BioMAP® Assay Panel for Test Agent Prioritization

Support for carcinogenicity – related assessments

AltoPredict

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Section 1: Method Description

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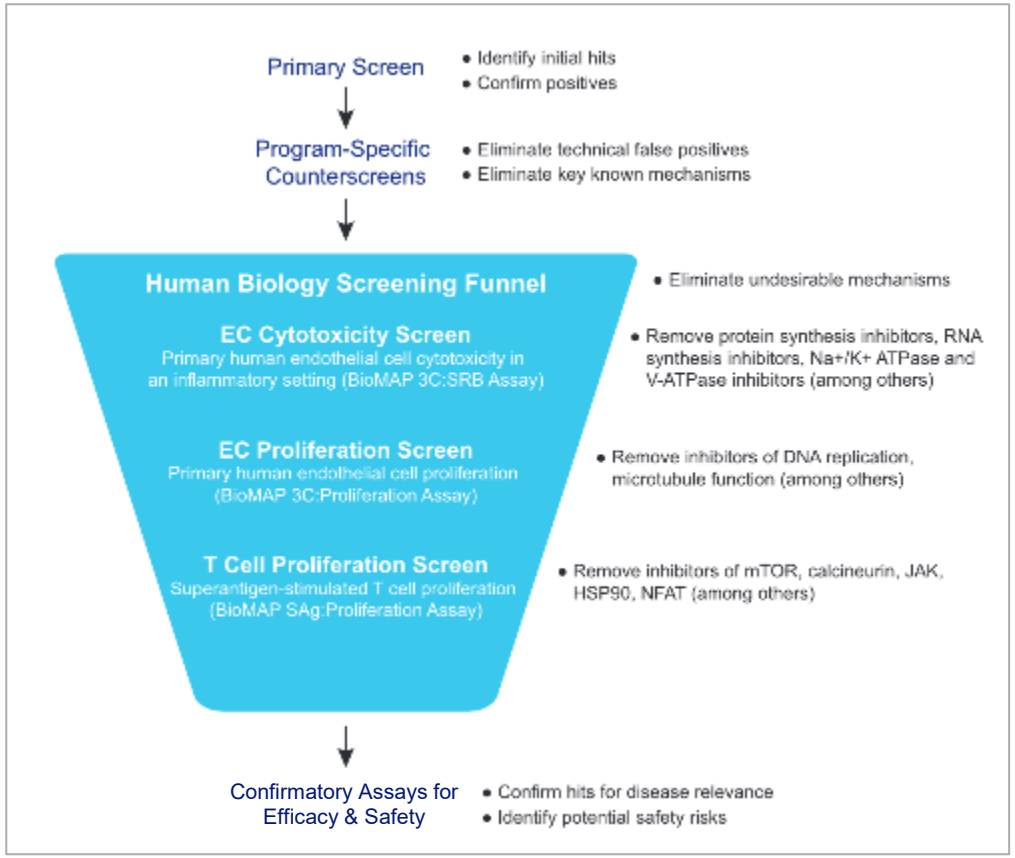
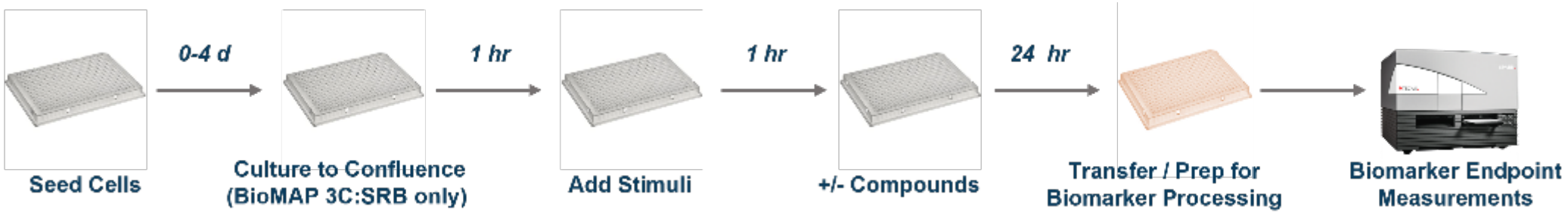
Section 1: BioMAP Assay Panel Method Description

- Panel of three assays
  - 3 human primary cell-based assays applied in serial for early triage of drug discovery leads
  - Human Biology Screening Funnel
- Commercial source
  - Eurofins Discovery
  - 3 of 148 assays within a commercial panel (BioMAP® Diversity PLUS)
- Historical data
  - Publications since 2004
  - EPA ToxCast program
- Application
  - Categorizing immunosuppression mechanisms



Berg (2021) The Future of Drug Discovery, Cell Chem Bio. https://doi.org/10.1016/j.chembiol.2021.01.010

Section 1: BioMAP Assay Panel Method Description



Assay Name	Cell Type		Stimulation		Endpoint
EC Cytotoxicity **BioMAP 3C:SRB*** BSK_3C_SRB**	Human Primary Endothelial Cells (HUVEC)	+	IL-1β + TNF-α + IFN-γ	→	Cytotoxicity (SRB – total protein)
EC Proliferation **BioMAP 3C:Prolif** BSK_3C_Proliferation	Human Primary Endothelial Cells (HUVEC)	+	(VEGF)	→	Proliferation (SRB – total protein)
T Cell Proliferation **BioMAP SAg:Prolif** BSK_SAg_Proliferation	Human Peripheral Blood Mononuclear Cells (PBMC)	+	Superantigens (TSST, SEB)	→	Proliferation (Alamar Blue)

** Commercial name, Eurofins Discovery*
*** Assay name in CompTox database*

Detailed methods in Shah (2017) Cell Chem Biol. https://doi.org/10.1016/j.chembiol.2017.06.003

Section 1: BioMAP Assay Panel Method Description

Assay Name	Mechanisms Detected	Example Carcinogens Detected	Key Characteristics***
EC Cytotoxicity **BioMAP 3C:SRB*** BSK_3C_SRB**	Alkylating agents DNA damaging agents Protein, RNA synthesis inhibitors	Chlorambucil Doxorubicin	KC2: Genotoxicity KC3: Genomic instability
EC Proliferation **BioMAP 3C:Prolif** BSK_3C_Proliferation	DNA replication inhibitors Inhibitors of microtubule function	Cisplatin Carboplatin Etoposide	KC7: Immunosuppression KC10: Proliferation
T Cell Proliferation **BioMAP SAg:Prolif** BSK_SAg_Proliferation	Immunosuppressants mTOR, calcineurin inhibitors, etc.	Azathioprine Cyclosporine A	KC7: Immunosuppression

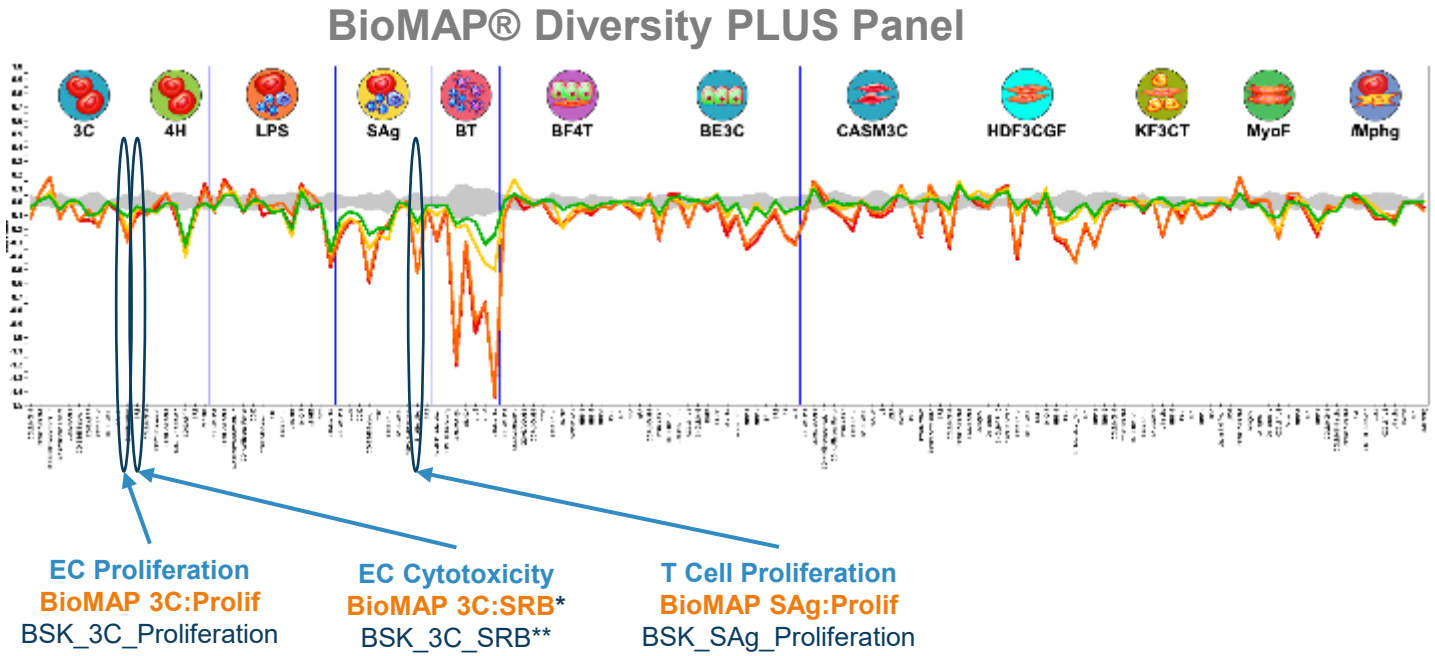
** Commercial assay name, Eurofins Discovery*
*** Assay name in CompTox database*
**** See slides 29 and 30 for AOPs and more details*

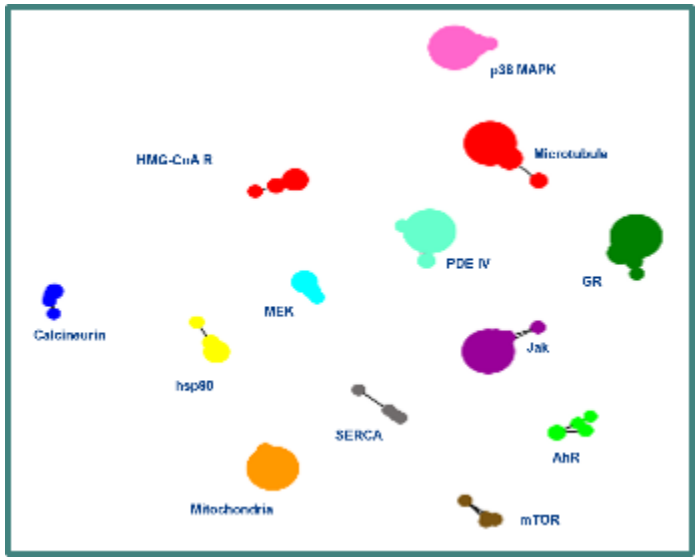
Section 1: BioMAP Assay Panel Method Description

- Advantages
  - High biological relevance to human safety
    - Human primary cells (vascular endothelial cells, peripheral blood mononuclear cells)
    - Three assays represent key biomarkers for toxicity signatures for acute toxicity, organ toxicity and immunosuppression (https://doi.org/10.3389/fdata.2019.00047)
    - Strength: classification of immunosuppressive mechanisms (distinguish immune cell-specific mechanisms from mechanisms relevant to acute toxicity and organ toxicity)
  - High quality, high throughput
    - Assays standardized, scaled, run under QA management process (>15 years)
    - Outstanding assay performance metrics
  - Well-managed donor-to-donor variability
    - Pooled donors, screened for pre-activation
  - Large body of historical data
    - Assays included in ToxCast Program

Section 1: BioMAP Assay Panel Method Description

- Proposed assay panel consists of 3 of the 148 assays from the Eurofins BioMAP Diversity PLUS panel
- This panel has been run in screening format (4 concentrations, singlicate testing) for > 15 years
- Large reference database has been employed for mechanism of action classification
- Note ability to distinguish GR agonists, JAK, calcineurin, mitochondria & mTOR inhibitors





https://doi.org/10.1186/s12885-022-09344-3

Section 1: BioMAP Assay Panel Method Description

- Advantages
  - 3 of 148 assays
    - Economical – but key informative assays for immunosuppression mechanisms
    - Distinguish immune cell specific mechanisms from those associated with acute toxicity, and organ toxicity
  - New format
    - 8 concentrations, triplicate replicates within experiment
    - Vehicle controls, key reference controls within experiment
    - Permits stand alone statistical tests (does not rely on historical data)

Section 1: BioMAP Assay Panel Method Description

- Limitations
  - Vehicle limitations
  - Agent limitations
    - Particle size (limited by pipet)
    - Light-sensitive agents
    - Volatile agents
    - Chemicals that interfere with Alamar blue
  - Compound metabolism
    - Cell types have limited metabolic capacity
  - Historical reference data limitations
    - Assays run in singlicate, data transformations incorporate vehicle controls (rather than keeping them separate), limited availability of full concentration-response data (required for no effect level determinations)

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- Berg, E.L., E.J. Kunkel, E. Hytopoulos, and I. Plavec. Characterization of compound mechanisms and secondary activities by BioMAP analysis. J Pharmacol Toxicol Methods, 2006, 53:67-74. https://doi.org/10.1016/j.vascn.2005.06.003

Section 2: Context of Use

Context of Use

A. How is your method intended to be used (e.g., chemical screening, hazard identification, potency evaluation, developing adverse outcome pathways (AOPs), point of departure identification for qualitative or quantitative risk assessment)?

- Chemical prioritization
- Hazard detection
- Mechanism of action
- Development of AOPs
- Point of departure
- Component assay for a combinatorial NAMs approach for replacement of animal testing (for human biomedical applications)

Context of Use

B. What regulatory testing need does your method address (e.g., replacing an animal assay, investigating mode of action or therapeutic target, or targeted endpoint of evaluation)?

- Investigating mode of action
- Supporting weight-of-evidence

Context of Use

C. What regulatory space does your method address (e.g., agrochemicals, pharmaceuticals, medical devices, cosmetics, food/food additives, industrial chemicals)?

- Agrochemicals
- Pharmaceuticals
- Devices (extracts, etc.)
- Cosmetics
- Food / food additives
- Industrial chemicals

Context of Use

D. Has data generated by your method been used for regulatory submissions?

- Not in the proposed format (8 concentrations, triplicate samples), but as components of the BioMAP Diversity PLUS panel, in screening mode (4 concentrations, singlicate samples).
- BioMAP data has been incorporated into regulatory submissions to support mechanistic questions and decisions (non-public information).

Section 3: Biological Relevance

Biological Relevance

A. Mechanistic understanding: How does the information provided by your method support known mechanistic knowledge of the carcinogenesis process (e.g., an AOP or toxicologically relevant biological process)?

- Assays have been mapped to mechanisms based on activity of reference compounds and drugs (see References on slide 10)
- See mapping to carcinogenesis key characteristics KCs (slide 30), and mapping to AOPs (slide 29)
- Assays provide mechanistic information that is downstream of AOP MIEs and early KEs, but closer to AOP outcomes

Biological Relevance

B. Reference compounds: What are well-characterized and understood compounds that can be used or were used to assess the scientific validity or transferability of your method?

- Key reference agents:
  - BioMAP 3C:SRB assay: cycloheximide, actinomycin D, digoxin, bortezomib, valinomycin
  - BioMAP 3C:Prolif assay: 5-fluorouracil, vincristine, cisplatin
  - BioMAP SAg:Prolif assay: sirolimus, cyclosporine, tacrolimus, mycophenolate, azathioprine
- List of 859 reference pharmaceuticals tested at Eurofins Discovery:
  - https://www.eurofinsdiscovery.com/biomap-recommended-benchmarks
  - https://docs.google.com/spreadsheets/d/1fH0lGBsk8Ym5_k7dtkpgsHCh6A-k84iH/edit?usp=share_link&ouid=115956796871474334710&rtpof=true&sd=true
- 1705 chemicals in CompTox Dashboard (ToxCast data):
  - https://comptox.epa.gov/dashboard/assay-endpoints/BSK_3C_SRB
  - https://comptox.epa.gov/dashboard/assay-endpoints/BSK_3C_Proliferation
  - https://comptox.epa.gov/dashboard/assay-endpoints/BSK_SAg_Proliferation

Biological Relevance

C. Comparison to existing laboratory animal methods: How does your method provide information that is equivalent or better than that from existing methods used for regulatory purposes? How does your method contribute to the reduction, refinement, or replacement of animal assays, and what complementary method development might be needed to comprehensively address carcinogenesis?

- Methods help provide confidence of human relevance (activity in human cell types). Testing in this method identifies chemicals that are not suitable for testing in animals having untoward mechanisms.
- Use of human primary cells provides the highest level of physiological relevance (retention of key pathways and pathway regulation).
- This method is highly complementary to orthogonal information gained from other assays, chemical properties, etc. and can help further characterize and categorize carcinogenesis mechanisms.

Section 4: Technical Characterization

Technical Characterization

A. How have the sources of variability (e.g., interference, culture conditions, technique, contaminants) been evaluated?

- Tracking of experimental variables and assay performance metrics (see slide 28) have been used to identify sources of variability.
- During assay development and as part of ongoing optimization, sources of variability such as cell and reagent lots, culture media, cell phenotypes (e.g., expression of activation markers), plate locations, laboratory personnel, equipment, various methodology options (e.g., cell banking methods, donor pools, plating methods) etc. have been tracked and associations with poor performance metrics or failure to pass acceptance criteria identified. In these cases, CAPA procedures have been used to investigate and mitigate technical variability (for example, by training and or improving processes).
- Methods to identify and mitigate cell contamination have been implemented, and quality management systems for ensuring quality of reagents, and performance of equipment as well as good documentation and training have helped reduce technical variability.
- Testing of various materials and vehicles has been performed to identify incompatible vehicles and materials
  - Compatible materials include small molecules, biologics, natural products, extracts, mixtures, nanomaterials, medical device extracts, cell preparations

Technical Characterization

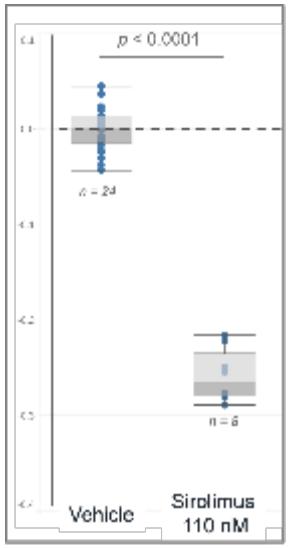
B. How has robustness (i.e., the ability of the method to be reproduced under different conditions or circumstances, without the occurrence of unexpected differences in the obtained results) been evaluated?

- The reproducibility of responses to positive and negative controls has been the primary method of assessing robustness, with assay performance metrics tracked continuously for > 15 years.
- Assay performance metrics are based on response to activation conditions (ratio of values with and without stimulation), response to reference control agents (e.g., colchicine, etc.), %CV of vehicle controls, and comparison to historical data. (See slide 28 for assay acceptance criteria details).

Technical Characterization

C. How has intra-laboratory reproducibility (i.e., the consistency of individual test results obtained within a laboratory using the same test protocol and test samples) been evaluated?

- Reproducibility has been assessed by measuring the response from testing a key reference control and comparing it to the vehicle control and evaluating for significance. In the figure below 8 independent experiments testing the reference control agent (sirolimus) in the T cell Proliferation assay (BioMAP SAg:Prolif) are shown.



Significance $p < 0.0001$ using unpaired t-test assuming unequal variances, t = 25.6; df = 30; CI between -0.232 and -0.273). Data are from independent experiments. The Y-axis represents a log-transformed ratio of the endpoint measurement (Alamar blue) for the drug-treated sample (n = 1) over vehicle controls (n ≥ 6). See methods slides 26-27.

Technical Characterization

D. How has transferability (i.e., the ability of the method to be accurately and reliably performed in different, competent laboratories) been evaluated (if relevant)?

- This has been partially addressed as the platform has been transferred to new facilities several times (including to a different US state) over the past 15 years. Transfer was measured by meeting quality management goals of 95% of assays (and plates) meeting assay acceptance criteria (note that data are not used if plates do not meet the assay acceptance criteria).

Supplemental Slides

Assay Methods

- Primary human cell types include human umbilical vein endothelial cells (HUVEC) and peripheral blood mononuclear cells, obtained from commercial sources. Stimulation conditions for the 3C system are IL-1b, 1 ng/ml; TNF-a, 5 ng/ml; and IFN-g, 20 ng/ml. Stimulation conditions for the T cell proliferation assay are a cocktail of superantigens (SAg) containing 0.02 ng/ml Staphylococcal enterotoxin B (SEB, Toxin Technologies, Sarasota, FL) and 0.02 ng/ ml Toxic shock syndrome toxin-1 (TSST-1, Toxin Technologies). HUVEC are cultured in 96-well plates to confluence prior to assay initiation. For the T cell proliferation assay, PBMC (7.5 x 104 cells/well) are added to wells. Assays are initiated by addition of compounds for 1 hr followed by addition of appropriate stimuli. Assay plates are then incubated at 37 degrees in 5% CO2 for 24hr (3C system). Cell proliferation is determined using sulforhodamine B (SRB) assay for adherent cell types or Alamar Blue for PBMC cells. For proliferation assays, individual cell types are cultured at sub-confluence and are read at specific times for different primary cell types (48, 72 or 96hr). After stimulation, plates and supernatants are harvested and biomarkers quantitated by ELISA and other methods (see below).
- Proliferation of PBMC (T cells) is assessed by Alamar blue reduction and proliferation of HUVEC is assessed by SRB staining. SRB is performed by addition of 0.1% sulforhodamine B to plates after fixation with 10% TCA, and reading wells at 560 nm (Ahmed et al., 1994). PBMC viability is assessed by adding Alamar blue to PBMC that had been cultured for 24 hours in the presence of stimuli and compounds and measuring its reduction after 8 hr.

Data Analysis Methods

- Measurement values for each endpoint measurement in a treated sample is divided by the mean value from eight DMSO control samples (from the same plate) to generate a ratio. All ratios were then log10 transformed. Significance prediction envelopes were calculated for historical controls (95%). Endpoints are identified as ‘‘annotated’’ if the log10 ratio values fall outside the 95% significance envelope at 2 or more concentrations, at least one of which has a minimum effect size of at least 20%. Overtly cytotoxic com- pounds are identified as generating profiles with one or more of the following readouts below the indicated thresholds: SRB < -0.3, PI or PBMC cytotoxicity <-0.3 in one or more systems. For analysis of profile similarities, overtly cytotoxic compound profiles are removed. Similar profiles are identified as those having the highest Pearson correlation above a selected threshold > 0.7. For analysis of signatures, a large database of BioMAP profiles was filtered for those profiles meeting the following criteria (1) Log ratio of PGE2 in the LPS system of >0.12 and TNFa in the LPS system below 0.09 (the upper limit of the 95% confidence envelope) or (2) Log ratio of sIL-2 >0.12 and sIL-6 > 0.12. Test agents that met these criteria at two or more concentrations were selected.

Assay Acceptance Criteria

- A BioMAP assay includes the multi-parameter data sets generated by the BioMAP platform for agents tested in the systems that make up the Diversity PLUS panel. Assays contain drug controls (e.g., legacy control test agent colchicine), negative controls (e.g., non-stimulated conditions), and vehicle controls (e.g., DMSO) appropriate for each system. BioMAP assays are plate-based, and data acceptance criteria depend on both plate performance (% CV of vehicle control wells) and system performance across historical controls for that system. The QA/QC Pearson Test is performed by first establishing the 1% false negative Pearson cutoff from the reference dataset of historical positive controls. The process iterates through every profile of system biomarker readouts in the positive control reference dataset, calculating Pearson values between each profile and the mean of the remaining profiles in the dataset. The overall number of Pearson values used to determine the 1% false negative cutoff is the total number of profiles present in the reference dataset. The Pearson value at the one percentile of all values calculated is the 1% false negative Pearson cutoff. A system will pass if the Pearson value between the experimental plate's negative control or drug control profile and the mean of the historical control profiles in the reference dataset exceeds this 1% false negative Pearson cutoff. Overall assays are accepted when each individual system passes the Pearson test and 95% of all project plates have % CV <20%.

AOPs

- Relevant AOPs

Assay Name	AOP ID	AOP Name
EC Cytotoxicity **BioMAP 3C:SRB*** BSK_3C_SRB**	509	Nrf2 inhibition leading to vascular disrupting effects through activating apoptosis signal pathway and mitochondrial dysfunction
EC Proliferation **BioMAP 3C:Prolif** BSK_3C_Proliferation	263	Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased cell proliferation
T Cell Proliferation **BioMAP SAg:Prolif** BSK_SAg_Proliferation	154	Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response
	315	Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response

Key Characteristics

- Relevant Key Characteristics

Assay Name	Number	Basis
EC Cytotoxicity **BioMAP 3C:SRB*** BSK_3C_SRB**	KC2: Genotoxicity KC3: Genomic instability	Agents with these mechanisms are active: Alkylating agents, DNA damaging agents, Protein, RNA synthesis inhibitors. Examples: chlorambucil, doxorubicin.
EC Proliferation **BioMAP 3C:Prolif** BSK_3C_Proliferation	KC7: Immunosuppression KC10: Proliferation	Agents with these mechanisms are active: DNA replication inhibitors, inhibitors of microtubule function. Examples: cisplatin, carboplatin, etoposide.
T Cell Proliferation **BioMAP SAg:Prolif** BSK_SAg_Proliferation	KC7: Immunosuppression	Agents with these mechanisms are active: Immunosuppressants mTOR, calcineurin inhibitors. Examples: azathioprine, cyclosporine A.

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