BSK_IMphg_MCP1

1. General Information

1.1 Assay Title: BioMAP Diversity Plus: IMphg Assay for Monocyte Chemoattractant Protein-1 (MCP1) Biomarker Activity

1.2 Assay Summary: BSK_IMphg is a cell-based, multiplexed-readout assay that uses venular endothelial cells and macrophages, a human vascular primary cell, with measurements taken at 24 hours after chemical dosing in a microplate: 96-well plate. BSK_IMphg_MCP1 is an assay component measured in the BSK_IMphg assay. It measures MCP-1 antibody related to regulation of gene expression using ELISA technology. Data from the assay component BSK_IMphg_MCP1 was analyzed at the endpoint, BSK_IMphg_MCP1, in the positive analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of binding reporter, gain or loss-of-signal activity can be used to understand protein changes. To generalize the intended target to other relatable targets, this assay endpoint is annotated to the 'cytokine' intended target family, where the subfamily is 'chemotactic factor'.

1.3 Date of Document Creation: September 05 2024

1.4 Authors and Contact Information:

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1.5 Assay Source: Bioseek is a division of DiscoveRx Corporation and developed the BioMAP system providing uniquely informative biological activity profiles in complex human primary cell systems.

1.6 Date of Assay Development: For date of assay development, see *Section 6: Bibliography*.

1.7 References: For complete list of references, see *Section 6: Bibliography*.

1.8 Proprietary Elements: The BioMAP platform uses both proprietary primary human cell-based assay design and integrated bioinformatics analysis platform to generate signature profiles.

1.9 Assay Throughput: 96-well plate. Assays were initiated by addition of chemical samples for 1 h followed by addition of appropriate stimuli. Assay plates were then incubated for 24 h unless otherwise indicated. The MyoF system was stimulated for 48 h, and the BT system was stimulated for either 72 h (soluble readouts) or 6 d (for measurement of secreted IgG). Concentrations of stimuli were as follows: cytokines (IL-1beta, 1 ng/mL, Peprotech 200–01B; TNFalpha, 5 ng/mL, Peprotech 300–01A; IFNgamma, 20 ng/mL, Peprotech 300–02; IL-4, 5 ng/mL, 200–04), activators (histamine, 10 uM, Sigma H7125; SAg, 20 ng/mL or LPS, 2 ng/mL, Sigma L7770), growth factors (TGF-beta, 5 ng/mL, R&D Systems 240-B/CF; EGF, Peprotech AF-100-15; basic-FGF, ThermoScientific 13256029; PDGF-BB, 10 ng/mL, Peprotech 100–14B; Zymosan, 10 ug/mL, Invivogen tlrl-zyn; Anti-IgM, 500 ng/mL). Superantigens (SAg), staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 (TSST-1) (staphylococcal enterotoxin F) from Staphylococcus aureus, and lipopolysaccharide (LPS) from Salmonella enteritidis were obtained from Sigma. The number of lymphocytes or macrophages added to the SAg, LPS, BT and Mphg systems were as follows for 96-well format: B cells (2.5 × 104), PBMC (7.5 × 104 cells/well for LPS and SAg systems or 2.5 × 104 cells/ well for BT system) or macrophages (7.5 × 104 cells/well). After stimulation, plates and supernatants were harvested and biomarkers quantitated by ELISA and other methods.

1.10 Status: The assay is fully developed, and data are publicly available in ToxCast's invitroDB.

1.11 Abbreviations:

AIC: Akaike Information Criterion
AOP: Adverse Outcome Pathway
CV: Coefficient of Variation
DMSO: Dimethyl Sulfoxide
ToxCast: US EPA's Toxicity Forecaster Program
tcpl: ToxCast Data Analysis Pipeline R Package
SSMD: Strictly Standardized Mean Difference

2. Test Method Description

2.1 Purpose: Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a chemoattractant cytokine (chemokine) that regulates the recruitment of monocytes and T cells into sites of inflammation. MCP-1 is categorized as an inflammation-related activity in the lMphg system modeling macrophage-driven Th1 vascular inflammation.

The BioMAP Diversity Plus panel includes 12 assays encompassing 148 endpoints particularly enriched with capabilities to detect modulators and effectors of vascular and immune biology. This panel has been extensively used in pharmaceutical and consumer products research for characterization of product candidates. Platform includes the T cell activation system (SAg) measuring multiple endpoints modulated by a cocktail of superantigens, and the B and T cell autoimmunity assay (BT) for T cell-dependent B cell activation and antibody production as key modulators of the innate and adaptive immune response, respectively. Additional assays include models of vascular inflammation, monocyte activation, lung inflammation and fibrosis, cardiovascular inflammation, and wound healing.

2.2 Scientific Principles: The BioMAP Diversity Plus panel can be used to model complex tissue and disease biology of organs (vasculature, immune system, skin, lung) and general tissue biology. Use of the BioMAP panel of human primary cell systems as (patho)physiologically relevant screening assays for evaluating adverse effects has previously been demonstrated through testing pharmaceuticals and clinical candidates as well as environmental chemicals in the EPA's ToxCast program. Beyond immunosuppression, significant bioactivity in human primary cells that correlated with mechanisms of action that may indicate potential for adverse effects in vivo. Future studies measuring the effects of environmental chemicals associated with immunotoxicity in the BioMAP co-culture systems, along with other human cell-based models of immune related effects including inflammation, may be useful for better defining the bioactivity profiles of non-pharmaceutical immunotoxic compounds and understanding mechanisms of putative immune related effects in human populations.

2.3 Experimental System: adherent venular endothelial cells and macrophages primary cell used. Primary human cell types used in BioMAP systems and their stimuli included the following: 3C System (HUVEC/IL-1beta, TNFalpha and IFNgamma), 4H System (HUVEC/IL-4 and histamine), LPS System (PBMC and HUVEC/LPS), SAg System (PBMC and HUVEC/TCR ligands), BT System (CD19+B cells and PBMC/anti-IgM + TCR ligands), BE3C System (bronchial epithelial cells/IL-1beta, TNFalpha and IFNgamma), BF4T System (bronchial epithelial cells and human dermal fibroblasts/TNFalpha and IL-4), HDF3CGF System (human dermal fibroblasts/IL-1beta, TNFalpha, IFNgamma, EGF, basic-FGF and PDGF-BB), KF3CT System (keratinocytes and dermal fibroblasts/IL-1beta, TNFalpha and IFNgamma), CASM3C System (coronary artery smooth muscle cells/IL-1beta, TNFalpha and IFNgamma), MyoF System (differentiated lung myofibroblasts/TNFalpha and TGFbeta), Mphg System (HUVEC and macrophages/TLR2 ligands)

2.4 Metabolic Competence: Primary cell types included in the BioMAP platform retain regulatory processes of their in vivo counterparts. All primary human cells utilized were obtained via commercially available sources and were used at early passage (≤ P4) or without passaging (in the case of PBMC and B cells) to minimize adaptation to cell culture and preserve physiological signaling responses. Xenobiotic biotransformation potential has not been characterized.

2.5 Exposure Regime: The levels of cell surface (or secreted, indicated by the prefix "s") biomarker endpoints were measured by ELISA. Overt cytotoxicity to cells in confluent adherent cultures (all systems other than the BT system) was assessed by measuring total protein levels using sulforhodamine B (SRB) staining in parallel cultures at the time of biomarker measurements, indicated as SRB endpoints. For proliferation assays for adherent cell types, individual cell types are cultured at sub-confluence and relative cell numbers quantified by SRB staining at time points optimized for each system (48 h: 3C and CASM3C systems; 72 h: BT and HDF3CGF systems; 96 h: SAg system). SRB was performed by staining cells with 0.1% SRB after fixation with 10% TCA and reading wells at 560 nm. Viability and proliferation of PBMC (T cells) was quantified by Alamar Blue reduction for the SAg and BT systems. For PBMC viability (referred to as PBMC Cytotoxicity within the assay endpoint names), cells were plated (75,000/well in a 96-well plate) and then chemical samples added for 1 h before addition of activators, SEB and TSST-1 (20 ng/mL final concentration each). Cells were incubated for 90 h. Then, Alamar Blue (20 uL/well) (Invitrogen, Cat DAL1100) was added for 6 h, and the plates were read with a fluorescence microplate

reader at 546/580 nm (excitation/emission). For PBMC proliferation, cells were plated and activated as above but incubated for only 16 h prior to addition of Alamar Blue. Plates were read after 6 h.

ASSAY DESIGN SUMMARY	
Nominal number of tested concentrations: 4	Target (nominal) number of replicates: 1
Standard minimum concentration tested: 2.2 µM	Standard maximum concentration tested: 60 µM
Key positive control: colchicine	Neutral vehicle control: DMSO
Baseline median absolute deviation for the assay (bmad): 0.023	
Response cutoff threshold used to determine hit calls: 0.079	
Detection technology used: ELISA (Fluorescence)	

2.6 Response: The Macrophage Activation (lMphg) system models chronic inflammation of the Th1 type and macrophage activation responses. This system is relevant to inflammatory conditions where monocytes play a key role including atherosclerosis, restenosis, rheumatoid arthritis, and other chronic inflammatory conditions.

2.7 Quality and Acceptance Criteria: Each assay may utilize different acceptance criteria and quality assurance methods as it pertains to the individual assay platform and implementation. Pre-processing transformations may indicate issues in plates or wells by setting well quality (wllq) values to 0. Analytical QC calls per sample and substance can also be considered to understand the applicability domain of the chemicals for screening.

2.8 Technical Limitations: ToxCast data can provide initial (screening) information about the capacity for a chemical to illicit a biological response; caution is advised with extrapolation of these results to organism-level responses. The potential for a chemical to elicit adverse health outcomes in living systems is a function of multiple factors, and this assay is not intended to provide predictive details regarding long term or indirect adverse effects in complex biological systems but can aid in the prioritization of compound selection for more resource intensive toxicity studies. See *Section 4.4*. for more information on the chemical applicability of the assay.

2.9 Related Assays: For related assays, consult the following assay lists or intended target families. This assay is present in the following assay lists:

NA

Additionally, this assay was annotated to the intended target family of cytokine.

3. Data Interpretation

The ToxCast Data Analysis Pipeline (tcpl) R package includes processing functionality for two screening paradigms: (1) single-concentration ("SC") and (2) multiple-concentration ("MC") screening. SC screening consists of testing chemicals at one concentration, often for the purpose of identifying potentially active chemicals to test in the multiple-concentration format. MC screening consists of testing chemicals across a concentration range, such that the modeled activity can give an estimate of potency, efficacy, etc. MC data is the focus of this documentation, with SC data processing metrics to be incorporated in the future.

3.1 Responses captured in prediction model: See *Section 2.6* for additional information on responses measured.

3.2 Data Analysis: Measurement values for each well (one biomarker per well) were divided by the mean value from 8 DMSO control samples (from the same plate) to generate a ratio. All ratios were then log10 transformed. Historical controls are the log10-ratios of DMSO control wells that are collected over time (23 experimental runs collected over 2 years). The transformed ratios for the 12-assay BioMAP panel were received by the EPA and loaded into the ToxCast database, invitrodb under the BioSeek assay source identifier, abbreviated as BSK. BSK was used for continuity in invitrodb and in public versions of ToxCast data despite more recent changes in the name and ownership of the assay technology (now owned by Eurofins Discovery and referred to as BioMAP systems).

Prior to the data processing, all the data must go through pre-processing to transform the heterogeneous data into a uniform format before it can be loaded into a database. Level 0 pre-processing is done in R by vendor/dataset-specific scripts with all manual transformations to the data documented with justification. Common examples of manual transformations include fixing a sample ID typo or changing well quality value(wllq) to 0 after identifying problems such a plate row/column missing an assay reagent.

Once data is loaded into the database, tcpl utilizes generalized processing functions provided to process, normalize, model, qualify, and visualize the data. To promote reproducibility, all method assignments must occur through the database and should come from the available list of methods for each processing level. Assigned multiple concentration processing methods include:

Level 2: Component-specific corrections include:

1: none (Use corrected response value (cval) as is; cval = cval. No additional mc2 methods needed for component-specific corrections.)

Level 3: Endpoint-specific normalization include:

1: none (Set the corrected response value (cval) as the normalized response value (resp); cval = resp. No additional mc3 methods needed for endpoint-specific normalization.)

Level 4: Baseline and required tcplFit2 parameters defined by:

1: bmad.aeid.lowconc.twells (Calculate the baseline median absolute value (bmad) as the median absolute deviation of normalized response values (rep) for test compound wells (wllt = t) with concentration index (cndx) equal to 1 or 2. Calculate one standard deviation of the normalized response for test compound wells (wllt = t) with a concentration index (cndx) of 1 or 2; onesd = sqrt(sum((resp - mean resp)^2)/sample size - 1). Onesd is used to establish BMR and therefore required for tcplfit2 processing.)

Level 5: Possible cutoff thresholds, where higher value for endpoint is selected, include:

1: bmad3 (Add a cutoff value of 3 multiplied by the baseline median absolute deviation (bmad) as defined at Level 4.), 4: log10_1.2 (Add a cutoff value of log10(1.2). Typically for fold change data.), 15: loec.coff (Method not yet updated for tcpl implementation: Identify the lowest observed effective concentration (loec) compared to baseline.)

Level 6: Cautionary flagging include:

5: modl.directionality.fail (Flag series if model directionality is questionable, i.e. if the winning model direction was opposite, more responses (resp) would have exceeded the cutoff (coff). If loss was winning directionality (top < 0), flag if count(resp < -1*coff) < 2*count(resp > coff). If gain was winning directionality (top > 0), flag if count(resp > coff) < 2*count(resp < -1*coff).), 6: singlept.hit.high (Flag single-point hit that's only at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at the highest tested concentration tested.), 7: singlept.hit.mid (Flag single-point hit that's not at the highest conc tested, where series is an active hit call (hitc >= 0.9) with the median response observed above baseline occurring only at one concentration and not the highest concentration tested.), 8: multipoint.neg (Flag multi-point miss, where series is an inactive hit call (hitc < 0.9) with multiple median responses observed above baseline.), 9: bmd.high (Flag series if modeled benchmark dose (BMD) is greater than AC50 (concentration at 50 percent maximal response). This is indicates high variability in baseline response in excess of more than half of the maximal response.), 10: noise (Flag series as noisy if the quality of fit as calculated by the root mean square error (rmse) for the series is greater than the cutoff (coff); rmse > coff.), 11: border (Flag series if borderline activity is suspected based on modeled top parameter (top) relative to cutoff (coff); |top| <= 1.2(coff) or |top| >= 0.8(coff).), 13: low.nrep (Flag series if the average number of replicates per concentration is less than 2; nrep < 2.), 14: low.nconc (Flag series if 4 concentrations or less were tested; nconc <= 4.), 15: gnls.lowconc (Flag series where winning model is gain-loss (gnls) and the gain AC50 is less than the minimum tested concentration, and the loss AC50 is less than the mean tested concentration.), 17: efficacy.50 (Flag low efficacy hits if series has an active hit call (hitc >= 0.9) and efficacy values (e.g. top and maximum median response) less than 50 percent; intended for biochemical assays. If hitc >= 0.9 and coff >= 5, then flag when top < 50 or max_med < 50. If hitc >= 0.9 and coff < 5, then flag when top < log2(1.5) or max_med < log2(1.5).), 18: ac50.lowconc (Flag series with an active hit

call (hitc >= 0.9) if AC50 (concentration at 50 percent maximal response) is less than the lowest concentration tested;if hitc >= 0.9 and ac50 < 10^logc_min, then flag.)

The following is an aggregate endpoint summary of the number of samples and chemicals tested, as well as active or inactive hit calls (hitc) and predicted winning models for all samples tested in this endpoint.

SAMPLE AND CHEMICAL COVERAGE	
Number of samples tested: 347	Number of chemicals tested: 164

ACTIVITY HIT CALLS		
Active hit count: hitc≥0.9 45	Inactive hit count: 0≤hitc<0.9 302	NA hit count: *hitc<0* *0*

WINING MODEL SELECTION	
Number of sample-assay endpoints with winning *hill* model:	7
gain-loss (gnls) model:	33
power(pow) model:	23
linear-polynomial (poly1) model:	121
quadratic-polynomial(poly2) model:	86
exponential-2 (exp2) model:	18
exponential-3 (exp3) model:	2
exponential-4 (exp4) model:	26
exponential-5 (exp5) model:	31

For each concentration series, several point-of-departure (POD) estimates are calculated for the winning model. The major estimates include: (1) the activity concentration at the specified benchmark response (BMR) (bmd), (2) the activity concentration at 50% of the maximal response (ac50), (3) the activity concentration at the efficacy cutoff (acc), (4) the activity concentration at 10% of the maximal response, and (5) the concentration at 5% of the maximal response.

3.3 Prediction Model: All statistical analyses were conducted using R programming language, employing the tcpl package to generate model parameters and confidence intervals. Each chemical concentration response series is fit to ten predictive models, encoded by the dependency package tcplfit2. The models include the constant, Hill, gain-loss, two polynomials (i.e. linear and quadratic), power, and four exponential variants. The polynomials, power, and exponential models are all based on BMDExpress2. The winning model (modl) is selected based on the lowest AIC value and is used to determine the activity (or hit call) for the concentration series. If two models have equal AIC values, then the simpler model (i.e. model with fewer parameters) wins. In invitrodb, levels 4 and 5 capture model fit information. mc4 captures summary values calculated for each concentration series, whereas mc4_param stores the estimated model parameters for all models fit to data in long format. mc5 captures the winning model selected and the activity hit call, whereas mc5_param stores the estimated model parameters for the selected winning model in long format. Activity for each concentration-response series is determined by calculating a continuous hit-call for the winning model, which is the product of three proportional weights. The first weight reflects whether there is at least one median response outside the efficacy cutoff band. Second, the top (or maximal change in the predicted response) is larger than the cutoff. The last weight reflects whether the AIC of the winning model is less than the constant model, i.e. the winning model is better fit than a flat line.

The continuous hit call value (hitc), fit category (fitc), and cautionary flags (mc6) can be used to understand the goodness-of-fit, enabling the user to decide the stringency with which to filter and interpret results. Hitc may

be further binarized into active or inactive, depending on the level of stringency required by the user; herein, hitc greater than or equal to 0.90 is active, hitc between 0 and 0.90 is inactive, and hitc less than 0 is not applicable, but different thresholds may be used. Fitc was summarize curve behavior relative activity, efficacy, and potency comparisons between the AC50 and the concentration range screened. Cautionary flags on fitting were developed in previous versions of tcpl and have been stored at level 6. These flags are programmatically generated and indicate characteristics of a curve that need extra attention or potential anomalies in the curve or data. Users may review these filtered groupings to understand high-confidence curves.

3.4 Software: The ToxCast Data Analysis Pipeline (tcpl) is an R package that manages, curve-fits, plots, and stores ToxCast data to populate its linked MySQL database, invitrodb. Data for invitrodb v4.2 was processed using the tcpl v3.2. See *Section 7: Supporting Information* on the ToxCast program and tcpl R package.

4. Test Method Performance

4.1 Robustness: The following assay performance metrics surmise the robustness of the method i.e. the reliability of the experimental results and the prediction capability of the model used.

NEUTRAL CONTROL (well type = "n")	
Neutral control well median response value, by plate: *nmed*	NA
Neutral control median absolute deviation, by plate: *nmad*	NA
Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100*	NA%
POSITIVE CONTROL (well type = "p")	
Positive control well median response value, by plate: *pmed*	NA
Positive control well median absolute deviation, by plate: *pmad*	NA
Z Prime Factor for median positive and neutral control across all plates: *(1 - ((3 * (pmad + nmad)) / abs(pmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for positive compared to neutral control wells: *((pmed - nmed) / sqrt($pmad^2$ + $nmad^2$)*	NA
Positive control signal-to-noise: *((pmed-nmed)/nmad)*	NA
Positive control signal-to-background: *(pmed/nmed)*	NA
NEGATIVE CONTROL (well type = "m")	
Negative control well median, by plate: *mmed*	NA
Negative control well median absolute deviation value, by plate: *mmad*	NA
Z Prime Factor for median negative and neutral control across all plates: *(1 - ((3 * (mmad + nmad)) / abs(mmed - nmed))*	NA
Strictly standardized mean difference (SSMD) for negative compared to neutral control wells: *((mmed - nmed) / sqrt($mmad^2$ + $nmad^2$)*	NA
Signal-to-noise (median across all plates, using negative control wells): *((mmed-nmed)/nmad)*	NA
Signal-to-background (median across all plates, using negative control wells): *(mmed/nmed)*	NA

4.2 Reference Chemical Information: Reference chemical curation is ongoing, and this section will be updated as more information becomes available.

4.3 Performance Measures and Predictive Capacity: The performance and predictivity for a given assay may be evaluated with a variety of performance statistics but is dependent upon available data. Predictive capacity (i.e. false negative, false positive rates) will be assessed when reference chemical information is available. Ideally,

assays will have sufficient data on reference chemicals (i.e. positive and negative controls) to enable estimation of accuracy statistics, such as sensitivity and specificity.

ToxCast targets may align to a range of event types in the Adverse Outcome Pathway (AOP) framework, however each assay technology may have specific limitations, which may require user discretion for more complex interpretations of the data.

The median root mean squared error (RMSE) across all winning models for active hits was calculated as: 31.

4.4 Chemical Library Scope and Limitations: The ToxCast Chemical Library was designed to capture a large spectrum of structurally and physicochemically diverse compounds. This chemical inventory incorporates toxicity data-rich chemicals, chemicals spanning major use-categories, and chemicals with exposure potential, including but not limited to pesticides, antimicrobials, fragrances, green chemistry alternatives, food additives, toxicity reference compounds and failed pharmaceuticals. In addition to environmental or exposure concerns, chemical selection criteria also consider practical constraints, such as commercial availability, dimethyl sulfoxide (DMSO) solubility and stability, and suitability for testing in automated or semi-automated systems (e.g., low volatility and moderate LogP values). Under these constraints, there were three major, interrelated drivers for chemical selection: availability of animal toxicity data or mechanistic knowledge, exposure potential, and EPA regulatory interest. The first driver would provide the necessary *in vivo* and mechanistic data to anchor and validate subsequent prediction modeling efforts, whereas the latter two were intended to provide coverage of the chemical landscape to which humans and ecosystems are potentially exposed and for which toxicity data are mostly lacking. Analytical QC calls per sample and substance should be considered to understand the applicability domain of the chemicals for screening.

5. Potential Regulatory Applications

5.1 Context of Use: Examples of end use scenarios could include, but are not limited to:

- *Support Category Formation and Read-Across:* The outcomes from the assay could be used to substantiate a hypothesis for grouping substances together for the purposes of read-across,
- *Priority Setting:* The assay might help prioritize substances within an inventory for more detailed evaluation,
- *Screening Level Assessment of a Biomarker or Mechanistic Activity or Response:* The screening level assessment may be sufficient to identify a hazard and provide a gauge of potency; or
- *Integrated approaches to testing and assessment (IATA):* The assay may form one component of an IATA.

6. **Bibliography:** Berg EL. Human Cell-Based in vitro Phenotypic Profiling for Drug Safety-Related Attrition. Front Big Data. 2019 Dec 11;2:47. doi: 10.3389/fdata.2019.00047. PMID: 33693370; PMCID: PMC7931891., Kunkel EJ, Plavec I, Nguyen D, Melrose J, Rosler ES, Kao LT, Wang Y, Hytopoulos E, Bishop AC, Bateman R, Shokat KM, Butcher EC, Berg EL. Rapid structure-activity and selectivity analysis of kinase inhibitors by BioMAP analysis in complex human primary cell-based models. Assay Drug Dev Technol. 2004 Aug;2(4):431-41. PubMed PMID: 15357924.

7. **Supporting Information:**

More information on the ToxCast program can be found at: https://www.epa.gov/chemical-research/toxicity-forecasting. The most recent version of downloadable data can be found at: https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data. The ToxCast Data Analysis Pipeline (tcpl) R package is available on CRAN or GitHub. Check out tcpl's vignette for comprehensive documentation describing ToxCast data processing, retrieval, and interpretation.