Research Article

A High-Throughput and Highly Automated Genotoxicity Screening Assay

Renxiang Chen[1,2,3], Yun-Tien Lin[1], Albert J. Fornace Jr.[1] and Heng-Hong Li[1]

[1]Department of Oncology, and Department of Biochemistry, Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC, USA; [2]Amelia Technologies LLC, Rockville, MD, USA; [3]Current address: RNAimmune Inc., Gaithersburg, MD, USA

Abstract

The increasing number of compounds under development and chemicals in commerce that require safety assessments pose a serious challenge for regulatory agencies worldwide. *In vitro* screening using toxicogenomic biomarkers has been proposed as a first-tier screen in chemical assessment and has been endorsed internationally. We previously developed, evaluated, and validated an *in vitro* transcriptomic biomarker responsive to DNA damage-inducing (DDI) agents, namely TGx-DDI, for genotoxicity testing in human cells and demonstrated the feasibility of using TGx-DDI in a medium-throughput, cell-based genotoxicity testing system by implementing this biomarker with the Nanostring nCounter system. In this current study, we took advantage of Nanostring nCounter Plexset technology to develop a highly automated, multiplexed, and high-throughput genotoxicity testing assay, designated the TGx-DDI Plexset assay, which can increase the screening efficiency eight-fold compared to standard nCounter technology while decreasing the hands-on time. We demonstrate the high-throughput capability of this assay by eliminating concentration determination and RNA extraction steps without compromising the specificity and sensitivity of TGx-DDI. Thus, we propose that this simple, highly automated, multiplexed high-throughput pipeline can be widely used in chemical screening and assessment.

1 Introduction

Chemicals that are capable of inducing genetic changes, such as mutations and chromosome damage, are defined as genotoxic (GTX) compounds. Genotoxicity is initiated by the covalent binding of chemicals or their metabolites to DNA as well as by non-covalent binding that also perturbs DNA and chromatin structure. If not repaired, DNA damage can lead to genomic instability and ultimately progress to cancer (Li et al., 2007; Birkett et al., 2019; Krewski et al., 2019; Brambilla et al., 2010). Therefore, genotoxicity testing is an essential part of safety assessment for predicting the carcinogenic potential of all drugs and chemicals.

Humans are continuously exposed to potential chemical hazards throughout their daily lives, including industrial chemicals, food, medicine, and consumer products. For many chemicals, insufficient toxicity information is available for risk assessment (Judson et al., 2010). The inventory of the TSCA (Toxic Substances Control Act) in the US has continued to grow since the initial reporting period and now lists more than 86,000 chemicals. The increasing number of compounds under development and chemicals in commerce that require safety assessments pose a serious challenge for regulatory agencies. Traditional toxicology tests in animals are not feasible for screening the large backlog of chemicals due to high costs and the lengthy times required. Thus, there are compelling needs for developing high-throughput screening (HTS) assays to assist regulatory agencies in making informed decisions and formulating policies regarding chemical safety.

The most commonly used *in vitro* genotoxicity testing batteries include the Ames test, mouse lymphoma assay (MLA), mammalian cell HPRT gene mutation assay, and *in vitro* chromosome damage (CD) assays consisting of micronucleus (MN) or chromosomal aberration (CA) assays. The specificity, sensitivity, and positive and negative predictivity of these assays were evaluated using data from a large database of over 700 rodent carcinogens and non-carcinogens (Kirkland et al., 2005). The mammalian cell-based testing batteries had high sensitivity (positive genotoxicity results with carcinogens) but low specificity (negative genotoxicity results for non-carcinogens), i.e., high false-positive results for tested chemicals, particularly in CD assays. Although Fowler et al. (2012a,b) reported that the false-positive

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Correspondence: Heng-Hong Li, MD, PhD
Department of Oncology, and Department of Biochemistry,
Molecular & Cellular Biology
Georgetown University Medical Center
3900 Reservoir Rd., N.W., Washington, DC, USA
(hl234@georgetown.edu)

rate could be reduced with careful selection of cell types, e.g., using p53-competent human cells or cell lines instead of the long-established, p53-defective rodent cell lines, a novel *in vitro* system with a balance between sensitivity and specificity for predicting genotoxicity is desirable.

Taking advantage of a modern toxicogenomic approach, we developed, evaluated, and validated an *in vitro* transcriptomic biomarker responsive to DNA damage-inducing (DDI) agents in human cells, called TGx-DDI, for genotoxicity testing (Li et al., 2015, 2017). While TGx-DDI does not detect the mode of action of aneugens due to the fact that damages caused by aneugens are at the chromosomal but not the DNA level (Li et al., 2015; Allemang et al., 2021), the TGx-DDI biomarker can readily discriminate DDI agents from non-DDI agents. Importantly, this biomarker can differentiate compounds with false-positive findings in CD assays from true DNA damaging agents (Li et al., 2017). The sensitivity and specificity of genotoxicity prediction by TGx-DDI were carefully validated in our laboratory and another laboratory using different technical platforms, such as microarray, nCounter, Tempo-seq, and qRT-PCR platforms (Buick et al., 2015, 2020; Cho et al., 2019; Li et al., 2017, 2019; Yauk et al., 2016). Moreover, we also demonstrated the feasibility of using TGx-DDI in a high-throughput-compatible cell-based genotoxicity testing system by implementing this biomarker with the Nanostring nCounter system, which is much more efficient than using a microarray platform (Li et al., 2017). A standardized experimental workflow, which includes a concentration optimization step followed by either microarray or nCounter analysis, has been previously proposed for TGx-DDI application in chemical testing (Li et al., 2015, 2017).

In the present study, we focused on developing a new highly-automated and high-throughput genotoxicity testing assay by integrating TGx-DDI with Nanostring nCounter Plexset™ technology, designated TGx-DDI Plexset assay, which can increase the screening efficiency eight-fold compared to the standard nCounter Codeset technology, which is referred to as the Codeset method hereafter, while decreasing hands-on time. Different from the Codeset method, which involves the measurement of a colored barcode tag for one sample in each nCounter assay, the Plexset technology combines eight samples, which are barcoded differently, in one assay.

In this study, we first evaluated the robustness, reproducibility, sensitivity, and specificity of the TGx-DDI Plexset assay to show that results are in concordance with the previously used approaches. Next, a panel of 17 chemicals with known mechanisms of action were analyzed to demonstrate the versatility of the TGx-DDI Plexset assay. Since this assay can directly measure gene expression from cell lysate, the concentration setting experiment and RNA isolation step were eliminated, and thus a simple, highly automated HTS pipeline is proposed for the safety assessment of chemical agents.

Currently, the U.S. Environmental Protection Agency (EPA) ToxCast program and the NIEHS Tox21 collaboration have been using *in vitro* HTS assays to screen chemicals for potential health effects and minimize the required animal-based toxicity tests. A battery of HTS assays, including both cell-free and cell-based assays, has been evaluated to survey a broad spectrum of bioactivities, such as cytotoxicity, cell growth, enzymatic activity, transcription factor activity, gene induction, and high-content imaging of cells, which are used as predictors of toxicity in prioritizing chemicals for more in-depth testing (Dix et al., 2007; Judson et al., 2010; Shukla et al., 2010). Unlike other *in vitro* assays that usually assess one specific endpoint, the TGx-DDI biomarker measures transcriptomic changes that reflect the complex cellular responses to genotoxic stress, which provides additional mechanistic information. Thus, the TGx-DDI Plexset assay with its high sensitivity and specificity provides a novel and highly efficient approach for genotoxicity screening and human health risk assessment.

2 Material and methods

Cell culture and treatment

TK6 cells, a spontaneously transformed human lymphoblastoid cell line, were grown in RPMI 1640 medium supplemented with 10% FBS as described previously (Li et al., 2015). This cell line was ordered from ATCC (ATCC® CRL-8015™). The vender provides a certificate of analysis. The TK6 cell line was expanded using a tiered cell banking procedure. To control the passage number and minimize genetic drift, cells with passage numbers 5 to 10 were stored as seed stock, passage numbers 10 to 15 are production stock. Assays in this work used cells of passages 16 and 17. Mycoplasma testing was carried out monthly using the MycoFluor™ Mycoplasma Detection Kit (ThermoFisher Catalog number: M7006). The cell culture density was maintained at a concentration of 1-10 × 10^5 cells/mL.

On the day before treatment, exponentially growing TK6 cells were seeded in a 96-well plate at a density of 5 × 10^4 cells/well. All chemicals were purchased from Sigma-Aldrich, and stocks were prepared according to the manufacturer's instructions. Cells were treated with the indicated chemical agent for 4 h over a broad dosage range (2 μM to 1 mM) or with its corresponding vehicle control (H_2O, DMSO, or EtOH) for 4 h, rinsed to remove the drug, and then lysed in 10 μL of RNA lysis buffer (Qiagen) to make a lysate containing 5,000 cells/μL. For ionizing radiation (IR) exposure, cells were irradiated at a dose of 4 Gy using a gamma ray (^{137}Cs) irradiator. For agents requiring metabolic activation, treatment of TK6 cells included S9 rat liver extract as described previously (Buick et al., 2015) with a modification. In brief, cells were exposed to agent in the presence of 1% 5,6 benzoflavone-/phenobarbital-induced rat liver S9 (BF/PB-induced S9) (Moltox, Boone, NC) with NADPH generating system cofactors for 3 h, rinsed to remove the drug and S9, incubated in fresh RPMI for 4 h, and then lysed in RNA lysis buffer as described above. All treatments were performed in triplicate. We will consider using alternative materials to replace FBS and rat liver S9, which are produced in association with animal suffering, in the future once the reproducibility and effectiveness of those alternative materials are fully validated.

TGx-DDI nCounter Plexset assay

The nCounter Plexset™ technology is based on proven nCounter technology to ensure highly multiplexed digital analysis. Methodological details of the nCounter technology have been published

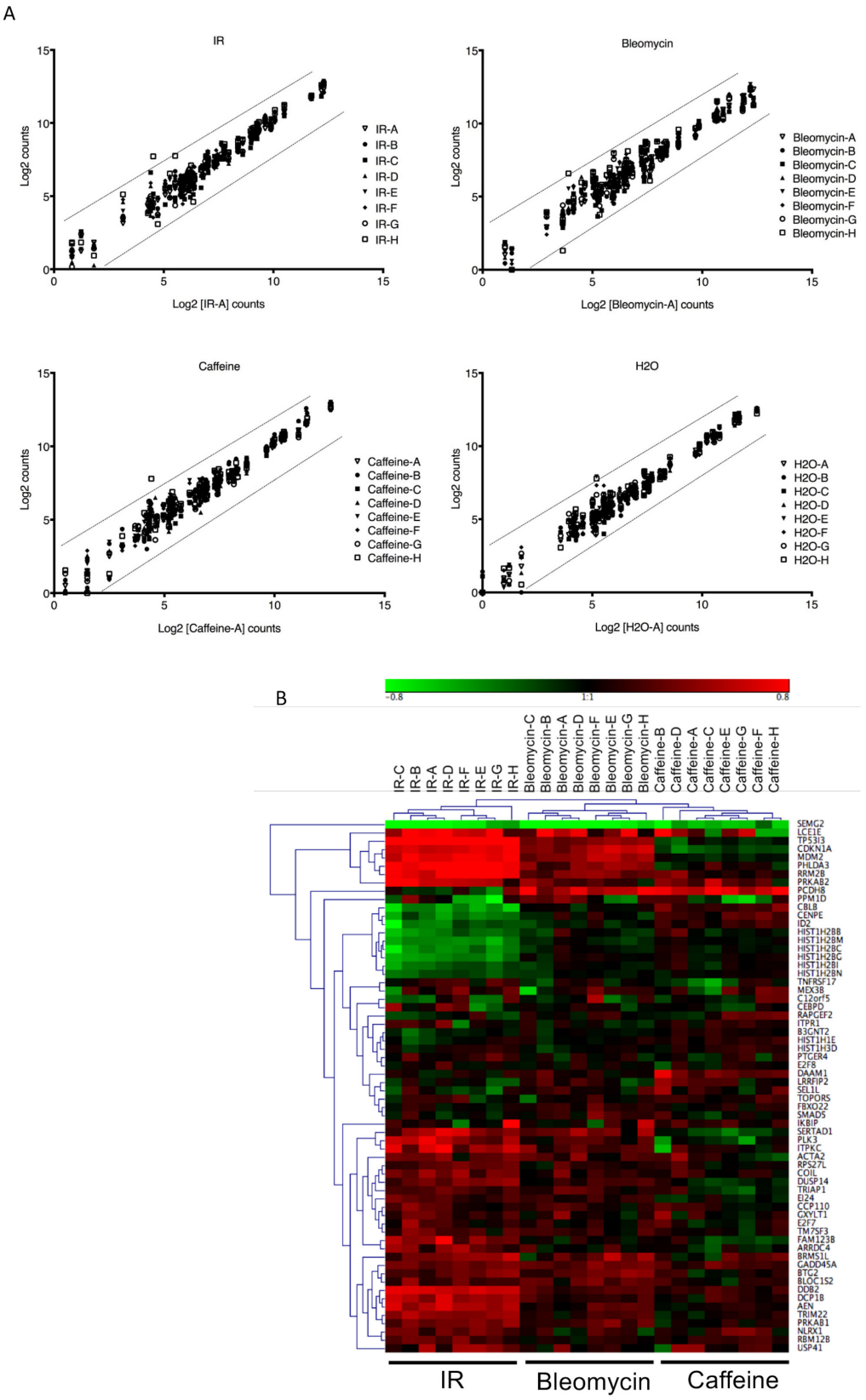


Fig. 1: Demonstration of technical robustness and reproducibility of nCounter Plexset assay

Two DDI agents, IR (4 Gy) and bleomycin (10 ng/mL), and one non-DDI agent, caffeine (2 mM), from our previous study (Li et al., 2017) were chosen to assess the reproducibility of the nCounter Plexset assay. A to H refers to Plexset A to H of the full set. (A) Scatter plot of normalized gene counts among biological replicates and PlexSet reagents. X-axis: log2 [counts] of sample hybridized to PlexSet A; Y-axis: log2 [counts] of Plexset A-H. (B) Two-dimensional clustering of each treatment/Plexset using fold-changes induced by treatments.

previously (Geiss et al., 2008). The standard nCounter Codeset gene expression assay includes 12 samples per run. In contrast, the Plexset™ assay enables evaluation of multiplexed targets for 96 samples per run. In short, one full PlexSet assay is comprised of 8 PlexSets, PlexSet A through H, and the target-specific oligonucleotide probes (probe A and B) for direct hybridization to RNA target. Each Plexset has a unique barcode that enables direct digital counting and multiplexing capabilities. The details of the nCounter Plexset technology can be found in the Nanostring PlexSet Reagents for Gene Expression user manual[1].

In this study, each set includes the TGx-DDI gene set and eight housekeeping genes – *G6PD*, *GUSB*, *HPRT1*, *LDHA*, *NONO*, *PGK1*, *PPIH*, and *TFRC* – selected based on stability and detectable expression levels. The optimized sequences for genes in the TGx-DDI panel were custom-designed and manufactured by NanoString, and probes A and B were designed by Nanostring and manufactured by IDT. The hybridization protocol followed the Nanostring PlexSet Reagents for Gene Expression user manual. Data collection was performed on an nCounter Digital Analyzer, where each fluorescent barcode was individually resolved and counted. The counts are separated (de-multiplexed) by nSolver Analysis (version 4.0) for further analysis. Normalized data were subjected to bioinformatics analysis. The nCounter Plexset assay was performed with cell lysate containing 5,000 cells/µL.

Bioinformatics analyses

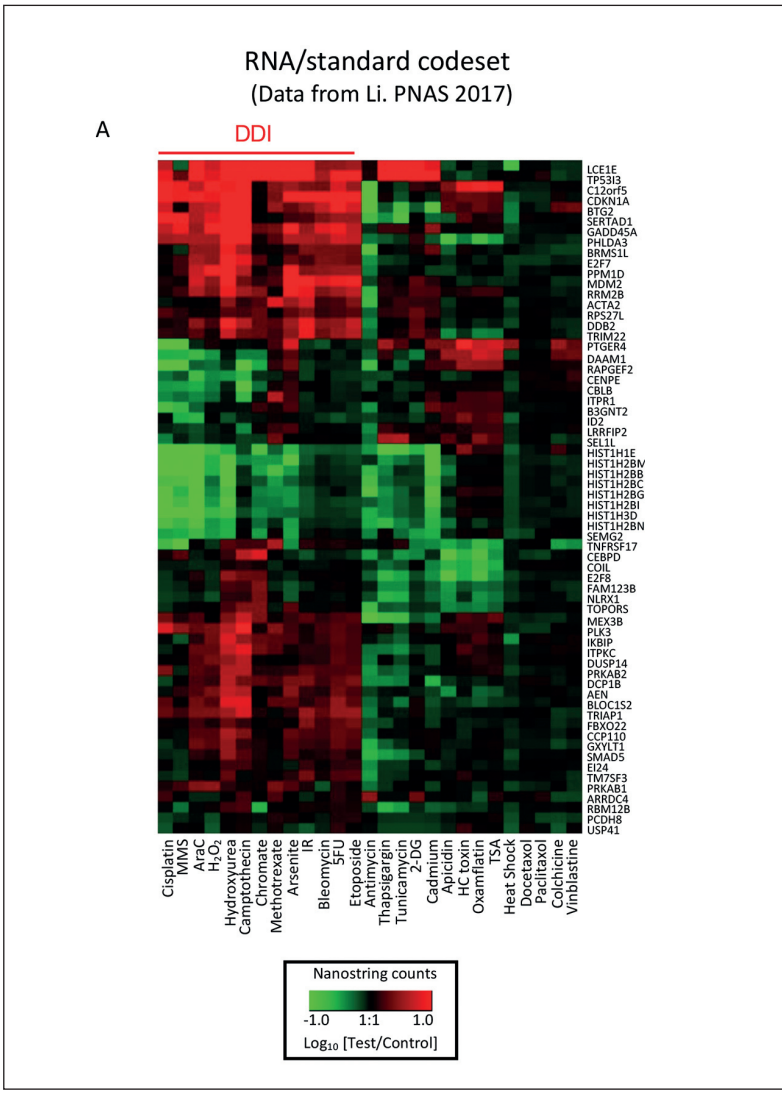
Gene expression data were exported from nSolver Analysis (version 4.0). Posterior probability analysis (PA) for test agents was performed given the classifier as described by Tibshirani et al. (2002) and implemented in the pamr package for R. By determining the extent of gene expression changes for each of the biomarker genes from DDI and non-DDI centroids, a DDI call was based on $p > 0.9$ of the compound being in that class, and *vice versa* for a non-DDI call. Two-dimensional clustering (2DC) was performed using Euclidean distances with average linkage by Genesis[2] . Principal component analysis (PCA) was performed using the prcomp function (Venables and Ripley, 2002) in R Bioconductor. Category assignment was determined by the position of the test agents in the tree structure of the dendrogram generated by 2DC, or in the PCA plot. A chemical was considered unclassified if it did not meet these criteria. An agent was classified as DDI if it gave a positive call in any one of the TGx-DDI biomarker analyses described above (2DC, PCA, or PA prediction) and was classified as non-DDI if it did not meet any of these criteria.

3 Results

3.1 Technical performance evaluation of TGx-DDI Plexset assay

In order to develop a high-throughput testing system, we integrated nCounter Plexset technology with our TGx-DDI transcriptomic biomarker to minimize the hands-on time and to increase the testing capacity. One full set of the nCounter Plexset assay includes 8 PlexSets, A through H, that contain unique barcodes for each set. Each set is comprised of identical genes, i.e., the 64 genes in TGx-DDI and eight housekeeping genes that were selected based on stability and expression level (Li et al., 2017).

To demonstrate the technical reproducibility and robustness of the nCounter Plexset assay and cell culture conditions, we conducted a series of experiments. We first evaluated the reproducibility of the assay and cell culture conditions using two DDI agents, IR and bleomycin, and one non-DDI agent, caffeine, to treat TK6 cells. In this experiment, TK6 cells were treated with these three agents using previous exposure conditions along with a concurrent vehicle control (H_2O) in eight independent replicates. Each replicate was then hybridized to the individual Plexset (Plexset A to H in Fig. 1) to assess the reproducibility of



[1] https://www.nanostring.com/wp-content/uploads/2020/12/MAN-10040-06_PlexSet_Reagents_User_Manual.pdf

[2] Genesis@genome.tugraz.at

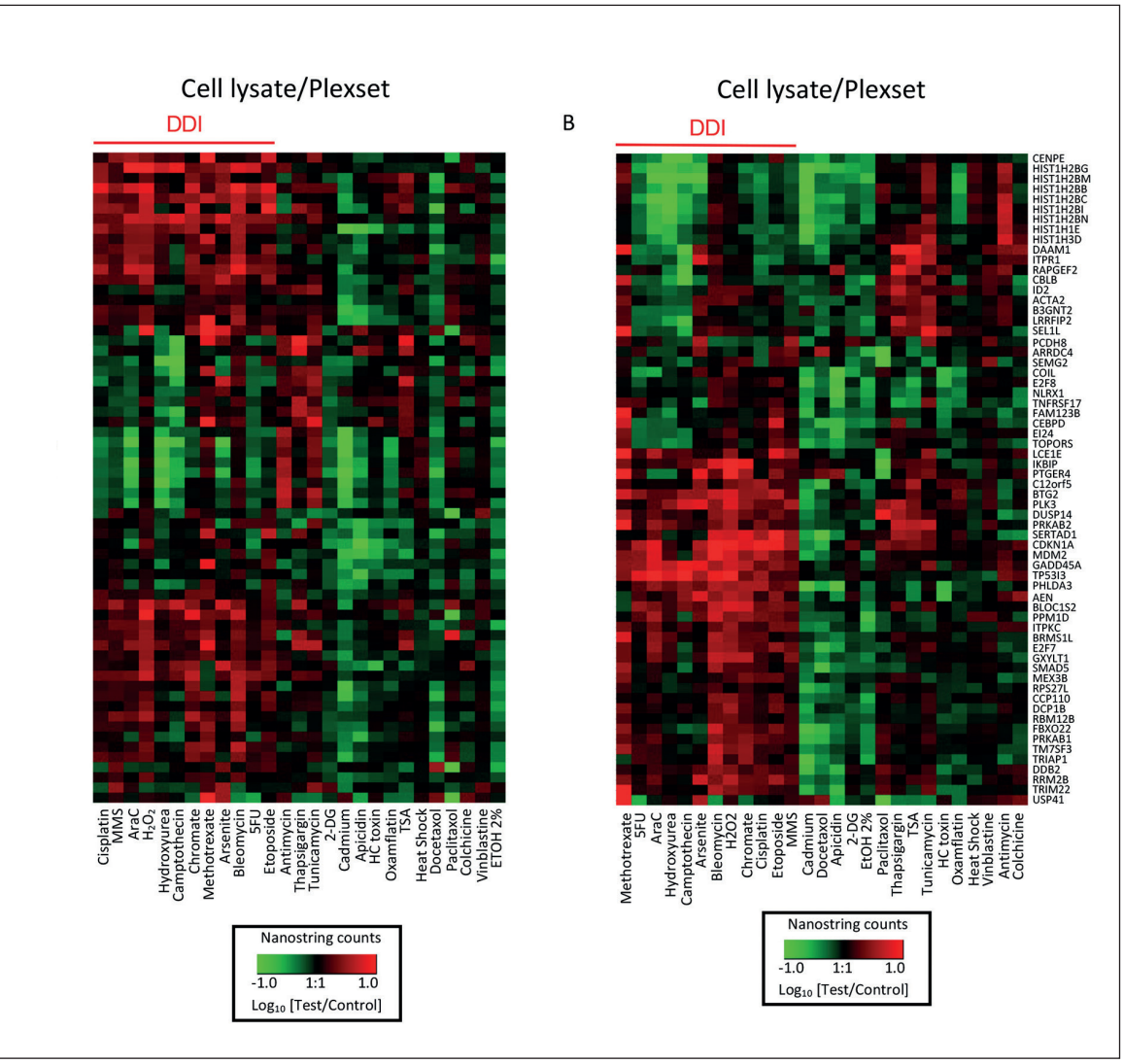


Fig. 2: Performance of TGx-DDI with the nCounter Plexset assay
(A) Comparison of nCounter Plexset with nCounter Codeset using original training set chemicals. On the left is a heatmap of TGx-DDI expression analysis using total RNA and standard nCounter Codeset. Data are from Li et al. (2017), and were visualized using Genesis[2]; on the right is a heatmap of TGx-DDI expression analysis using cell lysate and nCounter Plexset. The order of genes (vertical order) and chemicals (horizontal order) is the same in both panels.
(B) Heatmap of TGx-DDI expression analysis using previously tested chemicals and nCounter Plexset assay. All chemicals were classified as DDI or non-DDI using the same approach used in previous studies (Li et al., 2015, 2017).

the nCounter Plexset assay. As shown in Figure 1A, the scatter plot of gene counts for each treatment showed high consistency among each replicate/Plexset. Two-dimensional clustering of each treatment/Plexset using fold-changes induced by treatments also demonstrated that Plexset results clustered with each other in a single treatment (Fig. 1B), indicating high reproducibility among individual Plexsets within the full set.

We then assessed the robustness of the nCounter Plexset assay by comparing the results of training set agents in TK6 cells to those using total RNA and the standard nCounter codeset (Fig. 2). Using the same gene and treatment order as the previous nCounter assay, a similar gene expression profile was observed between the Plexset and standard codeset assays (Fig. 2A). Moreover, as anticipated, DDI and non-DDI agents could readily cluster with other agents in the same category (Fig. 2B). By applying our three-pronged analyses for classification as described in our previous study (Li et al., 2017), all compounds were classified into the correct category based on the nCounter Plexset assay results. The classification results of the three-pronged analyses and overall classification results are listed in Table 1. Taken together, these experiments demonstrated that the TGx-DDI Plexset assay can generate precise and reproducible data comparable to the standard Codeset assay, rendering it suitable for high-throughput genotoxicity testing.

3.2 TGx-DDI Plexset assay evaluation

To validate the sensitivity and specificity of DDI prediction of the TGx-DDI nCounter Plexset assay, we consulted with members of the National Toxicology Program, including the Genetic Toxicology Group in the Biomolecular Screening Branch, for advice on compound selection, and based on literature search (Kirkland et al., 2016) and *in vitro* genotoxicity profiles, we investigated 17 compounds with different characteristics. These compounds were categorized into three groups (Tab. 2). The criteria of classification were explained in detail previously (Kirkland et al., 2016). In brief, Group 1 included compounds that are

Tab. 1: The classification results of previously tested agents using the nCounter Plexset assay and three-pronged analysis
Previously tested training agents were used to treat TK6 cells; cell lysates were used to perform the TGx-DDI Plexset assay. Three-pronged analysis was performed to evaluate the accuracy of the TGx-DDI Plexset.

Agent	Three-pronged analysis			Overall
	2DC	PA	PCA	
5-FU	DDI	DDI	DDI	**DDI**
Ara-C	DDI	DDI	DDI	**DDI**
Arsenite	DDI	DDI	DDI	**DDI**
Bleomycin	DDI	DDI	DDI	**DDI**
Camptothecin	DDI	DDI	DDI	**DDI**
Chromate	DDI	DDI	DDI	**DDI**
Cisplatin	DDI	DDI	DDI	**DDI**
Etoposide	DDI	DDI	DDI	**DDI**
H_2O_2	DDI	DDI	DDI	**DDI**
Hydroxyurea	DDI	DDI	DDI	**DDI**
Methotrexate	DDI	DDI	DDI	**DDI**
MMS	DDI	DDI	DDI	**DDI**
2-DG	NDDI	NDDI	NDDI	NDDI
Antimycin	NDDI	NDDI	NDDI	**NDDI**
Apicidin	NDDI	NDDI	NDDI	**NDDI**
Cadmium	NDDI	NDDI	NDDI	**NDDI**
Colchicine	NDDI	NDDI	NDDI	**NDDI**
Docetaxol	NDDI	NDDI	NDDI	**NDDI**
EtOH 2%	NDDI	NDDI	NDDI	**NDDI**
HC toxin	NDDI	NDDI	NDDI	**NDDI**
Heat shock	NDDI	NDDI	NDDI	**NDDI**
Oxamflatin	NDDI	NDDI	NDDI	**NDDI**
Paclitaxol	NDDI	NDDI	NDDI	**NDDI**
Thapsigargin	NDDI	NDDI	NDDI	**NDDI**
TSA	NDDI	NDDI	NDDI	**NDDI**
Tunicamycin	NDDI	NDDI	NDDI	**NDDI**
Vinblastine	NDDI	NDDI	NDDI	**NDDI**

DDI, DNA-damage inducing; NDDI: non-DNA-damage inducing

positive in both Ames and CD assays and are referred to as "true positives" in Table 2. Five out of six agents in this group need metabolic activation to produce metabolites that directly interact with DNA. Therefore, this group of agents serves as a positive control for detection of direct DNA-reactive mechanisms. Agents in Group 2 are negative in both Ames and CD assays and are referred to as "true negatives". This group of agents serves as the negative control for testing the assay. Group 3 was comprised of compounds that should be negative in *in vitro* mammalian cell genotoxicity tests but were reported to induce positive results typically in chromosomal damage assays, often at high concentrations (Kirkland et al., 2016). Compounds in this group usually are negative *in vivo* and are negative in the Ames assay, and thus are considered false-positives in the CD assays.

In our previous study, the nCounter TGx-DDI assay used isolated RNA and the standard Codeset (Li et al., 2017) following concentration determination. In order to achieve high-throughput capability, we eliminated the concentration-determination and RNA preparation steps and measured the gene count directly from crude cell lysate. In this assay, cytotoxicity was determined over a broad dosage range (from 2 µM to 1 mM) using a standard MTT assay (Fig. S1[3]). Four concentrations were selected to proceed to the TGx-DDI Plexset assay for each agent based on the cytotoxicity results. Three low concentrations, 2 µM, 16 µM and 125 µM, were consistent among all agents. The top concentration of each agent was determined according to the cytotoxicity. If there was no cytotoxicity, a concentration of 1 mM was used; otherwise, the top concentration was set to lead to a cell survival rate of 45% ± 5%.

Figure 3 shows the TGx-DDI heatmap for compounds in all three groups. 2DC, PCA and PA results using TGx-DDI biomarker are shown by colored boxes above each heatmap. Yellow and blue represent positive and negative results, respectively, and grey indicates indeterminable results. The three-pronged analysis strategy was used to minimize the chance of false-negatives. As described in our previous study (Li et al., 2017), a compound was classified as DDI if it was determined positive in any of the three classification analyses and was classified as non-DDI if all analyses produced negative results. 2DC results and the PCA plot for each treatment are shown in Figure S2[3]. The classification of Group 1 agents is shown in Figure 3A. In this group, benzo[a]pyrene (BaP) was tested in parallel as a positive control for S9 activation. A concentration-dependent response was observed for BaP, and it was classified as DDI when cells were treated with BaP at the concentration of 10 µg/mL.

As shown in Figure 3A, Group 1 compounds that require metabolic activation were classified as DDI in the presence of S9. Without metabolic activation, 2-acetylaminofluorene (2-AAF) and 2,4-diaminotoluene (2,4-DAT) were also classified as DDI at the highest concentration of 1 mM. Moreover, cyclophosphamide and PhIP were classified as non-DDI at the lowest concentration in the presence of S9. Interestingly, 7,12-dimethylbenzanthracene (DMBA) was classified as non-DDI at the lowest concentration but was predicted as DDI at other concentrations with or without S9 presence. Lastly, 4-chloroaniline, which does not need metabolic activation, was classified as non-DDI at the three lower concentrations but classified as DDI at the highest concentration. Figure 3B shows the classification of Group 2 compounds. All compounds in this group were classi-

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Tab. 2: Groups of test compounds
Group 1 collects true positive compounds, with both Ames and chromosome damage (CD) positive; Group 2 collects true negative compounds, with both Ames and CD negative; Group 3 collects "irrelevant positives", with Ames negative and CD positive.
Y, metabolic activation is required; N, metabolic activation is not required.

Compound	Class	Genotoxic profile		Metabolic activation
		Ames	CA	
2-Acetylaminofluorene	Group 1	+	+	Y
2,4-Diaminotoluene	Group 1	+	+	Y
4-Chloroaniline	Group 1	+	+	N
7,12-Dimethyl-benzanthracene	Group 1	+	+	Y
Cyclophosphamide	Group 1	+	+	Y
PhIP-HCl	Group 1	+	+	Y
Ethionamide	Group 2	-	-	N
Lead acetate	Group 2	-	-	N
Trimethyl-ammonium chloride	Group 2	-	-	N
Tolterodine	Group 2	-	-	N
Sulfisoxazole	Group 2	-	-	N
Acrylamide	Group 3	-	+	N
Chlorpheniramine maleate	Group 3	-	+	N
Ethyl acrylate	Group 3	-	+	N
p-Nitrophenol	Group 3	-	+	N
Resorcinol	Group 3	-	+	N
Tertiary-butyl hydroquinone	Group 3	-	+	N

fied as non-DDI at all concentrations. The prediction of Group 3 compounds is shown in Figure 3C. Four out of six agents in this group were classified as non-DDI agents at all concentrations and only one compound, tertiary-butyl hydroquinone (TBHQ), was classified as DDI at all concentrations. Additionally, ethyl acrylate gave non-DDI calls at the three lower concentrations but was classified as DDI at the highest concentration.

4 Discussion

The objective of this study was to validate the specificity and versatility of this high-throughput genotoxicity testing assay for assessment of genotoxic hazard and de-risking compounds with false-positive *in vitro* chromosome damage findings. In our previous study, we demonstrated the feasibility of using the TGx-DDI biomarker and nCounter system in a high-throughput cell-based genotoxicity testing system (Li et al., 2017). An ideal high-throughput assay requires 1) a high degree of multiplex, 2) high accuracy, 3) automated workflow to reduce human errors and lessen the labor cost, and 4) low cost so that large-scale screening is affordable. In order to achieve the above goals, we integrated NanoString nCounter Plexset technology with our TGx-DDI biomarker to develop a high-throughput TGx-DDI testing assay, named the TGx-DDI Plexset assay. NanoString's nCounter Plexset technology enables direct, multiplexed, high-throughput detection of gene expression levels that does not involve reverse transcription or require amplification. By using nCounter Plexset technology, in which 8 sets of reagents, Plexset A to Plexset H, are included in a full set, we can test up to 96 samples per nCounter run.

We first assessed the robustness and reproducibility of the TGx-DDI Plexset assay and demonstrated that it can generate comparable, robust, and reproducible data. Due to the cost and low throughput of standard transcriptomic methods such as microarray and RNAseq, it is not always feasible to test multiple concentrations when a large number of compounds are subjected to toxicity screening. Thus, a concentration optimization procedure had been applied to determine the concentration for the transcriptomic assay (Li et al., 2015, 2017). We here demonstrate the high-throughput capability of this assay by eliminating concentration determination and RNA extraction steps for the classification of DDI and non-DDI agents.

In our previous study, we developed a TGx-DDI biomarker based on an experimental and analytical protocol for distinguishing DDI agents from non-DDI agents and evaluated its performance across different platforms (Li et al., 2017; Buick et al., 2015). Moreover, by integrating the TGx-DDI biomarker with nCounter technology, we demonstrated the feasibility of developing a highly automated high-throughput genotoxicity testing system (Li et al., 2017). The nCounter Plexset technology provides an excellent technical platform for this purpose, since it can analyze up to 96 samples per nCounter run, which is eight times more than the Codeset technology.

Therefore, the robustness, reproducibility, and sensitivity of the TGx-DDI Plexset assay needed to be assessed. The results showed that the nCounter Plexset assay can generate comparable, robust, and reproducible data for each Plexset (Fig. 1). Moreover, the output of the TGx-DDI Plexset assay is comparable to that of the standard nCounter assay (Fig. 2; Tab. 1), demonstrating that this multiplexed technology can increase testing capacity without compromising sensitivity. Hence, the TGx-DDI Plexset assay is an excellent automated HTS system.

To validate the TGx-DDI Plexset assay, we assessed 17 agents in three groups with broad *in vitro* genotoxicity profiles. Our previous TGx-DDI-based experimental protocol requires a concentration-determination step ahead of the microarray or nCounter assay (Li et al., 2017; Buick et al., 2015). In this HTS assay, TK6 cells were treated with these agents over a wide concentration range to eliminate the qRT-PCR-based concentration-determination process (Li et al., 2017). Following our data analysis workflow, the data from the TGx-DDI Plexset assay were interpreted

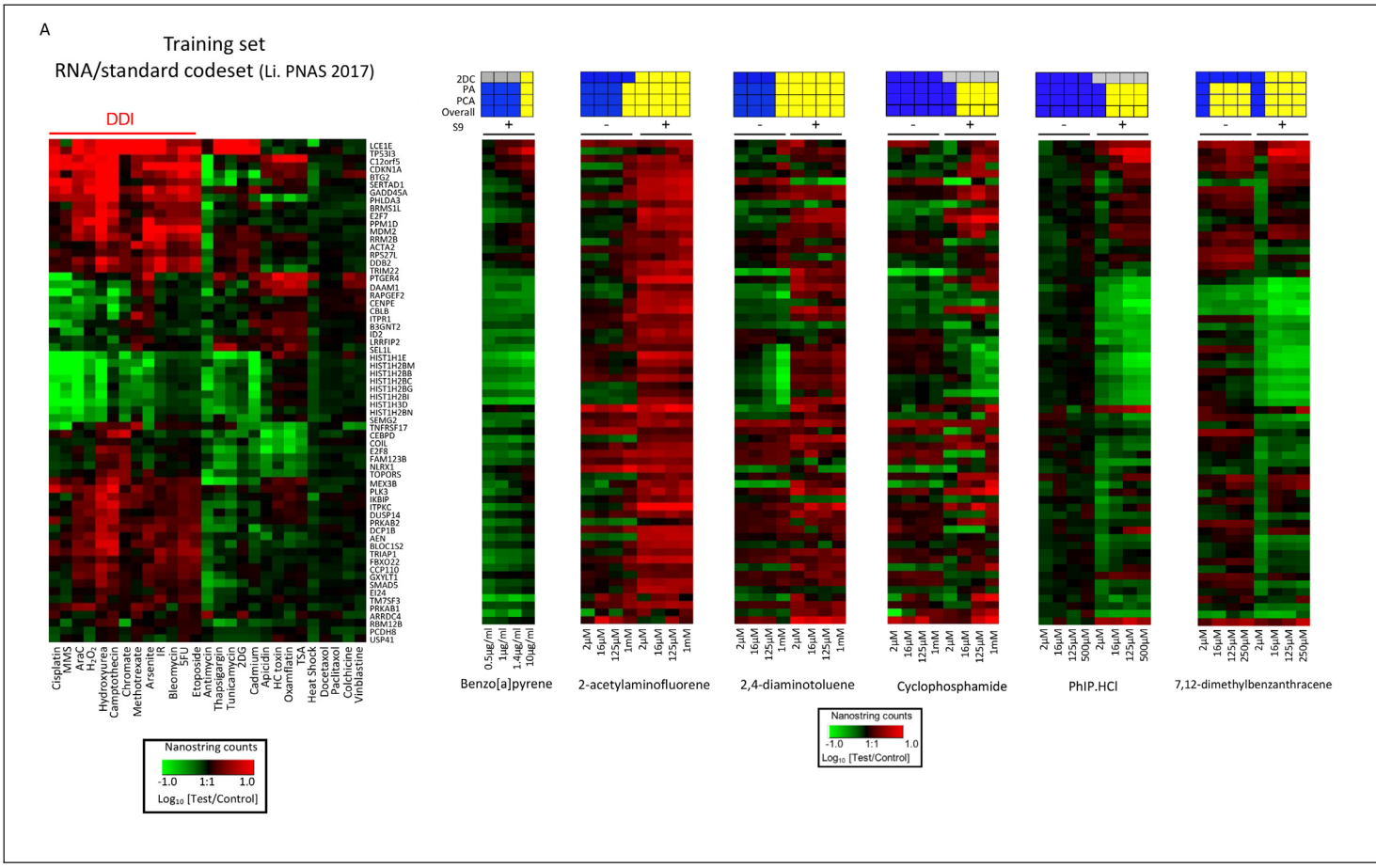


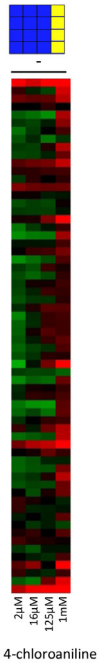
Fig. 3: Prediction of chemical toxicity using nCounter Plexset assay

Nineteen chemicals were grouped based on their toxicology profile (Tab. 1). Group 1 includes chemicals that were positive in both Ames and CD assays. Five out of six test chemicals require metabolic activation; Group 2 chemicals are both Ames- and CD-negative chemicals; Group 3 chemicals are Ames-negative and CD-positive chemicals. (A-C) Heatmaps of group 1-3 chemicals. The TGx-DDI transcriptomic biomarker panel from our previous study (Li et al., 2017) is on the left, the one on the right is the prediction of each compound. The results of 2DC, PA, PCA, and overall are displayed above each heat map. Three methods were used to predict DDI positivity (yellow), and the overall prediction (bottom) is based on positive results with any of these three methods. Yellow and blue indicate positive and negative findings, respectively; grey indicates indeterminable findings.

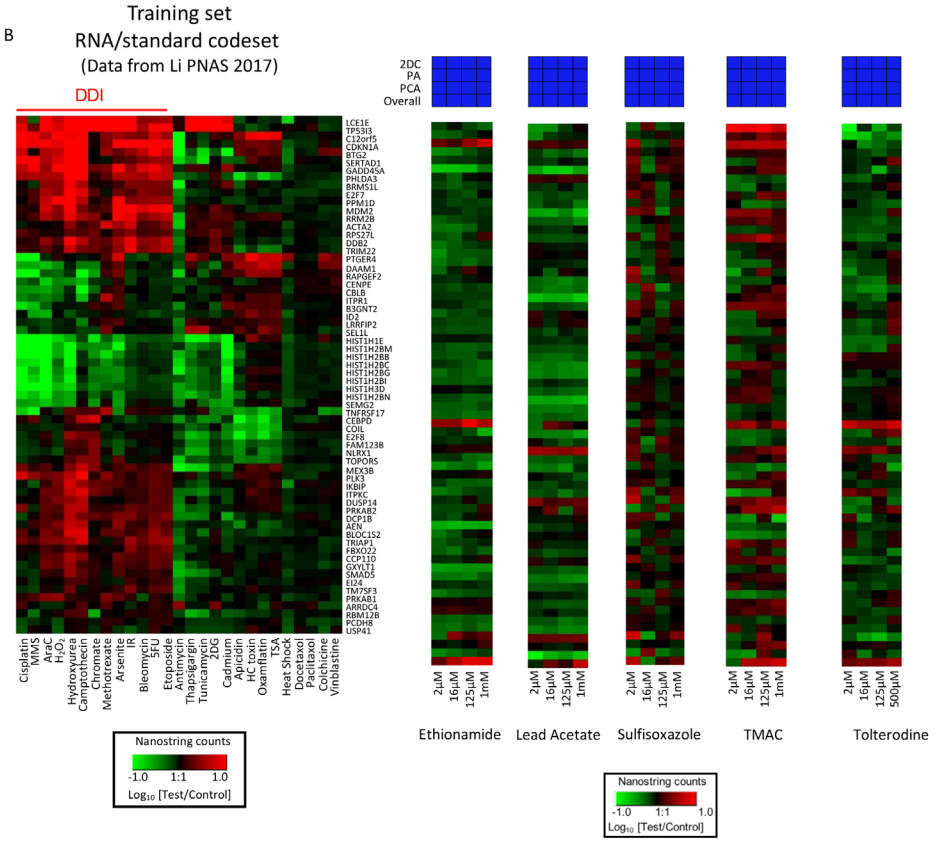
using three statistical approaches, 2DC, PA, and PCA, to ensure robust data analysis and minimize false-negative results (Li et al., 2017).

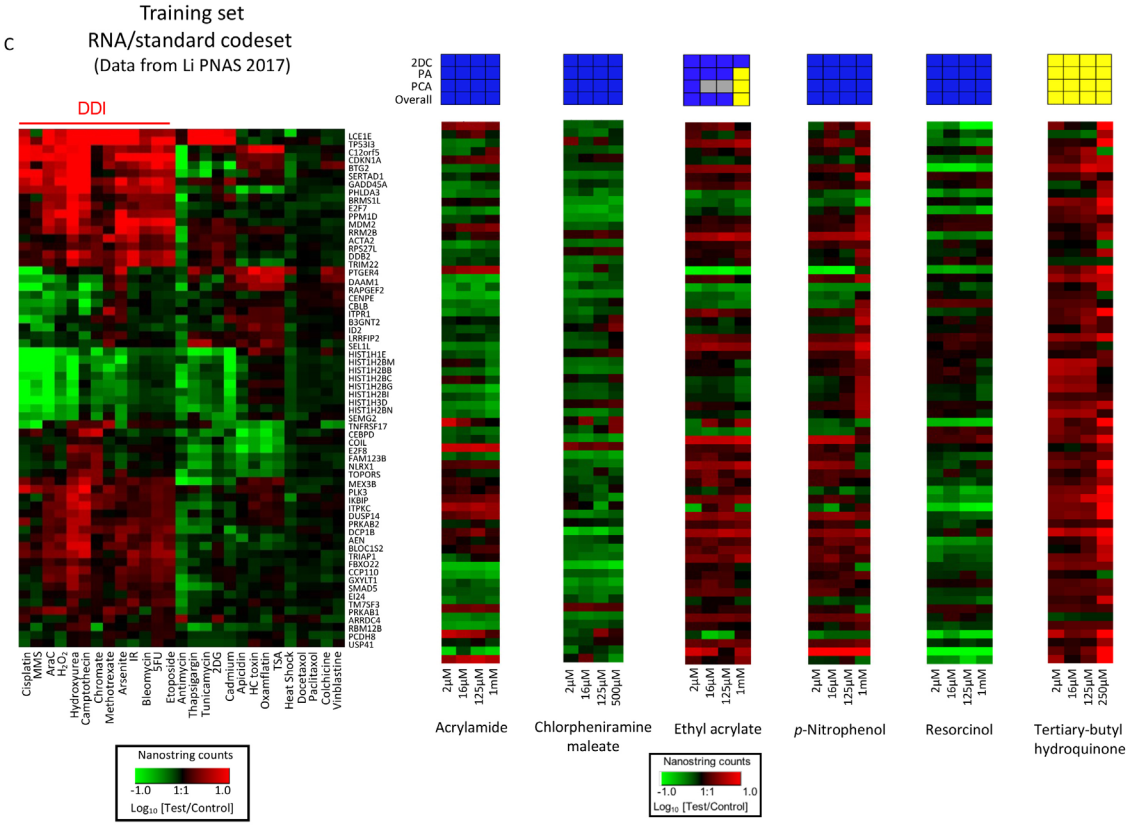
Figure 3 summarizes the results of the TGx-DDI Plexset assay for 17 agents. As shown in Figure 3A, consistent with their *in vitro* genotoxicity profiles, five out of six agents that require metabolic activation in Group 1 were classified as DDI in the presence of S9 at various concentrations, and 4-chloroaniline, which does not need metabolic activation, was also classified as DDI at the highest concentration. In addition, all agents in Group 2 were classified as non-DDI at all concentrations, which is consistent with the findings of *in vitro* CA and Ames assays (Fig. 3B). Without the concentration-determination step by which we selected only one concentration based on the induction of three indicator genes (*ATF3*, *CDKN1A*, and *GADD45*), this HTS assay enables us to assess the genotoxicity of agents in a dynamic range so that we can classify agents in a concentration-dependent manner. For example, 2-AAF and 2,4-DAT were classified as DDI at 1 mM without S9 activation (Fig. 3A). If only one concentration had been tested, this information might have been missed.

As the positive control of S9 activation in this assay, BaP was classified as DDI only at the concentration of 10 µg/mL. In our previous study using total RNA to measure gene expression levels, BaP was classified as DDI at both 1.4 µg/mL and 10 µg/mL (Li et al., 2017). This discrepancy could be due to the S9 activation time. In the previous study, cells were exposed to agents in the presence of S9 for 4 h, then recovered in fresh medium for 4 h, and then were collected for RNA isolation (Li et al., 2017). In this assay, we eliminated the RNA isolation step and instead measured gene expression levels directly from crude cell lysates, which requires a higher percentage of viable cells. Al-



4-chloroaniline





though variations in cell number can be normalized, big differences in the number of viable cells may compromise the precision of results. In a time course experiment of BaP testing the cytotoxicity of S9, the MTT assay of BaP-treated cells showed that there was no difference between 2- and 3-h treatments, but S9 became more cytotoxic at 4 h (Fig. S3[3]). Therefore, we modified our previous protocol by reducing S9 exposure to 3 h to compensate for its cytotoxicity.

Four out of six agents in Group 3 were classified as non-DDI at all concentrations, while TBHQ was classified as DDI at all concentrations (Fig. 3C). TBHQ is a phenolic antioxidant used in foods and by the cosmetic industry. Many antioxidants have been shown to act as either antioxidant or pro-oxidant under certain conditions, which could be caused by the generation of reactive oxygen intermediates (Black, 2002; Fujisawa et al., 2002), and this activity could potentially explain the DDI exhibited by TBHQ. Moreover, ethyl acrylate was classified as non-DDI at 3 lower concentrations and as DDI at 1 mM. It has been showed that ethyl acrylate has potential for genotoxicity in mammalian cells through a clastogenic mechanism and is "possibly carcinogenic to humans" (EFSA Panel on Food Contact Materials et al., 2017; Suh et al., 2018). Increased sister chromatid exchanges (SCE) and chromosomal aberrations (CA) in Chinese hamster ovary (CHO) cells with or without metabolic activation were reported (Moore et al., 1988; Loveday et al., 1990; Tennant et al., 1987). Our results showed that ethyl acrylate only induces DNA-reactive genotoxicity at very high concentrations, which demonstrated that our assay can readily derisk irrelevant findings for a single agent with this multi-concentration assay and can add significant value to the current genotoxicity testing battery.

Overall, these results demonstrated that the TGx-DDI Plexset assay can assess genotoxicity in a simple and rapid way with high-throughput capacity by combining dose optimization and transcriptomic analysis into one assay. The ability to include a wide range of concentrations is another strength and can highlight positive effects only seen at very high, irrelevant concentrations. Not only has this assay reduced hands-on time and allowed a more automated workflow, but it also enhanced specificity without compromising sensitivity, fulfilling the criteria for an ideal high-throughput testing assay mentioned earlier. As a transcriptomic biomarker that reflects the cellular responses to genotoxicity, we propose using the TGx-DDI assay to provide additional mechanistic information to augment current genotoxicity hazard assessment as stated previously (Li et al., 2017). The high-throughput potential developed in the current study will facilitate and expedite the genotoxicity screening of chemical agents.

References

Allemang, A., De Abrew, K. N., Shan, Y. K.. et al. (2021). A comparison of classical and 21st century genotoxicity tools: A proof of concept study of 18 chemicals comparing in vitro micronucleus, ToxTracker and genomics-based methods (TGx-DDI, whole genome clustering and connectivity mapping). *Environ Mol Mutagen 62*, 92-107. doi:10.1002/em.22418

Birkett, N., Al-Zoughool, M., Bird, M. et al. (2019). Overview of biological mechanisms of human carcinogens. *J Toxicol Environ Health B Crit Rev 22*, 288-359. doi:10.1080/10937404.2019.1643539

Black, H. S. (2002). Pro-oxidant and anti-oxidant mechanism(s) of BHT and beta-carotene in photocarcinogenesis. *Front Biosci 7*, d1044-55. doi:10.2741/a830

Brambilla, G., Mattioli, F., Robbiano, L. et al. (2010). Genotoxicity and carcinogenicity testing of pharmaceuticals: Correlations between induction of DNA lesions and carcinogenic activity. *Mutat Res 705*, 20-39. doi:10.1016/j.mrrev.2010.02.004

Buick, J. K., Moffat, I., Williams, A. et al. (2015). Integration of metabolic activation with a predictive toxicogenomics signature to classify genotoxic versus nongenotoxic chemicals in human TK6 cells. *Environ Mol Mutagen 56*, 520-534. doi:10.1002/em.21940

Buick, J. K., Williams, A., Gagné, R. et al. (2020). Flow cytometric micronucleus assay and TGx-DDI transcriptomic biomarker analysis of ten genotoxic and non-genotoxic chemicals in human HepaRG™ cells. *Genes Environ 42*, 5. doi:10.1186/s41021-019-0139-2

Cho, E., Buick, J. K., Williams, A. et al. (2019). Assessment of the performance of the TGx-DDI biomarker to detect DNA damage-inducing agents using quantitative RT-PCR in TK6 cells. *Environ Mol Mutagen 60*, 122-133. doi:10.1002/em.22257

Dix, D. J., Houck, K. A., Martin, M. T. et al. (2007). The ToxCast program for prioritizing toxicity testing of environmental chemicals. *Toxicol Sci 95*, 5-12. doi:10.1093/toxsci/kfl103

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Silano, V., Bolognesi, C. et al. (2017). Safety of ethyl acrylate to be used as flavouring. *EFSA J 15*, e05012. doi:10.2903/j.efsa.2017.5012

Fowler, P., Smith, K., Young, J. et al. (2012a). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type. *Mutat Res 742*, 11-25. doi:10.1016/j.mrgentox.2011.10.014

Fowler, P., Smith, R., Smith, K. et al. (2012b). Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement. *Mutat Res 747*, 104-117. doi:10.1016/j.mrgentox.2012.04.013

Fujisawa, S., Atsumi, T., Kadoma, Y. et al. (2002). Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity. *Toxicology 177*, 39-54. doi:10.1016/s0300-483x(02)00194-4

Geiss, G. K., Bumgarner, R. E., Birditt, B. et al. (2008). Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat Biotechnol 26*, 317-325. doi:10.1038/nbt1385

Judson, R. S., Houck, K. A., Kavlock, R. J. et al. (2010). In vitro screening of environmental chemicals for targeted testing prioritization: The ToxCast project. *Environ Health Perspect 118*, 485-492. doi:10.1289/ehp.0901392

Kirkland, D., Aardema, M., Henderson, L. et al. (2005). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. *Mutat Res*

584, 1-256. doi:10.1016/j.mrgentox.2005.02.004

Kirkland, D., Kasper, P., Martus, H. J. et al. (2016). Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. *Mutat Res Genet Toxicol Environ Mutagen 795*, 7-30. doi:10.1016/j.mrgentox.2015.10.006

Krewski, D., Bird, M., Al-Zoughool, M. et al. (2019). Key characteristics of 86 agents known to cause cancer in humans. *J Toxicol Environ Health B Crit Rev 22*, 244-263. doi:10.1080/10937404.2019.1643536

Li, H. H., Aubrecht, J. and Fornace, A. J. (2007). Toxicogenomics: Overview and potential applications for the study of non-covalent DNA interacting chemicals. *Mutat Res 623*, 98-108. doi:10.1016/j.mrfmmm.2007.03.013

Li, H. H., Hyduke, D. R., Chen, R. et al. (2015). Development of a toxicogenomics signature for genotoxicity using a dose-optimization and informatics strategy in human cells. *Environ Mol Mutagen 56*, 505-519. doi:10.1002/em.21941

Li, H. H., Chen, R., Hyduke, D. R. et al. (2017). Development and validation of a high-throughput transcriptomic biomarker to address 21st century genetic toxicology needs. *Proc Natl Acad Sci U S A 114*, E10881-E10889. doi:10.1073/pnas.1714109114

Li, H. H., Yauk, C. L., Chen, R. et al. (2019). TGx-DDI, a transcriptomic biomarker for genotoxicity hazard assessment of pharmaceuticals and environmental chemicals. *Front Big Data 2*, 36. doi:10.3389/fdata.2019.00036

Loveday, K. S., Anderson, B. E., Resnick, M. A. et al. (1990). Chromosome aberration and sister chromatid exchange tests in Chinese hamster ovary cells in vitro. V: Results with 46 chemicals. *Environ Mol Mutagen 16*, 272-303. doi:10.1002/em.2850160409

Moore, M. M., Amtower, A., Doerr, C. L. et al. (1988). Genotoxicity of acrylic acid, methyl acrylate, ethyl acrylate, methyl methacrylate, and ethyl methacrylate in L5178Y mouse lymphoma cells. *Environ Mol Mutagen 11*, 49-63. doi:10.1002/em.2850110107

Shukla, S. J., Huang, R., Austin, C. P. et al. (2010). The future of toxicity testing: A focus on in vitro methods using a quantitative high-throughput screening platform. *Drug Discov Today 15*, 997-1007. doi:10.1016/j.drudis.2010.07.007

Suh, M., Proctor, D., Chappell, G. et al. (2018). A review of the genotoxic, mutagenic, and carcinogenic potentials of several lower acrylates. *Toxicology 402-403*, 50-67. doi:10.1016/j.tox.2018.04.006

Tennant, R. W., Margolin, B. H., Shelby, M. D. et al. (1987). Prediction of chemical carcinogenicity in rodents from in vitro genetic toxicity assays. *Science 236*, 933-941. doi:10.1126/science.3554512

Tibshirani, R., Hastie, T., Narasimhan, B. et al. (2002). Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A 99*, 6567-6572. doi:10.1073/pnas.082099299

Venables, W. N. and Ripley, B. D. (2002). *Modern Applied Statistics with S*. New York, USA: Springer.

Yauk, C. L., Buick, J. K., Williams, A. et al. (2016). Application of the TGx-28.65 transcriptomic biomarker to classify genotoxic and non-genotoxic chemicals in human TK6 cells in the presence of rat liver S9. *Environ Mol Mutagen 57*, 243-260. doi:10.1002/em.22004

Conflict of interest

The authors declare that they have no conflicts of interest.

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