ASSAY DEVELOPMENT IN DRUG DISCOVERY

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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

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

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

“HITS”

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

- Drug Discovery approaches
- 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 case studies
Drug Discovery Approaches

The two approaches are not mutually exclusive: Drug Discovery programs can use two-pronged approach.

**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
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
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

Automation

Cost

“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 read-outs, 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
  - Increased specificity of compounds
  - Can measure compound characteristics (e.g. Ki etc)

- **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 ➔ no FRET
  *Close*: Energy transfer from donor to acceptor ➔ FRET

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

- **Advantages**:
  - Reduced assay time and cost
  - Homogenous assay format
  - 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-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.

- **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.)

Un-labeled compounds

Screen

ATP binding pocket

Cy3 labeled compound

FP assay

FP read

Southern Research Drug Discovery
Luminescence-based assays

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

**Example:** Luciferase reporter assay:

Luciferin + ATP → Luciferyl adenylate + PPI

Luciferyl + O2 → Oxyluciferin + AMP + Light
Examples of biochemical assays
Assay for kinase activity

ADP Hunter assay

- Kinase
- Substrate
- ATP

Kinase target
Substrate → Phosphorylated Substrate + ADP

Inhibitors
Substrate → Phosphorylated Substrate + ADP

Fluorescent product
Ex: 530 nm, Em: 590 nm

Reduced signal

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Assay for protein-protein interaction

AlphaScreen technology

*Amplified Luminescent Proximity Homogenous Assay*

Excitation at 680 nm

Alpha Donor Bead

\[ {^1}O_2 \]

4 µsec

Alpha Acceptor Bead

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

![Diagram](image)

*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
High Content Screening

**High content screens:**
- Analysis of whole cells or components of cells with simultaneous readout of several parameters
- 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
- Facilitates validation of effects of compounds at the cellular and subcellular level (expression, localization, morphological changes in cells)
**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.

![Graph showing Cytochalasin-D concentration response on cell migration](image)

Cytochalasin-D 48hrs
IC50 = 0.12uM

Cell Invasion Ratio

- Concentration (uM): 10, 1, 0.1, 0.01
- % Inhibition: 0, 25, 50, 75, 100

![Graph showing percentage inhibition vs concentration](image)
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

- 96-well: 100-200 µl
- 384-well: 25-50 µl
- 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*

• 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
  
  \[ Z = 1 - \frac{3 \text{SD of sample} + 3 \text{SD of control}}{\text{mean of sample} - \text{mean of control}} \]
  
  \( Z > 0.5 \)

- **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
  
  \( S/B = \frac{\text{Mean of sample}}{\text{Mean of control}} \)

  \( S/N = \frac{\text{Mean of sample} - \text{Mean of control}}{\text{Standard Deviation of control}} \)

  Will be covered in HTS lecture
ASSAY DEVELOPMENT CASE STUDIES
CASE 1: 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\(_{50}\))**
1. Concentration response in primary assay for potency (IC\(_{50}\))
2. Cytotox assay to eliminate compounds with toxicity

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

**Secondary assay:** TXNIP qPCR

**Chemistry analysis**

13 Scaffolds of interest

**Structure Activity Relationship (SAR)**

LEAD COMPOUNDS

Test in other confirmatory assays at UAB
CASE 2: Identifying compounds that induce 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 (IC$_{50}$)**
- Counter screen using a mutant form of the HO-1 promoter
- Concentration response in primary assay for potency (IC$_{50}$)
- Cytotox assay to eliminate compounds with toxicity
- ~ 2000 "Hits"

**Chemical clustering analysis**
- ~ 747 "Hits"
- 28 "Hits" representing six chemical clusters

**Secondary assays**
- HO-1 high content screen
- "Analoging"/SAR purchase of analogs of hits/chemical synthesis

**LEAD COMPOUNDS**

Test in other confirmatory assays (Activity assays)
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
Solving the world’s hardest problems.