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

Target Identification

Target Validation

Assay Development

HTS

Lead Identification

Lead Optimization

Development

"Hit" validation

Chemistry Structure-Activity Relationship (SAR) bioavailability (PK, ADME), toxicity

In vivo efficacy

Pre-clinical GLP-Tox Clinical

Assay development

Primary assays

Secondary assays
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

Roadblocks to faster assay development

Why is this a bottleneck?

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 exercise
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 projects 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, Ribavirin

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
Key Considerations in Assay Development

- Relevance
- Reproducibility
- Quality
- 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
  - Enzymes (e.g. kinases, proteases)
  - Receptors (e.g. Nuclear receptors, Kinase receptors, ion channels, GPCRs)
  - Hormones

Cell-based assays
- Phenotype-based
  - Transcriptional read-outs
  - Second messenger levels
  - Protein interactions
  - Protein expression and localization
  - Cell viability (cell death/apoptosis)
  - Proliferation
Biochemical Assays

- Measure function of a purified target
- Identify compounds that modulate the activity 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

- Provide a functional read-out of compound activity
- Useful for follow up of biochemical assays

**Examples:**

*Transcriptional activity:* reporter-based assays  
*Cell proliferation:* MTT etc. for oncology  
*Viability:* viral CPE, ATP content  
*Apoptosis:* Caspase assays, TUNNEL assays  
*GPCR activity:* second messenger levels (cAMP, Ca)  
*Motility/Migration:* Bacterial viability assays, mammalian cell motility (wound healing and oncology)
## Biochemical versus Cell-based assays

### Biochemical Assays

**Advantages:**
- Simple
- More consistency
- High adaptability to HTS
- Increased specificity of compounds
- Wide variety of commercially available read-outs and endpoints

**Disadvantages:**
- May be non-physiological
- Not possible to determine compound characteristics

### Cell-based assays

**Advantages:**
- More physiological
- Can simultaneously assay for compound characteristics such as efficacy, toxicity, membrane permeability, off-target effects.

**Disadvantages:**
- Complex
- High rate of noise and artifacts, variability
- Less adaptable to HTS
- 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
- Bioluminescence
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
  - Advantages: High sensitivity, ease of operation, various read-out modes
  - 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 → no FRET
  *Close:* Energy transfer from donor to acceptor → FRET

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

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

- **Disadvantage:**
  - High background

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

**TR-FRET: Time Resolved-Fluorescence 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.
Fluorescence-based assays (contd.)

FP assay

ATP binding pocket

Cy3 labeled compound

FP read

Un-labeled compounds

Screen
Bioluminescence-based assays

- **Bioluminescence** is the production and emission of light by a living organism (e.g. luciferase by firefly)

```
Luciferin + ATP → Luciferyl adenylate + PPi
Luciferyl + O2 → Oxyluciferin + AMP + Light
```
Examples of biochemical assays
Assay for kinase activity

ADP Hunter assay

Kinase target

Substrate

Phosphorylated Substrate + ADP

Inhibitors

Fluorescent product

Ex: 530 nm
Em: 590 nm

Reduced signal

Fluorescent product

Fluorescent product
Assay for protein-protein interaction

**AlphaScreen technology**
Amplified Luminescent Proximity Homogenous Assay

Excitation at 680 nm

Emission at 520-620 nm

$^{1}\text{O}_2$

200 nm

4 μsec
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
Assay for protease cleavage activity

A. Protease recognition sequence

B. Protease digestion

Inactive GloSensor™-10F Protein (Low Luminescence)  Active GloSensor™-10F Protein (High Luminescence)
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
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 $G_q$)
- Assays are designed to measure second messengers such as cAMP, Ca$^{2+}$
High Content Screening

**High content imaging:**
- Cell analysis at the single cell level
- Used to study protein expression, localization and cellular morphology
  - Apoptosis
  - Intracellular trafficking
  - Autophagy
  - Mitochondrial function
  - Cell cycle
  - Cytotoxicity

**High content screens:**
- Well-level plate reads:
  Intensities are averaged across all cells in a well.
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

96-well
100-200µl

384-well
25-50µl

1536-well
4-10 µl

3456-well
1-2 µl
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

• Z-factor is an indication of your assay’s suitability for HTS
Statistical Analysis: Z Factor

The Z-factor is a measure of the quality or power of a HTS assay; it is an attempt to quantify the suitability of a particular assay for use in a full-scale, high-throughput screen; to be acceptable for HTS, the Z factor has to be >0.5

\[
Z = 1 - \frac{3\text{SD of sample} + 3\text{SD of control}}{\text{mean of sample} - \text{mean of control}}
\]

Clearly, the larger SD (standard deviation) of the positive and negative signals is, the smaller the Z factor, which means the assay is less reliable.

# Z factor: Example

**Experiment “A”**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (arbitrary activity units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg of Neg Ctrl</td>
<td>200</td>
</tr>
<tr>
<td>Avg of Pos Ctrl</td>
<td>68</td>
</tr>
<tr>
<td>Fold-diff bwn Neg &amp; Pos Ctrl</td>
<td>2.9</td>
</tr>
<tr>
<td>Std. Deviation of Neg Ctrl</td>
<td>13</td>
</tr>
<tr>
<td>Std. Deviation of Pos Ctrl</td>
<td>3</td>
</tr>
</tbody>
</table>

**Experiment “B”**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (arbitrary activity units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg of Neg Ctrl</td>
<td>381</td>
</tr>
<tr>
<td>Avg of Pos Ctrl</td>
<td>68</td>
</tr>
<tr>
<td>Fold-diff bwn Neg &amp; Pos Ctrl</td>
<td>5.6</td>
</tr>
<tr>
<td>Std. Deviation of Neg Ctrl</td>
<td>48</td>
</tr>
<tr>
<td>Std. Deviation of Pos Ctrl</td>
<td>6</td>
</tr>
</tbody>
</table>
### Z factor: Example

#### Experiment “A”

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (arbitrary activity units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg of Neg Ctrl</td>
<td>200</td>
</tr>
<tr>
<td>Avg of Pos Ctrl</td>
<td>68</td>
</tr>
<tr>
<td>Fold-diff bwn Neg &amp; Pos Ctrl</td>
<td>2.9</td>
</tr>
<tr>
<td>Std. Deviation of Neg Ctrl</td>
<td>13</td>
</tr>
<tr>
<td>Std. Deviation of Pos Ctrl</td>
<td>3</td>
</tr>
</tbody>
</table>

**Z = 0.64**

#### Experiment “B”

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (arbitrary activity units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg of Neg Ctrl</td>
<td>381</td>
</tr>
<tr>
<td>Avg of Pos Ctrl</td>
<td>68</td>
</tr>
<tr>
<td>Fold-diff bwn Neg &amp; Pos Ctrl</td>
<td>5.6</td>
</tr>
<tr>
<td>Std. Deviation of Neg Ctrl</td>
<td>48</td>
</tr>
<tr>
<td>Std. Deviation of Pos Ctrl</td>
<td>6</td>
</tr>
</tbody>
</table>

**Z = 0.48**
Other parameters in Assay Optimization

- **Coefficient of variation (CV)**
  \[
  \%CV = \frac{SD}{Mean} \times 100
  \]

- **Signal to background (S/B)**
  - Mean of sample
  - Mean of control

- **Signal to noise (S/N)**
  - Mean of sample - Mean of Control
  - SD of control

Measure of dispersion of a probability distribution

Measures of signal strength
Assays in drug discovery: The hit identification and validation process

**Example:** Identification of a small molecule inhibitor of mitotic spindle bipolarity

Identification of Monastrol, inhibitor of mitotic kinesin, Eg5

Hypothetical screening cascade

Assay development
Primary and secondary assays

HTS primary screen
(single dose)

“Hits”

Hit validation and determination of potency
(\(IC_{50}/EC_{50}\))
(counter screens, concentration response curves)

Selected hits \(IC_{50}\) or \(EC_{50}\) < 10 µM

Secondary phenotypic assays

Confirmed hits

In vitro DMPK, toxicity, permeability selectivity profiling

“Optimized hits”

SAR/analoging

Compound chemistry assessment

“Leads”

In vivo PK, toxicology and efficacy studies

Candidate selection
Case: Hypothetical cancer target

- **Cancer target: Protein kinase**
- Involved in a pathway which promotes tumor growth
- Overexpressed in cancer cells
- Exists in two forms: in cancer cells, it is complexed with other proteins; in normal cells it exists in a free uncomplexed form
- Kinase activity is critical for its function
- There are commercially available inhibitors which bind to ATP-binding pocket
- What are the types of assays that would enable identification of inhibitors?
Assays for hypothetical target

- **Primary assay:**
  - **Options:**
  1. **Exploit Kinase activity:**
     - a. Develop kinase activity assay for the purified target in a complexed form (specific for cancer cells)-primary screen
     - b. Develop kinase activity assay for purified recombinant target (not specific for cancer cells)-counter screen
  2. **Exploit availability of commercial compounds that bind to the ATP-binding site to design a fluorescence-based binding assay.**
    - Example FP (Fluorescence Polarization) assay: Synthesize fluorescently labeled commercial compound which binds to the target; design a screen which identifies compounds which can “compete-off” the fluorescent compound. When fluorescent compound is bound to the target, light emitted will be in the same plane as the excitation light; If the compound is competed off, then there will be a “shift” in fluorescence due to rotation of the fluorophore.

- **Secondary assays:**
  - Assay for expression of proteins “downstream” of target: e.g. ELISA
  - Assay for cell proliferation in cancer cell line
  - Assay for toxicity in non-cancer cell line
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:

• http://assay.nih.gov/assay/index.php/Table_of_Contents
Conclusion:

“The subject is not exhausted but we are.”

George Bernard Shaw