Tools for Cell Metabolism:
Bioluminescent NAD(P)/NAD(P)H-Glo™ Assays

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Metabolic Pathways Regulate Basic Needs of Living Cells

The metabolic requirements of proliferating cells are different from differentiated cells.

Differentiated cell supports catabolic requirements

Proliferating cell supports catabolic and anabolic requirements
Cancer Cells Rewire their Metabolic Pathways to Adjust to Changing Requirements

- Cancer is a disease of uncontrolled cell growth
- Metabolic pathways have to balance energy production with an increased need for biomolecule biosynthesis and to provide protection against increased ROS production

- Increase glucose uptake
- Shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect)
- Increase lactate production
- Activates pentose phosphate pathway
- Increase anabolic reactions
"Information about a cell’s metabolic state is integrated into the regulation of epigenetics"

Oncogenic and Metabolic Pathways Have to Work Together

- Alterations in signal transduction
  - Signaling pathway
  - Transcription
  - DNA

- Metabolic reprogramming
  - Metabolic pathway
  - Change in metabolites
  - Transcription
  - DNA

- The principal mechanisms underlying this metabolic reprogramming by oncogenes and tumor suppressors is still poorly understood

- Key metabolites can serve as direct signatures of metabolic changes and provide a functional readout of cellular state
Rapid and Easy to Use Detection Assays are Needed to Elucidate the Role of Metabolic Pathways

Key metabolic needs of cancer cells:

- Support favorable energetics
  - Increase glucose and glutamine uptake
  - Altered ATP/ADP/AMP ratios
- Manage excessive reactive oxygen species (ROS)
- Regulate the redox potential
  - GSH/GSSG, \textit{NAD(P)}/\textit{NAD(P)H}
- Satisfy the anabolic demands of macromolecular biosynthesis
  - \textit{NADPH} is the major currency for macromolecular biosynthesis

Oncogenes and Tumor Suppressors

Metabolic Adaptation

- Energy
- Biosynthesis
- Redox

Assays

- ATP/ADP/AMP Glucose uptake
- ROS NADPH
- GSH/GSSG NAD(P)/NAD(P)H
Nicotinamide Adenine Dinucleotides: NAD, NADH, NADP, NADPH

- Cofactors for dehydrogenases – class of enzymes regulating major metabolic pathways

- Work in pairs
  - \[ \text{NAD} \leftrightarrow \text{NADH} \]
  - \[ \text{NADP} \leftrightarrow \text{NADPH} \]

- Each pair has distinct functions

\[ \text{NAD}^+ \text{ oxidized} \]
\[ \text{NADPH} \text{ reduced} \]

\[ \text{Nicotinamide} \]
\[ \text{Ribose} \]
\[ \text{Adenine} \]

Missing in \[ \text{NAD}^+ \text{ and NADH} \]
NADPH Plays a Key Role in Altered Cancer Metabolism

- NADP/NADPH provide reducing equivalents
  - For biosynthetic reactions (anabolic processes)
  - GSSG reduction and ROS defense

- Measuring NADP/NADPH in cells is the most challenging
  - NADP/NADPH levels are ~10x lower compared to NAD/NADH
  - Cells respond by changing the NADPH/NADP ratio rather than total nucleotide levels
  - The Reduced form (NADPH) is predominant in cells and very unstable
NAD is an Important Redox Factor and Substrate in Various Signaling Pathways

NAD/NADH – redox reactions in energy metabolism and mitochondria function

NAD as signaling molecule – NAD levels are regulated by consumption and production

- DNA repair
- Gene expression
- MAPK regulation
- Ca²⁺ signaling

Precursors containing pyridine moiety

Produce → NAD^+

Consume

PARPs
MARTs
SIRTs
CD38

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Methods for Measuring Nicotinamide Adenine Dinucleotides

Detection of reduced forms NADH and/or NADPH – suited for biochemical assays

(I) Direct absorbance/fluorescence

- Reduced forms but not oxidized absorb light at 340 nm and have intrinsic fluorescence Ex350/Em460

(II) Enzymatic oxidation

- Clostridium Diaphorase couples oxidation of reduced forms with reduction of various dyes

\[ \text{NADPH} \xrightarrow{\text{Diaphorase}} \text{Resorufin (fluorophore)} \]
Detection of Oxidized and Reduced Forms in Cells/Tissues and Biochemical assays

(III) Enzyme coupled cycling reactions

- Detects oxidized and reduced forms present in the sample
- Specific for non-phosphorylated (NAD+ + NADH) or phosphorylated (NADP+ + NADPH) pairs through the choice of cycling enzymes

(IV) Metabolite analysis/profiling by LC-MS

(V) Methods for selective monitoring of compartmental responses
- Genetically-encoded fluorescent sensors
- Indirect calculation of NAD/NADH ratio from the concentration of redox couples (for example, lactate and pyruvate)
Challenges of NAD(P)/NAD(P)H Detection: preserving the natural state of nucleotides

✓ Accurate and reliable nucleotide detection depends on:
  - Rapidly stopping cellular metabolic activity
  - Setting up an extraction method that is not destructive
  - Using robust, reproducible methods for metabolite separation and quantitation

✓ Improper sample preparation can lead to misleading results:
  - 90% of nucleotides can be degraded upon cell lysis leading to inaccurate detection:
    - Reported NAD/NADH levels vary from 0.02 to 2 fmole/cell

✓ Two types of sample preparations are commonly used:
  - Extraction into organic buffers
  - Extraction into acid/base buffers

✓ Both methods require multiple handling steps and due to the lack of sensitivity and robustness, they do not allow direct in-well nucleotide detection:
  - Not adaptable to different plate formats (96-; 384-; 1536-well plates) and HTS applications
Physics and Chemistry of Bioluminescence Translates into Preferred Features for Assay Development

One chemistry – multiple detection systems

Bioluminescence is not affected by:
- Excitation and emission wavelength overlap
- Fluorescent chemicals in media
- Background fluorescence

Superior performance for plate-based assays
- Rapid and easy to use
- Increased sensitivity, lower background
- Broad linear range
- Less interference from fluorescent compounds
- Ability to multiplex with fluorescent assays

Luciferase Activity

Luciferin Generation

Light
A novel Proluciferin Substrate was Combined with Specific Cycling Enzymes to Develop Three Assays

NAD(P)H-Glo™ Detection System - detects NADH and NADPH biochemically

NAD/NADH-Glo™ Assay – detects non-phosphorylated forms in cells

NADP/NADPH-Glo™ Assay – detects phosphorylated forms in cells
All Three Assays Use the Same “add&read” Protocol but Measure Different Nucleotides

Step 1: Make Detection Reagent

Step 2: Add to the sample, 1:1 ratio

Incubate 30-60 min.

Step 3: Read luminescence

Apply to various upfront sample preparations

NAD/NADH-Glo™ Assay measures:
- Cellular levels
  - Total
  - NAD and NADH
  - NAD/NADH
- Enzyme Activity

NADP/NADPH-Glo™ Assay measures:
- Cellular levels
  - Total
  - NADP and NADPH
  - NADP/NADPH
- Enzyme Activity

NAD(P)H-Glo™ Detection System measure:
- NADH and/or NADPH
- Activity of enzymes that produce or use NADH or NADPH
NAD(P)H-Glo Detection System Measures Reduced Nicotinamide Adenine Nucleotides

50µl Nucleotide standards in PBS + 50µl Detection Reagent

• High Sensitivity: LOD ~5-50nM
• Wide linear range: 50nM-25µM
• Good S/B: Max S/B ~500; at 1µM S/B >10

• Signal becomes stable when all of the NAD(P)H is consumed
• If production of NAD(P)H is not stopped, the signal continues to increase until all reductase substrate is consumed
Dehydrogenase Activity Detection with NAD(P)H-Glo™

Measure NAD(P)H production

Alternative: measure utilization as decrease in signal

\[ \frac{S/N}{\text{Signal-to-Noise Ratio}} = \frac{\text{Mean}_{\text{signal}} - \text{Mean}_{\text{background}}}{\text{SD}_{\text{background}}} \]
**NAD(P)H-Glo™ is Easily Automated**

![Graph showing RLU vs Well Number with [C]=10µM, S/B=795; Z'=0.89, [C]=5µM, S/B=396; Z'=0.89, and [C]=1µM, S/B=72; Z'=0.87.]

- Scalable reaction volumes
- Good Z’ values
- Excellent plate uniformity
- Detection reagent stable at room temperature for at least 8 hours

**384 well plates**

8µl NADH + 8µl Detection Reagent
30 min reaction time

\[ Z' = 1 - \frac{(3 \times \text{Std}_{\text{high-control}} + 3 \times \text{Std}_{\text{low-control}})}{\text{(Mean Control}_{\text{high}} - \text{Control}_{\text{low}})} \]
NAD/NADH-Glo™ and NADP/NADPH-Glo™ Couples NAD(P)H Detection with Enzyme Cycling Reaction

- **NAD(P)** is converted to **NAD(P)H** by cycling enzymes and is utilized by Reductase to produce luciferin.
- The reaction cycles to increase sensitivity with the light output remaining proportional to the starting amount of nucleotides.
- The cycling continues until all reductase substrate is consumed.
- To achieve a stable “Glo-type” signal the cycling reaction can be stopped with stopping solution.
NAD(P)/NAD(P)H Detection in Cells

Rapid in-well, one-step, add & read protocol without sample processing

- Luciferase Detection Reagent contains detergents for efficient cell lysis/endogenous enzyme inhibition
- Assays are optimized to run reductase and enzyme cycling reactions together with luciferase detection
- Modifications allowed to simplify protocol
  - Detection reagents can be pre-made and added directly to the cells
  - Detection system is compatible with different media and buffers (relative light output varies)
- Assay sensitivity enables detection of total NAD+NADH or NADP+NADPH directly in 96/384 wells

384-well plates
25µl Cells+25µl Detection Reagent

(NAD^+ + NADH)

Add Detection Reagent
Incubate RT 30min

Light
Read Luminescence
NAD/NADH-Glo and NADP/NADPH-Glo assays are specific

NAD/NADH-Glo™ detects

NAD + NADH

NADP/NADPH-Glo™ detects

NADP + NADPH
Bioluminescence Provides Improved Sensitivity and Broader Linearity than other Detection Methods

NAD/NADH-Glo™ Assay

<table>
<thead>
<tr>
<th>Bioluminescence</th>
<th>Fluorescence</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (S/B &gt;3)</td>
<td>4nM</td>
<td>250nM</td>
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<tr>
<td>Linearity</td>
<td>0.004-0.5µM</td>
<td>0.06-4µM</td>
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<tr>
<td>Signal Window (S/B)</td>
<td>481</td>
<td>16</td>
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</tbody>
</table>

Reactions were performed following recommended protocols: 60min for luminescence; 90min for fluorescence and 120min for absorbance
Bioluminescence Provides Improved Sensitivity and Broader Linearity than other Detection Methods

NADP/NADPH-Glo™ Assay

<table>
<thead>
<tr>
<th></th>
<th>Bioluminescence</th>
<th>Fluorescence</th>
<th>Absorbance</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity (S/B &gt;3)</td>
<td>7nM</td>
<td>125nM</td>
<td>4,000nM</td>
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<tr>
<td>Linearity</td>
<td>0.004-1µM</td>
<td>0.06-4µM</td>
<td>N.D.</td>
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<tr>
<td>Signal Window (S/B)</td>
<td>430</td>
<td>16</td>
<td>3</td>
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</table>
Adaptable to different plate formats and HTS applications

NAD/NADH-Glo™ Assay

- [C]=100nM; S/B=20; Z'=0.89
- [C]=250nM; S/B=53; Z'=0.85

NADP/NADPH-Glo™ Assay

- [C]=100nM; S/B=29; Z'=0.85
- [C]=250nM; S/B=80; Z'=0.82

384-LV well plates
8µl NADH + 8µl Detection Reagent
40 min reaction time

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Direct in-well dinucleotide detection using simple add and read protocol

✓ To measure NAD+NADH levels the NAD/NADH-Glo™ reagent is added directly to cells at 1:1 ratio
✓ To measure NADP+NADPH levels the NADP/NADPH-Glo™ reagent is added directly to cells at 1:1 ratio
Nucleotide Quantitation based on externally added dinucleotide recovery

- Light output is directly proportional to the amount of nucleotides present in the sample but relative light units are dependent on the media/buffer

- To calculate \( \text{NAD}^+ + \text{NADH} \):
  - Standard curves must be performed under the same conditions
  - \( \text{NAD}^+ + \text{NADH} \) amounts also can be calculated by “spiking” the samples with known nucleotide concentrations

384 well plate:
30µl A549 (2,5000 cells/well) in F12-K media or PBS + 30µl NAD/NADH-Glo™ Reagent
Sensitivity and Linearity Depend on Cycling Time

**MCF-7 Cells plated in 96-well plate**
50µl cells + 50µl NAD/NADH-Glo™ Detection Reagent

**Plate read at 15min**
- Linearity from 6,250 (S/B= 5) to 50,000 (S/B=58) cells per well

**Re-read at 45 min**
- Linearity from 1,563 (S/B=5) to 25,000 (S/B=84) cells per well

<table>
<thead>
<tr>
<th>Cell/50µl</th>
<th>50,000</th>
<th>25,000</th>
<th>12,500</th>
<th>6,250</th>
<th>3,125</th>
<th>1,563</th>
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<tr>
<td>15min RLU</td>
<td>3,946,450</td>
<td>1,669,227</td>
<td>715,710</td>
<td>364,489</td>
<td>206,243</td>
<td>131,774</td>
<td>68,014</td>
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<tr>
<td>S/B</td>
<td>58</td>
<td>25</td>
<td>11</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
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<tr>
<td>45min RLU</td>
<td>6,982,047</td>
<td>6,256,761</td>
<td>2,820,788</td>
<td>1,350,112</td>
<td>662,929</td>
<td>340,881</td>
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<tr>
<td>S/B</td>
<td>93</td>
<td>84</td>
<td>38</td>
<td>18</td>
<td>9</td>
<td>5</td>
<td>1</td>
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Measuring Total NAD+ NADH in Rat Blood Cells

Detection of Total NAD+ NADH using the NAD/NADH-Glo™ Assay

- RBC and WBC were isolated from 15ml of whole rat blood in EDTA using HISTOPAQUE 1083 and resuspended in PBS
- 50µl of cells were transferred into white 96-well plate and 50µl of NAD/NADH-Glo™ Reagent was added
- Luminescence was read after 30 minutes incubation at room temperature
Detecting Oxidized and Reduced Forms Individually

For measuring total oxidized and reduced forms or if only one form is present in the sample, the detection reagents are added directly to the sample.

If both oxidized and reduced forms are present in the sample and only one form is to be measured, the other form is destroyed before adding detection reagents.
Standard Protocols for Measuring Individual Nucleotides

- Oxidized forms are destroyed in basic solutions upon heating
- Reduced forms are destroyed in acidic solutions

Considerations:
- Starting sample: one well versus two wells
- Stability of oxidized forms in base buffers without heating
- Differential treatment of the sample (heat versus no heat)
- Errors in calculation when ratio is calculated by subtraction
**Improved Protocol for Measuring Individual Dinucleotides**

**Cells in PBS (50ul)**

- **NAD**
- **NADP**
- **NADH**
- **NADPH**

Add Basic Lysis buffer (50ul)

Divide the sample (100ul)

**For measuring oxidized (50ul)**

- Add 0.4N HCl (25ul)
- Heat at 60C for 15 min

**NAD**

**NADP**

- Cool down for 10 min
- Add Tris base (25ul)

**For measuring reduced (50ul)**

- Heat at 60C for 15 min

**NADH**

**NADPH**

- Cool down for 10 min
- Add Tris base (25ul) + 0.4N HCl (25ul)

**Samples are ready for dinucleotide detection**

**Improvements**

- ✔ Less sample required
- ✔ In-well detection
- ✔ Starting sample is from the same well
- ✔ Samples are in the same buffers at the end of treatment
- ✔ The ratio (NAD/NADH or NADP/NADPH) can be calculated directly based on RLU values
Selective Degradation of NAD, NADH, NADP and NADPH standards

All dinucleotide standards were diluted in PBS/Bicarbonate/0.5%DTAB buffer

- All forms are stable in this buffer
- Oxidized forms are degraded upon heating (60°C for 15min)
- Reduced form are degraded by adding 0.4N HCl and heating (60°C for 15min)
Individual measurement of cellular NAD, NADH, NADP and NADPH in Jurkat cells

- Nucleotide ratio can be calculated directly from RLU values
- The amount is calculated from calibration curve or from a spike of known nucleotide amounts

- The amounts of NADPH/NADP in Jurkat cells are ~20-fold lower in comparison with NAD/NADH
- 6,000 cells/well was used for NAD/NADH detection and 48,000 cells/well for NADP/NADPH detection
- All measurements were done from the same sample of cells collected in PBS
Monitor effect of FK866, an inhibitor of a key enzyme in the NAD biosynthesis pathway

Selective decrease in NAD (after NADH destruction)

Decrease in NAD with no change in viability

- PC3 cells (15K/well) were treated with increasing FK866 concentrations for 30 hours
- NADH was destroyed by acid treatment and NAD levels were determined using the NAD/NADH-Glo Assay
- Changes in cell viability were measured in separate wells using the CellTiter-Glo Viability Assay
Changes in cellular dinucleotide levels can be detected rapidly in a high-throughput format

- Rapidly monitor changes in cellular \( \text{NAD(P)} + \text{NAD(P)H} \) levels using an easy in-well, add-and-read protocol
- Observe correct pharmacology (\( \text{IC}_{50} \) values 0.7-4.5 nM)

**One step assay for total NAD+NADH**

- 25µl cells (~5K)/well
- Add FK866 NAD biosynthesis inhibitor
- Treat 28h
- Add 25µl NAD/NADH-Glo™ Detection Reagent
- Incubate 30-60min
- Record Luminescence
Measuring biochemical activity of NAD producing/consuming enzymes

✓ Enzymes involved in NAD biosynthesis or NAD-dependent signaling reactions (sirtuins, PARPs) can be assayed using NAD/NADH-Glo
  ❖ NADH is not present in those reactions
  ❖ NAD detection is sensitive (4nM-500nM) and robust (Z’>0.8 at 100nM NAD)
  ❖ The assay linearity can be extended up to 20-40uM

384 well plate:
15μl NAD + 15μl NAD/NADH-Glo Detection Reagent
Reaction time: 30 minutes
Measuring biochemical activity of NAD producing/consuming enzymes

- Measuring NADH conversion to NAD (increase in signal)
  - NADH is degraded by acids
  - 10% conversion of NADH to NAD can be detected with S/B>5
  - The sensitivity depends on the purity of NADH

Set up of conversion curve

<table>
<thead>
<tr>
<th>% NADH to NAD</th>
<th>25</th>
<th>20</th>
<th>15</th>
<th>10</th>
<th>5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH, uM</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>NAD, uM</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
**NAD(P)/NAD(P)H-Glo can be used with samples prepared by other methods**

- **Commonly used sample preparations**
  - Organic extraction
    - Hot EtOH/Hepes
  - Acid/base extractions

- **Outline for comparison of dinucleotide extractions**
  - EtOH/Hepes
  - Dried
  - PBS
  - PBS
  - 50 mM Tris, pH 7.5
  - Total amount (NAD+NADH and NADP+NADPH)
  - Bicarb/DTAB/+/−HCl
  - Individual NAD, NADH, NADP, NADPH

- **Yes, if**

- **Nucleotides are quantitatively recovered**
- **Final buffer composition does not interfere with Detection Reagents**
  - Dissolve organic extraction samples after drying in PBS or PBS/Bicarbonate/DTAB buffer
  - If acid/base method is used adjust pH 8-9
  - Test interference using dinucleotide titration curves
Example of using NAD/NADH-Glo and NADP/NADPH-Glo with samples prepared using organic extraction

NAD/NADH-Glo™ Assay

- Rapid dinucleotide degradation upon hypotonic cell lysis
- No significant difference between the total amounts determined after organic extraction and direct measurement in PBS
- Decrease in recovery of reduced forms after organic extraction

NADP/NADPH-Glo™ Assay
NAD, NADH, NADP and NADPH serve as important target-independent nodes

They link the metabolic state of cells with energy homeostasis and gene regulation

NAD(P)/NAD(P)H-Glo™ Detection Assays meet the need for rapid and robust measurement of nicotinamide adenine dinucleotides
Three assay formulations were developed to measure NAD, NADH, NADP, NADPH

- The assays are based on using a novel proluciferin compound that can be coupled to NAD(P)H oxidation by a reductase enzyme and light production by luciferase.

- The sensitivity and large signal window enable rapid in-well measurement of cellular nucleotides without sample pre-processing.

- Upstream events in cancer cell metabolism that are coupled to NAD(P)/NAD(P)H production can be studied rapidly with higher precision.
Bringing bioluminescence to cell metabolism
Thank You!

Bringing bioluminescence to cell metabolism

Other assays are under construction

Contact: jvidugir@promega.com