ASSAY DEVELOPMENT IN DRUG DISCOVERY

Indira Padmalayam, Ph.D.

Drug Discovery Division, Southern Research Institute, Birmingham, Alabama 35205
The Drug Discovery Process: The Assay Development Stage

HTS
- Target Identification
- Target Validation
- Assay Development
- Lead Identification
- Lead Optimization
- Development

Non-HTS
- Target Identification
- Target Validation
- Assay Development
- Lead Optimization
- Development

Assay development
- Primary assays
- Secondary assays

“Hit” validation
- Chemistry
- Structure-Activity Relationship (SAR)
- bioavailability (PK, ADME),
- toxicity
- In vivo efficacy

Pre-clinical
- GLP-Tox
- Clinical
**Assay development:** A critical part of the “hit” discovery process

A “hit” is a compound which has the desired activity in a compound screen and whose activity is confirmed upon retesting.

Adapted from: Hughes, J.P. et al. (2011), British Journal of Pharmacology, 162 1239-1249
Assay Development

Why is this a bottleneck?

Assay Development=months
HTS=weeks

Roadblocks to faster assay development

- Lack of high quality reagents and cell lines (44%)
- Novel or complex targets (21%)
- Assays robust enough for HTS (17%)
- Communication outside HTS lab (8%)
- Capacity and cost (10%)

Drug Discovery World, Summer 2010
Topics to be covered

• Paradigms in Drug Discovery
• Significance of Assay Development in Drug Discovery
• Assay types
• Assay formats
• Optimizing assays for HTS
  Factors to be considered (will be covered in detail in HTS lecture)
• Assay development examples
Paradigms in Drug Discovery

**Physiology-based**
- Target is unknown
- Physiological/phenotypic read-outs
- Cell-based assays

**Target-based**
- Known target
- Read-outs are based on activity or expression of target
- Biochemical or Cell-based assays

The two paradigms are not mutually exclusive: Drug Discovery programs can use two-pronged approach.
Physiology-based drug discovery

Example: Viral CPE (Cytopathic Effect) assay

Green: uninfected cells
Red: host cells + virus
Blue: host cells + virus treated with an antiviral compound, Ribavarin

Slide: courtesy, Jim Noah, SRI
Target-based Drug Discovery

- **Enzymes:**
  - Kinases
    - Receptor Tyrosine Kinase
    - Non-receptor tyrosine kinase
    - Serine Threonine kinase
  - Phosphatases
  - Proteases:
    - Serine proteases
    - Zinc proteases
- **Receptors:**
  - Ion channel receptors
  - GPCRs
  - Nuclear receptors

*Science, 2000*
To ensure successful Assay Development:
- Ask the right “question” *(the biological problem being addressed)*
- Make sure that the “wording of the question” is correct *(choose the correct assay, read-out and sequence of assays)*.

**Key Questions:**
1. Are we trying to inhibit or activate the target?
2. What function of the target are we trying to regulate?
3. What are the types of assays that are available to measure the function?
4. Primary assay vs Secondary assays
5. Which assay will work best in terms of translatability to HTS
6. Which are the post-HTS assays that will drive Structure Activity Relationship (SAR)
Key Considerations in Assay Development

The three “Rs”
- Relevance
- Robustness
- Reliability/Reproducibility

Practicality/Feasibility

Cost

Automation

“The quality of an assay determines the quality of the data: compromising on assay development can have substantial downstream consequences”
Types of Assays

Assays in Drug Discovery

Biochemical assays

- **Target-based**
  - Measure function of a purified target
  - **Activity assays**: Enzymes (e.g. kinases, proteases)
  - **Binding assays**: Receptors
    (e.g. Nuclear receptors, Kinase receptors, ion channels, GPCRs)
  - Identify compounds that modulate activity / binding of the target protein
  - Recombinant (engineered) proteins, proteins isolated from crude cell lysates
  - Monitor a surrogate read-out
  - **Examples**: Kinase/ATPase assays, protease assays, protein interaction assays

Cell-based assays

- **Phenotype-based**
  - Measure function of the target in the context of the cell
    - Transcriptional readouts, second messenger levels, cell viability (cell death/apoptosis), proliferation
  - Measure expression of the target
    - mRNA levels, protein expression and localization
  - Provide a functional read-out of compound activity (as a functional consequence of target engagement)
  - **Examples**: reporter assays, viability assays, GPCR and ion channel assays, qPCR
Biochemical versus Cell-based assays

Biochemical assays

- Advantages:
  - Simple
  - More consistency
  - Direct measurement of target engagement
  - Can measure compound characteristics such as $K_d$, $K_i$ etc.
  - Increased specificity of compounds

- Disadvantages:
  - May be non-physiological
  - Not possible to determine compound properties such as membrane permeability, toxicity, off-target effects

Cell-based assays

- Advantages:
  - More physiological, amenable to systems approach
  - Can simultaneously assay for compound properties (membrane permeability, toxicity, off-target effects)

- Disadvantages:
  - Complex
  - High rate of noise
  - Exclusion of less soluble/permeable compounds
Causes of Assay Variation

Biochemical Assays

- pH
- Temperature
- Ion concentration
- Reagent Solubility
- Reagent Stability
- Reagent Aggregation
- Order of reagent addition
- Instrumentation

Cell Based Assays

Same as for biochemical assays
Plus:

- Cell culture plastics
- Culture media
- Culture conditions
- Serum
- Cell cycle
- Passage number
Common Assay formats

- Fluorescence
- Luminescence
Fluorescence-based assays

- Based on excitation of a fluorophore
- Variety of assays using fluorescence
  - Simple assays where protein of interest is conjugated to fluorophore, or where the protein of interest generates a fluorescent product
  - Reporter assays

- **Advantages:** High sensitivity, ease of set-up and operation
- **Disadvantages:** Prone to false positives due to auto fluorescence of compound

**Examples:**
- FRET, TR-FRET
- Fluorescence polarization (FP)
Fluorescence-based assays (contd.)

FRET: Fluorescence Resonance Energy Transfer

- **Principle**: Two fluorophores: Donor and Acceptor
  - Based on transfer of energy between donor and acceptor
  - Distance is critical

  *Far*: No energy transfer $\rightarrow$ no FRET

  *Close*: Energy transfer from donor to acceptor $\rightarrow$ FRET

- **Use**: Protein-protein, antigen-antibody, DNA-DNA, DNA-protein

- **Advantages**:
  - Homogenous assay format
  - Reduced assay time and cost (HTS friendly)

- **Disadvantage**:
  - Short half life of fluorophore results in high background

Half life: 1 to 10 nano secs
Fluorescence-based assays (contd.)

TR-FRET: Time Resolved-Florescence Resonance Energy Transfer

• “Improved” version of FRET
  - Uses long lived fluorophores and time-resolved detection to reduce background
  - Rare earth elements (Lanthanides): Samarium (Sm), Europium (Eu), Terbium (Tb), and Dysprosium (Dy)

• **Advantage:**
  - Low background; better signal to noise

• **Disadvantage:**
  - Lanthanides have poor ability to absorb light, so have to be complexed with organic moieties that can harvest light and transfer it to them.
Fluorescence-based assays (contd.)

Fluorescence Polarization (FP) assay

- **Principle:**
  Small, unbound fluorophore: fast rotation, light is emitted in a plane different from excitation light.
  Fluorophore bound to protein: Slow rotation, light is emitted in the same plane as excitation light.

- **Applications:** Study molecular interactions e.g. protein-protein, receptor-ligand, DNA-protein, tyrosine Kinase assays.

- **Advantages:** Highly sensitive (low picomolar range), homogenous assays, multiple measurements can be made on the same sample, because there is no change in samples during the assay.

- **Disadvantage:** More optimization of assay may be needed to ensure saturation of all target binding sites with the fluorophore-labeled ligand (to ensure displacement by unlabeled ligand)
Fluorescence-based assays (contd.)

FP assay

ATP binding pocket
Cy3 labeled compound

Un-labeled compounds
Screen

FP read
Luminescence-based assays

• Chemical reaction produces light
• Bioluminescence is the production and emission of light by a living organism (e.g. luciferase by firefly)

Luciferin + ATP $\rightarrow$ Luciferyl adenylate + PPI
Luciferyl + O2 $\rightarrow$ Oxyluciferin + AMP + Light

Example: Luciferase reporter assay:

1. Determine promoter response elements (RE) of interest.
2. Clone RE upstream of the firefly luciferase (Luc) gene.
3. Transfer construct into cell.
4. Add luciferase detection reagent and measure firefly luciferase activity.
5. Add firefly luciferase activity. Validate transcription factor activity and "X" may be due to brightness.
Examples of biochemical assays
**Assay for kinase activity**

**ADP Hunter assay**

Substrate $\rightarrow$ Kinase $\rightarrow$ Phosphorylated Substrate $\rightarrow$ Inhibitors $\rightarrow$ Phosphorylated Substrate

- **Kinase target**
- **Substrate**
- **Phosphorylated Substrate**
- **Inhibitors**
- **Reduced signal**
Assay for protein-protein interaction

AlphaScreen technology
Amplified Luminescent Proximity Homogenous Assay

Excitation at 680 nm

\[ {^{1}}O_2 \]

4 µsec

Emission at 520-620 nm

200 nm
Alphascreen Assay: Measuring Tau-Fyn interactions in Alzheimer’s disease

- Interactions between Tau and Fyn are implicated in Alzheimer’s disease
- Donor bead labeled with glutathione
  - Binds to Fyn-GST
- Acceptor bead chelated to nickel
  - Binds to a His-tag on Tau protein

Figure: Courtesy Erik Roberson, ADDA Tau-Fyn project
Examples of cell-based assays
Cell based assays

- **Proliferation**
  - Fluorescent dyes

- **Viability**
  - Assays that measure ATP content (Cell Titer Glo), viral CPE (bioluminescence-based)
  - Apoptosis assays (bioluminescence/fluorescence)

- **Migration**
  - Scratch assay

- **Reporter Gene assays**
  - Transcriptional activity/expression

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Influenza CPE assay - Preliminary CV Plate

Cell Proliferation (non-specific dye)

Cell viability (CPE assay)

Cell migration “scratch assay”
Assays for G protein-coupled receptors (GPCRs)

- GPCRs are the targets for majority of best-selling drugs and approx 40% of prescription drugs (e.g. Zantac, Clarinex, Zyprexa)
- GPCRs are proteins with 7 transmembrane domains
- Assay will depend on the type of GPCR being targeted. 3 type of G proteins ($G_s$, $G_i/G_0$ and $G_q$)
- Non-G protein mediated responses ($\beta$- Arrestin recruitment)
- Assays are designed to measure second messengers such as cAMP, Ca$^{2+}$
- Measurement of proximal or distal responses
- HTS friendly assays
High Content Screening

High content screens:

- Facilitates validation of effects of compounds at the cellular and subcellular level (expression, localization, morphological changes in cells)
- Macromolecules (e.g. proteins, RNA) are labeled with fluorescent tags
- Technology uses automated digital microscopy, flow cytometry and IT-systems for analysis and storage of data
- Slower than HTS
- Popular as secondary screens in drug discovery programs
High Content Screening in Drug Discovery

**Example 1:** Cell migration assays: Oncology

Cytochalasin-D concentration response on cell migration

MDA-MB-231 breast cancer cells were co-cultured with 3T3L1 fibroblasts and treated with Cytochalasin-D, an inhibitor of actin polymerization. Migration of cells through the matrigel layer was measured at various heights of the Z stack.
High Content Screening in Drug Discovery

Example 2: Neurite Outgrowth Assay: Alzheimer’s disease

Aβ concentration response on neurite outgrowth
Assay development: From the bench to HTS

When you go

From This

To This

THE RULES CHANGE
Everything is done in Microtiter Plates
96, 384, 1536, 3456

<table>
<thead>
<tr>
<th>Well Size</th>
<th>Volume</th>
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<tr>
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<td>4-10 μl</td>
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<tr>
<td>3456-well</td>
<td>1-2 μl</td>
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</tbody>
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Assay development: From the bench to HTS

- What are you aiming for in an HTS assay:
  - *To have a reasonable chance, to believe the results of a single determination, i.e. one well*

- For that you need:
  - *Reproducibility from well to well*
  - *Reproducibility from assay plate to assay plate*
  - *Reproducibility from day to day*

- How do you know if your assay is ready for HTS?
ASSAY QC PARAMETERS

- **“Z” factor:** Key measure of readiness of an assay for HTS
  \[ Z = 1 - \frac{3\text{SD of sample} + 3\text{SD of control}}{\text{mean of sample} - \text{mean of control}} \quad \text{Z} > 0.5 \]

- **Coefficient of variance (CV):** Measure of dispersion
  \[ \% \text{CV} = \frac{\text{SD}}{\text{Mean}} \times 100 \]

- **Signal to background (S/B) and Signal to noise (S/N):** Measures of signal strength
  \[ \text{S/B} = \frac{\text{Mean of sample}}{\text{Mean of control}} \]
  \[ \text{S/N} = \frac{\text{Mean of sample} - \text{Mean of control}}{\text{Standard Deviation of control}} \]

Will be covered in HTS lecture next week
ASSAY DEVELOPMENT CASE STUDIES
Case 1: Identification of a small molecule inhibitor of mitotic spindle bipolarity

Identification of Monastrol, inhibitor of mitotic kinesin, Eg5

**CASE 2: Identifying compounds that inhibit TXNIP expression**

ADDA project: TXNIP as a target for diabetes
TXNIP = Thioredoxin-interacting protein

### Assay development
Primary and secondary assays

### HTS primary screen
(single dose)

**“Hits”**

### Hit validation and determination of potency (IC$_{50}$)
(counter screens, concentration response curves)

1. Cytotox assay to eliminate compounds with toxicity
2. Concentration response in primary assay for potency (IC$_{50}$)
3. Chemistry analysis to identify scaffolds of interest

**~ 1258 “Hits”**

### Counter screen: To eliminate non-specific luciferase inhibitors

**651 “Hits”**

### Secondary assay: TXNIP qPCR

**157 “Hits”**

### Chemistry analysis

**37 “Hits”**

Test in other confirmatory assays at UAB
**CASE 3: Identifying compounds that activate HO-1 expression**

ADDA project: HO-1 as a target for multiple conditions (chronic kidney disease, transplant rejection)
HO-1 = Heme Oxygenase-1

**Assay development**
- Primary and secondary assays

**HTS primary screen**
- (single dose)
- "Hits"

**Hit validation and determination of potency (IC50)**
- (counter screens, concentration response curves)

**Secondary assays**
- HO-1 qPCR and Western blot

**"Analoging"**: selection and purchase of analogs of hits

**80 Compounds**

Test in other confirmatory assays (qPCR and In Cell Western)
NCGC Online Resources

- The National Chemical Genomics Center provides a comprehensive online manual for assay development and validation with HTS in mind.

For more information go to:

- Manuscript helpful for HTS Assay Development:
  Inglese, J. et.al. (2007), Nature Chemical Biology 3(8) 466-479
“The subject is not exhausted but we are.”

George Bernard Shaw