# CCTE_Mundy_HCI_hNP1_Casp3_7

## 1. General Information

*1.1* Assay Title: CCTE's Caspase-Glo 3/7 Assay in human neuroprogenitor cells for Apoptosis, Mundy Lab

*1.2* Assay Summary: CCTE_Mundy_HCI_hNP1_Casp3_7 is a multiplexed, cell-based-readout assay that uses neural stem cells derived from a neuroepithelial cell lineage of WA09 embryonic cells with measurements taken at 26.5 hours after chemical dosing in a microplate: 96-well plate. CCTE_Mundy_HCI_hNP1_Casp3_7 is one component of the CCTE_Mundy_HCI_hNP1_Casp3_7 assay. It measures apoptosis related to caspase activation using Luminescent Reporter. Data from the assay component CCTE_Mundy_HCI_hNP1_Casp3_7 was analyzed at the endpoint CCTE_Mundy_HCI_hNP1_Casp3_7_gain in the positive analysis fitting direction relative to DMSO as the negative control and baseline of activity. Using a type of viability reporter, gain -of-signal activity can be used to understand viability effects.

*1.3* Date of Document Creation: September 05 2024

*1.4* Authors and Contact Information:

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*1.5* Assay Source: The Mundy Lab at the EPA Center for Computational Toxicology and Exposure utilizes high content imaging to characterize chemical effects in neurodevelopment.

*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: CellTiter-Glo 2.0 Assay (Promega G9242), and apoptosis, Caspase-Glo 3/7 Buffer (Promega G810A), and Caspase-Glo 3/7 Substrate (Promega G811A), are patented. This assay is considered part of the developmental neurotoxicity in vitro battery. See the OECD Initial Recommendations on Evaluation of Data from the Developmental Neurotoxicity (DNT) In Vitro Testing Battery document: https://one.oecd.org/document/ENV/CBC/MONO(2023)13/en/pdf.

*1.9* Assay Throughput: 96-well plate. The methods described here are described for a 96 well plate format. Typically, 18 plates can be made in one culture (Six for Proliferation, six for apoptosis, six for cytotoxicity), which allows testing 16 compounds in triplicate technical replicates. With thawing and expansion plating can occur every 14 days, allowing 32 compounds in triplicate at multiple concentrations to be screened per month

*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: Changes in the caspase activation are indicative of cell death.

The CCTE_Mundy_HCI_hNP1_Casp3_7 assay is designed to investigate changes in apoptosis in response to chemical exposure in human neuroprogenitor cells (hNP1) using a high-content imaging (HCI) technology. Apoptosis is one of several key processes of neurodevelopment. This endpoint measures intensity of luminescent signal produced by caspase 3/7 cleavage of a detection reagent. The signal produced is proportional to the number of apoptotic cells. An increase as compared to control is indicative of increased apoptosis.

2.2 Scientific Principles: Apoptosis is a form of programmed cell death that also plays a critical role in development of the nervous system. In particular, cells that fail to become integrated into neural networks during neural development often undergo apoptosis. Chemical exposure can result in changes in apoptosis and such changes may, like changes in proliferation, alter the number of cells in the nervous system, resulting in developmental

neurotoxicity. Cells are plated onto 96 well clear and opaque plates and allowed 40-44 hr for attachment to the substrate and recovery. Between 40 and 44 hours all plates are exposed to the chemical ("Dose"). Twenty-four hours after exposure, cells are either fixed or assayed.

2.3 Experimental System: adherent hNP1 primary cell used. Human neural progenitor cells (hNP1) were obtained as cryopreserved cells from ArunA Biomedical, Athens Georgia. Cells are plated on pre-coated poly-L-ornithine/laminin clear bottom and opaque 96 well plates (day 0).

2.4 Metabolic Competence: Metabolic competence has not been characterized extensively to date in this assay.

2.5 Exposure Regime: Human neural progenitor cells (hNP1) were obtained as cryopreserved cells from ArunA Biomedical, Athens Georgia. Cells are plated on pre-coated poly-L-ornithine/laminin clear bottom and opaque 96 well plates (day 0). Plates are placed in a 37oC, 5% CO2 incubator for 40 to 44 hours at which time plates are removed from the incubator and 10 uL of a freshly made chemical solution is added to each well. Apoptosis was measured based on detection of activated caspase-3/7 using the Caspase-Glo 3/7 assay kit (Promega). Twenty-four hours after chemical treatment, 50 ul of Caspase-Glo 3/7 assay buffer was added to each well and the plate incubated for 30 min at room temperature. Luminescence was then recorded in each well using a FLUOstar Optima plate reader.

| ASSAY DESIGN SUMMARY | |
|---|---|
| Nominal number of tested concentrations: 10 | Target (nominal) number of replicates: 3 |
| Standard minimum concentration tested: 0.00313 µM | Standard maximum concentration tested: 3000 µM |
| Key positive control: staurosporine (0.1 uM) | Neutral vehicle control: DMSO or water |
| Baseline median absolute deviation for the assay (bmad): 4.659 | |
| Response cutoff threshold used to determine hit calls: 30 | |
| Detection technology used: Luminescent Reporter (Fluorescence) | |

2.6 Response: The Caspase-Glo 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. These members of the cysteine aspartic acid-specific protease (caspase) family play key effector roles in apoptosis in mammalian cells. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Addition of Caspase-Glo 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

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:

DNT-IVB: Assays included in the developmental neurotoxicity in vitro battery, see https://www.regulations.gov/document/EPA-HQ-OPP-2020-0263-0006

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

**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: With the lid removed from the plate, read in the BMG FLUOstar OPTIMA Fluorescence/Luminescence Microplate Reader following the instructions in OP-NHEERL/ISTD/SBB/TMF/2013-007-r0 using the "Caspase Glo" protocol (set "Gain" at 3500). All data and calculations are recorded in an Excel spreadsheet and stored in a laboratory drive.

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:*

5: resp.pc (Calculate the normalized response (resp) as a percent of control, i.e. the ratio of the difference between the corrected (cval) and baseline (bval) values divided the difference between the positive control (pval) and baseline (bval) values multiplied by 100; resp = (cval-bval)/(pval-bval)*100.), 6: resp.multneg1 (Multiply the normalized response value (resp) by -1; -1*resp.), 11: bval.apid.nwlls.med (Calculate the baseline value (bval) as the plate-wise median, by assay plate ID (apid), of the corrected values (cval) for neutral control wells (wllt = n).), 32: pval.zero (Set the positive control value (pval) to 0; pval = 0.)

*Level 4: Baseline and required tcplFit2 parameters defined by:*

2: bmad.aeid.lowconc.nwells (Calculate the baseline median absolute value (bmad) as the median absolute deviation of normalized response values (resp) for neutral control wells (wllt = n). Calculate one standard deviation of the normalized response for neutral control wells (wllt = n); 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.), 19: pc30 (Add a cutoff value of 30. Typically for percent of control data.)

*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.), 20: no.med.gt.3bmad (Flag series where no median response values are greater than baseline as defined by 3 times the baseline median absolute deviation (bmad); nmed_gtbl_pos and nmed_gtbl_neg both = 0, where nmed_gtbl_pos/_neg is the number of medians greater than 3*bmad/less than -3*bmad.)

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: 346 | | Number of chemicals tested: 307 |
| **ACTIVITY HIT CALLS** | | |
| Active hit count: hitc≥0.9<br>85 | Inactive hit count: 0≤hitc<0.9<br>261 | NA hit count: *hitc<0*<br>*0* |
| **WINING MODEL SELECTION** | | |
| Number of sample-assay endpoints with winning *hill* model: | 20 | |
| *gain-loss (gnls)* model: | 42 | |
| *power(pow)* model: | 30 | |
| *linear-polynomial (poly1)* model: | 70 | |
| *quadratic-polynomial(poly2)* model: | 45 | |
| *exponential-2 (exp2)* model: | 10 | |
| *exponential-3 (exp3)* model: | 6 | |
| *exponential-4 (exp4)* model: | 99 | |
| *exponential-5 (exp5)* model: | 24 | |

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* | 74034.5 |
| Neutral control median absolute deviation, by plate: *nmad* | 3418.876 |
| Coefficient of variation (CV%) in neutral control wells: *(nmad/nmed)*100* | 4.58% |
| **POSITIVE CONTROL (well type = "p")** | |
| Positive control well median response value, by plate: *pmed* | 199269.5 |
| Positive control well median absolute deviation, by plate: *pmad* | 8058.672 |
| 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($pmad^2$ + $nmad^2$ )* | 12.845 |
| 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* | 71359.75 |
| Negative control well median absolute deviation value, by plate: *mmad* | 3663.875 |
| 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($mmad^2$ + $nmad^2$)* | 0.055 |
| 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: 24.

*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:** Harrill JA, Freudenrich T, Wallace K, Ball K, Shafer TJ, Mundy WR. Testing for developmental neurotoxicity using a battery of in vitro assays for key cellular events in neurodevelopment. Toxicol Appl Pharmacol. 2018 Sep 1;354:24-39. doi: 10.1016/j.taap.2018.04.001. Epub 2018 Apr 5. PMID: 29626487., Carstens, K. E., Carpenter, A. F., Martin, M. M., Harrill, J. A., Shafer, T. J., & Paul Friedman, K. (2022). Integrating Data From In Vitro New Approach Methodologies for Developmental Neurotoxicity. Toxicological sciences : an official journal of the Society of Toxicology, 187(1), 62–79. https://doi.org/10.1093/toxsci/kfac018

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.