New cell based assays and tools for epigenetics, biologics, metabolic markers and more.....

Craig Malcolm: Product Manager Cell Analysis & Proteomics

Cell-Based Assay Tour - March 2014
Outline

- Brief outline of Promega’s epigenetics portfolio
  - new isozyme-selective cell-based histone deacetylase assays

- Cell based assays for Biologics characterisation
  - Reporter gene bioassays for antibody-dependent cellular cytotoxicity (ADCC)
  - Additional reporter gene assays for other biologic therapeutic mAb’s

- Recently launched suite of luminescence read-out metabolite assays

- New assays for oxidative stress (ROS-Glo)
Epigenetics - definition

- heritable changes in gene expression that arise from changes in chromosomes without alteration of DNA sequence
- changes occur throughout all stages of development or in response to environmental factors such as exposure to toxins or chronic stress and are implicated in diseases such as cancer
- Epigenetic mechanisms of gene regulation, which collectively make up the epigenome, include modifications to DNA and histone components of nucleosomes as well as expression of noncoding RNAs (ncRNAs)
- modifications can affect gene accessibility to DNA-binding and regulatory proteins such as methyl-CpG-binding proteins, transcription factors, RNA polymerase II and other components of the transcriptional machinery, ultimately altering transcription patterns, often in tissue- and cell-specific ways
Epigenetic mechanisms involved in regulation of gene expression

Cytosine residues within DNA can be methylated, and lysine and arginine residues of histone proteins can be modified. Me = methylation, Ac = acetylation, P = phosphorylation, Ub = ubiquitination.
Promega’s epigenetics portfolio - I

- Non-cell based assays

  - **MethylEdge™ Bisulfite Conversion System**
    - efficient DNA conversion and recovery with reduced template fragmentation using a protocol that can be completed in less than two hours
    - kit includes desulphonation and cleanup. Component
    - no additional cleanup kit required

  - **Methylation-Specific Restriction Enzymes** *eg.* Hpall, MboI, MspI, and Sau3AI
    - valuable tools for studying DNA methylation patterns
    - coupled with robust DNA purification and PCR reagents - allow for detection of methylation-specific events
Promega’s epigenetics portfolio - II

- Methyltransferase (MTase-Glo) – in development
- Universal assay
- Based on production of s-adenosylhomocysteine (SAH)
- Designed for characterising the effects of compounds on DNA, protein and small molecule methyltransferases
Promega’s epigenetics portfolio - III

- **Cell-based & biochemical assays** - simple luminescence-based assays for
  - **histone deacetylase (HDAC) & sirtuin (SIRT) activities**
    - HDAC-Glo™ Assays are used to determine histone deacetylase activity in cell-based or biochemical formats
    - flexible, sensitive bioluminescent assays provide a highly predictive, easy-to-use method for accurate and efficient inhibitor profiling.

- **DUB-Glo™ Protease Assay**
  - homogeneous, bioluminescent assay that measures the activity of numerous deconjugating enzymes including deubiquitinating (DUB), deSUMOylating (SENP) and deneddylating (NEDP) proteases
  - these proteases reverse the protein modification by ubiquitin and ubiquitin-like proteins (Ubl proteins) and thus are integral components in the complex mechanisms of post-translational protein regulation in eukaryotes.
New HDAC-Glo™ Class IIa and 2 selective assays

- HDAC-Glo™ Class IIa and 2 selective assays launched 31/01/14
- Single-reagent-addition, homogeneous, luminescent assays that measure the relative activity of histone deacetylase (HDAC) Class IIa and Class I enzyme 2, respectively, from cells, extracts or purified enzyme sources
- Assays use an isozyme-selective acetylated, live-cell-permeant, luminogenic peptide substrate that can be deacetylated by HDAC activities
- Deacetylation of the peptide aminoluciferin substrate is measured using a coupled enzymatic system in which a protease in the Developer Reagent cleaves the deacetylated peptide from the aminoluciferin substrate, releasing aminoluciferin
- Aminoluciferin is quantified in a reaction using Ultra-Glo™ Recombinant Luciferase (firefly)
- Signal from the assay reaction can be measured within 15–45 minutes after reagent addition with no sample manipulation
- HDAC-mediated luminescent signal is persistent, with a half-life of greater than 2 hours, allowing batch processing of multiwell plates.
**HDAC-Glo™ Class Ila - mechanism**

**Note:** The three enzymatic events occur in a coupled, nearly simultaneous reaction that is proportional to deacetylase activity.

**Assay Advantages**
- **Simple Measurement of Deacetylating Activities:** Single-reagent-addition, homogeneous “add-mix-measure” protocol.
- **Sensitive:** The assay provides 100-fold or better sensitivity than comparable fluorescence methods.
- **Utility:** The assay may be used with recombinant enzyme sources or in a cell-based format.
- **Fast Data Acquisition:** Collect maximal signal in as few as 20 minutes with persistent, “glow-type” steady state signal half-life.
HDAC-Glo Class IIa data

- biochemical assay format
- compare HDAC 4, 5, 7 and 9
- 384-well format
- assay volume 20µl

- cell-based assay format
- compare lytic/non-lytic assay
- using K562 cells as HDAC IIa source
- 2,500 cell/well (lytic)
- 10,000 cells/well (non-lytic)
- 384-well format
- assay volume 40µl
Z’ analysis in 384-well format (HDAC-Glo™ IIa assay)

Biochemical assay format using purified HDAC 7 & PXD-101 inhibitor

<table>
<thead>
<tr>
<th>Compound Test Condition (n = 96)</th>
<th>Signal:Background</th>
<th>% CV</th>
<th>Z’</th>
</tr>
</thead>
<tbody>
<tr>
<td>no PXD-101 (1% DMSO)</td>
<td>N.A.</td>
<td>5.6</td>
<td>N.A.</td>
</tr>
<tr>
<td>0.5μM PXD-101</td>
<td>1.4</td>
<td>5.5</td>
<td>0.10</td>
</tr>
<tr>
<td>5μM PXD-101</td>
<td>4.6</td>
<td>8.3</td>
<td>0.72</td>
</tr>
<tr>
<td>50μM PXD-101</td>
<td>16.7</td>
<td>8.7</td>
<td>0.81</td>
</tr>
</tbody>
</table>
**HDAC-Glo™ Class 2 - mechanism**

**Note:** The three enzymatic events occur in a coupled, nearly simultaneous reaction that is proportional to deacetylase activity.

**Assay Advantages**
- **Simple Measurement of Deacetylating Activities:** Single-reagent-addition, homogeneous “add-mix-measure” protocol.
- **Sensitive:** The assay provides 100-fold or better sensitivity than comparable fluorescence methods.
- **Utility:** The assay may be used with recombinant enzyme sources or in a cell-based format.
- **Fast Data Acquisition:** Collect maximal signal in as few as 20 minutes with persistent, “glow-type” steady state signal half-life.
HDAC-Glo Class 2 data

- biochemical assay format
- Recombinant HDAC 2
- Inhibitors SAHA & LBH-589
- 384-well format
- assay volume 20µl

- cell-based assay format
- compare lytic/non-lytic assay
- using K562 cells as HDAC 2 source
- 750 cells/well (lytic)
- 5,000 cells/well (non-lytic)
- 384-well format
- assay volume 40µl
Z’ analysis in 384-well format (HDAC-Glo™ 2 assay)

Biochemical assay format using purified HDAC 2 & SAHA inhibitor
Tools for biologics – ADCC reporter assays and other cell-based assays for mAb characterisation
Antibody-dependent cell-mediated cytotoxicity (ADCC) is the main MOA of antibodies through which virus-infected or other diseased cells are targeted for destruction by components of the cell-mediated immune system, such as NK cells.

1. Antibodies bind antigens on the surface of target cells.
2. NK cell CD16 Fc receptors recognise cell-bound antibodies.
3. Cross-linking of CD16 triggers degranulation into a lytic synapse.
4. Tumour cells die by apoptosis.

Classification of therapeutic antibodies based on their putative mechanisms of action

**Class I**: mAbs bind cell surface antigens and the Fc effector function is part of the MOA

**Class II**: mAbs bind cell surface antigens but MOA does not involve Fc effector function

**Class III**: mAbs bind and neutralize soluble antigens, blocking ligand from binding to its cognate receptor (e.g., Avastin)
Antibody structure

Fab domain - binds to antigen expressed on target cells e.g. cancer cells, virus infected cells

Fc domain – binds to Fc receptor (e.g. FcγIIIR, CD16) on immune system effector cells
**Traditional ADCC Assays**

**Effector cells**
- PBMCs (peripheral blood mononuclear cells)
- Natural killers cells (NK) from PBMCs
- NK cell lines (e.g., NK92)

**Target cells**
- Load with chromium-51 or Eu
- Monitor cell lysis (LDH, Calcein AM, GAPDH, CytoTox-Glo™)

- difficult to isolate & purify, variable yields & efficacy
- stability of Fc receptor expression an issue
- spontaneous release, radioactive
- additional assay step

*CytoTox-Glo™ Cytotoxicity Assay*
**Classic ADCC assay vs ADCC reporter bioassay**

**Classic ADCC assay**
- **Signal is from target cell**
- High variability of assay - mainly due to primary NK cells
- Spontaneous lysis of target & effector cells results in high background

**Reporter-based ADCC bioassay**
- **Signal is from effector cell**
- Reduced variability by replacing NK cells with genetically engineered stable cell line
  - FcγRIIIa (V158)
  - NFAT-RE luc2
- Improved bioassay performance with robust reagents and assay design
Scientific basis of ADCC reporter bioassay

Target-cell bound Ab binds to FcγRIIIa on effector cell – activating pathway

Luciferase reporter is readout of pathway activation state

New reporter gene bioassay measures a step earlier in the pathway

Image source: Leibson-PJ, Immunity 1997
ADCC reporter bioassay - development

### Low Variability NFAT-RE luciferase bioassay

Developed & tested using:
- CD20 and Her2 Ab drugs
- CD20+ and Her2+ target cells
- Frozen, thaw-and-use, or continuously cultured cells
- Extensive ‘alpha’ evaluations:
  - tested in multiple global biopharma & biotechs
  - tested in multiple systems

1. Effector cells are engineered to express FcγRIIIa (V158) and NFAT-RE luc2 luciferase
2. ‘Cells as reagents’ (thaw-and-use)
3. Homogeneous assay format – simple ‘add-mix-read’ bioluminescent assay
4. Optimized and robust assay reagents and protocol
5. Performance characteristics that meet needs of stability testing, lot release and Ab characterization
ADCC reporter bioassay protocol

**Single day bioassay – no cell preparation!**

1. Incubate control, reference or test antibody with target cells.

2. Add engineered effector cells containing:
   - FcγRIIIa (V158)
   - NFAT-RE luc2 luciferase

3. Incubate to allow for pathway activation (as short as 6 hours).

4. Add luciferase detection reagent and measure luminescence.
ADCC reporter bioassay – typical results

Assay protocol:

CD20+ WIL2-S cells
+ 
Rituximab dilution series
+ 
Engineered Jurkat effectors
↓
Induction (22 hours)
↓
Quantification of luciferase activity

Specifics

- **E:T ratio = 6:1**
  (150k effector cells:25k WIL2-S target cells, per well)

FI=30

Log$_{10}$ [Rituximab], g/ml

Bioluminescence (RLU)

EC$_{50}$=4.8x10$^{-9}$ g/ml
Use of different target cells

Suspension or adherent target cells can be used

Rituximab (anti-CD20)

CD20\(^+\) B cell lines (suspension) as target cells

Trastuzumab (anti-Her-2)

Her2\(^+\) breast cancer cell lines (adherent) as target cells
An ideal bioassay…

- Reflective of the mechanism of action (MOA) of the biological product
- Well controlled (precise, accurate, robust, reproducible)
- Stability-indicating
- Usable as a QC lot-release assay
ADCC reporter bioassay is specific and reflective of the mechanism of action of the biologic material.

Target cells, effector cells and specific antibody
- No Target cells
- No Effector cells or no FcγRIIIa
- No antibody or non-specific antibody

Assay signal is dependent on:
- Presence of Target cells
- Presence of FcγRIIIa receptor
- Appropriate specific antibody

Reflects correct MOA
Bioassay characteristics - ICH guideline Q2 [R1]

Validation of Analytical Procedures:
- Accuracy
- Precision:
  - Repeatability (intra-assay precision)
  - Intermediate precision (day to day, analyst-to-analyst)
  - Reproducibility (lab to lab)
- Specificity
- Linearity
- Range
- Robustness

Assay is well controlled (precise, accurate, robust, reproducible)
Stability indicating for Fc effector function – activity of heat-treated antibody drugs

**Rituximab**

- 4 degrees C control: $EC_{50} = 5.77 \text{ng/ml}$
- 1 day 65 degrees C
- 2 days 65 degrees C
- 3 days 65 degrees C

**Tositumomab**

- Control at 4 degrees C: $EC_{50} = 31.0 \text{ng/ml}$
- 5' at 65 degrees C
- 10' at 65 degrees C
- 15' at 65 degrees C

**Activity of heat-treated antibody drugs**

**Trastuzumab**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>1.284e-008</td>
</tr>
<tr>
<td>65°C, 1 day</td>
<td>1.902e-008</td>
</tr>
<tr>
<td>65°C, 3 days</td>
<td>2.031e-008</td>
</tr>
<tr>
<td>65°C, 5 days</td>
<td>3.139e-008</td>
</tr>
</tbody>
</table>

Assay is stability-indicating & usable as a QC lot-release assay
ADCC reporter bioassay activity correlates with amount of antibody N-glycosylation

Rituximab and Trastuzumab:
Linear correlation obtained between percentage of N-glycosylated antibody in blended antibody samples and relative luciferase reporter activity in ADCC reporter bioassay.

Small differences in Fc effector activity in ADCC pathway activation are easily distinguished in the ADCC reporter bioassay.
To be more flexible to research needs, we offer multiple kit formats:

1. **Core Kits:**
   1X kit – Cat.# G7017
   5X kit – Cat.# G7018

2. **Complete Kits:**
   Includes control antibody and target cells plus effector/target cells and reagents

3. **Target Kits:** Control antibody and target cells

**Note:** the ADCC Bioassay Effector Cells alone are also available for propagation and banking under a license agreement.
Other cell-based assays for therapeutic mAb characterisation

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cell type</th>
<th>Antibodies</th>
<th>Readout</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα blocker assay</td>
<td>U937 cells</td>
<td>Adalimumab, Infliximab</td>
<td>Caspase 3/7 activation</td>
</tr>
<tr>
<td>VEGF blocker assay</td>
<td>HEK 293</td>
<td>Avastin</td>
<td>NFAT/luc2P reporter assay</td>
</tr>
<tr>
<td>CTLA-4 receptor blocker assay</td>
<td>Jurkat</td>
<td>Yervoy</td>
<td>IL2 promoter/luc2P reporter assay</td>
</tr>
</tbody>
</table>

These are just examples – more assays for biologics are being added all the time....
TNFα blocker assay

Apoptosis is a mechanism of action of TNFα and suitable as a bioassay readout.

- TNFα
- TNFα receptor
- Apoptotic cell death
- Cytoplasm
- Nucleus
- Cell survival
- Cell differentiation
- NF-κB activation
- IκBα degradation
- Caspase 3
- JNK translocation
- AP-1
- MKK7
- JNK
- Bid
- NF-κB translocation
- MEKK1
- ASK1
- TRADD
- TRAF 2
- Rip
Bioluminescent TNFα blocker apoptosis bioassay

**Protocol:**

1. TNFα and anti-TNFα Ab are co-incubated; the TNFα and anti-TNFα mix is added to thaw-and-use U937 cells in assay medium.

2. Response is induced in as little as 2.5hr.

3. Caspase-Glo® 3/7 Assay is added and luminescence is measured immediately.

**Apoptosis readout:** quantifying blocker activity against TNFα
**TNFα blocker apoptosis bioassay can be used to quantify activity of on-market TNFα blocker drugs**

**A.** Fully human adalimumab demonstrates significant improved potency in TNFα blocking biological activity compared with a mouse mAb.

**B.** Good potency discrimination is obtained using a series of infliximab preparations of different theoretical potencies.

![Graph showing potency comparison](attachment:image.png)

<table>
<thead>
<tr>
<th></th>
<th>Final Anti-TNF (μg/ml)</th>
<th>Final infliximab (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adalimumab</td>
<td>0.005997</td>
<td>0.05798</td>
</tr>
<tr>
<td>monoclonal</td>
<td>0.04813</td>
<td>0.03743</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Anti-TNF (μg/ml)</td>
<td>300,000, 250,000, 200,000, 150,000, 100,000, 50,000, 0</td>
</tr>
<tr>
<td>Final infliximab (μg/ml)</td>
<td>300,000, 250,000, 200,000, 150,000, 100,000, 50,000, 0</td>
</tr>
</tbody>
</table>
VEGF blocker assay principle
Avastin titration

Raw data

Fold increase

Incubate cells with Avastin titration for 30 minutes prior to adding VEGF at EC_{80} concentration (14 ng/ml)

IC_{50} of Avastin = 92 ng/ml
Yervoy/CTLA-4 assay principle

- Activation of T cells and cytokine release such as IL2 production require CD3 antibody induced TCR activation and CD80/CD86 co-stimulatory signals to CD28.
- CTLA-4 receptor expression sequester ligands away from CD28, thus preventing co-stimulatory signals from being received.
- Yervoy Antibody blockade of CTLA-4/CD80/CD86 interactions promote T cell activation
Jurkat/IL2-luc2P/CTLA-4 Delta stable cell line performance

Raw data

Fold increase

Ec50 = 6.64ug/mL
New assays for metabolic markers
A novel Proluciferin Substrate was Combined with Specific Cycling Enzymes to Develop Three Assays

**NAD(P)H-Glo™ Detection System** - detects reduced forms NADH and NADPH

- NAD(P)H → Reductase → Luciferin → Light
  - Luciferin Detection Reagent (LDR)

**NAD/NADH-Glo™ Assay** - detects non-phosphorylated forms

- NAD Cycling Substrate → NADH → Reductase → Luciferin

**NADP/NADPH-Glo™ Assay** - detects phosphorylated forms

- NADP Cycling Substrate → NADPH → Reductase → Luciferin
**Features of the assays: sensitivity, specificity and wide assay window**

<table>
<thead>
<tr>
<th></th>
<th>NAD(P)H-Glo Detection system</th>
<th>NAD/NADH-Glo Detection system</th>
<th>NADP/NADPH-Glo Detection system</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limit of Detection (LOD)</strong></td>
<td>25nM (625fmol/25μl)</td>
<td>1nM (25fmol/25μl)</td>
<td>1nM (25fmol/25μl)</td>
</tr>
<tr>
<td><strong>Linearity</strong></td>
<td>25nM – 50μM</td>
<td>1 – 500nM</td>
<td>1 – 500nM</td>
</tr>
<tr>
<td><strong>Signal-to-background (S/B max)</strong></td>
<td>~400</td>
<td>~250</td>
<td>~250</td>
</tr>
<tr>
<td><strong>Cells/well for Total dinucleotides</strong></td>
<td>NA</td>
<td>500 -25,000</td>
<td>500 – 12,000</td>
</tr>
<tr>
<td><strong>Cells/well for Individual dinucleotides</strong></td>
<td>NA</td>
<td>2,000 -100,000</td>
<td>2,000 – 100,000</td>
</tr>
</tbody>
</table>
Sample preparation for measuring NAD+ /NADH or NADP/NADPH individually

Both assays employ an sample acidification or heat-treatment (60°C) step to selectively destroy a specific metabolite.
ROS-Glo™ H₂O₂ Assay
**ROS-Glo™ H₂O₂ assay**

- ROS-Glo™ H₂O₂ Assay is a homogeneous, fast and sensitive bioluminescent assay that measures the level of hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS), directly in cell culture or in defined enzyme reactions.

- A derivatised luciferin substrate is incubated with sample and reacts directly with H₂O₂ to generate a luciferin precursor.

- Addition of ROS-Glo™ Detection Solution converts the precursor to luciferin and provides Ultra-Glo™ Recombinant Luciferase to produce light signal that is proportional to the level of H₂O₂ present in the sample.
**ROS-Glo™ mechanism & protocol**

1. **H₂O₂ Substrate**
   - Reaction with H₂O₂

2. **Luciferin Precursor**
   - ROS-Glo™ Detection Solution

3. **Luciferin**
   - Light

---

**Protocol Steps**

1. Treat samples. Add H₂O₂ Substrate Solution. Incubate for up to 6 hours.
2. Add ROS-Glo™ Detection Solution. Incubate for 20 minutes.
3. Read luminescence.
**ROS-Glo™ assay data**

- Hep G2 cells
- 96-well plate format
- 20µM menadione
- 10,000 cells/well
- 2 hour incubation with menadione
- Add ROS-Glo™ Detection Solution
- Luminescence read after 20 minutes
Multiplexing a fluorescent cytotoxicity readout with luminescence ROS-Glo™ to obtain both readings from the same population of cells (Hep G2 plated at 2,000 cells/well in 384-well plate)

After 2 hours compound treatment, ROS-Glo™ assays are conducted on media aliquoted into a separate plate followed by addition of CellTiter-Glo viability reagent to original plate. (Hep G2 plated at 2,000 cells/well in 384-well plate)
Questions??