample input in a hybridization assay with the GPS_200 CodeSet (GARDskin) or GARDair50 CodeSet (GARDair). Hybridization was performed using a MiniAmp thermocycler (ThermoFisher

Scientific) using a block temperature of 65°C and a lid temperature of 70°C for a duration of 24 ± 0.5 hours. The hybridized samples were added to an nCounter Sprint cartridge and individual transcripts quantified using a Nanostring nCounter Sprint Profiler Digital Analyzer.

GARDskin and GARDair Classification – GDAA Analyses

Raw data exported from the Nanostring nCounter Sprint Profiler were analyzed using the respective GARD Data Analysis Application for dermal (GDAA v2.2.1; https://senzagen.shinyapps.io/GDAA_v2_2_1/) and respiratory (GDAAair v1.2.1; https://senzagen.shinyapps.io/GDAAair_1_2_1/) sensitizer classifications. The nanostring RNA expression files and sample annotation files were uploaded into the GDAA cloud application. These files were automatically analyzed for quality followed by normalization of expression values based on reference genes to facilitate prediction of sensitization potential based on the appropriate support vector machine prediction algorithm. The GDAA software generated a mean Decision Value (DV) based on the average of the DV for the replicates for each test compound. Mean DV values greater than zero resulted in a classification of respiratory sensitizer for the test compound.

Results and Summary

Phthalic anhydride and phthalic acid were nominated by the USEPA for dermal and respiratory hypersensitivity testing. The test compounds were procured by BRT. *In vitro* methods were used to determine the potential for dermal hypersensitivity using GARDskin and respiratory hypersensitivity using GARDair assays.

Both test compounds were soluble in DMSO at 500 µM. Dose finding assays did not show cytotoxicity resulting in Main Runs being performed at 500 µM for GARDskin and GARDair.

Classifications for dermal and respiratory sensitization are provided in Table 4. Phthalic anhydride was positive in both assays indicating that it is a dermal and respiratory sensitizer. In contrast, phthalic acid was negative in both assays and was classified as a non-sensitizer.

Table 4: GARD™air classifications of test compounds.

Test Compound ID	CAS RN®	Tested Concentration (µM)	Mean DV	Classification
GARDskin Classifications				
Phthalic anhydride	85-44-9	500	3.60	Positive
Phthalic acid	88-99-3	500	-0.229	Negative
GARDair Classifications				
Phthalic anhydride	85-44-9	500	4.96	Positive
Phthalic acid	88-99-3	500	-0.26	Negative

DV – Decision Value; individual sample and control DVs for all compounds are available in Appendix II.

Description of Circumstances Affecting Data Quality or Integrity

BRT is not aware of any circumstances that would impact data quality or integrity.

Maintenance of Raw Data and Records

All raw data generated at BRT were labeled with the Burleson Research Technologies study number. All raw data, protocol and amendments, and Final Report and amendments generated by the Testing Facility will be archived at study completion and retained in the archive of the Testing Facility until transferred to the NTP Archive within 240 days of signing the final report, unless alternative arrangements are made with the Sponsor.

All raw data not specific to this study (e.g., instrument logs, CVs, etc.) are archived by the Testing Facility.

Appendix I: GARD™skin protocol version 1.0 from SenzaGen.

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GARDskin and GARDpotency

Assay Protocol

INDEX

PART A. INTRODUCTION

ASSAY PROTOCOL NAME

GARD™skin and GARD™potency assay protocol v.1.0
Effective date: 2020-03-02

CONTACT

techsupport@senzagen.com

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A. VERSION HISTORY

Version Number	Description
1.0	**Main change:** GARD assay protocol v.06.1 have been combined with GARDpotency amendment v.01 and GARDskin Solvent Selection v.1.1.2 to become GARDskin and GARDpotency assay protocol v.1.0 **Minor changes:** • Part A *Protocol Introduction* have been shortened, the following sections have been removed: *Resume*, *Experimental description*, *Discussion*, *Status*, *Proprietary and/or Confidentiality Issues* and the following sections have been added: *Intellectual property rights*, *Trademarks*, *Patents*, *Copyright* and *Disclaimer*. • In Part B *Technical description,* the section *Handling of the Test substances* have been moved from *Materials and Preparations* to *Method*. The section *Solubility testing* and *Serial dilution* have been moved to individual sections and are not found directly under the section *GARD Input Finder*. • The protocol is re-written as if one test substance is to be analyzed instead of a campaign (several test substances). • Information and references to CASI have been removed in the entire document. • Addition of the *1-step dilution method* in the sections *Solubility testing* and *Serial dilution* as a result of evaluation in the developing laboratory. • Clarifications of GARD *Acceptance criteria* to enable analysis of a single test substance. • Centrifugation of cell debris removed in section *RNA Isolation* as a result on an evaluation of its effect in the developing laboratory. • A clarification of the purpose of the controls was added in section *Controls GARDskin and GARDpotency*. • Information and references to *GARD supporting protocol* have been removed in the entire document. • Addition of GARD Potency Prediction Signature CodeSets in Table 3. • The definition of MCB and WCB were removed in section *Cell or Experimental system*. • Information of assessing FBS and mAbs have been added in section *Preparations*. • Suggested volumes of the mAbs were removed in Table 5. • Appendix 3 have been removed. • Removed information about how to make a CDF file using the CDF template. • Changed from Arial to DinPro Font and added a general SenzaGen layout.

B. ABSTRACT

Genomic Allergen Rapid Detection (GARD) is an *in vitro* assay designed to predict the ability of chemical substances to induce sensitization. GARD is based on the analysis of the relative expression levels of specific biomarker signatures following the chemical stimulation of a human myeloid leukemia cell line (SenzaCell), acting as an *in vitro* model of human dendritic cells. The readout of the assay is a transcriptional quantification of the genomic predictors using NanoString nCounter technology.

In the GARDskin prediction model chemicals 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. The endpoint value of each GARDskin measurement is a derived decision value (DV) from the SVM model.

GARDpotency is an add-on functionality to the GARDskin assay. GARDpotency builds on the GARDskin method description but differs with respect to the usage of GARDpotency specific genomic predictors for the NanoString endpoint measurements.

Whereas the GARDskin assay provides a binary prediction for substances being either skin Sensitizers or Non-sensitizers, GARDpotency adds a further dimension by discriminating skin sensitizing substances as being class 1A and 1B as defined by to the Globally Harmonized System (GHS)/CLP sensitizing potency classification system for the classification and labelling of substances for skin sensitization.

The SenzaCells are available under a license agreement upon request. The IP rights of the GARD biomarker signatures and any assay utilizing the signatures, in its entirety or parts thereof, are owned by SenzaGen AB.

This method description document is based on the protocol *GARD assay v.06.0* currently under review at the scientific committee (ESAC) of the European Center for the Validation of Alternative Methods (ECVAM) for a regulatory validation of GARDskin and GARDpotency at OECD.

C. ABBREVIATIONS

BSA – Bovine Serum Albumin
DMF – Dimethylformamide
DMSO – Dimethyl Sulfoxide
DV – Decision Value
FBS – Fetal Bovine Serum
GARD – Genomic Allergen Rapid Detection
GDAA – GARD Data Analysis Application
GHS – Globally Harmonized System
GM-CSF – Granulocyte Macrophage Colony Stimulating Factor
GPS – GARD Prediction Signature
mAbs – Monoclonal antibodies
PBS – Phosphate Buffered Saline
RCC – Reporter Code Count
RLF – Reporter Library File
Rv90 – Concentration Inducing 90% Relative viability
SVM – Support Vector Machine
TS – Test substance

PART B. TECHNICAL DESCRIPTION

A. 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 which are sensitizing, acutely toxic and environmentally hazardous. When supplied, always read the SDS of each chemical substance and follow the precautions stated for each substance. In case of an unknown test substance consider it as a sensitizer and a highly toxic compound and wear maximum protection.

The TRIzol reagent is corrosive and carcinogenic and should be handled according to instructions provided by the supplier, read available safety data sheet (SDS).

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

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

Positive control and solvents (negative controls), see CAS number and Catalog number in Table 3, read available SDS.

SENZAGEN

B. 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 a test substance are occurring for two reasons: first in the *GARD Input Finder* to identify a relevant stimulation concentration and secondly in the *GARD Main Stimulation* to harvest RNA.

In the *GARD Input Finder*, the test substance is screened for cytotoxic effects, and this is done to identify an appropriate concentration (i.e. the concentration that yields a Relative viability of ~90%) to be used as the Input concentration in the *GARD Main Stimulations*. 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 the test substance is found, cells are exposed again to the test substance in the *GARD Main Stimulations* with the concentration identified in the *GARD Input Finder*. This step is repeated three times to achieve three biological replicate samples. Thus, every GARD assessment of a test substance is based on three replicate *GARD Main Stimulations*.

The endpoint measurements of GARD, i.e. the quantification of the GARD biomarker signature mRNA transcripts, is performed on total RNA purified from cells from the *GARD Main Stimulation*. The quantification is performed using the NanoString nCounter instrument and endpoint specific biomarker CodeSets. The same RNA samples can be used for quantification of different endpoint specific biomarker signatures. The result is analyzed with the *GARD Data Analysis Application* (GDAA).

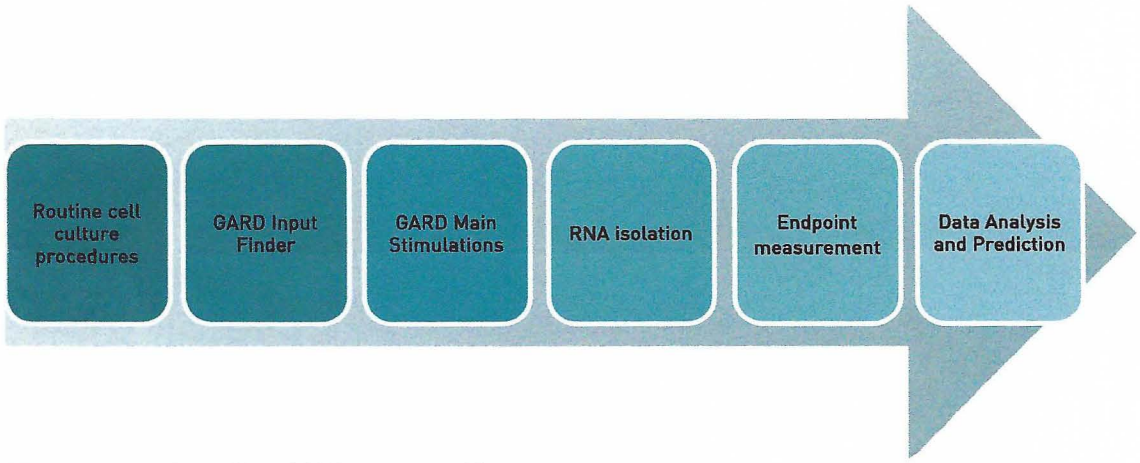

Figure 1. Overview of the GARD assay workflow.

GARDSKIN AND GARDPOTENCY

The GARDskin and GARDpotency assays for the assessment of skin sensitizers employs a tiered prediction approach. In an initial step, the GARDskin assay is used to assess the test substances by classifying their inherent ability to induce skin sensitization using a biomarker signature comprising 200 genes. Only substances that are classified as skin sensitizers are considered further for potency assessment. The GARDpotency assay consists of an additional binary prediction model that classifies skin sensitizers as weak sensitizers (1B) or strong sensitizers (1A) using a biomarker signature comprising 51 genes.

It is of importance to note that GARDpotency does not discriminate between Non-sensitizers and Sensitizers. Thus, it is of utmost importance that only test substances that have previously been determined to be skin sensitizers, as classified by e.g. GARDskin or other complementary sources of information, are further analyzed using GARDpotency. Analyzing test substances with GARDpotency that have no prior sensitizing classification data will by default result in a sensitizing classification (1A or 1B), potentially being a false positive result.

C. MATERIALS AND PREPARATIONS

CELL OR EXPERIMENTAL SYSTEM

The human myeloid leukemia cell line SenzaCell is provided by SenzaGen AB and sent to the licensed CRO on dry ice. The provided vial should be stored in liquid nitrogen. The vial should be expanded and frozen in liquid nitrogen as a homogenous cell bank according to instructions from the developing laboratory.

EQUIPMENT

Listed in Table 1-3 are the fixed equipment, consumables, and Medium, Serum, Buffers, Reagents and Chemicals needed to perform the GARDskin and GARDpotency assays.

Table 1. Fixed Equipment

Equipment	Manufacturer*
Sterile (LAF) hood for cell culture work, Class II	-
Fume hood for handling of chemicals	-
Heating block (operating at 37°C±5°C)	-
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, 2-8°C	-
Centrifuge adapters for 5/15/50 ml tubes (adapters for 96 well plates if plates are used in flow cytometry analysis)	
Freezer (operating at-18°C to -22°C)	-
Ultra-low freezer (operating at -70°C to -90°C)	-
Refrigerator (operating at 2-8°C)	-
Flow cytometer (minimum equipped with a blue laser, e.g. 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
Thermocycler**	-
Centrifuge adapters for 96 well plates**	-
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 currently cannot be exchanged.

**Equipment at NanoString Facility if not available in-house.

Table 2. Consumables

Product	Company*	Catalog Number
TC Flask, 162-175 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
Cell Medium		
MEM/Alpha Modification with L-glut, Ribo-& Deoxyribo	Thermo Scientific	SH30265.01
Fetal Bovine Serum (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
Buffers & Solvents		
D-PBS, HyClone	GE Healthcare	SH30028.02
Bovine Serum Albumin (BSA), Cohn fraction V	-	-
TRIzol	Ambion	15596018
Ethanol, 95-100%, Undenatured	Solveco	1015
*Antibodies*** & Staining*		
Mouse anti-human CD86-FITC	BD	555657
Mouse anti-human HLA-DR-FITC	BD	347400
Mouse anti-human CD34-FITC	BD	555821
Mouse anti-human CD1a-FITC	Agilent Dako	F714101-2
Mouse anti-human CD54-PE	BD	555511
Mouse anti-human CD14-PE	Agilent Dako	R086401-2
Mouse anti-human CD80-PE	BD	340294
Mouse polyclonal anti-IgG1-FITC	BD	555748
Mouse polyclonal anti-IgG1-PE	BD	555749
Propidium Iodide, 50 µg/ml	BD	556463
Trypan Blue Solution, 0.4%	Thermo Scientific	15250061
Reagents & Kits		
Direct-zol RNA MiniPrep	Zymo Research	R2052
RNA 6000 Nano Kit	Agilent	5067-1511
nCounter master kit	NanoString	NAA-AKIT-###
GPS200_v2 CodeSet (GARDskin)	NanoString	(Contact SenzaGen)
Potency_v1 CodeSet (GARDpotency)	NanoString	(Contact SenzaGen)
Chemicals and solvents	*CAS no*	*Catalog no****
p-Phenylenediamine (PPD)	106-50-3	695106
DMSO 100%	67-68-5	D5879
Acetone	67-64-1	34850
Ethanol, 95-100%	64-17-5	-
Dimethylformamide	68-12-2	1.03053
Isopropanol	67-63-0	I9516
Glycerol	56-81-5	G5516

*Listed are 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, except for the NanoString products, which are specifically required in this protocol.
**Each lot of FBS needs to be assessed according to recommended instruction from the developing laboratory.
***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

Serum, GM-CSF and antibodies

Prior to commencing a study, FBS needs to be assessed and antibodies need to be titrated according to recommended instructions from the developing laboratory. The FBS can be aliquoted and stored long-term at -18°C or below. The GM-CSF (150 µg/ml) should be prepared, aliquoted and stored long-term at -18°C or below. The GM-CSF can be stored short-term at 2-8°C for maximum 1 week.

Cell medium

The cell medium for the SenzaCells is prepared by supplementing MEM/Alpha FBS to a final FBS concentration of 20%. FBS-supplemented media is referred to as *semi-complete medium* and is stored at 2-8°C for maximum 30 days. Fresh GM-CSF is added 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 are frozen and stored in liquid nitrogen in complete medium supplemented with DMSO to a final DMSO concentration of 10%.

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.

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 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 mAbs HLA-DR-FITC and CD54-PE. For all flow cytometry analysis, the flow rate is set to 60-120 µl/min.

Controls GARDskin and GARDpotency

For each GARDskin and GARDpotency study, relevant controls are analyzed in each of the three replicate *GARD Main Stimulations.* The controls are listed in Table 4, with relevant information for the laboratory work. The GARD input concentration of the positive control, p-Phenylenediamine (PPD), should be determined in a *GARD Input Finder* experiment.

Note:

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

Table 4. List of controls used in the GARDskin and GARDpotency assays.

Substance ID	Control	GARDskin prediction*	GARDpotency prediction**	Solvent	GARD Input conc (µM)	Relative/Absolute viability at GARD Input
pos ctrl (GARDskin) 1A ctrl (GARDpotency)	p-Phenylenediamine (PPD)	S	1A	DMSO	As determined in an Input Finder	84.5%-95.4% (Relative)
neg ctrl	Test substance solvent***	NS	Not analyzed	-	Used in-well concentration***	≥95.5% (Relative)
unstim ctrl	Unstimulated cells (Medium)	-	-	-	-	≥84.5% (Absolute)

*GARDskin prediction as S (Sensitizer) or NS (Non-sensitizer).
**GARDpotency prediction as 1A (strong) or 1B (weak).
***See Table 7 for available solvents and corresponding maximum concentration.

The controls have the following purpose:

Unstimulated control

Samples of unstimulated cells are used for normalization of the dataset. By removing batch-effects the dataset is aligned to the prediction models training-set. The unstimulated control is used in both GARDskin and GARDpotency data analysis.

Negative control

The purpose of the negative control, which is defined as the test substance solvent, in the GARDskin assay is to show that the cells have not become activated in any steps of the GARD assay's experimental procedures and should be accurately classified as Non-sensitizer. The negative control should not be analyzed in GARDpotency.

Positive control

The purpose of the positive control (PPD) sample is to demonstrate that the SenzaCells used during an experiment are responsive and can become activated when exposed to a skin sensitizer. The positive control should be accurately classified as Sensitizer in GARDskin and classified as 1A in GARDpotency.

It is important to note that the purpose of the control samples in the GARDskin and GARDpotency assays are not to validate the performance of the prediction model, but to assert that the cells used for an experiment are valid. The models are fixed equations not affected by the assay and the validity of the prediction models has been performed in discovery and validation studies.

D. 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 300-315x*g*, 5 min, 2-8°C. All incubations are performed at 37°C±1°C and 5%±0.5% CO_2.
Cell 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 cm^2. For volumes of 10-40 ml, use TC Flask 75 cm^2. For volumes of 40-100 ml, use TC Flask 162-175 cm^2. Note that for large cultures, more than one TC 162-175 cm^2 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 the cells.
- Remove supernatant by decantation. Resuspend cell pellet in 5 ml semi-complete medium. Add 0.26 µl GM-CSF 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 cm^2) and incubate the cell culture (i.e. cell passage number P0).
- The next day, transfer the cell culture from the cell culture flask to a 50 ml tube. Centrifuge.
- 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 10^5 cells /ml.
- Add 0.26 µl GM-CSF per 1 ml of cell suspension to the cell culture. The cell culture medium is now referred to as complete. Incubate the cell culture (i.e. cell passage number P1).

Maintenance of cells

Every 3-4 days the cells are counted and split to 2 x 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 appropriate tubes. Centrifuge.
- 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 x 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% CO_2.

Freezing of cells

- To freeze the cells, transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge.
- 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 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).
- 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 300-315x*g*, 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 x 10^5 cells in each sample.

- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315x*g*, 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	Isotype PE
Sample 2	CD86-FITC	CD54-PE
Sample 3	HLA-DR-FITC	CD80-PE
Sample 4	CD34-FITC	CD14-PE
Sample 5	CD1a-FITC	
Sample 6	Propidium Iodide (PI)	

- Incubate in dark at 2-8°C for ~15 min.
- Wash the cells once by adding ~1 ml Wash buffer and centrifuge the samples.
- 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.

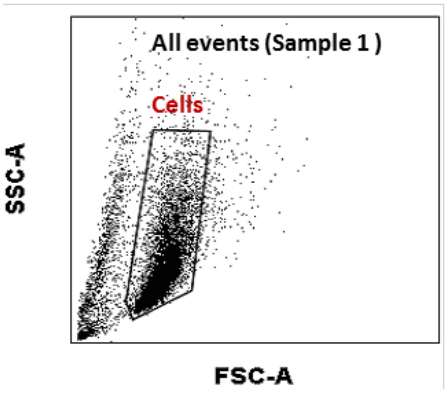

Figure 2. Instructions for setting the gate for the SenzaCell population.

Analysis of Phenotypic Quality Control markers

Show the "Cells" population in a PE/FITC scatter plot. Set quadrants for PE and FITC positive and negative cells using Sample 1 (isotype controls) as Figure 3A. Apply the quadrant from the isotype control sample 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.

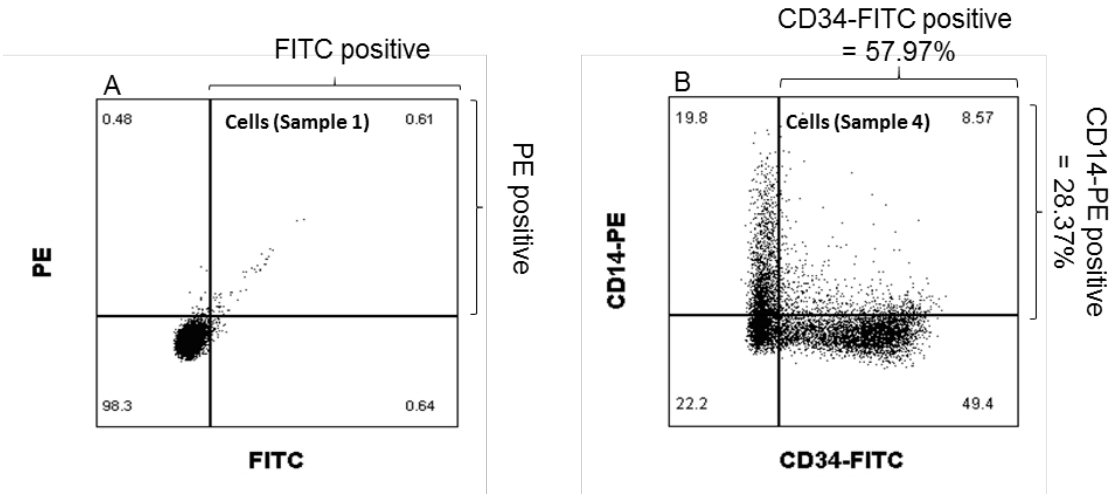

Figure 3. Instructions for setting the quadrants for PE and FITC positive cells (A). Apply the preset gate and quadrants to record the fraction of positive cells for each phenotypic marker (B).

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.

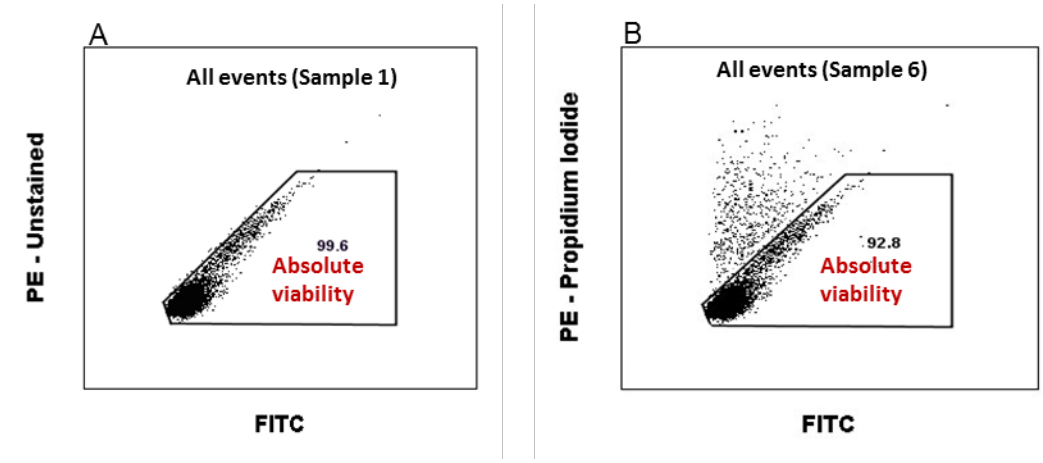

Figure 4. Instructions for setting the gate for "Absolute viability" (A). Apply the gate "Absolute viability" on Sample 6 (B).

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 should not be used for the purpose of cell stimulation at that timepoint.

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 PI 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 are known to be heterogenous, and variations within the accepted ranges from given examples are expected.

Cell seeding for test substance stimulation

Cells are seeded for stimulation directly following a cell split, i.e. 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

Handling of the test substance

A chemical that is to be tested for sensitization in the GARD assay is referred to as a "test substance". The test substance should be stored according to instructions from the supplier, in order to ensure its stability. Weighing of the test substance can be performed prior to the day of cell stimulation if stored correctly and the stability of the substance can be ensured. Dissolved test substance should be prepared fresh on the day of cell stimulation. Test substances should be dissolved in a compatible solvent as appropriate stocks of target in-well concentration, in this document referred to as a Stock A concentration, depending of maximum possible solvent in-well concentration.

To prepare a *solid* test substance, calculate the weight (see Note below about minimum weight of the scale) needed for an appropriate volume 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.

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

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

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

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:

- 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.

Solubility Testing

Solubility of test substances should be ensured by a visual inspection of the solution. Extensive vortex and heat (37°C±5°C) can be applied to achieve complete dissolution.

Consider the physiochemical properties of the test substance and use the GARDskin and GARDpotency compatible solvents and its maximum in-well concentrations listed in Table 7.

1. Find a solvent that solubilize the test substance to the maximum *GARD Input Finder* dilution series in-well concentration of 500 µM.

2. If the test substance is not soluble to the maximum in-well concentration of 500 µM, use the solvent and solvent in-well concentration that generates the highest test substance in-well concentration, minimum 1 µM in-well concentration is recommended.

Table 7. The GARDskin and GARDpotency compatible solvents and the highest in-well concentrations.

Solvent*	Max in-well concentration (%)
Acetone	0.1
DMF	0.1
DMSO	0.5
EtOH	0.1
Glycerol	1
Isopropanol	0.25
DMF:Glycerol 4:1 (v/v %)	0.25
Water	0.1
Complete medium	100

*All the solvents should be of high grade (purity ≥99.5%).

Serial dilution

- Perform a serial dilution in the chosen solvent to get a range of Stock A concentrations (see example in Figure 5). Vortex well between each dilution step. Extensive vortex and heat (37°C±5°C) can be applied.
- From Stock A, prepare a range of Stock B concentrations by adding appropriate volume of Stock A to semi-complete medium. Extensive vortex and heat (37°C±5°C) can be applied. 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 chosen solvent (neg ctrl) in semi-complete medium to achieve the corresponding in-well concentration of the solvent.

1-step dilution method

If a test substance has a maximum solubility in Stock B which is lower than expected with respect to the maximum solubility in Stock A (i.e. displays solubility issues in Stock B), and **is found to be non-toxic**, i.e. the relative viability is above 95.4 %, the operator should try to increase the in-well concentration by bypassing the Stock B step as follows:

1. Prepare Stock A at the maximum possible concentration and prepare a serial dilution.
2. Add appropriate volume of Stock A dilution directly to complete medium.
3. Note the highest possible test substance concentration in complete medium that does not have solubility issues i.e. completely solved.
4. If the highest possible concentration is higher than that in Stock B, prepare cells as described in 'cell seeding' in the sections below.

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 dilution range can be scaled down.
- A test substance with solubility issues should be used from the highest possible concentration, down to 1 µM in-well concentration of the dilution range.

GARD Input Finder

The GARD Input concentration should be established for the test substance in a *GARD Input Finder* stimulation experiment. For an efficient workflow, multiple test substances can be assessed in each stimulation experiment. The controls (unstim ctrl and neg ctrl) are included in each *GARD Input Finder* stimulation experiment.

To determine the GARD Input concentration for a test substance, cell stimulations are performed using in-well concentrations ranging from 500 µM, or highest soluble concentration, to 1 µM (see example in Figure 5). Test substances should be dissolved in appropriate solvent in 1.5 ml micro tubes. For details about weighing and calculation, see section *Handling of the test substance*.

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315x*g* for 5 min at 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 2.22 x 10^5 cells /ml (final cell concentration in wells after addition of test substance will be 2 x 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 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 and number of plates, include two wells of unstimulated cells (unstim ctrl) and one well with the solvent (negative control) (Figure 5).

- **Test substance**: add 200 µl of Stock B to the 1.8 ml cell suspension seeded in 24-well plates for the dilution range of each test substance. Mix well by carefully pipetting up and down. Final cell concentration in wells is 2 x 10^5 cells /ml.
- If the 1-step dilution method is used:
  - Add 2 µl of each Stock A dilution that did not have solubility issues in complete medium to wells containing 1.8 ml cells.
  - Add 198 µl semi-complete medium to the same wells.
- **Negative control**: add 200 µl of relevant solvent Stock B to the 1.8 ml cell suspension, ending up with the appropriate in-well concentration.
- **Unstimulated control**: add 200 µl 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°C±1°C and 5%±0.5% CO_2.

Example of preparation of a serial dilution and a *GARD Input Finder* stimulation for one test substance is shown in Figure 5.

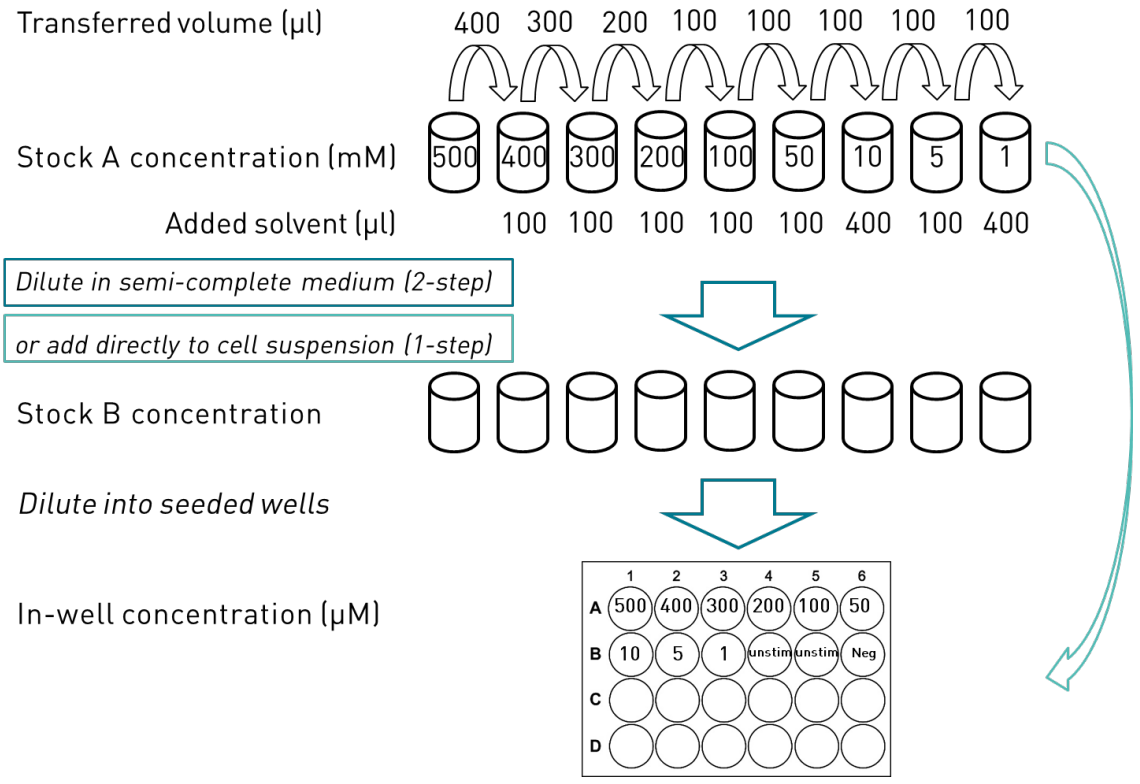

Figure 5. Example of a schematic description of preparation of dilution series of one test substance for GARD Input Finder.

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

- Duplicate flow cytometry samples of each test substance stimulation for the dilution range
- Duplicate flow cytometry samples of negative control
- 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.
- Remove the supernatant by aspiration, resuspend in 1 ml wash buffer and centrifuge.
- 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. 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 is used to keep track of the placement of the cell population in the FSC/SSC scatter plot, see Annex 1 *Cell population* for a common pitfall. A low "Cells" population can give a false percentage "Absolute viability".

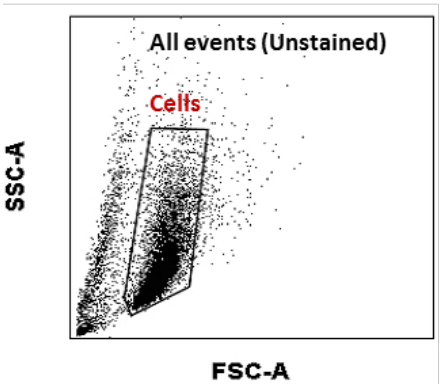

Figure 6. Instructions for setting the gate for the SenzaCells.

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.

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

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 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 as following:

1. A test substance that induces 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 GARD Input concentration.

2. 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 different concentrations. Interpolation between data points is not recommended, as linearity cannot be assumed.
3. A test substance that is not cytotoxic (Relative viability ≥95.5%) is used for *GARD Main Stimulation* at 500 µM or highest soluble concentration.
4. A test substance that has solubility issues in Stock A or Stock B and is not cytotoxic should be re-evaluated to control if any other solubility method (e.g. 1-step dilution) or solvent can be used to increase the in-well concentration (maximum 500 µM).

GARD Main Stimulation

Once the GARD Input concentration for the test substance is established, *GARD Main Stimulation* should be repeated three times with individual preparations of the test substance and controls, and individual cell cultures to achieve three biological replicate samples (see Table 4 for details of the controls). The three *GARD Main Stimulations* can either be run in parallel or sequentially. If several test substances are to be analyzed in the same GARD experiment, the same controls can be used.
In Figure 8, a schematic example of a stimulation experiment with 8 TS and 3 controls are visualized, including one extra well with unstimulated controls.

Preparation of test substance and controls

- Prepare appropriate volume of Stock A of each substance (i.e. test substance and positive control, see Table 4) in the appropriate solvent as established in section *Solubility Testing* and according to the equations in the section *Handling of test substance*. Vortex well. Carefully apply heat (37°C±5°C) if necessary, to achieve complete dissolution.
- Prepare the Stock B concentration by adding appropriate volume of Stock A to semi-complete cell medium (depending on in-well concentration of solvent). Vortex well. Carefully apply heat (37°C±5°C) if necessary, to achieve complete dissolution.
- If the 1-step dilution is used, prepare appropriate volume of Stock A of the test substance in the appropriate solvent as established in section *Solubility Testing* and according to the equations in the section *Handling of the test substance*. Vortex well. Carefully apply heat (37°C±5°C) if necessary, to achieve complete dissolution.
- In addition, prepare the negative control to achieve appropriate in-well concentration.

Note:
- Take into consideration the solubility of the test substance and the strategy to achieve the GARD Input concentration (section *Solubility Testing* above).

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315x*g* for 5 min in 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 2.22 x 10^5 cells /ml (final cell concentration in wells after addition of substance will be 2 x 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 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 controls (see Figure 8).

Cell stimulations

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

- **Test substance**: 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 x 10^5 cells /ml.
- If the 1-step dilution method is used:
  - Add 4 µl of each Stock A to wells containing 3.6 ml cells.
  - Add 396 µl semi-complete medium to the same wells.
- **Positive control**: add 400 µl of Stock B to the 3.6 ml cell suspension, ending up with the appropriate in-well concentration.
- **Negative control**: add 400 µl of Stock B to the 3.6 ml cell suspension, ending up with the appropriate in-well concentration.
- **Unstimulated control**: 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°C±1°C and 5%±0.5% CO_2.

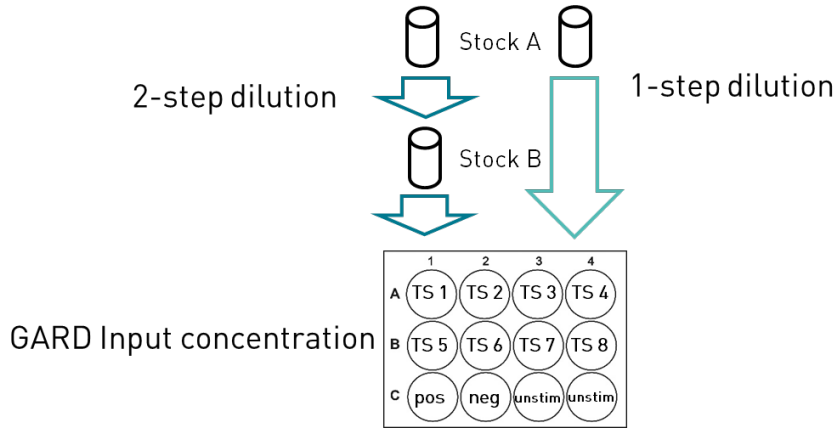

Figure 8. Example of preparation of a GARD Main Stimulation.

After 24 h±0.5 h incubation, harvest and prepare for each substance stimulation (i.e. test substance and 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°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°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°C.
- Harvest all samples from all plates before continuing.
- Centrifuge TRIzol samples at 300-315x*g* for 5 min at 2-8°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 500 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 -18°C or below (stable for one month) or -80°C±10°C (stable for one year).
- Wash the flow cytometry samples by adding ~1 ml Wash buffer and centrifuge at 300-315x*g*, 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 in 50 µl Wash buffer.
- Incubate in dark at 2-8°C for ~10 min.
- Wash the cells by adding approximately 1 ml Wash buffer and centrifuge at 300-315x*g*, 5 min, 2-8°C.
- Remove the supernatant by aspiration and 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 the test substance and controls show a relative viability within the Quality Control criteria described below.

Relative viability Quality Control criteria

- The unstimulated control should have an Absolute viability of ≥84.5%.
- The negative control should have a Relative viability of ≥95.5%.
- The positive control should have a Relative viability of ≥84.5%-95.4%.
- Test substances that are expected to induce cytotoxicity should have a Relative viability between 84.5%-95.4%.
- Test substances that are assayed at 500 µM, or at the highest soluble concentration, should have a Relative viability of ≥84.5%.

If a test substance or control does not pass the Quality Control, see below 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 the test substance and controls, discarding the samples from the failed stimulation.
- If the Quality Control criteria is not reached for **the positive control** (Relative viability 84.5%-95.4%) a new *GARD Main Stimulation* should be performed with the test substance and controls, discarding the samples from the failed stimulation.
- If the Quality Control criteria is not reached for **the negative control** (Relative viability ≥95.5%), a new *GARD Main Stimulation* should be performed with the test substance and controls, discarding the samples from the failed stimulation.
- If the Quality Control criteria for the Relative viability is not reached for a **test substance,** discard its generated samples from the failed stimulation and include the test substance in a new *GARD Main Stimulation*. This is to achieve a total of three biological replicates for each test substance for further analysis.
- If the Quality Control criteria for the Relative viability is not reached for a **test substance,** because of an **incorrect GARD Input concentration**, the test substance is re-tested to find the correct GARD Input concentration.

Part result:
For *each* test substance and control, 3 TRIzol replicate samples with passed Relative viability Quality Control are generated from each of the three *GARD Main Stimulations*. Only one TRIzol sample from each of the three *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 used for RNA isolation should be assigned a unique Sample ID, i.e. three Sample ID's for the three TRIzol samples from each test substance and control in order to keep track of the RNA samples isolated and QC assessed. Avoid extensive use of special characters for the Sample ID. 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.

Total RNA, including mRNA, is isolated from the TRIzol samples using a commercially available kit and reagents. Direct-zol RNA MiniPrep, Zymo Research, specified in Table 3, 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 (10,000-16,000x*g*) to avoid RNA Wash Buffer residues in the eluate.
- Elute RNA by adding 25 µl DNase/RNase free water directly to the column matrix and centrifuge at 16,000x*g* 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±10°C.

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

RNA quantification and quality control

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

RNA Quality Control criteria

- The RNA concentration of a qualified sample should be ⩾20 ng/µl.
- 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.

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

ENDPOINT MEASUREMENT

GARDskin and GARDpotency

The endpoint measurement of GARDskin is the mRNA quantification of the GARDskin prediction signature and GARDpotency is the mRNA quantification of the GARDpotency prediction signature, using the NanoString nCounter system. The custom made CodeSets (i.e. sets of oligonucleotide probes representing the genes of each corresponding prediction signature) have been developed by SenzaGen and NanoString. To place an order for a batch of the CodeSets, please contact SenzaGen AB. If a study is to be analyzed using more than one batch of the same endpoint specific CodeSet, the controls should be analyzed with those CodeSet batches. 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.

Cartridge Definition File

A Cartridge Definition File (CDF) needs to be created by the NanoString facility prior to any NanoString run. It is used to map the reads from each lane in the cartridge to sample specific attributes.

Note:

- The unique Sample ID chosen and specified in the CDF 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 x*g* for more than 30 seconds. Do not "pulse" with the microcentrifuge 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 ProbeSet 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. Use RNase-free 0.2 ml tubes. Label each tube with its Sample ID. Mix by flicking and inverting 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 inverting 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 its 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 inverting and spin down with a minicentrifuge.
- Add 2 µl of the Capture ProbeSet to each hybridization tube. Close the tubes with plastic lids and carefully mix by flicking and inverting and spin down with a minicentrifuge.
- Place the hybridization tubes in the thermocycler and incubate for 24 h (±0.5 h).

Note:

- Program the thermocycler using 15 µl volume, at temperatures stated above. Do not set the thermocycler 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 2000x*g* 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 the plastic lid and close the metal lid.
7. Initiate the run by following on-screen instructions. If reagents or plastics are not inserted correctly the Prep Station will inform on the screen. Check before leaving the station.
8. Once the Prep Station protocol is finished, after approximately 3 h, carefully remove the cartridge and place it on a lab-tissue, seal the cartridge with provided tape.
9. Discard used material in the Prep Station.
10. Proceed to *Analysis with Digital Analyzer*.

Analysis with Digital Analyzer

1. Place 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 quantifying probes, the analysis takes 5 hours and the instrument can be left 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, 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. The cartridge can be rescanned within two weeks.

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 control probes included in the CodeSet.

For NanoString facilities not using the GARD Data Analysis Application (GDAA), each acquired RCC-file should be quality controlled. The quality control is performed to assure that the NanoString analysis has been successful. Samples that fail any of the below described critical quality criteria should not be used for further analysis in the GARD™skin data analysis pipeline. The critical quality metrics are imaging quality, linearity of the spike-in RNA control probes, limit of detection (LOD), and binding density. The imaging quality is calculated as the ratio between the number Fields of Views (FOV) (predefined in the CDF file as 555) and the number of successfully counted FOVs. A ratio above 0.75 (>0.75) is required for a sample to pass the imaging quality control. The linearity of the positive spike-in controls is calculated using the positive control probes (POS_A-E) and their known RNA concentrations. The acquired counts for the positive control probes and their respective concentrations should be logarithmized (log2) before calculating the R^2 value of a linear fit to the data points. An R^2 value above 0.95 (R^2>0.95) is required for a sample to pass the linearity quality control. The LOD quality control uses all the negative controls (NEG_A-H) and the positive control E (POS_E). The LOD is defined as the mean counts of the negative control probes plus 2 standard deviations of the counts, see Equation 4.

$$LOD = \mu + 2 * \sigma \qquad \text{(Equation 4)}$$

Where μ and σ are the mean value and the standard deviation of the negative control probes' counts respectively. For a sample to pass the LOD quality control, the positive control probe POS_E must be above the estimated LOD (POS_E > LOD). The binding density is a measure of the number of probes observed per cartridge surface area during the gene expression acquisition in the Digital Analyzer. For a sample to pass the binding density quality control, the binding density must be above 0.05 and below 2.25 (0.05< binding density <2.25). For summary of the critical quality control parameters, see Table 8.

Table 8. Summary of the critical RCC-file quality control parameters

Quality Metric	Critical parameter
Imaging Quality	>0.75
Linearity	>0.95
Limit of Detection	POS_E > LOD
Binding Density	0.05< *AND* <2.25

In addition to the above described critical quality control parameters, it is also recommended to count the number of endogenous probes with 0 observed gene counts. If any samples contain multiple endogenous genes with 0 observed counts, a plausible explanation could be that the NanoString cartridge was analyzed with the wrong version of the RLF file. If this is the case, the affected cartridge should be rescanned (within two weeks) with the correct version of the RLF file to maintain sample integrity, and the previously generated RCC files should be discarded. If no apparent cause could be identified for the presence of multiple endogenous genes with 0 observed gene counts, no additional action needs to be taken.

DATA ANALYSIS

Follow instructions in appropriate version of the document *User Manual GDAA* and analyze the samples with the appropriate version of the GDAA for data analysis of GARDskin and GARDpotency. Sample IDs and Substance IDs are needed for preparing the Annotation file for data analysis as described in the GDAA user manual, as well as the GARD Input concentration in µM (only for GARDpotency).

Important: The files for the test substance should be analyzed together with relevant control files i.e. only control samples (unstim, neg and pos) that are stimulated in the same *GARD Main Stimulations* as the test substance.

Part result:
Following complete analysis, each RCC file (except unstim ctrl) analyzed with the GARDskin prediction model by GDAA will receive a DV.
Following complete analysis, each RCC file (except unstim ctrl) analyzed with the GARDpotency prediction model by GDAA will receive a DV.

ACCEPTANCE CRITERIA

For the final GARD™skin prediction to be valid for a test substance, the following acceptance criteria must be met by the test substance:

1. Solubility
    a. It should have been soluble in a solvent presented in Table 7 resulting in an in-well concentration between 1-500 µM.
2. *GARD Input Finder*
    a. It should have been included in a *GARD Input Finder* that was performed with SenzaCells that passed the Phenotypic Quality Control.
3. *GARD Main Stimulation*
    a. It should have been included in three *GARD Main Stimulations* that were performed with SenzaCells that passed the Phenotypic Quality Control, where each *GARD Main Stimulation* generated one replicate.
    b. The generated replicates should have passed **Relative** viability Quality Control.
4. RNA and NanoString Quality Control
    a. At least two replicates should have passed the RNA Quality Control.
    b. At least two replicates should have passed the NanoString Quality Control
5. **GARDskin** prediction
    a. Each replicate must have been derived from a *GARD Main Stimulation* that **also** generated:
        i. An unstimulated control that passed **Absolute** viability-, RNA- and NanoString Quality Control.
        ii. A positive control that passed **Relative** viability-, RNA- and NanoString Quality Control.
        iii. A negative control that passed **Relative** viability-, RNA- and NanoString Quality Control.
    b. The positive and negative control should be accurately classified as Sensitizer and Non-sensitizer, respectively.
6. **GARDpotency** prediction
    a. Each replicate must have been derived from a *GARD Main Stimulation* that **also** generated:
        i. An unstimulated control that passed **Absolute** viability-, RNA- and NanoString Quality Control.
        ii. A positive (1A) control that passed **Relative** viability-, RNA- and NanoString Quality Control.
    b. The positive (1A) control should be accurately classified as 1A.

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

PREDICTION MODEL

GARDskin

For GARDskin 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**".

GARDpotency

For GARDpotency predictions, the prediction model is defined as:

"If the median DV of biological replicate samples is ⩾ 0, the substance is classified as a **1A**. If the median DV of biological replicate samples is < 0, the substance is classified as a **1B**".

RECORDING OF DATA

Recommended documentation regarding test substance information and essential results are listed below:

Test substance

- Substance ID, i.e. name of the test substance or a simplified code of your choice (avoid special characters) used throughout the study to identify the substance. For the controls use "pos ctrl", "neg ctrl" and "unstim ctrl"
- Molecular weight, density and purity used in the calculations
- Selected solvent and solvent in-well concentration
- Max screened in-well concentration
- GARD Input Concentration (µM)
- Relative viability at 90% (Yes/No)

Test method conditions

- Cell passage number at cell stimulation
- Phenotypic Quality Control passed (Yes/No)

GARD Input Finder

- Determined GARD Input concentration (µM)
- Relative (or Absolute for unstim ctrl) viability at GARD Input concentration
- Relevant comments, e.g. if Stock B was bypassed with a 1-step dilution

GARD Main Stimulations

- In-well test substance concentration (µM)
- Relative (or Absolute for unstimulated control) viability (%)

RNA Isolation and RNA QC

For each RNA Sample:

- RNA concentration (ng/µl)
- RNA Integrity Number (RIN)

Endpoint measurement

For each RNA sample analyzed with NanoString:

- NanoString Quality Control passed (Yes/No)

Data analysis

- Save the rendered report files of the predictions

Results

- Number of replicates meeting acceptance criteria with respect to Relative viability, RNA QC and NanoString QC
- GARDskin prediction results
- GARDpotency prediction results

E. ANNEXES

Annex 1. Cell population
Annex 2. SenzaCell Phenotype

Annex 1. Cell population

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

A

B

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

Annex 2. SenzaCell Phenotype

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

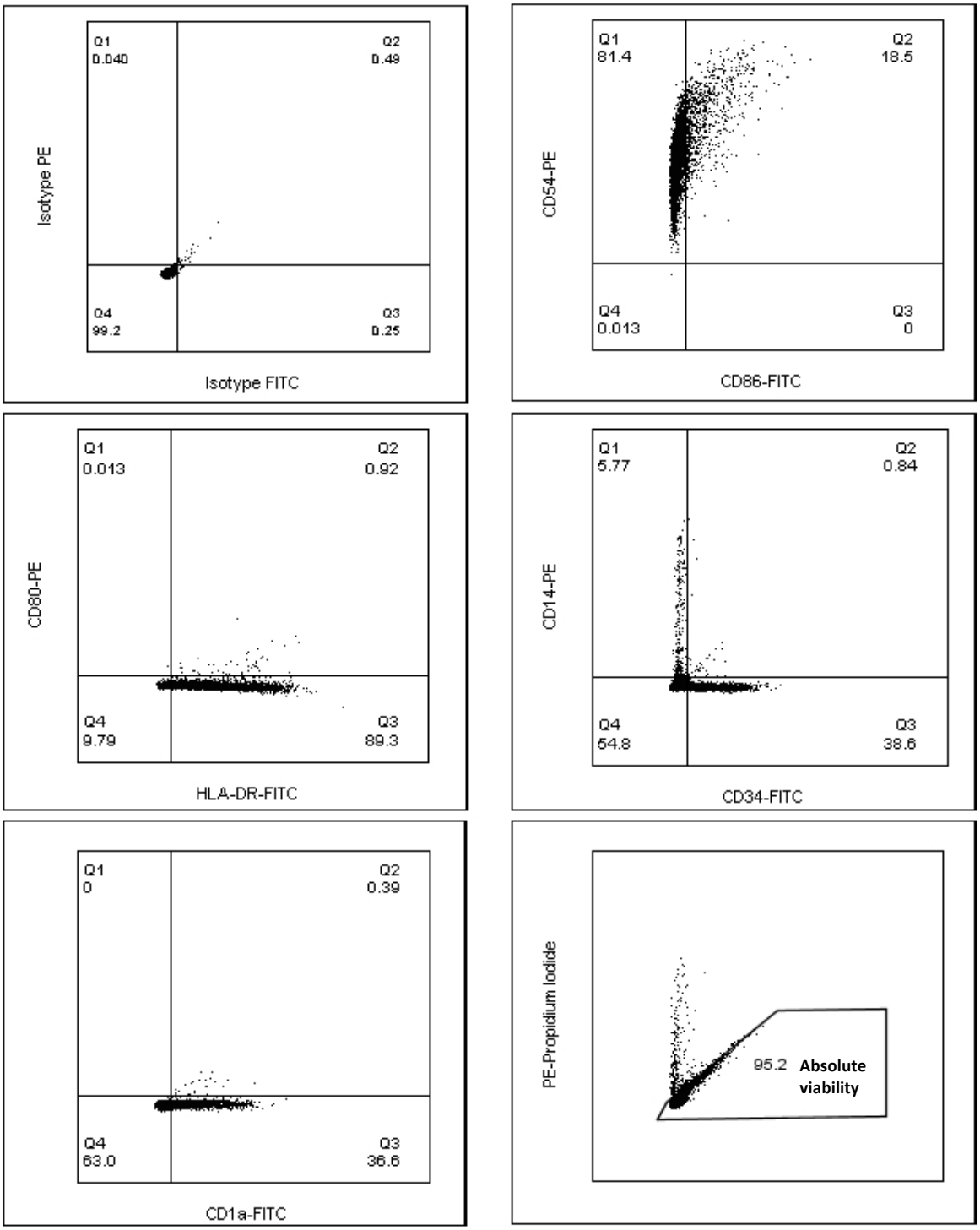

Figure 2. PE/FITC scatter plots of mAb stained SenzaCells for phenotypic control.

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Appendix II: GARD™air protocol version 1.2 from SenzaGen.

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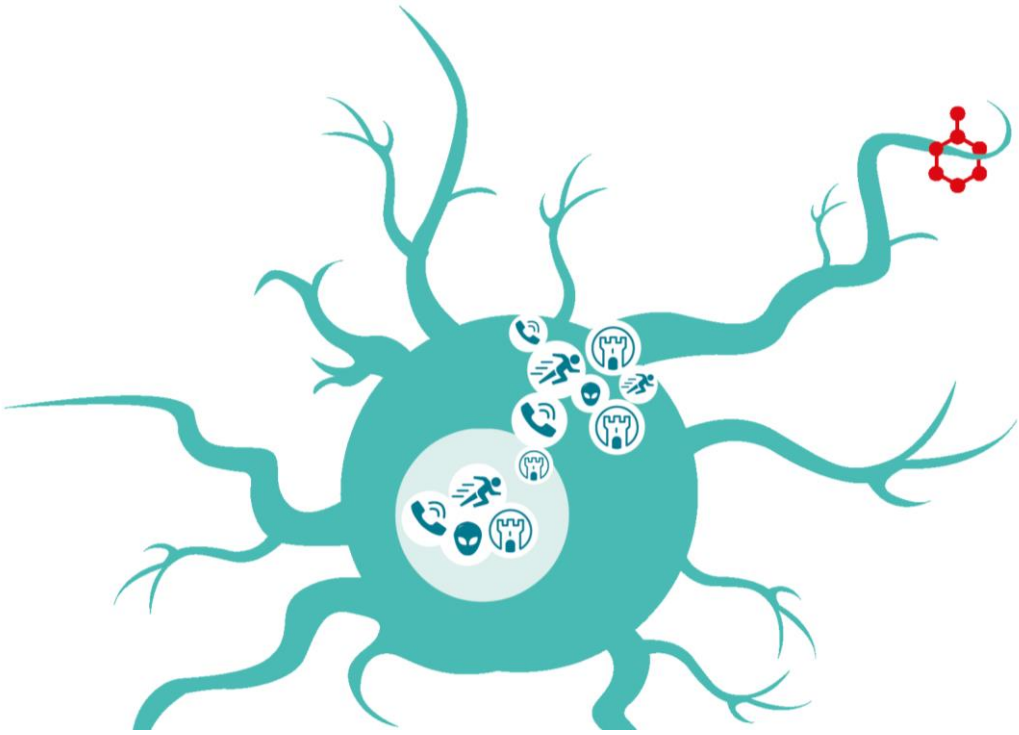
GARD®air

Genomic Allergen Rapid Detection air



INDEX

1 PROTOCOL INTRODUCTION

1.1 PROTOCOL NAME

GARDair v.1.2
Date: 2023-APR-12

1.2 CONTACT

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1.3 ABSTRACT

Genomic Allergen Rapid Detection (GARD) is an *in vitro* assay designed to predict the ability of chemical substances to induce sensitization, e.g. skin and respiratory sensitization. GARD is based on the analysis of the relative expression levels of specific biomarker signatures following the chemical stimulation of a human myeloid leukemia cell line (SenzaCell™), acting as an *in vitro* model of human dendritic cells. The readout of the assay is a transcriptional quantification of the genomic predictors using Nanostring nCounter technology. Chemicals 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. The endpoint value of each GARD measurement is a derived decision value (DV) from the SVM model.

The SenzaCell cells are available under a license agreement upon request. The IP rights of the GARD biomarker signatures and any assay utilizing the signatures, in its entirety or parts thereof, are owned by SenzaGen AB.

GARDAIR

GARDair is an application of the GARD assay, and is designed to predict the ability of chemical substances to induce respiratory sensitization. GARDair is based on the analysis of the relative expression levels of a GARDair specific respiratory biomarker signature and an SVM model that is trained using data from cell stimulations with chemicals having known respiratory sensitizing properties.

This method is based on the OECD Test Guideline No. 442E, Annex 4, with the following exceptions: four main stimulations are performed, and another gene expression profile is analyzed.

1.4 ABBREVIATIONS

BSA – Bovine Serum Albumin
CASI – Campaign and Sample Information
csv file – comma-separated values file
DMSO – Dimethyl Sulfoxide
DV – Decision Value
FBS – Fetal Bovine Serum
GARD – Genomic Allergen Rapid Detection
GDAA – GARD Data Analysis Application
GM-CSF – Granulozyte Macrophage Colony Stimulating Factor
mAbs – Monoclonal antibodies
PBS – Phosphate Buffered Saline
RCC – Reporter Code Count
RLF – Reporter Library File
Rv90 – Concentration inducing 90% Relative viability
SVM – Support Vector Machine
TS – Test substance

1.5 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(s) which are sensitizing, acute toxic and environmental hazardous. When supplied, always read the SDS of each chemical substance and follow the precautions stated for each substance. In case of an unknown test substance consider it as a sensitizer and a 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

Positive control and negative control chemicals, see CAS number and Catalog number in Table 3, read SDS at www.sigmaaldrich.com.
TRIzol™ reagent (Catalog number 15596026), read SDS at www.thermofisher.com
Propidium Iodide (Catalog number 556463), read SDS at www.bdbioscience.com

2 GARDAIR ASSAY

2.1 PROCEDURE

Figure 1 describes the workflow of the GARDair 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: first in the *GARD Input Finder* to identify a relevant stimulation concentration and secondly in the *GARD Main Stimulation* to harvest RNA.

In the *GARD Input Finder*, every test substance is screened for cytotoxic effects, and this is done to identify an appropriate concentration (i.e. one that yields ~90% Relative viability) to be used as the Input concentration in the *GARD Main Stimulations*. 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 in the *GARD Main Stimulations* with the concentration identified in the *GARD Input Finder*. This step is repeated four times to achieve four biological replicate samples. Thus, every GARD assessment of a test substance is based on four replicate *GARD Main Stimulations*. Depending on the number of test substances to be assayed, the four *GARD Main Stimulations* can either be run in parallel or sequentially.

The endpoint measurements of GARD, i.e. the quantification of the GARD biomarker signature mRNA transcripts, is performed on total RNA purified from cells from the *GARD Main Stimulation*. The quantification is performed using the Nanostring nCounter instrument and the result is analyzed with the *GARDair Data Analysis Application* (GDAAair). Each test substance is binary predicted as a Respiratory Sensitizer or a Respiratory Non-sensitizer.

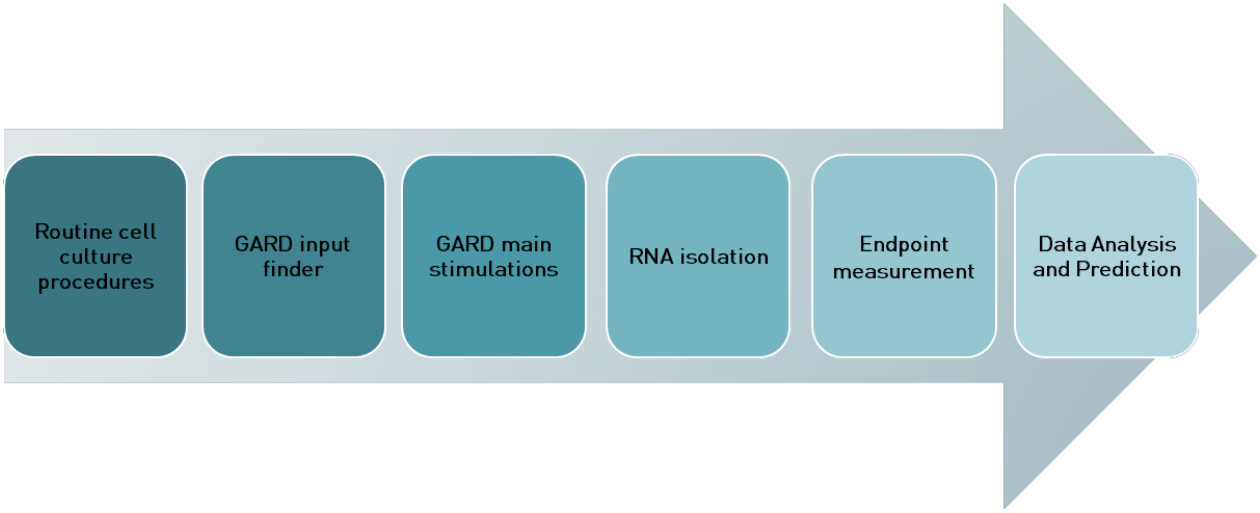

Figure 1. Overview of the GARD assay workflow.

2.2 MATERIAL

The Test System used in the GARDair assay is the human myeloid leukemia cell line SenzaCell provided by SenzaGen AB and sent to licensed CRO on dry ice. The provided vial should be stored in liquid nitrogen. The vial should be expanded and frozen in liquid nitrogen as a homogenous cell bank according to instructions from the developing laboratory.

For equipment, consumables, and reagents, see Table 1-3.

Table 1. 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, e.g 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 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, 175 cm^2	Corning	431080
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**
Cell Medium		
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
Buffers & Solvents		
D-PBS, HyClone	GE Healthcare	SH30028.02
BSA, Cohn fraction V	-	-
TRIzol	Ambion	15596018
Ethanol, 95-100%, Undenatured	Solveco	1015
Water, HyClone Molecuar Biology Grade Water	GE LifeSciences	SH30538.02
*Antibodies*** & Staining*		
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
Reagents & Kits		
Direct-zol RNA MiniPrep	Zymo Research	R2052
RNA 6000 Nano Kit	Agilent	5067-1511
nCounter master kit	Nanostring	NAA-AKIT-###
Biomarker specific codeset	Nanostring	(Contact SenzaGen)
Chemicals	*CAS no*	*Catalog no****
Reactive Black 5 (pos ctrl)	17095-24-8	306452
DMSO 100%	67-68-5	D5879
Acetone (≥99.5%)	67-64-1	179124
Dimethylformamide (DMF) (ACS reagent)	68-12-2	319937
Ethanol (Pharma grade, ≥99.7%)	64-17-5	1065 (Solveco)
Glycerol (for molecular biology, >99%)	56-81-5	G5516
Isopropanol (electronic grade)	67-63-0	733458
Molecular Biology Grade water	Corning	46-000-CM

*Listed are 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 instructions from the developing laboratory.

***Each lot of mAbs needs to be titrated to determine antibody concentration giving saturation according to instructions from the developing laboratory.

****Catalog numbers at Sigma-Aldrich/Merck or Solveco (Ethanol) for guidance of e.g. purity of each chemical.

2.3 METHOD PREPARATIONS

MEDIUM AND ENDPOINT ASSAY SOLUTIONS

Serum and antibodies
Prior to commencing a study, FBS needs to be assessed and antibodies need to be titrated. For specific instructions, contact the developing laboratory.

Cell medium
The cell medium for the SenzaCells is prepared by supplementing 500 ml MEM/Alpha with 125 ml FBS (final FBS concentration is 20%). FBS-supplemented media is referred to as *semicomplete 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 are 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.

Controls
For each GARDair assay, relevant controls are analysed in each of the four replicate *GARD Main Stimulations*. The controls are listed in Table 3 and 4, with relevant information for the laboratory work. The GARD Input concentration of the positive control, Reactive black 5, can be determined in the GARD Input Finder experiment. The controls have following purpose:

Unstimulated control
Samples of unstimulated cells are used for normalization of the dataset. By removing batch-effects the dataset is aligned to the prediction models training-set.

Negative control
The purpose of the negative control, which is defined as the test substance solvent, in the GARDair assay is to show that the cells have not become activated in any steps of the GARD assay's experimental procedures and should be accurately classified as Respiratory Non-sensitizer.

Positive control
The purpose of the positive control (Reactive Black 5) sample is to demonstrate that the SenzaCells used during an experiment are responsive and can become activated when exposed to a respiratory sensitizer.

Table 4. List of Reference Items

Substance ID	Reference Items	S/NS*	Solvent	Maximum GARD Input concentration	Relative viability
pos ctrl	Reactive black 5	S	water	To be determined	≥84.5%
neg ctrl	Acetone	NS	-	0.1% (v/v)	≥95.5%
neg ctrl	DMF	NS	-	0.1% (v/v)	≥95.5%
neg ctrl	DMSO	NS	-	0.1% (v/v)	≥95.5%
neg ctrl	Ethanol	NS	-	0.1% (v/v)	≥95.5%
neg ctrl	Glycerol	NS	-	0.1% (v/v)	≥95.5%
neg ctrl	Isopropanol	NS	-	0.25% (v/v)	≥95.5%
neg ctrl	Water	NS	-	0.1% (v/v)	≥95.5%
unstim ctrl	Unstimulated cells	NS	-	-	≥84.5%**

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

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 titrated 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

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 is recommended to be accompanied by a Campaign and Sample information document hereafter referred to as CASI (see Appendix1, LO FRM 17 CASI.xlsx). 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 a 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 10 test substances. Each of the RNA samples are designated a unique **Sample ID** corresponding to a row in the “Sample Information” sheet in the CASI document and will be analyzed 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 (e.g. customer name), optional

2.4 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 300-315x*g*, 5 min, 2-8°C. All incubations are performed at 37°C±1°C and 5%±0.5% CO_2. Cell 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 cm^2. For volumes of 10-45 ml, use TC Flask 75 cm^2. For volumes of 45-100 ml, use TC Flask 162-175 cm^2. Note that for large cultures, more than one TC 162-175 cm^2 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 300-315x*g*, 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 cm^2). Incubate at 37°C±1°C and 5%±0.5% CO_2 (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 300-315x*g*, 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 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 medium is now referred to as complete. Incubate in cell culture flasks at 37°C±1°C and 5%±0.5% CO_2 (i.e. cell passage number P1).

Recording of data:
For a thawed cell batch, record into the CASI document (sheet “Cell maintenance log”): -
- Identity of the cell batch
- Date of thawing
- At each cell passage, record passage number, 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 x 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 300-315x*g*, 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 x 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% CO_2.

Freezing of cells

- To freeze the cells, transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge cells at 300-315x*g*, 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.
- 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 300-315x*g*, 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 made 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 performed to ensure cells are maintained in an inactivated state and to detect phenotypic drift.

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

- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315x*g*, 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 300-315x*g*, 5 min, 2-8°C.
- Remove the supernatant by aspiration and 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.

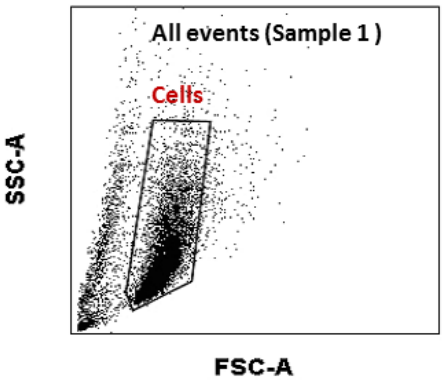

Figure 2. Instructions for setting the gate for the SenzaCell population.

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.

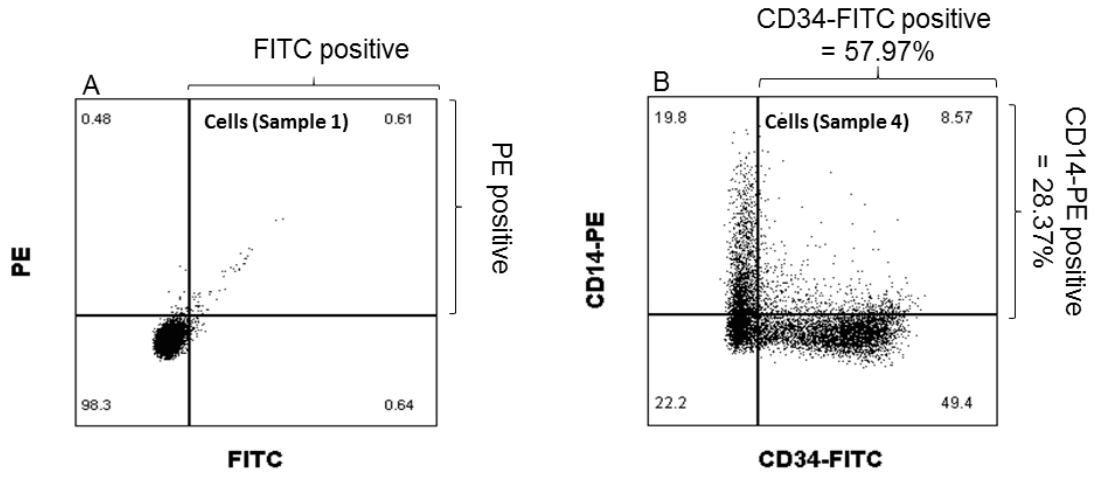

Figure 3. Instructions for setting the quadrants for PE and FITC positive cells (A). Apply the preset gate and quadrants to record the fraction of positive cells for each phenotypic marker (B).

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.

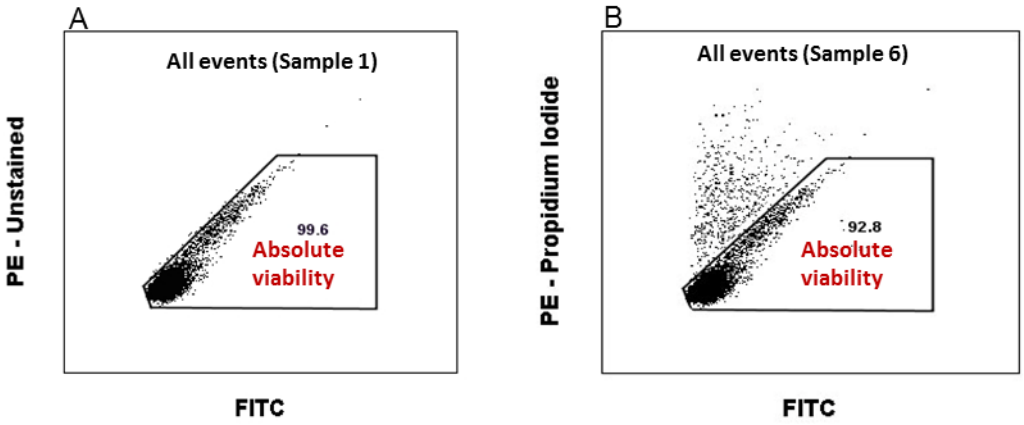

Figure 4. Instructions for setting the gate for “Absolute viability” (A). Apply the gate “Absolute viability” on Sample 6 (B).

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, consult with the developing laboratory. A representative example of a typical SenzaCell phenotype is presented in Appendix2 SenzaCell Phenotype.

Table 6. Acceptance range for expression of phenotypic biomarkers of the SenzaCell cells.

Phenotypic biomarker	Accepted range of positive cells (%)*
CD86	10-40
CD54	>0
HLA-DR	>0
CD80	<10
CD34	>0
CD14	>0
CD1a	>0
Phenotypic biomarker	**Accepted range of negative cells (%)**
Absolute viability (PI negative cells)	≥84.5

*. An entirely positive cell population is not required.

Recording of data:
Record into the CASI document (sheet "GARD Input Finder or GARD Main Stimulation"):
- Identity of the cell batch
- Date of thawing
- Purpose of the Phenotypic QC
- Passage number
- Date of Phenotypic QC
- The positive fraction of cells (%) for each phenotypic marker
- Absolute viability (%) (PI negative fraction)

Accepted Cell passages 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

Handling of Test substances
Chemicals that are to be tested for respiratory 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 their stability. Weighing of the substances can be performed prior to the day of cell stimulation if they are stored correctly and the stability of the substances 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 the highest target in-well concentration, in this document referred to as a Stock A. Solubility of test substances should be ensured by a visual inspection of the solution. Extensive vortex and heat (37°C±5°C) can be applied to achieve complete dissolution, see section *Solubility Test and Solvent Selection* below for assessment of solubility and appropriate 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.
- If a molecular weight range is given, use the upper limit for the calculations.
- If a density range is given for a liquid, use the lower limit for the calculations.
- If the purity of the substance is not available, use best available knowledge to approximate the purity of the test substance, or assume 100% purity.
- 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.

Recording of data:

- Record the molecular weight and density used in the calculations
- Record the calculations

Solubility Test and Solvent Selection

For selection of solvent follow the procedure 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°C±5°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 **water** according to the equations in the section *Handling of Test substances*. Vortex well. Carefully apply heat (37°C±5°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**, other solvents may be used, see Table 4 for compatible solvents and maximum tolerated in-well concentrations.
5. 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.

GARD INPUT FINDER

The GARD Input concentration should be established for each test substance in a *GARD Input Finder* stimulation experiment. For an efficient workflow, it is suggested to establish the GARD Input concentration for up to 10 test substances in each stimulation experiment. The negative and unstim controls are included in each *GARD Input Finder* stimulation experiment.

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 appropriate solvent as e.g. 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*.

Example of Serial dilution

- Perform a serial dilution from 500 mM 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.
- Prepare a stock B OR do a direct dilution of stock A in the cell culture well.
    - *Option 1: Stock B – 2-step dilution*

      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 and carefully apply heat (37°C±5°C) if necessary to achieve complete dissolution. 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 or Water (neg ctrl) in semi-complete medium to achieve the in-well concentration of 0.1% DMSO or 0.1% Water.

- *Option 2: Direct dilution of stock A – 1-step dilution*
  Dilute stock A is used directly in the cell culture well to generate a final concentration of 0.1% of the vehicle. The negative control(s) are added in the same way.

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315x*g* for 5 min at 2-8°C.
- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells.

Option 1: Stock B – 2-step dilution

- Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2.22 x 10^5 cells /ml (final cell concentration in wells after addition of test substance will be 2 x 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 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).

Option 2: Direct dilution of stock A – 1-step dilution

- Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2 x 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 medium is now referred to as complete.
- Use 24-well plates and seed **2 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 for each negative ctrl (solvent used to dissolve the Test Substance) (Figure 5).

Option 1: Stock B – 2-step dilution

- Test substances: add 200 µl 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 x 10^5 cells /ml.
- Negative control: add 200 µl of solvent Stock B to the 1.8 ml cell suspension. The solvent in-well concentration should correspond to the final solvent concentration in the well with Test Substance.
- Unstimulated control: add 200 µl 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°C±1°C and 5%±0.5% CO_2.

Option 2: Direct dilution of stock A – 1-step dilution -example

- Test substances: add e.g 2 µl of Stock A to the 2 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.

- Negative control: add e.g 2 µl of solvent Stock A to the 2 ml cell suspension. The solvent in-well concentration should correspond to the final solvent concentration in the well with Test Substance.
- Unstimulated control: leave as is.
- Incubate for 24 h (±0.5 h) at 37°C±1°C and 5%±0.5% CO_2.

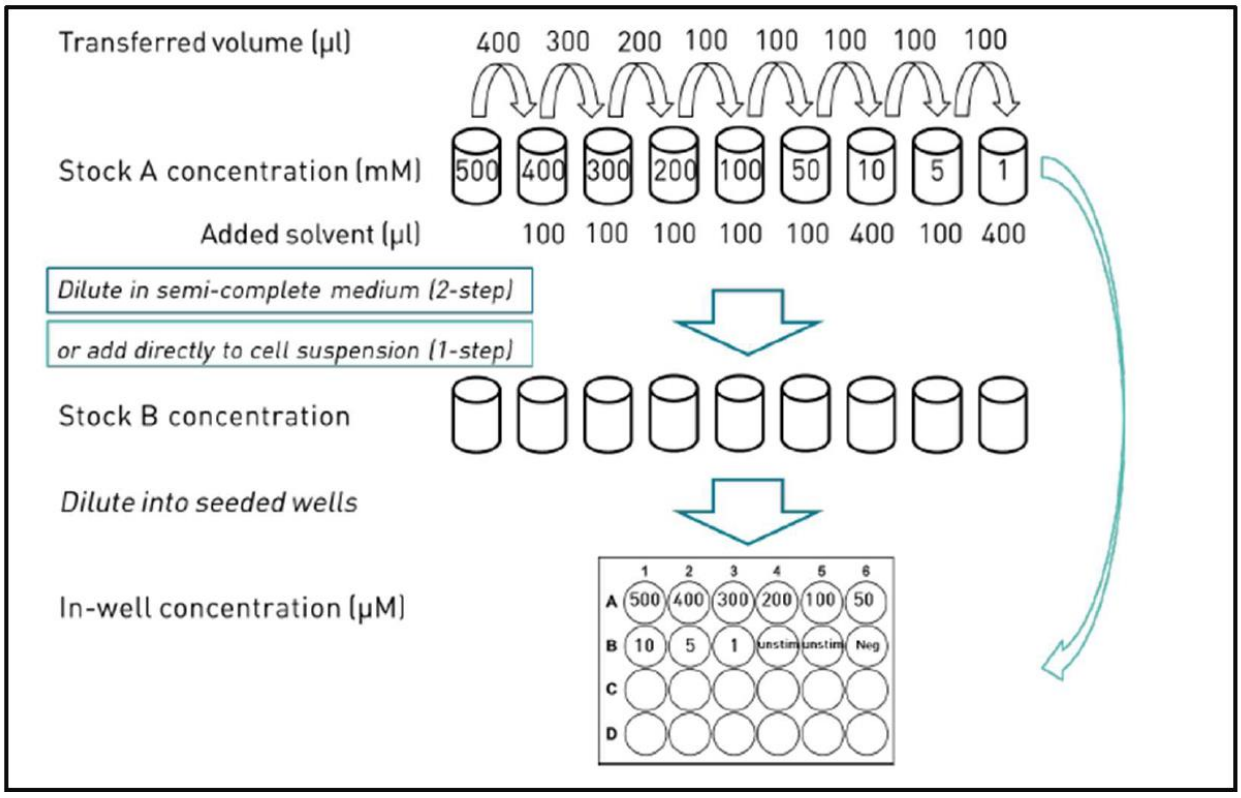

Figure 5. Schematic description of preparation of dilution series of one test substance for *GARD Input Finder*.

Cell harvest for flow cytometry analysis

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

- Duplicate flow cytometry samples of each test substance stimulation for the whole dilution range and for the negative control.
- Four flow cytometry samples of unstimulated cells (unstim ctrl)
- Mix the wells by carefully pipetting up and down and split each well into duplicate samples.
- Wash the cells by adding ~1 ml Wash buffer and centrifuge at 300-315x*g*, 5 min, 2-8°C.
- Remove the supernatant by aspiration and repeat the washing step.
- Prepare a staining solution (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 300-315x*g*, 5 min, 2-8°C.
- Remove the supernatant by aspiration and 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 Appendix3 *Cell population* for a common pitfall. A low “Cells” population can give a false percentage “Absolute viability”.

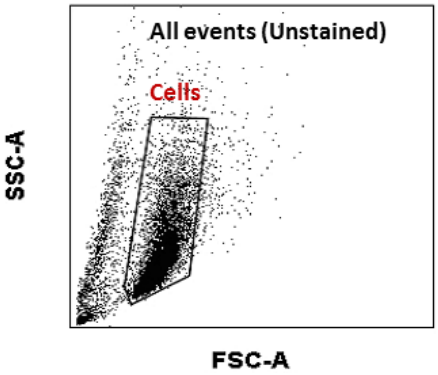

Figure 6. Instructions for setting the gate for the SenzaCells.

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).

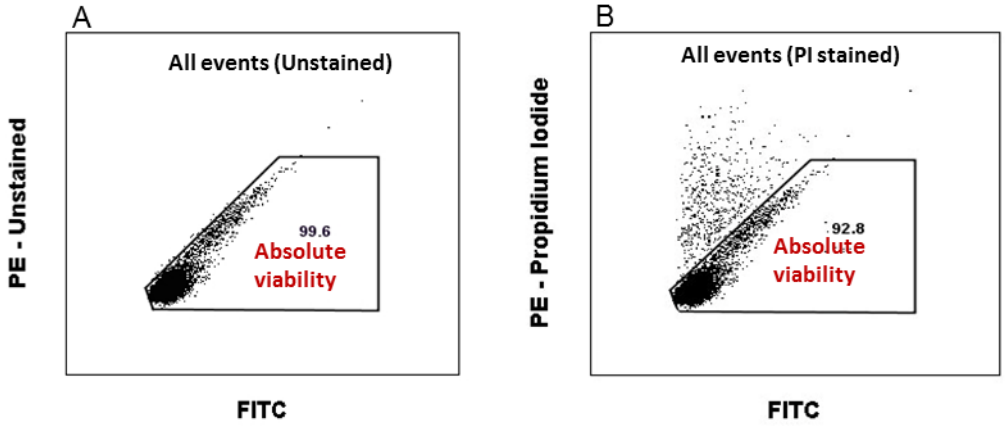

Figure 7. Instructions for setting the gate for “Absolute viability” (A). Apply the gate “Absolute viability” on PI stained samples (B).

Calculation of Relative viability

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.

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

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 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 procedure:

1. The Test substances concentration that induces cytotoxicity (84.5%-95.4%. Relative viability) is used as guidance for the *GARD Main Stimulation.* If multiple concentrations fulfill the acceptance criterion, the concentration that yields the Relative viability **closest to** 90% is chosen as the GARD Input concentration.
2. 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.
3. Test substances that are soluble in Stock B at a concentration that corresponds to the highest concentration achieved in Stock A and are not cytotoxic (Relative viability ≥95.5%) are used for *GARD Main Stimulations* at the highest permissible in-well concentration.

Recording of data:

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

- Date of experiment/experiments
- *GARD Input Finder* number
- Selected solvent
- Maximum solubility of Stock A (mM)
- Maximum screened in-well concentration (µM)
- Determined GARD Input concentration (µM)
- Relative (or Absolute for unstim ctrl) viability at GARD Input concentration
- Relevant comments, e.g. if Stock B was bypassed with a 1-step dilution

GARD MAIN STIMULATION

Once the GARD Input concentration for each test substance has been established, *GARD Main Stimulation* should be repeated four times for all test substances, positive and negative controls, and unstimulated controls to achieve four biological replicate samples (see Table 4 for details of the positive and negative controls). Note: if the selected test substance concentration cannot be confirmed during the main stimulations, another concentration may be appropriate. However, the same concentration should be used in all four Main stimulations.

In Figure 8, a schematic example of a stimulation experiment with 8 test substances and 3 reference items is visualized, including two wells with the unstimulated controls.

Preparation of Test substances and Reference Items

- Prepare Stock A with a c_T of e.g. 1000x the GARD Input concentration of each test substances and the positive control (see Table 4) in the appropriate solvent as established in section *Solubility Test and Solvent Selection* and according to the equations in the section *Handling of Test substances*. Vortex well. Carefully apply heat (37°C±5°C) if necessary to achieve complete dissolution.
- If applicable prepare Stock B of the Test Substance, positive control and negative control(s) as above.

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 (see section *Solubility Test and Solvent selection* above)**.**

Cell seeding

- Transfer cell culture from cell culture flasks to 50 ml tubes. Centrifuge at 300-315x*g* for 5 min in 2-8°C.
- Remove supernatant by decantation. Resuspend the cells in an appropriate volume of semi-complete medium.
- Count the cells.

  Option 1: Stock B – 2-step dilution
  - Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2.22 x 10^5 cells /ml (final cell concentration in wells after addition of test substance will be 2 x 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 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 Reference Items (see Figure 8).

  Option 2: Stock A – 1-step dilution
  - Resuspend cell culture in semi-complete medium to a volume corresponding to a cell concentration of 2 x 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 medium is now referred to as complete.
  - Use 12-well plates and seed **4 ml** of cell suspension into the number of wells needed for test substances and Reference Items (see Figure 8).

Cell stimulations

For each cell stimulation, independently of number of test substances, include two wells of unstimulated cells (unstim ctrl) and one well for each negative ctrl (solvent used to dissolve the Test Substance) and one well with the positive control (Figure 8).

Option 1: Stock B – 2-step dilution

- Test substances: add 400 µl of Stock B to the 3.6 ml cell suspension seeded in 12-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 x 10^5 cells /ml.
- Negative control: add 400 µl of solvent Stock B to the 3.6 ml cell suspension. The solvent in-well concentration should correspond to the final solvent concentration in the well with Test Substance.
- Unstimulated control: 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°C±1°C and 5%±0.5% CO_2.

Option 2: Direct dilution of Stock A – 1-step dilution

- Test substances: add e.g 4 µl of Stock A to the 4 ml cell suspension seeded in 12-well plates for the entire dilution range of each test substance. Mix well by carefully pipetting up and down.
- Negative control: add e.g 4 µl of solvent Stock A to the 4 ml cell suspension. The solvent in-well concentration should correspond to the final solvent concentration in the well with Test Substance.
- Unstimulated control: leave as is.
- Incubate for 24 h (±0.5 h) at 37°C±1°C and 5%±0.5% CO_2.

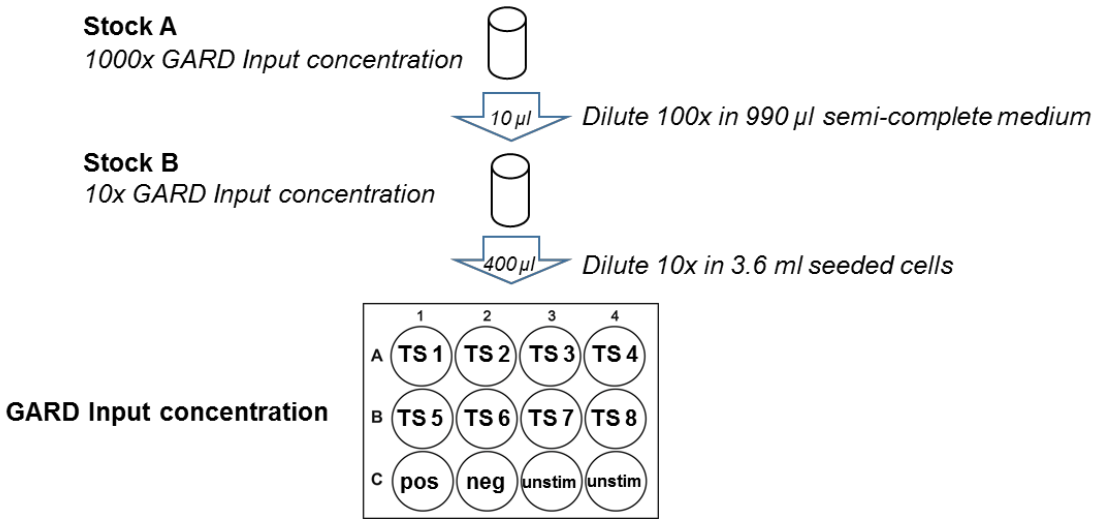

Figure 8. Example of preparation of a *GARD Main Stimulation (2-step dilution)*.

Cell harvest for flow cytometry analysis and TRIzol

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

- Mix the cell culture in each well by pipetting up and down and harvest 3 x 1 ml from each substance, into 3 RNase-free 1.5-2 ml micro tubes (for TRIzol samples).
- Store the tubes at 2-8°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°C.
- Harvest all samples from all plates before continuing.

- Centrifuge TRIzol samples at 300-315x*g* for 5 min at 2-8°C. Remove supernatant carefully by aspiration.
- Quickly add 500 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°C±5°C (stable for one month) or -80°C±10°C (stable for one year).
- Wash the flow cytometry samples by adding ~1 ml Wash buffer and centrifuge at 300-315x*g*, 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 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 300-315x*g*, 5 min, 2-8°C.
- Remove the supernatant by aspiration and 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 to ensure that test substances show the same Relative viability as in the *GARD Input Finder (cytotox test items Rv* 84.5 % - 95.4 %., non-cytotoxc and ≥95.5) and that the Reference Items show the expected Relative (or Absolute for unstim ctrl) viability as described in Table 4. If the Test Substance do not induce the same Relative viability (eg.

Relative viability Quality Control criteria

Each Test and Reference Item should yield four replicate Main Stimulation cell samples, each from an individual Main Stimulation, that have fulfilled the following acceptance criteria:

- The positive control and a Test Item expected to induce cytotoxicity, should have a relative viability of 84.5 % - 95.4 %.
- The negative control and a Test Item not expected to induce cytotoxicity, should have a relative viability of ≥95.5 %. The unstimulated control should have an Absolute viability of ≥84.5 %.

If a test substance or Reference Items do not pass the Quality Control, see below 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 Reference Items, discarding the samples from the failed stimulation.
- If the Quality Control criteria is not reached for **all positive controls** (Relative viability ≥84.5%), a new *GARD Main Stimulation* should be performed with all test substances and Reference Items, discarding the samples from the failed stimulation.
- If the Quality Control criteria is not reached for **the negative control** (Relative viability ≥95.5%), a new *GARD Main Stimulation* should be performed with all test substances and Reference Items, discarding the samples from the failed stimulation.
- If the Quality Control criteria for the Relative viability is not reached for a **test substance,** discard its generated samples from the failed stimulation and include the test substance in a following *GARD Main Stimulation.*

- If the Quality Control criteria for the Relative viability is not reached for a **test substance,** the selected test substance concentration may be changed in the GARD Main stimulation. However, the same concentration should be used in all four Main stimulations.

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 four *GARD Main Stimulations* for each substance (see example in the CASI document).

For each test substance and Reference Items, record:
- Date of cell stimulation
- *GARD Main stimulation* number
- In-well test substance concentration (µM)
- Relative (or Absolute for unstim ctrl) viability (%)
- If the Relative viability QC is passed
- If the sample is chosen for RNA isolation
- Relevant comments

Part result:
For *each* test substance and Reference Items, three TRIzol replicate samples with passed Relative viability Quality Control are generated from each of the *GARD Main Stimulations.* Only one TRIzol sample from each of the *GARD Main Stimulations* will initially 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 used for RNA isolation 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 Reference Items) 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:
For each Sample ID, record into the CASI document (sheet 'Sample Information'):
- RNA isolation date
- RNA isolation run number

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 (10,000-16,000x*g*) to avoid RNA Wash Buffer residues in the eluate.
- Elute RNA by adding *25 µl* DNase/RNase free water directly to the column matrix and centrifuge at 16,000x*g* for 30 seconds. Optional: 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±10°C.

It is recommended that a small aliquot (2 µl) 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.

RNA Quality Control criteria

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

If the sample do not 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 the Sample ID with the with the addition of "-p" ("pooled").

Recording of data:

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

- Bioanalyzer run date
- Bioanalyzer run number
- RNA concentration (ng/µl)
- RNA Integrity Number (RIN)
- If the sample passed RNA QC
- If the sample is chosen for Nanostring endpoint measurements
- Additional comments, e.g. if pooled TRIzol samples were used

The CASI document will automatically calculate how the RNA should be diluted for the Nanostring endpoint measurements and in the two columns under the heading 'Nanostring input volume calculations' (in the 'Sample Information' sheet) it will display the RNA sample volume (µl) to be diluted in the indicated volume (µl) of water vehicle. The calculations are done to yield a final RNA concentration of 20 ng/µl in a final volume that is dependent on the starting RNA concentration of the sample (between 7 and 20 µl).

Part result:
For each test substance and Reference Items, four RNA samples each identified with a Sample ID and passing the RNA Quality Control is generated.

ENDOINT MEASUREMENT

The endpoint measurement of GARDair is the mRNA quantification of the GARDair prediction signature, using the Nanostring nCounter system. A custom "CodeSet" (i.e. a set of oligonucleotide probes representing the genes of the GARDair prediction signature) has been developed by SenzaGen and Nanostring. To place an order for a batch of the GARDair prediction signature CodeSet, contact SenzaGen AB. 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 analysis

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 2000x*g,* 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.

1. Heat the thermocycler to 65°C, with a lid temperature of 70°C. If a time program is required, set it to infinity.
2. 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.
3. 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.
4. 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.
5. 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.
6. Distribute 8 µl of master mix to each hybridization tube.
7. Add 5 µl of RNA sample to each hybridization tube. Carefully mix by flicking and inversion and spin down with a minicentrifuge.
8. 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.
9. Place the hybridization tubes in the thermocycler and incubate for 24h (±0.5 h).

Note:

- Program the thermal 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 2000x*g* 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 Code set specific RLF file.
3. Upload the created CDF-file (specific for the Nanostring run) and the RLF file (specific for the batch of CodeSet) in the Digital Analyzer.
4. Follow the on-screen instructions to start quantifying 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.
5. 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 GDAAair* as described below and failed samples are automatically excluded before further analysis.

For Nanostring facilities not using the GDAAair (GARD Data Analysis Application air), each acquired RCC-file should be quality controlled. The quality control is performed to assure that the Nanostring analysis have been successful. Samples that fail any of the below described critical quality criteria should not be used for further analysis in the GARDair data analysis pipeline. The critical quality metrics are: imaging quality, linearity of the spike-in RNA control probes, limit of detection (LOD), and binding density. The imaging quality is calculated as the ratio between the number Fields of Views (FOV) (predefined in the CDF file as 555) and the number of successfully counted FOVs. A ratio above 0.75 (>0.75) is required for a sample to pass the imaging quality control. The linearity of the positive spike in controls is calculated using the positive control probes (POS_A-E) and their known RNA concentrations. The acquired counts for the positive control probes and their respective concentrations should be logarithmized (log2) before calculating the R^2 value of a linear fit to the data points. An R^2 value above 0.95 (R^2>0.95) is required for a sample to pass the linearity quality control. The LOD quality control uses all the negative controls (NEG_A-H) and the positive control E (POS_E). The LOD is defined as the mean counts of the negative control probes plus 2 standard deviations of the counts, see Equation 4.

$$LOD = \mu + 2 * \sigma \qquad \text{(Equation 4)}$$

Where μ and σ are the mean value and the standard deviation of the negative control probes' counts respectively. For a sample to pass the LOD quality control, the positive control probe POS_E must be above the estimated LOD (POS_E > LOD). The binding density is a measure of the number of probes observed per cartridge surface area during the gene expression acquisition in the Digital Analyzer. For a sample to pass the binding density quality control, the binding density must be above 0.05 and below 2.25 (0.05-2.25). For summary of the critical quality control parameters, see Table 7.

Table 7. Summary of the critical RCC-file quality control parameters

Quality Metric	Critical parameter
Imaging Quality	>0.75
Linearity	>0.95
Limit of Detection	(POS_E / LOD) >1
Binding Density	0.05-2.25

In addition to the above described critical quality control parameters, we also recommend counting the number of endogenous probes with 0 observed gene counts. If any samples contain multiple endogenous genes with 0 observed counts, a plausible explanation could be that the Nanostring cartridge was analyzed with the wrong version of the RLF file. If this is the case, the affected cartridge should rapidly be rescanned with the correct version of the RLF file to maintain sample integrity, and the previously generated RCC files should be discarded. If no apparent cause could be identified for the presence of multiple endogenous genes with 0 observed gene counts, no additional action needs to be taken.

2.5 DATA ANALYSIS

The Nanostring raw data files are imported and analyzed on any desktop computer using GARDair Data Analysis Application (GDAAair), accessible through a web browser. Authentication is required to access GDAA on the web. First time users will need to contact a SenzaGen representative (see contact persons) in order to obtain the required login details.

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 biomarker signature. One ZIP file is created for each Nanostring run and one RCC-file is generated for each analyzed Sample ID.

In the GDAAair, a normalization algorithm adjusts the test set to remove batch effects between the test set and the training set. During the normalization process, each *GARD Main Stimulation* is independently normalized. The adjustment is performed in PCA space and uses the unstimulated samples in the data set. A support vector machine (SVM) model, previously defined using a training dataset, is used to generate decision values (DV) for each new sample. The mean DV of a test substance is used to classify a substance. The control substances are used to validate the experiment, see acceptance criteria below.

PREDICTIONS USING GDAAAIR

1. To classify the raw data files with GDAAair, an annotation file in ".csv" or ".txt" 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 columns "Sample ID", "Substance ID" and "Main Stim #" and paste them into the new Excel workbook and save as "Annotation_file_[campaign name].[csv|txt]", i.e. use CSV (Comma delimited) or Text (Tab delimited). Ensure that the Substance IDs of

the control samples are “unstim ctrl” for the unstimulated controls, “neg ctrl” for the negative controls and “pos ctrl” for the positive controls.

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 8 below for an example of a compatible layout for an annotation file.

Table 8. Example of an annotation file compatible with the functions used in the data analysis.

	A	B	C
1	Sample ID	Substance ID	Main Stim #
2	EN-001	TestSubstance 1	1
3	EN-002	TestSubstance 2	1
4	EN-003	pos ctrl	1
5	EN-004	neg ctrl	1
6	EN-005	unstim ctrl	1

6. Open an internet browser and login to GDAAair app.

7. The left panel of GDAAair provides navigation through the application by 4 different tabs; “1. Data Upload”, “2. Quality Control”, “3. Classify” and “4. Information”. The upper right corner of the application displays the logged in user’s name and associated laboratory. A logout link can be accessed by clicking on the upper right corner.

8. On the data upload tab, upload the ZIP/RCC files in the “RCC files” browser and the annotation file in the “Annotation file” browser. A study ID and comments for the predictions can be given in respective text input field. Any text entered will be included in the final prediction report. The box on the lower left part of the screen shows summarized information of the uploaded files. Make sure that the expected number of samples was uploaded and that all samples were successfully mapped to the annotation file. Any errors or warnings encountered during the data processing will be displayed on a box on the lower right part of the screen.

9. After uploading the files, navigate to the quality control tab. The first table describes the predictions on the control samples. Control samples that have at least two valid replicates will receive a prediction based on their mean values. Control samples that fail to meet this criterium will not receive a prediction. The bottom table show the RCC data quality control metrics. Samples that fail the RCC data quality control will be displayed with a red background, and the value of the “Passed?” column will be “No”.

   *NOTE: The GDAAair application was created based on a protocol where a mean DV value of at least two valid replicates results in a prediction. However, in this updated protocol the predictions of the positive and negative controls are based on a mean DV of the **four** replicates.*

10. Continue to the classify tab to obtain the predictions on the test substances. The table shows the predictions on the uploaded test substance. Test substances with at least two valid replicates will receive a prediction as either “Respiratory Sensitizer” or “Non-Respiratory Sensitizer”. Lastly, download the prediction report by clicking the “Download Report” button. Clicking the button will render a pdf-report of the predictions. The report will also contain information about the uploaded files and the RCC data quality control.

    NOTE: The GDAAair application was created based on a protocol where a mean DV value of at least two valid replicates results in a prediction. However, in this updated

*protocol the prediction of the Test substance is based on mean DV of the **four** replicates.*

Recording of data:
- Save the rendered pdf-report of the predictions.

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

2.6 ACCEPTANCE CRITERIA

1. SOLUBILITY

a. The Test Substance should have been soluble in an assay compatible solvent (see Table 4) between 1-500 mM.

2. GARD INPUT FINDER

a. The SenzaCells used should have passed the Phenotypic Quality Control.

3. GARD MAIN STIMULATION

a. The SenzaCells used should have passed the Phenotypic Quality Control.
b. The cell stimulations with Test Substance, positive control, negative control and unstimulated control should have passed the Relative viability Quality Control in four Main Stimulations.
c. The selected GARD Input concentration should be identical in all four replicates.

4. RNA AND NANOSTRING QUALITY CONTROL

a. From each of the four Main stimulations one RNA sample should be generated from cells stimulated with the Test Substance, positive control, negative control and unstimulated control, i.e. 4 Test Substance RNA samples, 4 positive control RNA samples, 4 negative control RNA samples and 4 unstimulated control RNA samples. All of these samples should pass the RNA and Nanostring Quality Control acceptance criteria.

2.7 PREDICTION MODEL

For binary classification, the prediction model is defined as:

If the mean DV of biological replicate samples is **≥ 0**, the Test Substance and the Positive control are classified as **Respiratory Sensitizers**. If the mean DV of biological replicate samples is **< 0**, the Test Substance and the Negative control are classified as **Respiratory Non-sensitizers**.

Recording of data:
For each substance summarize the result into the CASI document (sheet “Data report”).
- Selected Solvent
- Max screened in-well concentration
- GARD Input Concentration
- Relative viability at 90% (Yes/No)
- Number of replicates meeting QC criteria with respect to Rv, RNA and Nanostring

GARDair prediction results; prediction, Mean Decision Values

2.8 BIBLIOGRAPHY

SCIENTIFIC PUBLICATIONS

Johansson H., Gradin R., Forreryd A., Agemark M., Zeller K., Johansson A., Larne O., van Vliet E., Borrebaeck C., Lindstedt M., Evaluation of the GARD assay in a blind Cosmetics Europe study. ALTEX Online first, 2017.

Forreryd A., Zeller K., Lindberg T., Johansson H., Lindstedt M. From genome-wide arrays to tailor-made biomarker readout – Progress towards routine analysis of skin sensitizing chemicals with GARD. Toxicolgy In Vitro, 2016.

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Lindstedt M., Borrebaeck C.A.K. Pattern rules: biomarker signatures for sensitization as an alternative to animal testing. Biomark Med, 2011.

Johansson H., Albrekt A.S., Lindstedt M., Borrebaeck C.A.K. A genomic biomarker signature can predict skin sensitizers using a cell-based in vitro alternative to animal tests. BMC Genomics, 2011.

Larsson K., Lindstedt M., Borrebaeck C.A.K. Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells. Immunology, 2006.

FILED PATENTS

GARDskin (ANALYTICAL METHODS AND ARRAYS FOR USE IN THE SAME) (PCT/GB2011/052082)

GARDpotency (ANALYTICAL METHODS AND ARRAYS FOR USE IN THE SAME) (PCT/EP2017/056878)

GARDair (ANALYTICAL METHODS AND ARRAYS FOR USE IN THE SAME) (PCT/IB2013/053321)

SenzaCell
(PCT/EP2018/075829; TW107132270)

Granted patents

GARDskin CN103429756B; EP2633077; HK1189245

2.9 APPENDIX 1. CASI

CASI, Excel document: “LO FRM 17 CASI.xltx”

2.10 APPENDIX 2. SENZACELL PHENOTYPE

An example of the SenzaCell phenotype is visualized in Figure 1. Note that SenzaCells are known to be heterogenous, and deviations from given examples are expected.

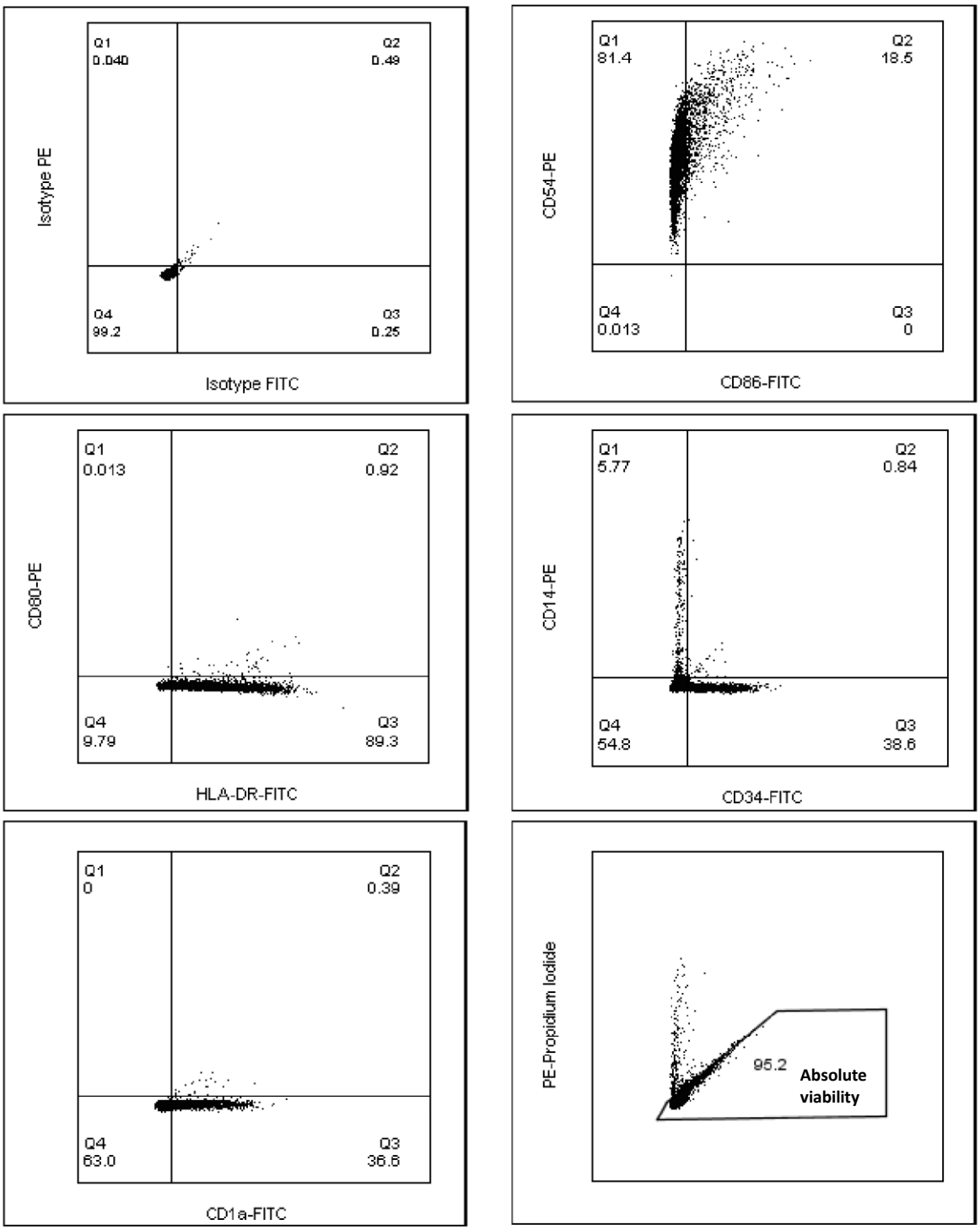

Figure 1. PE/FITC scatter plots of mAb stained SenzaCells for phenotypic control.

2.11 APPENDIX 3. 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 µM (A) and 100 µM (B), respectively. A low fraction of “Cells” can give a false percentage of Absolute viability, see Figure 1A.

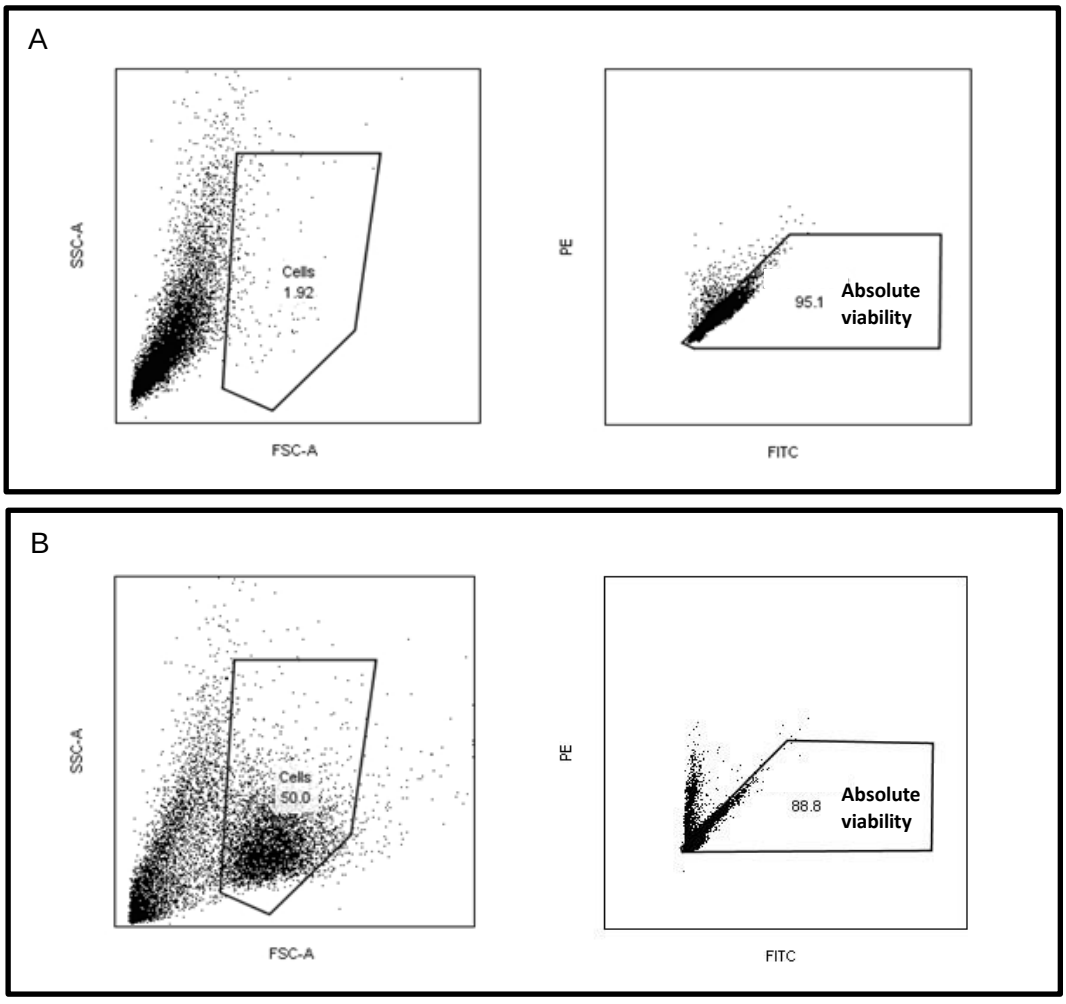

Figure 1. FCS/SSC and PE/FITC scatter plots of cells stimulated with a test substance at 400 µM (A) and at 100 µM (B).

Appendix II: GDAA™skin classification output files.

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GDAA Prediction Report

SenzaGen AB

Contents

Information

This prediction report was generated using GDAA version 2.2.1.

Study ID
NIEHSO 20221118 – Experiment H (GARDskin)

Time
2025-04-23 15:52:27 EST

User
Amber Edwards

Laboratory
Burleson Research Technologies, Inc.

Comments
N/A

Data Upload Summary
Assay: GARDskin
RLF: GPS200_v2_C11371
Number of uploaded RCC files: 15
Number of failed QC: 0
Number of unmapped RCC files: 0
Number of Unstimulated control samples: 3
Number of Negative control samples: 3
Number of Positive control samples: 3

Predictions

Control Samples

Table 1: Predictions on control samples.

Sample	Prediction	Mean Decision Value	Valid Replicates
Unstimulated control			3
Negative control	Non-sensitizer	-1.06	3
Positive control	Sensitizer	7.20	3

Test Substances

Table 2: Predictions on test substances.

Sample	Prediction	Mean Decision Value	Valid Replicates
BRTGA-21	Sensitizer	3.60	3
BRTGA-99	Non-sensitizer	-0.229	3

Prediction Details

Table 3: Prediction details.

Substance ID	Sample ID	Decision Value
neg ctrl	H003p	-0.381
neg ctrl	H009p	-1.15
neg ctrl	H015p	-1.66
pos ctrl	H005p	9.70
pos ctrl	H011p	5.26
pos ctrl	H017	6.62
UnstimCtrl	H006p	-1.03
UnstimCtrl	H012p	-1.14
UnstimCtrl	H018	-1.22
BRTGA-21	H001p	5.59
BRTGA-21	H007p	4.70
BRTGA-21	H013p	0.495
BRTGA-99	H002p	0.582
BRTGA-99	H008p	-0.463
BRTGA-99	H014	-0.804

Uploaded Data

RCC Files

RLF Version
GPS200_v2_C11371

Table 4: Uploaded RCC files.

File Name	MD5 Checksum	Sample ID
20250415_30103024880626-01_H001p_01.RCC	b0f2059e86f03e57febf474ae1dfab07	H001p
20250415_30103024880626-01_H002p_02.RCC	d1b58e1e27c9bd4e0964336f5be21efa	H002p
20250415_30103024880626-01_H003p_03.RCC	401e4dfac3b4db56bef4b6732a4e085b	H003p
20250415_30103024880626-01_H005p_04.RCC	72cfe943ad3b8962b44307fe3c6c4093	H005p
20250415_30103024880626-01_H006p_05.RCC	76e4e0dc424da8121ea6d99a044cecc4	H006p
20250415_30103024880626-01_H007p_06.RCC	14ce8dd2cedc3e7a08abffaee5d1eea0	H007p
20250415_30103024880626-01_H008p_07.RCC	8e622b72679c1edfe40d1a76c709a0a1	H008p
20250415_30103024880626-01_H009p_08.RCC	2b5a3114381580cb603b415d92bc99a2	H009p
20250415_30103024880626-01_H011p_09.RCC	39332ee22d234e9d99ddec4445111000	H011p
20250415_30103024880626-01_H012p_10.RCC	59ac1701a8d07d6a3c53ce691dfa3769	H012p
20250418_30103026070726-01_H013p_01.RCC	6a140f6ae3adad192e8d77b8f10ea333	H013p
20250418_30103026070726-01_H014_02.RCC	3caf917095b039d9b782d2443496ed2a	H014
20250418_30103026070726-01_H015p_03.RCC	9f982ab4bba80969f4e9eace855b7aba	H015p
20250418_30103026070726-01_H017_04.RCC	4ba29328863e55cfb3885162dbb26cf1	H017
20250418_30103026070726-01_H018_05.RCC	8571f9b3b2562346c14b63834006ec01	H018

Annotation File

File Name
Annotation_file_ExpH_GARDskin.csv

MD5 Checksum
e8659ff96def12e8e8c9351a7ea42d3a

Table 5: Uploaded annotation file.

Sample ID	Substance ID	Main Stim #
H001p	BRTGA-21	1
H002p	BRTGA-99	1
H003p	neg ctrl	1
H005p	pos ctrl	1
H006p	UnstimCtrl	1
H007p	BRTGA-21	2
H008p	BRTGA-99	2
H009p	neg ctrl	2
H011p	pos ctrl	2
H012p	UnstimCtrl	2
H013p	BRTGA-21	3
H014	BRTGA-99	3
H015p	neg ctrl	3
H017	pos ctrl	3
H018	UnstimCtrl	3

RCC Data Quality Control

See page below.

Table 6: RCC data quality control.

Sample ID	Imaging Quality	Binding Density	POS_E / LOD	Linearity	Zero Count Genes	Endogenous Below LOD	Passed
H001p	1.0000000	0.45	9.549848	0.9996411	0	4	yes
H002p	1.0000000	0.93	4.945069	0.9986712	0	8	yes
H003p	1.0000000	0.69	7.097353	0.9993224	0	6	yes
H005p	1.0000000	1.50	4.486698	0.9992652	0	2	yes
H006p	0.9948454	1.01	4.440817	0.9990763	0	9	yes
H007p	1.0000000	0.87	3.847116	0.9981989	0	6	yes
H008p	1.0000000	1.17	4.514562	0.9979113	0	1	yes
H009p	0.9948454	0.82	5.690141	0.9989918	0	7	yes
H011p	1.0000000	0.68	5.434146	0.9980936	0	8	yes
H012p	1.0000000	0.92	5.588416	0.9994421	0	4	yes
H013p	0.9845361	0.89	4.505359	0.9991907	0	10	yes
H014	0.9948454	0.88	4.170781	0.9966271	0	7	yes
H015p	1.0000000	0.98	3.858133	0.9958919	0	6	yes
H017	1.0000000	0.88	5.102840	0.9970885	0	2	yes
H018	1.0000000	1.03	5.072828	0.9978937	0	4	yes

Runtime Messages

No runtime messages were generated.

Appendix II: GDAA™air classification output files.

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GDAAair

SenzaGen AB

Contents

Information

This prediction report was generated using GDAAair version 1.2.1.

Study ID
NIEHSO 20221118 - Experiment C (BRTGA-21)

Time
2025-12-01 08:20:05 EST

User
Amber Edwards

Laboratory
Burleson Research Technologies, Inc.

Comments
N/A

Data Upload Summary
Assay: GARDair
RLF: GARDair50_C10168
Number of uploaded RCC files: 16
Number of failed QC: 0
Number of unmapped RCC files: 0
Number of Unstimulated control samples: 4
Number of Vehicle control samples: 4
Number of Positive control samples: 4

Predictions

Test item: BRTGA-021

Classification: **Respiratory sensitizer**

Warnings: No warnings were encountered.

Test item information

- Sample ID: 108p, 236, 261p, C267p.
- Main stim: 2, 7, 9, 10.
- Decision values: 4.32, 9.13, 2.80, 3.58.
- Mean decision value: 4.96

Associated control sample information

Unstimulated control

- Sample ID: 156p, 242, 266p, C284p.
- Main stim: 2, 7, 9, 10.

Negative control

- Sample ID: 152p, 239, 263p, C281p.
- Main stim: 2, 7, 9, 10.
- Decision values: -4.18, -0.461, -1.28, 1.37.
- Mean decision value: -1.14.
- Classification: Non-sensitizer.
- Warnings: No warnings were encountered.

Positive control

- Sample ID: 154, 240, 264, C282.
- Main stim: 2, 7, 9, 10.
- Decision values: 6.55, -4.84, -14.8, 19.8.
- Mean decision value: 1.68.
- Classification: Respiratory sensitizer.
- Warnings: No warnings were encountered.

Prediction Details

Table 1: Prediction details.

Sample	Sample ID	Main Stimulation	Decision Value
BRTGA-021	C267p	10	3.58
BRTGA-021	108p	2	4.32
BRTGA-021	236	7	9.13
BRTGA-021	261p	9	2.80
neg ctrl	C281p	10	1.37
neg ctrl	152p	2	-4.18
neg ctrl	239	7	-0.461
neg ctrl	263p	9	-1.28
pos ctrl	C282	10	19.8
pos ctrl	154	2	6.55
pos ctrl	240	7	-4.84
pos ctrl	264	9	-14.8
unstim ctrl	C284p	10	
unstim ctrl	156p	2	
unstim ctrl	242	7	
unstim ctrl	266p	9	

Uploaded Data

RCC Files

RLF Version
GARDair50_C10168

Table 2: Uploaded RCC files.

File Name	MD5 Checksum	Sample ID
20240529_30102981971125-01_152p_06.RCC	1f9c410108f2821dcfc716460635d759	152p
20240529_30102981971125-01_154_08.RCC	29077428e04999da0c18e91c81353535	154
20240529_30102981971125-01_156p_09.RCC	7732aca6389e64682e5dd663c0874e73	156p
20240719_30103023870626-01_108p_03.RCC	e4720a841d86315d885a92f1bc9e4018	108p
20240724_30103023890626-01_236_12.RCC	60f01dff11a77f6baef284a83d219185	236
20240725_30103023910626-01_239_03.RCC	75a0726225aefdf5fb1cf27269447985	239
20240725_30103023910626-01_240_04.RCC	c41cf95b392c8cf35869327ca6e6a703	240
20240725_30103023910626-01_242_05.RCC	95fbb0c915106a24bee26c79992a4238	242
20240816_30103023950626-01_264_09.RCC	2dc7f9dd1a67057ac7c397640af5b7a1	264
20240820_30103024400626-01_261p_05.RCC	3e6f7f521de69bf2c41d78c2b5cecf1c	261p
20240820_30103024400626-01_263p_07.RCC	50b36d1e7d3435de9f46153c3f720578	263p
20240820_30103024400626-01_266p_08.RCC	2250950d2bc6d2b776b96328e0818a19	266p
20241029_30103024350626-01_C267p_05.RCC	b1d239e9b19b484e1e421d831f177f0f	C267p
20241030_30103024340626-01_C281p_06.RCC	4073bafe7169644d11023dccb0bc015b	C281p
20241030_30103024340626-01_C282_07.RCC	917c8126b063d491c4f06bbf37863260	C282
20241030_30103024340626-01_C284p_08.RCC	4028940a3b6255ec0a41e790f27ae18c	C284p

Annotation File

File Name
Annotation_file_ExpC_DMSO.csv

MD5 Checksum
89090af0984a48666dd24d4400c6ca5b

Table 3: Uploaded annotation file.

Sample ID	Substance ID	Main Stim #
108p	BRTGA-021	2
152p	neg ctrl	2
154	pos ctrl	2
156p	unstim ctrl	2
236	BRTGA-021	7
239	neg ctrl	7
240	pos ctrl	7
242	unstim ctrl	7
261p	BRTGA-021	9
263p	neg ctrl	9
264	pos ctrl	9
266p	unstim ctrl	9
C267p	BRTGA-021	10
C281p	neg ctrl	10
C282	pos ctrl	10
C284p	unstim ctrl	10

RCC Data Quality Control

See page below.

Table 4: RCC data quality control.

Sample ID	Imaging Quality	Binding Density	POS_E / LOD	Linearity	Zero Count Genes	Endogenous Below LOD	Passed
152p	0.995	0.230	9.11	0.972	0	0	yes
154	1	0.210	10.4	0.988	0	1	yes
156p	1	0.230	5.36	0.983	0	2	yes
108p	1	0.150	11.0	0.984	0	0	yes
236	1	0.190	4.59	0.983	0	2	yes
239	1	0.160	4.05	0.952	0	3	yes
240	1	0.180	3.20	0.956	0	3	yes
242	1	0.080	4.84	0.956	1	17	yes
264	1	0.330	3.89	0.957	0	4	yes
261p	1	0.360	14.9	0.999	0	0	yes
263p	1	0.270	14.6	0.997	0	0	yes
266p	0.995	0.300	21.5	0.998	0	0	yes
C267p	0.995	0.270	17.5	0.999	0	0	yes
C281p	1	0.370	13.4	0.999	0	0	yes
C282	1	0.310	8.44	0.997	0	0	yes
C284p	1	0.360	11.0	0.999	0	0	yes

Runtime Messages

No runtime messages were generated.

GDAAair

SenzaGen AB

Contents

Information

This prediction report was generated using GDAAair version 1.2.1.

Study ID
NIEHSO 20221118 - Experiment H (DMSO)

Time
2025-10-09 09:08:34 EST

User
Amber Edwards

Laboratory
Burleson Research Technologies, Inc.

Comments
BRTGA-099

Data Upload Summary
Assay: GARDair
RLF: GARDair50_C11220
Number of uploaded RCC files: 16
Number of failed QC: 0
Number of unmapped RCC files: 0
Number of Unstimulated control samples: 4
Number of Vehicle control samples: 4
Number of Positive control samples: 4

Predictions

Test item: BRTGA-099

Classification: **Non-sensitizer**

Warnings: No warnings were encountered.

Test item information

- Sample ID: H002p, H008p, H014, H020.
- Main stim: 1, 2, 3, 4.
- Decision values: -0.760, -0.482, -1.58, 1.78.
- Mean decision value: -0.260

Associated control sample information

Unstimulated control

- Sample ID: H006p, H012p, H018, H024p.
- Main stim: 1, 2, 3, 4.

Negative control

- Sample ID: H003p, H009p, H015p, H021.
- Main stim: 1, 2, 3, 4.
- Decision values: -4.31, -1.14, -1.53, -0.176.
- Mean decision value: -1.79.
- Classification: Non-sensitizer.
- Warnings: No warnings were encountered.

Positive control

- Sample ID: H004p, H010p, H016p, H022p.
- Main stim: 1, 2, 3, 4.
- Decision values: 15.2, 10.9, 16.7, 7.80.
- Mean decision value: 12.7.
- Classification: Respiratory sensitizer.
- Warnings: No warnings were encountered.

Prediction Details

Table 1: Prediction details.

Sample	Sample ID	Main Stimulation	Decision Value
BRTGA-099	H002p	1	-0.760
BRTGA-099	H008p	2	-0.482
BRTGA-099	H014	3	-1.58
BRTGA-099	H020	4	1.78
neg ctrl	H003p	1	-4.31
neg ctrl	H009p	2	-1.14
neg ctrl	H015p	3	-1.53
neg ctrl	H021	4	-0.176
pos ctrl	H004p	1	15.2
pos ctrl	H010p	2	10.9
pos ctrl	H016p	3	16.7
pos ctrl	H022p	4	7.80
UnstimCtrl	H006p	1	
UnstimCtrl	H012p	2	
UnstimCtrl	H018	3	
UnstimCtrl	H024p	4	

Uploaded Data

RCC Files

RLF Version
GARDair50_C11220

Table 2: Uploaded RCC files.

File Name	MD5 Checksum	Sample ID
20250415_30103024680626-01_H002p_02.RCC	5fdd1100cda8be02b2929253a2426c7d	H002p
20250415_30103024680626-01_H003p_03.RCC	a110e6f92051b20c742b176edd5244fb	H003p
20250415_30103024680626-01_H004p_04.RCC	64611ea175b08fd5371be196766f5033	H004p
20250415_30103024680626-01_H006p_05.RCC	7ad707ed30a8bcab2982d38169629056	H006p
20250415_30103024680626-01_H008p_07.RCC	a7489547c3dee1bfcd80b6d1a5a42c84	H008p
20250415_30103024680626-01_H009p_08.RCC	7a5640e3f4e78a39ab9b7c6295d1268b	H009p
20250415_30103024680626-01_H012p_10.RCC	e0518b921a05c5f8fb295ec014a80801	H012p
20250422_30103024670626-01_H010p_11.RCC	190abb1ce541844eaeb13e475b3c878d	H010p
20250422_30103024670626-01_H014_02.RCC	37a5f2682b0e45a18441ea45c96c706d	H014
20250422_30103024670626-01_H015p_03.RCC	767b6ec4f57cb1d341a9288067b7252a	H015p
20250422_30103024670626-01_H016p_04.RCC	1dda3c2b09fe657c295b59d1a4061f35	H016p
20250422_30103024670626-01_H018_05.RCC	e70743409c1bb72cd7abb781a9032a2b	H018
20250422_30103024670626-01_H020_07.RCC	baa936ff9589cd1464d99e1f2d5ade49	H020
20250422_30103024670626-01_H021_08.RCC	56f594fef61e96cb275b3c929780cdd5	H021
20250422_30103024670626-01_H022p_09.RCC	6a0f5c66ba5ecc4dafedf9f8527f5366	H022p
20250422_30103024670626-01_H024p_10.RCC	876e1d9bf75844e9682ae4cb4371bb01	H024p

Annotation File

File Name
Annotation_file_ExpH_GARDair.csv

MD5 Checksum
ff98a50d931deed8ed81d8a072081b9b

Table 3: Uploaded annotation file.

Sample ID	Substance ID	Main Stim #
H002p	BRTGA-099	1
H003p	neg ctrl	1
H004p	pos ctrl	1
H006p	UnstimCtrl	1
H008p	BRTGA-099	2
H009p	neg ctrl	2
H010p	pos ctrl	2
H012p	UnstimCtrl	2
H014	BRTGA-099	3
H015p	neg ctrl	3
H016p	pos ctrl	3
H018	UnstimCtrl	3
H020	BRTGA-099	4
H021	neg ctrl	4
H022p	pos ctrl	4
H024p	UnstimCtrl	4

RCC Data Quality Control

See page below.

Table 4: RCC data quality control.

Sample ID	Imaging Quality	Binding Density	POS_E / LOD	Linearity	Zero Count Genes	Endogenous Below LOD	Passed
H002p	1	0.440	13.1	0.999	0	0	yes
H003p	1	0.360	13.4	0.999	0	0	yes
H004p	1	0.410	15.7	0.999	0	0	yes
H006p	0.990	0.430	15.7	0.999	0	0	yes
H008p	1	0.590	10.7	0.998	0	0	yes
H009p	1	0.390	10.6	0.998	0	0	yes
H012p	1	0.450	13.9	0.998	0	0	yes
H010p	1	0.370	13.5	0.999	0	0	yes
H014	1	0.430	11.8	0.997	0	0	yes
H015p	1	0.400	13.5	0.997	0	0	yes
H016p	1	0.510	12.1	0.999	0	0	yes
H018	1	0.400	9.49	0.997	0	0	yes
H020	1	0.350	11.5	0.997	0	0	yes
H021	1	0.390	12.5	0.998	0	0	yes
H022p	1	0.430	10.8	0.997	0	0	yes
H024p	1	0.510	18.7	0.999	0	0	yes

Runtime Messages

No runtime messages were generated.