Walter+ Eliza Hall Institute of Medical Research DISCOVERIES FOR HUMANITY | National Drug Discovery Centre

Screening assay requirements

The first section of this document identifies criteria to determine whether your assay is suitable and ready for transfer to the high-throughput robotic screening platform at the National Drug Discovery Centre (NDDC).

The second section provides basic guidance to assist with development of a suitable assay. If you are considering a future application to the NDDC and have not yet developed an assay, you are encouraged to confirm with us that the selection of assay technology is appropriate before initiating development.

1. Assay readiness criteria

To be eligible to apply to the NDDC, the screening assay must meet the requirements listed in the table below. If the assay does not fully meet the requirements, please indicate in your application what resources and assistance are needed to bring the assay up to the required standard. If you have developed a validated 96-well assay but do not have access to 384-well microtiter-compatible plate readers or need advice on assay development in general, please contact the NDDC. We are happy to advise and assist in the miniaturisation to 384-well format. Further miniaturisation to 1536-well format may be investigated as part of the assay transfer stage.

A data review by NDDC screening staff will determine whether the assay is fit for purpose and ready for evaluation for uHTS platform transfer.

	Required	Preferred
Assay format	Demonstrated in 96-well format and/or 384-well format	384 well format or 1536 well format
Readout technology This list will be updated as new technologies are integrated within the Centre. So, if your technology of choice is not available, please contact us.	• Absorbance • Fluorescence • Fluorescence polarisation • FRET • Time-resolved FRET • Luminescence • High-throughput flow cytometry • High-content imaging	We recommend avoiding fluorescence polarisation technology; if it is the only technology available, a far-red dye is preferable.
Signal-to-background ratio (ratio of high control signal to low control signal)	> 2	Ideally > 10
Z' (Robustness) $Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{\lvert \mu_p - \mu_n \rvert}$ where σ_p, σ_n are the standard deviations and μ_p, μ_n are the means of the positive (*p*) and negative (*n*) controls. Use at least 16 control wells.	≥ 0.4 Obtained on at least 3 independent whole-plate experiments (using adequate screening controls *eg* with DMSO)	≥ 0.6 Obtained on at least 3 independent whole-plate experiments (using adequate screening controls *eg* with DMSO)

	Required	Preferred
Reaction stability over projected assay time	Conduct time-course experiments to determine the range of acceptable times for each incubation step in the assay Incubation step > 5 min	Incubation step > 60 min
DMSO tolerance	DMSO tolerance tested up to 5% Max DMSO tolerance > 0.4%	Max DMSO tolerance > 1 %
Storage requirement of each component (eg proteins, cell lines etc.)	Identify conditions under which reagent aliquots can be stored for more than 3 months without loss of activity Test freeze-thaw cycle	Stable when stored for more than 6 months
Stability requirement of each component (eg proteins, cell lines etc.)	Test the stability of the working reagent solution during assay (varying temperature, time on bench, etc.) Working reagent solution stable on the bench for more than 1 hour	Working reagent solution stable on the bench for more than 3 hours
Cell lines	• Mycoplasma-free (provide document of testing) • Tested for passage-number effects • Bulk freeze of adequate number of aliquots for screen • When thawed, cell viability should be > 90%	When thawed, cell viability should be > 95%
Protein	Protein > 90% pure Evidence of protein supply, reproducibility and stability upon storage for > 3 months If there is insufficient protein available due to stability constraints, you must provide timelines for production and evidence of reproducibility.	Sufficient protein available to complete both the pilot and primary screen
Reference controls		Validate the assay using known reference compounds
Assay development report	• Provide a report of the assay development results with evidence of optimised assay conditions, for example: – Assay components concentration optimisation – Buffer optimisation – Incubation time optimisation – Order of reagent addition – Incubation temperature optimisation • Provide raw data of the whole-plate (at least 96 well plate) experiments and reference compound testing • Provide a detailed standard operating procedure	

2. Quick assay-development road map including key experiments to perform

a. <u>All assays</u> (optimisation experiments that are common to all assay development)

- Buffer optimisation (following is a non-exhaustive list of considerations that could be tested depending on the type of assay developed):
  - Reducing agent (*eg* DTT, TCEP…) is recommended to minimise protein oxidation and to avoid selection of thiol-reactive screening compounds
  - Detergent (*eg* Triton, CHAPS…) is highly recommended to prevent selection of aggregator hits and reduce non-specific binding to assay plates
  - Divalent cations (*eg* Ca^{2+}, Mg^{2+}, Mn^{2+})
  - pH
  - Buffer source (*eg* HEPES *vs* acetate)
  - Salts (*eg* NaCl, KCl)
  - Chelating agents if necessary (*eg* EDTA)
  - Bovine serum albumin (or another carrier protein to reduce background *eg* casein, BGG, ovalbumin)
- DMSO titration to determine the DMSO tolerance of the assay
- Assay volume miniaturisation (must at least be compatible with a 96-well plate format)
- Whole-plate experiment including at least 16 wells for each positive and negative control

b. <u>Enzymatic assays</u>

- Detection linearity and standard curve
  - The assay should be performed within the linear portion of the kit/instrument capacity
- Enzyme titration and kinetic read
  - The enzyme concentration and reaction time should be adjusted within the linear phase of the reaction and where less than 10% of substrate conversation has occurred
  - Note that the assay detection limit is dependent on the enzyme concentration (IC_{50} detection limit = ½ [enzyme])
- Enzyme reaction temperature optimisation (ideally to be run during the enzyme titration and kinetic read stage)
- Substrate(s) K_m determination; final substrate concentration should be adjusted depending on the mode of action targeted:
  - $[S] > K_m$ uncompetitive inhibitors
  - $[S] < K_m$ for competitive inhibitors
  - $[S] = K_m$ will detect both competitive and uncompetitive inhibitors
- Stop reagent identified if possible
- Order-of-addition optimisation (compound / enzyme / substrate)

c. <u>Protein-protein interaction assays</u>

- PPI complex cross-titration to determine the optimum concentration of both components
- Detection reagent concentration / volume optimisation (*eg* AlphaScreen beads / TR-FRET beads) to be in the linear phase below the hooking point
- Incubation time optimisation
- Order-of-addition optimisation
- Competition experiment with at least 1 untagged binding partner

d. Cell viability assays

- Cell density and cell incubation time optimisation should be performed simultaneously
- Detection reagent concentration/volume optimisation (*eg* CellTiter-Glo volume addition)
- Stimulating agent optimisation (if needed)
- Carrier-protein titration (*eg* FCS); the assay should use the minimum tolerated
- Order-of-addition optimisation (compound / cells)

For more detailed guidelines on the development of HTS-amenable assays, please refer to: Sittampalam GS, Coussens NP, Brimacombe K, et al., editors. *Assay Guidance Manual* [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK53196/