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Screening assay requirements

The first section of this document identifies criteria to determine whether your assay is suitable to apply for a subsidised high-throughput screen 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 get in touch with us before initiating development.

Assay readiness criteria

To be eligible to apply to the NDDC for a subsidised screen, your assay must meet the minimum 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 need advice on assay development in general, please contact the NDDC. We are happy to advise and assist. Assays validated in a 96-well format will be miniaturised to a 384-well format by NDDC staff as part of the subsidised service if your application is successful. Further miniaturisation to 1536-well format may be investigated as part of the assay transfer stage.

	Minimum acceptable	Preferred
Assay format	Demonstrated in 96-well format	Demonstration in 96-well format is perfectly acceptable for most assay technologies. Demonstration in 384- or 1536-well format may be advantageous in some circumstances but is not a requirement.

Readout technology
 	
This is a **non-exhaustive** list of the recommended technologies.
	• Binding assay
	  ○ TR-FRET
	  ○ AlphaScreen®
	• Reporter assay
	  ○ Nano-Glo® Dual-Luciferase®
	• Cytokine release/in-cell western
	  ○ TR-FRET
	  ○ AlphaLISA®
	• Kinase
	  ○ ADP-Glo™
	  ○ FRET assays
	  ○ Alpha reagents (Revvity)
	• Cell viability
	  ○ CellTiter-Glo®
	  ○ High-content imaging
		We recommend aiming to avoid fluorescence polarisation technology due to high rate of false positives. If it is the only technology available, a far-red dye is preferable.

	Minimum acceptable	Preferred

	• Methyltransferase assay
	  ○ TR-FRET (EPIgeneous, AptaFluor®)
	  ○ Luminescence (MTase-Glo™)
	• GPCR
	  ○ LANCE® Ultra cAMP kit
	
	Many others are acceptable. Prospective applicants are encouraged to speak with NDDC staff.	

Signal-to-background ratio *(ratio of high control signal to low control signal)*	> 2	Ideally > 10
Z' (Robustness) *(reference formula available below)*	≥ 0.4 Obtained on at least 3 independent whole-plate experiments (using adequate screening controls) *Note: To ensure reliable estimation of sample standard deviation, each plate should contain a minimum of 8 low control wells and 8 high control wells.*	≥ 0.6 Obtained on at least 3 independent whole-plate experiments (using adequate screening controls)
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 steps should not be shorter than 10 minutes.	Very short incubation steps are non-preferred.
DMSO tolerance	DMSO tolerance tested up to 5% (Note that DMSO does not need to be tolerated up to that level.) For cell-based assays: DMSO tolerance > 0.2% For biochemical assays: DMSO tolerance > 0.4%	For cell-based assays: DMSO tolerance > 0.4% For biochemical assays: DMSO tolerance > 1 %
Storage requirement of each component (e.g. *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 (e.g. *proteins, cell lines etc.)*	Test the stability of the working reagent solution during assay (*e.g.* time on bench). Working reagent solution stable on the bench for more than1 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 • When thawed, cell viability should be > 90%	When thawed, cell viability should be > 95%

	Minimum acceptable	Preferred
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 the screening campaign
Experiment data	Evidence of optimised assay conditions, for example: • Assay components concentration optimisation • Buffer optimisation • Incubation time optimisation • Order of reagent addition • Incubation temperature optimisation	
Normalisation controls	Any method for generating high and low assay signals and calculating assay robustness statistics (*e.g.* +/- enzyme)	A reference compound or unlabelled competitor peptide to generate minimum (or maximum) signal
Reference controls		Validate the assay using known reference compounds
Assay development report	• Provide a presentation-file summary, including graphs of all the assay development experiments performed to date • Provide raw data of the whole-plate experiments and reference compound testing • Provide a detailed standard operating procedure	

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 (*e.g.* DTT, TCEP, βME, glutathione) is recommended to minimise protein oxidation and to avoid selection of thiol-reactive screening compounds
  - Detergent (*e.g.* Triton, CHAPS) is highly recommended to prevent selection of aggregator hits and reduce non-specific binding to assay plates
  - Divalent cations (*e.g.* Ca^{2+}, Mg^{2+}, Mn^{2+})
  - pH
  - Buffer source (*e.g.* HEPES *vs* acetate)
  - Salts (*e.g.* NaCl, KCl)
  - Chelating agents if necessary (*e.g.* EDTA)
  - Bovine serum albumin (or another carrier protein to reduce background *e.g.* 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. Enzymatic assays

- 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. Protein-protein interaction assays

- PPI complex cross-titration to determine the optimum concentration of both components and ensure signal is in the linear phase, below the hook point
- Detection reagent concentration / volume optimisation (*e.g.* AlphaScreen beads / TR-FRET dyes)
- 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 (*e.g.* CellTiter-Glo volume addition)
- Stimulating agent optimisation (if needed)
- Carrier-protein titration (*e.g.* 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/

Z'-factor (robustness) reference formula and definitions

$$Z' = 1 - \frac{3(s_p + s_n)}{|\bar{x}_p - \bar{x}_n|}$$

Where:

- $\bar{x}_p$ = **mean** of the **positive control** values
- $\bar{x}_n$ = **mean** of the **negative control** values
- s_p = **sample standard deviation** of the **positive control** values $= \sqrt{\frac{\sum_{i=1}^{n_p}(x_{p,i} - \bar{x}_p)^2}{n_p - 1}}$
- s_n = **sample standard deviation** of the **negative control** values $= \sqrt{\frac{\sum_{i=1}^{n_n}(x_{n,i} - \bar{x}_n)^2}{n_n - 1}}$