Screening Assay Development: How to avoid pitfalls

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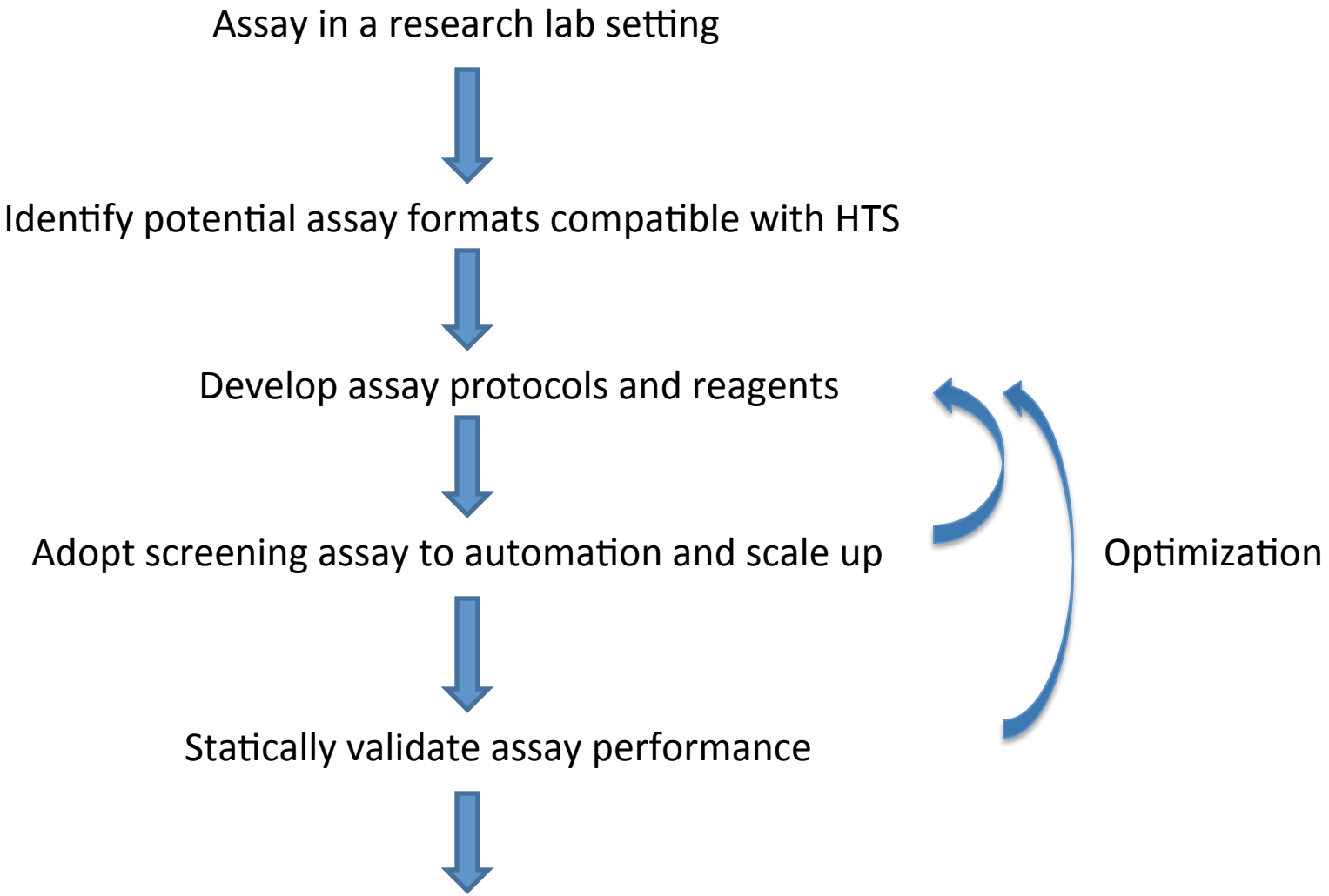
General Flow of Assay Development



What to know before coming to a HTS facility:

1. Know your choice of target for screen
   - Disease relevance

   - Chemical tractability:
     --- certain target class are easier to inhibit than others
     such as, GPCRs, nuclear hormone receptors, kinases etc.
     --- targets that work via protein-protein interactions have a lower probability of being successful in HTS

   - Screenability:
     --- ion channels are more difficult to establish as screens than GPCRs, kinases, proteases, nuclear hormone receptors

2. Have a good assay

3. Know your data analysis strategies, hit selection tools and follow up assays

Characteristics of good assays for HTS

- High sensitivity
- Low variability (reproducibility) -- well to well; plate to plate; day to day
- High signal to background ratio (Z')
- Large dynamic range
- Simple steps (liquid-handling compatibility)
- DMSO Tolerance
- Positive controls

z factor or z prime

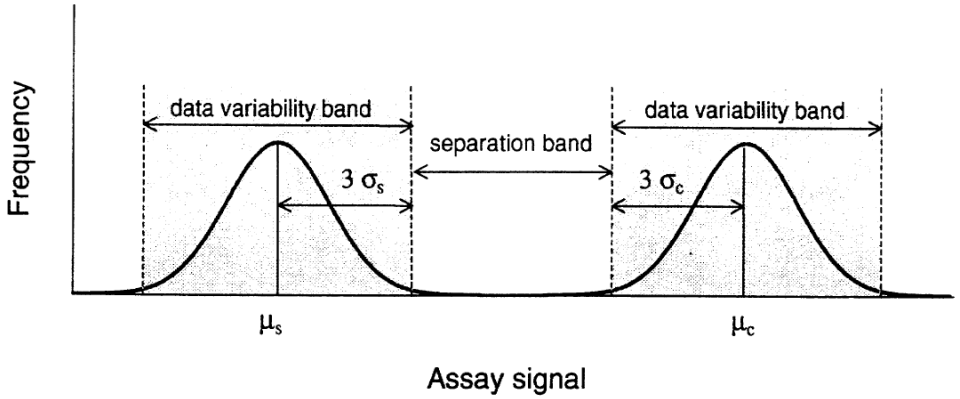
----- a screening window coefficient

$$Z factor = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

data variation

signal dynamic range

μ: mean
σ: standard deviation
p: positive controls
n: negative controls

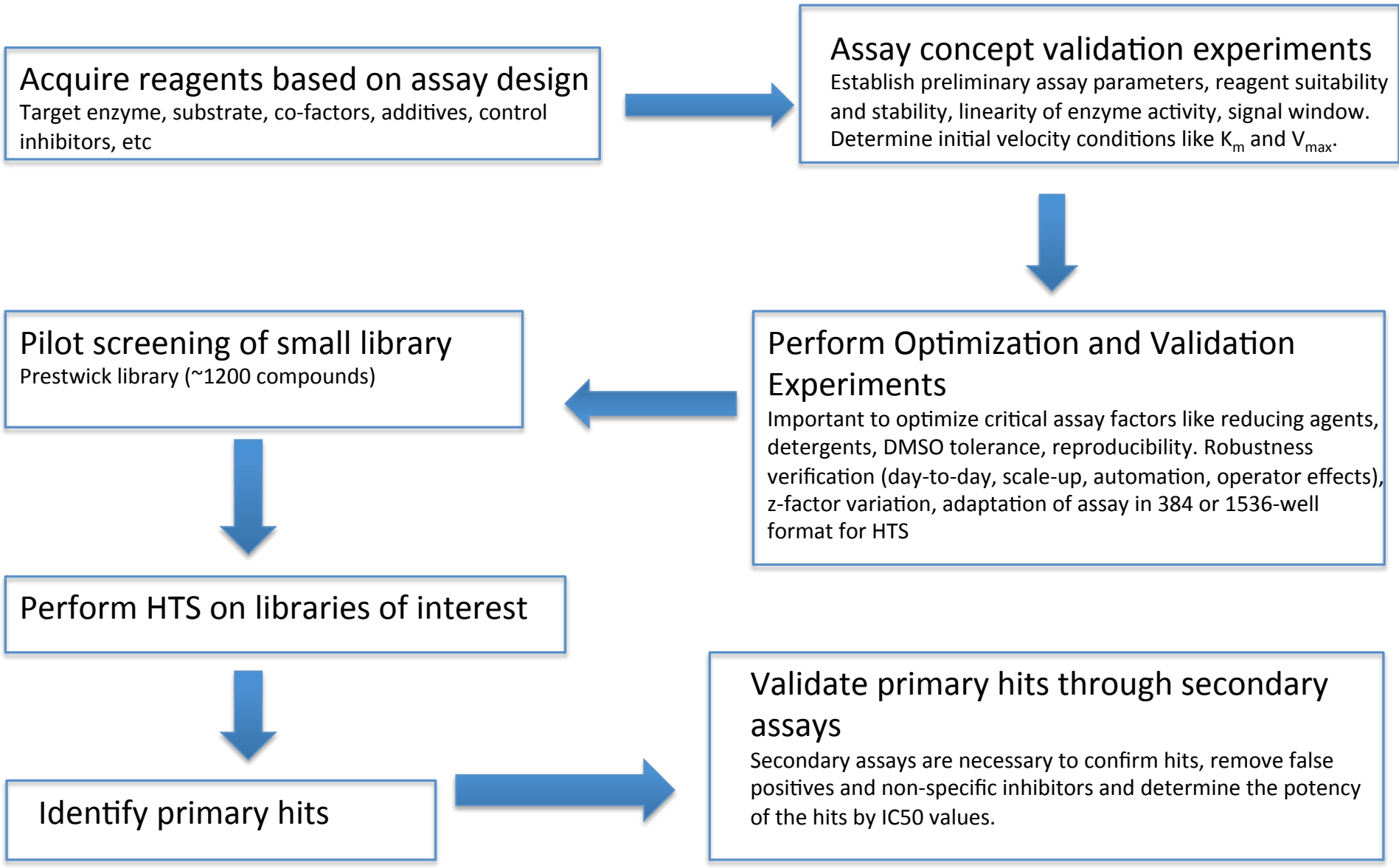


z' = 1.0 an ideal assay
0.5 ≤ z' <1.0 an excellent assay
z' <0.5 a marginal assay

** **Z'** is a measurement of the quality or suitability of a HTS assay.

Zhang JH et. al. *J Biomol Screen.* 1999;4(2):67-73

General flow of Enzyme Assay Development in Small Molecule Discovery

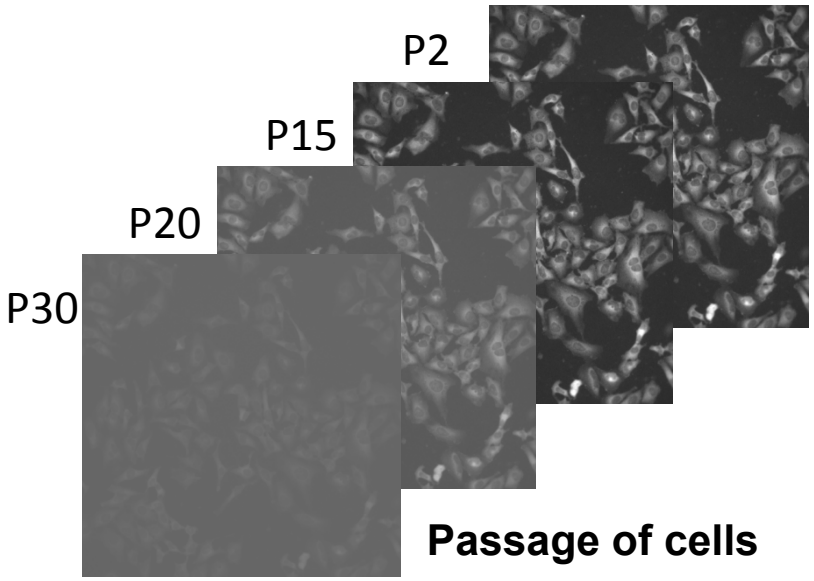


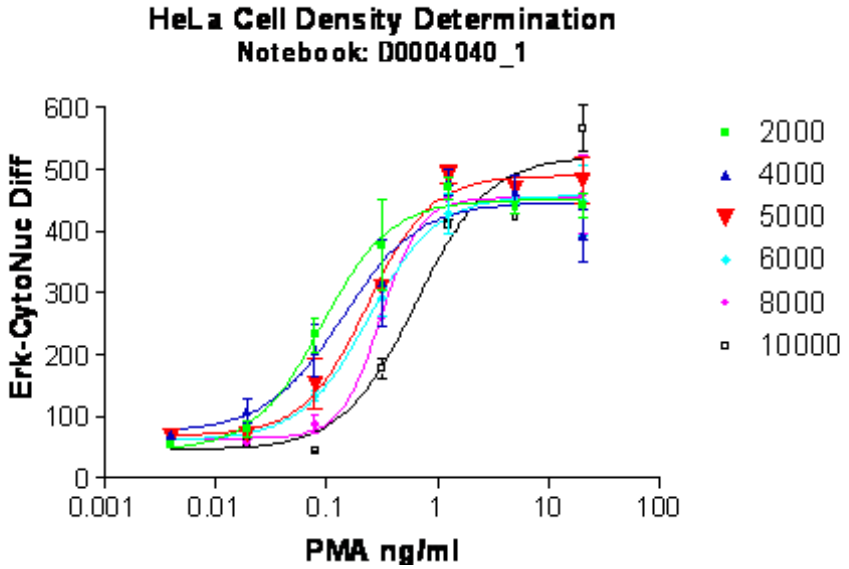
Biochemical Assays –Common Pitfalls

- Assay Interference
  - absorbance assays – compounds can absorb in the detection range. Preferable to have detection range >500nm
  - fluorescence assays – compounds can have intrinsic fluorescence in the detection range
- Generation of false positives
  - non-specific inhibitors, compounds that react with the detection reagents, compounds that inhibit coupling enzymes
- Cost of reagents
  - cost of protein production, substrates, co-factors, additives, etc need to be estimated for any HTS screen. Each library is run in duplicate and so for a 25K library, 50,000 reactions will be performed in ~4 days
- Sensitivity (Z-factor)
  - for a reliable HTS the z-factor needs to be >0.5
- Enzyme stability
  - enzymes need to be stable in the assay buffer conditions for extended periods
- Temperature sensitivity
  - preferable to run assays at room temperature (22 ^{o}C)
- DMSO tolerance
  - library compounds are dissolved in DMSO and hence it is necessary to determine the effect of DMSO on the enzyme reaction
- Effect of additives
  - it is important to determine the effect of common assay ingredients like triton, reducing agents, BSA etc on enzyme activity
- Too many steps
  - too much washing or too many additions are not compatible in a HTS setting.

Cell-based assays – Assay Development

- Establish liquid handling and detection protocols
- Source and evaluate reagents
- Test to establish cell and assay specifics:
  - Choice of cell line
  - Cell culture-Passage of Cells
  - Cell culture-Cell Banking
  - Cell Seeding Density
  - DMSO tolerance
  - Implementation of controls
  - Time course experiments
  - Z plates
  - Run pilot library (1-10 plates)
  - Develop data handling SOP and analysis pipeline





Cell-based assays - Assay Development *(notes)*

Choosing the cell line – have to balance characteristics amenable to screening with biological relevance.
1. Primary cells – most relevant but have many characteristics that make them difficult to use in large scale screens.
2. Established cell lines – good for screening but not always relevant.
3. Stably transfected cell lines – Commercially available vs make your own.

Cell banking – very important, need to use same batch of cells through entire screen if possible, cell banking ensures: Consistent quality of material, Consistent passage range between experiments, Cell line characteristics are retained, Decreased consumables/costs.

Cell passage – need to establish working passage range, particularly with stably transfected cell lines the number of usable passages may be very limited.
Maintaining physiological characteristics of the cells is critical for screening.
Confluency and passage control ensures maintenance of the phenotype and limits response drift.

Cell seeding density – Cell density is determined as part of assay development needs to be established using positive controls – more cells are not always better. Density recommendations may not be appropriate for different cell types and require additional density studies.

Source and Evaluate reagents – kits vs DIY: May not be specifically your target. May not work on your cell type.

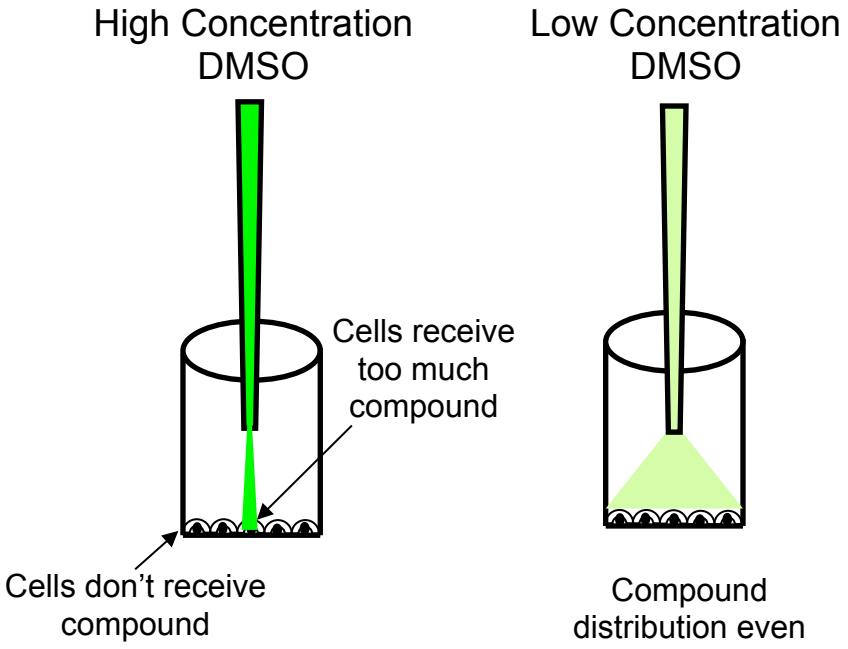
Implementation of controls – need to be biologically relevant.

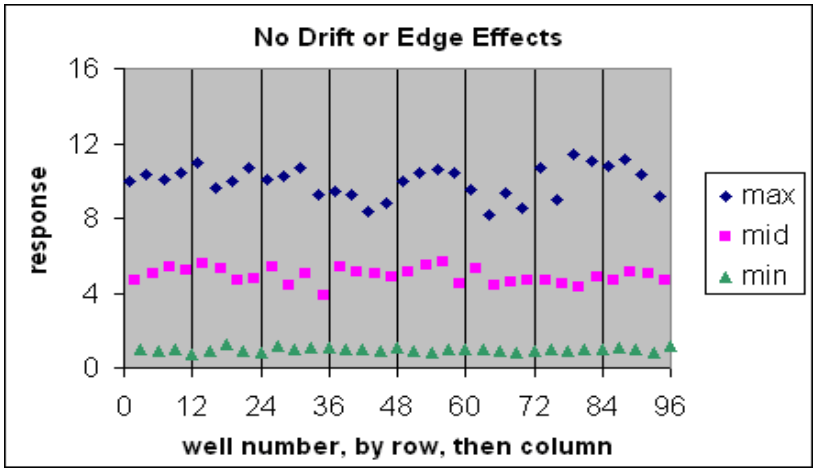
Time course experiments – establish assay window.

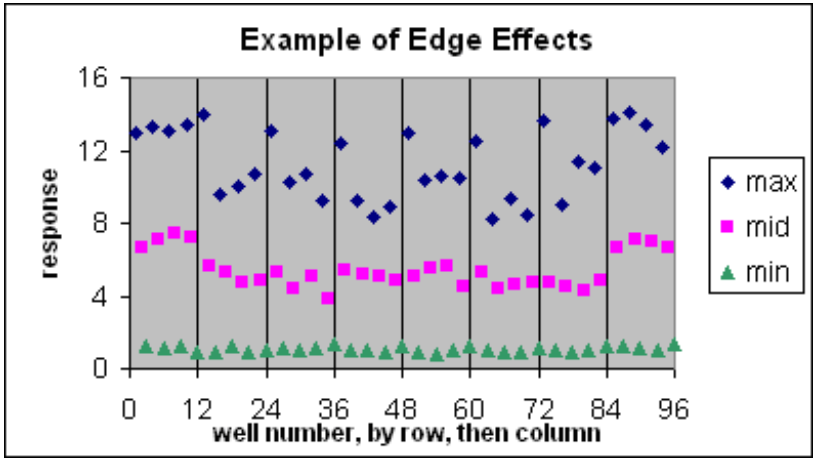
DMSO tolerance – small molecule libraries typically dissolved in DMSO, needs to be done early in assay development, controls should always be in DMSO, typically less than 1%, usually closer to 0.1%

Cell based Assays – Common Pitfalls

- Loss of cells
- Edge Effects
- Signal drift across and between plates
- Signal drift over time
- Uneven compound delivery







Cell based Assays – Common Pitfalls *(notes)*

Loss of cells

Too much washing can lead to cell loss, try to use no wash assays, also can evaluate different ECMs (extra cellular matrix)

Edge effects

Due to evaporation or uneven temperature across plate. Location in incubator and air permeable seals can usually eliminate. Minimizing length of assay will also help (plate more cells at start to avoid long incubations to reach confluency).

Signal drift

If optimized cell banking and handling procedures followed usually can avoid. Expand enough cells at start in order to use same batch of cells for entire screen.

Uneven compound delivery

"Liquid plumber" effect. Can eliminate by pre-diluting compounds in media or using liquid handling robotics that reduce or eliminate effect (eg Echo).

Assay development for RNAi screen

1. A large or small screen?

2. Documentation cell culture details, such as cell passage and cell health.

3. Confirmation of transfection efficiency or infection rate
   --- It is a major source of variability for RNAi screen.

4. Converting assays from low-density to high-density formats if possible

5. A pilot screen is valuable for testing the data analysis pipeline

6. Off-target effect

7. Follow-up assays are crucial to success.
   --- Testing independent reagents targeting the same gene to confirm the true hits
   --- Rescue experiment introducing a copy of the targeted gene that has been mutated to no longer be recognized by the RNAi reagent
   --- Using non-RNAi methods to demonstrate the biological role of the gene

Web Resources

❑**Assay Guidance Manual from the National Center for Advancing Translational Sciences**:
http://www.ncbi.nlm.nih.gov/pubmed/22553861

❑ **General Enzyme Kinetics**

http://themedicalbiochemistrypage.org/enzyme-kinetics.php
http://www.ultranet.com/~jkimball/BiologyPages/E/EnzymeKinetics.html

❑ **A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays**

http://jbx.sagepub.com/content/4/2/67 (doi: 10.1177/1087057199004002O6)

❑ **Other types of biochemical assays**

Thermal shift assays – for enzyme with no viable detection methods for assay development
http://thermofluor.org/resources/PTI-Fluorescence-basedThermalShiftAssay.pdf
Alpha-screen for Protein-Protein Interaction Assays
http://www.perkinelmer.com/Catalog/Category/ID/AlphaScreen+Assays+and+Reagents

❑ **Explanation of High Content Screening**

http://www.cellomics.com/home/three-worlds-buttons/what-is-high-content.html

❑ **RNAi global initiative**

http://www.rnaiglobal.org/Home/