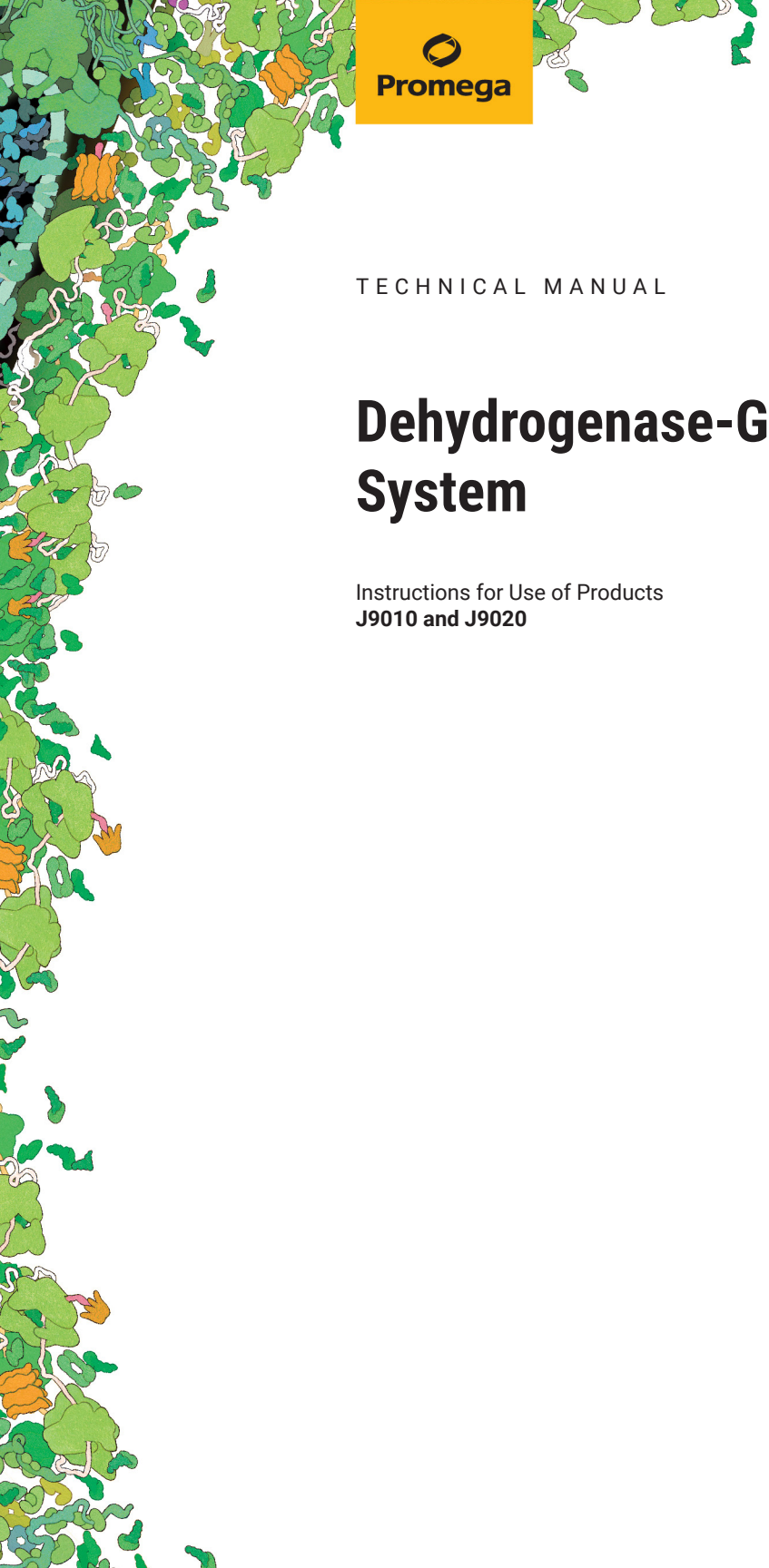

TECHNICAL MANUAL

Dehydrogenase-Glo™ Detection System

Instructions for Use of Products
J9010 and J9020

11/23
TM710

Dehydrogenase-Glo™ Detection System

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Dehydrogenase-Glo™ Detection System[(a)] provides a core bioluminescent NAD(P)H detection technology (1,2) that can be rapidly implemented to measure dehydrogenase activity in cell lysates, tissue homogenates and other biological samples. The Dehydrogenase-Glo™ Detection System provides only the reagents required for bioluminescent NAD(P)H detection. To build a specific dehydrogenase assay, you will need to identify and source the dehydrogenase substrate.

To measure dehydrogenase activity, the sample is combined at a 1:1 ratio with detection reagent containing a dehydrogenase-specific substrate, cofactor (NAD for NAD-dependent and NADP for NADP-dependent dehydrogenases), Reductase, Reductase Substrate and Ultra-Glo™ rLuciferase. The detection reagent also contains detergents to provide complete cell and tissue lysis, while the endogenous dehydrogenases remain active. The enzyme-coupled reactions shown in Figure 1 begin and progress simultaneously. The luminescent signal generated is proportional to the amount of dehydrogenase in the sample. The signal increases until all reductase substrate is consumed. When the substrate has been consumed, subsequent activity and corresponding signal are no longer within the linear range of the assay.

When measuring the activity of specific dehydrogenase in complex biological samples, the background activity of other dehydrogenases must be considered. The sensitivity of bioluminescent detection permits measuring dehydrogenase activity with low sample input, minimizing nonspecific background and the addition of excess dehydrogenase substrate drives specific activity of the dehydrogenase of interest. To determine the background activity, prepare a control containing the required cofactor (NAD or NADP) without the dehydrogenase-specific substrate and subtract this signal when calculating dehydrogenase-specific activity.

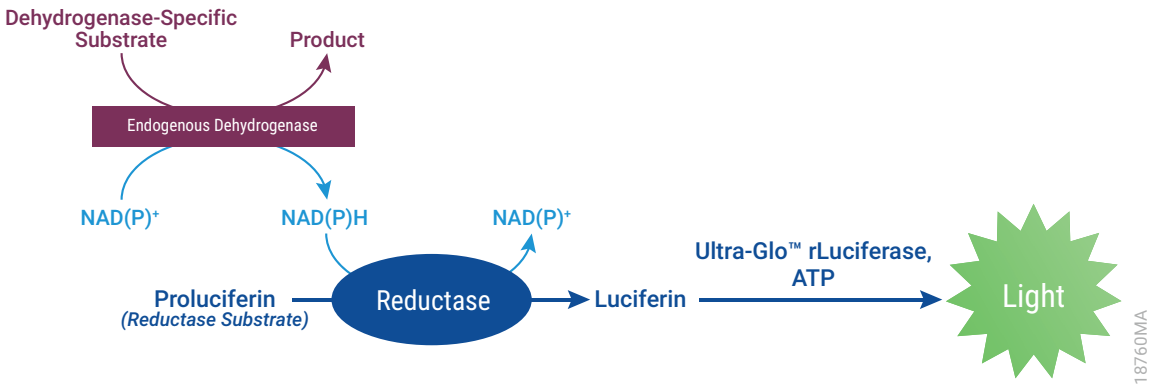

Figure 1. Schematic diagram of the Dehydrogenase-Glo™ Detection System principle. An endogenous dehydrogenase catalyzes the oxidation of a dehydrogenase-specific substrate with concomitant reduction of $NAD(P)^+$ to NAD(P)H. In the presence of NAD(P)H, Reductase enzymatically reduces a proluciferin Reductase Substrate to luciferin. Luciferin is detected using Ultra-Glo™ Recombinant Luciferase, and the amount of light produced is proportional to the NAD(P)H produced through specific dehydrogenase activity in the sample.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Dehydrogenase-Glo™ Detection System	**5ml**	**J9010**

The system contains sufficient reagents to perform 100 reactions in 96-well plates. Includes:

- 5ml Luciferin Detection Solution
- 55µl Reductase
- 55µl Reductase Substrate
- 275µl NAD
- 100µl NADP

PRODUCT	SIZE	CAT.#
Dehydrogenase-Glo™ Detection System	**50ml**	**J9020**

The system contains sufficient reagents to perform 1,000 reactions in 96-well plates. Includes:

- 50ml Luciferin Detection Solution
- 275µl Reductase
- 275µl Reductase Substrate
- 1ml NAD
- 500µl NADP

Storage Conditions: Store the Dehydrogenase-Glo™ Detection System at less than –65°C. Alternatively, store the Reductase Substrate at less than –65°C protected from light and all other components at –30°C to –10°C. Do not freeze-thaw the kit components more than three times. As needed, dispense kit components into single-use aliquots to minimize freeze-thaw cycles.

3. Assay Considerations

The Dehydrogenase-Glo™ Detection System was developed for measuring activity of endogenous dehydrogenases present in biological samples. Purified dehydrogenase activity can also be measured using the kit.

3.A. Sample Preparation

For measuring dehydrogenase activity in mammalian cells, the cells can be collected, resuspended in PBS and transferred to 96-well assay plates for immediate detection. In some cases, the dehydrogenase activity can be measured directly in cultured cells without medium removal. However, the presence of medium can result in a significant increase in assay background and must be determined empirically.

If the activity is not measured immediately, we recommend collecting the sample in storage buffer (200mM Tris [pH 7.3], 10% glycerol and 1% bovine serum albumin) and storing at ≤–20°C until assayed. For enzymes with limited stability (e.g., isocitrate dehydrogenase), store samples at ≤–65°C for best results.

3.B. Assay Linearity and Dilution Recommendations

When measuring dehydrogenase activity in biological samples, small molecules and endogenous dehydrogenases present can interfere with results. Therefore, we recommend testing several dilutions of unknown samples, running a control without the dehydrogenase-specific substrate and measuring luminescence kinetically as shown in Figure 2. For samples with high nonspecific background, subtract the no-substrate control signal from sample relative light unit (RLU) values prior to calculating dehydrogenase activity.

Assay specificity can be enhanced by diluting the sample to minimize background dehydrogenase activity and by providing excess dehydrogenase-specific substrate. For the assays performed in this technical manual, 200µM of dehydrogenase substrate and 200µM of dinucleotide cofactor (NAD or NADP) are sufficient. For some dehydrogenases, additional optimization of substrate and dinucleotide concentrations may be required.

3.C. Assay Incubation Time

We recommend recording luminescence 30–90 minutes after adding dehydrogenase detection reagent. We do not recommend taking readings before 30 minutes to ensure that the coupled enzyme reactions reach equilibrium. Incubation times longer than 60 minutes will increase assay sensitivity, but it is important to confirm that the assay results are still in the linear range and that the luminescence signal continues to increase.

3.D. Specific Activity Calculation

There is a linear relationship between luminescence and dehydrogenase activity. Luminescent measurements can be described simply in terms of relative light units (RLU) and the data can be expressed as the relative change in luminescence between the experimental controls and test conditions.

If needed, the dehydrogenase specific activity can be calculated by including a 10µM NADH or NADPH control. NADH (Sigma Cat.# N6660) and NADPH (Sigma Cat.# N9910) are not included in this kit and should be freshly reconstituted in PBS to make a 200µM stock and diluted to 10µM prior to use.

When calculating specific activity, we recommend measuring the luminescence signal in kinetic mode and choosing two time points in the linear range of the assay. For NADH and NADPH controls, after adding detection reagent, the luminescence signal will increase until all NADH or NADPH is converted to luciferin. The signal will then plateau, indicating complete conversion. Once plateaued, the NADH or NADPH signal can be used to calculate dehydrogenase specific activity.

3.E. Measuring Dehydrogenase Activity

Materials to Be Supplied By the User

- phosphate-buffered saline (PBS; e.g., Sigma Cat.# D8537 or GIBCO™ Cat.# 14190)
- 96-well assay plates (opaque white with white or clear bottom; e.g., Corning® Cat.# 3903 or 3912)
- luminometer (e.g., GloMax® Discover System, Cat.# GM3000)
- NADH or NADPH (freshly prepared, Sigma Cat.# N6660, N9910)
- dehydrogenase substrate

4. Measuring Dehydrogenase Activity

4.A. Reagent Preparation

We recommend performing reactions with 50µl of sample and 50µl of dehydrogenase detection reagent in a 96-well plate. Alternative volumes can be used provided the 1:1 ratio of dehydrogenase detection reagent volume to sample volume is maintained (e.g., 12.5µl of sample and 12.5µl dehydrogenase detection reagent in a 384-well plate format).

1. Thaw all components on ice or at room temperature. Once thawed, equilibrate the Luciferin Detection Solution to room temperature. Place all other components on ice. Mix thawed components prior to use.
2. Calculate the volume of detection reagents required. You will need 50µl for each assay in a 96-well plate with 50µl of sample. We recommend preparing additional reagent to compensate for pipetting error.
3. Prepare 10mM dehydrogenase-specific substrate in water or compatible buffer.
4. Prepare both dehydrogenase detection reagent and no-substrate detection reagent by combining components as shown in the table below.

   Note: Prepare only the volume of detection reagent calculated in Step 2. Unused dehydrogenase detection reagent cannot be stored.

Component	Volume Per Reaction	Volume Per 100 Reactions
Luciferin Detection Solution	50µl	5ml
Reductase Substrate	0.25µl	25µl
Reductase	0.25µl	25µl
NAD, 40mM*	0.25µl	25µl
NADP, 20mM*	0.50µl	50µl
Dehydrogenase Substrate, 10mM**	1µl	100µl

*Add required dinucleotide (dependent on the dehydrogenase of interest).

**Omit when preparing the no-substrate detection reagent.

5. Mix by gently inverting five times.

4.B. Recommended Controls

Include the recommended controls on every assay plate and process the controls the same as experimental samples.

- **Buffer-Only Control:** Triplicate wells of sample preparation buffer alone to determine assay chemistry background.
- **No-Substrate Controls:** Triplicate wells for each sample to determine nonspecific assay background. To assay these controls, use no-substrate detection reagent.
- **Optional NADH or NADPH Control:** Triplicate wells of 10µM NADH or NADPH for calculating dehydrogenase-specific activity. Either NADH or NADPH can be used, regardless of the requirements of the dehydrogenase of interest since the full conversion of 10µM NADH or NADPH into luciferin results in the same light output. NADH or NAPDH should be freshly reconstituted in PBS and diluted to 10µM in the same buffer as the sample prior to use. To assay these controls, prepare a detection reagent lacking both dehydrogenase substrate and dinucleotide (NAD or NADP).

4.C. Assay Protocol

When performing dehydrogenase assays with the Dehydrogenase-Glo™ Detection System, be sure to use assay plates that are compatible with your luminometer. See Section 6.B for more information.

1. Transfer 50µl of samples to a 96-well plate. Include buffer-only and NADH or NADPH controls, if required (Section 4.B).
   Note: Each sample should be tested in triplicate for dehydrogenase-dependent activity and nonspecific background activity (no-substrate control).
2. Prepare the dehydrogenase detection reagent and the no-substrate detection reagent as described in Section 4.A.
3. Add 50µl of dehydrogenase detection reagent to each sample well and to buffer-only control wells.
4. Add 50µl of no-substrate detection reagent to no-substrate control wells.
5. Mix the plate for 30–60 seconds by shaking.
6. Incubate at room temperature for 30–90 minutes.
7. Record luminescence every 15 minutes using a plate-reading luminometer, following the instrument manufacturer's instructions.
8. Calculate dehydrogenase-specific activity by subtracting no-substrate control RLU for each time point, then using the equation below:

$$\text{pmol NAD(P)H/cell/minute} = \frac{(RLU_{time\ 2} - RLU_{time\ 1}) \times \text{sample volume (µl)} \times [NAD(P)H]\ (\mu M)}{RLU_{NAD(P)H} \times \Delta\text{time} \times \text{cell number}}$$

5. Examples of Measuring Dehydrogenase Activity in Biological Samples

5.A. K562 Cells

Here we show an example of measuring select dehydrogenase activity in K562 cells. The chosen dehydrogenases are regulators of key metabolic pathways, and their activity can be measured by Dehydrogenase-Glo™ Detection System using dehydrogenase-specific substrates as shown in Table 1.

Table 1. Dehydrogenase and Substrate Information. K562 cells were collected and diluted to 2.5 × 10^4 cells/ml, followed by twofold dilutions in PBS, including a PBS-only control. Dehydrogenase detection reagents were prepared for each dehydrogenase measured as described in Section 4.A. Since the activity of both NAD- and NADP-dependent dehydrogenases was measured, no-substrate detection reagents were prepared for each dinucleotide cofactor (NAD only, NADP only) and were used to determine nonspecific assay background.

Dehydrogenase	Dehydrogenase Substrate	Dinucleotide (NAD/NADP)	Pathway
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	D-glyceraldehyde 3-phosphate (Sigma Cat.# 39705)	NAD	Glycolysis
Glucose 6-phosphate dehydrogenase (G6PDH)	Glucose 6-phosphate (Sigma Cat.# 10127645001)	NADP	Pentose Phosphate Pathway
6-phosphogluconate dehydrogenase (6PGDH)	6-phosphogluconate (Sigma Cat.# P7877)	NADP	Pentose Phosphate Pathway
Isocitrate dehydrogenase (IDH)	D-isocitrate (Sigma Cat.# 58790)	NADP	TCA Cycle

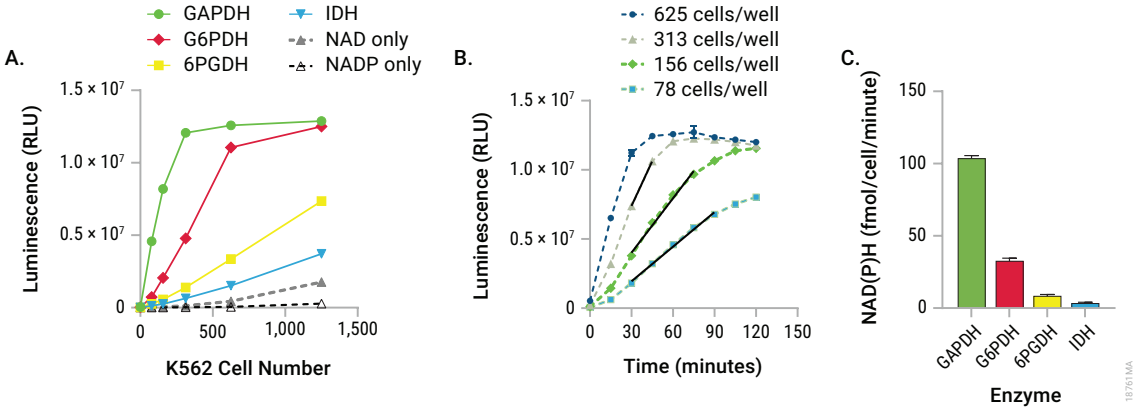

Figure 2. Measuring dehydrogenase activity in K562 cells. K562 cells were diluted twofold in PBS, starting at 2.5 × 10^4 cells/ml, and 50µl was transferred into a 96-well assay plate. Dehydrogenase detection reagent was prepared (Section 4.A) for each dehydrogenase and 50µl was added to sample wells. No-substrate controls (NAD only and NADP only) were included to determine background from nonspecific enzyme activity in K562 cells. **Panel A.** Luminescence was measured for dehydrogenases and controls after 60 minutes at different cell densities. **Panel B.** Time-dependent increase in GAPDH activity was shown at different cell densities. Luminescence was read every 15 minutes for 120 minutes. Solid black lines indicate linear range for each cell number. **Panel C.** Specific activity for each dehydrogenase was calculated as described in Section 4.C. Data represent the average of three replicates with error bars indicating one standard deviation.

As shown in Figure 2, the assay background and specific dehydrogenase activity will depend on the dehydrogenase activity as well as the cell number used in the assay and needs to be optimized for each dehydrogenase. The assay background is influenced by the number of cells and endogenous dehydrogenase activity of those cells. To start, we recommend using 100–2,000 cells per 50µl. Higher cell numbers might increase assay background, thereby decreasing assay window for measuring specific dehydrogenase activity.

In Figure 2, Panel A, the nonspecific assay background was measured at different cell densities using no-substrate detection reagents containing only NAD (for NAD-dependent dehydrogenases) or NADP (for NADP-dependent dehydrogenases) cofactors. For dehydrogenase-specific activity, each detection reagent was supplemented with the appropriate dehydrogenase substrate. The specific dehydrogenase signal was above background for all dehydrogenases after 60 minutes. However, while IDH and 6PGDH remain linear at higher cell densities, GAPDH and G6PDH were out of linear range above 313 and 625 cells per well, respectively. As shown in Figure 2, Panel B, the optimal number of cells for GAPDH was between 78–156 cells/well with luminescence signal being linear between 30–60 minutes as preferred for this assay. At 60 minutes, >150-fold increase in signal above nonspecific background was measured with 156 cells per well.

The data indicate that the Dehydrogenase-Glo™ Detection System can be used to measure key metabolic pathways (e.g., glycolysis, TCA cycle, pentose phosphate pathway, etc.) following simple assay optimization with minimal sample input and robust light output.

5.B. Dehydrogenase Inhibition in Cell Lysates

The sensitivity and robustness of the Dehydrogenase-Glo™ Detection System makes it well suited for high-throughput applications, including inhibitor screening. Figure 3 shows an example of using the Dehydrogenase-Glo™ Detection System to measure glucose-6-phosphate dehydrogenase (G6PDH) inhibition in K562 cell lysates. In this experiment, K562 cells were lysed by sonication and pre-incubated with G6PDi-1, a known inhibitor of G6PDH (3), before adding G6PDH detection reagent to measure glucose-6-phosphate dehydrogenase activity. The experiment was performed with optimized conditions, shown in Figure 2, using 150 cells/well and reading luminescence after a 60-minute incubation with G6PDH detection reagent. With these assay conditions, the signal is within the linear range of the assay. With more than a tenfold assay window, it is well suited for inhibitor screening (Figure 3).

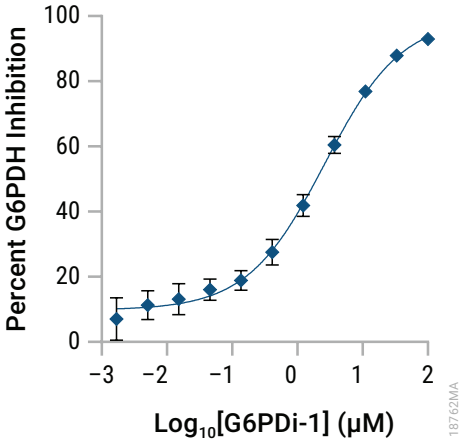

Figure 3. Inhibiting glucose 6-phosphate dehydrogenase with G6PDi-1. K562 cells were collected and resuspended to 7.5×10^5 cells/ml in storage buffer and lysed by sonication. Cell lysates were further diluted in storage buffer and 150 cells in 90µl were transferred to a 96-well assay plate. Various concentrations of G6PDi-1 inhibitor (10µl each; 16nM–1mM) were added to the samples and incubated for 2.5 hours at room temperature. The G6PDH assay was then performed as described in Section 4.C. Percent inhibition of G6PDH was calculated based on DMSO-treated control for each G6PDi-1 concentration tested. The IC_{50} calculated for G6PDH inhibition, using G6PDi-1 and 150 cells, is 2.6µM. Data represent the average of three replicates with error bars indicating one standard deviation.

5.C. Tissue Homogenates

Figure 4 shows an example of measuring specific activity of multiple dehydrogenases in tissue homogenates. Dehydrogenases and corresponding substrates were selected as described in Section 4.C and Table 1.

To measure dehydrogenase activity in tissues, first homogenize tissue to 5–15mg/ml in PBS or storage buffer (200mM Tris [pH 7.3], 10% glycerol and 1% bovine serum albumin). For more information on storing samples, see Section 3.A. Since the assay linearity and background activity will vary, depending on dehydrogenase activity and tissue type, the optimal amount of tissue and assay timing should be determined for each dehydrogenase.

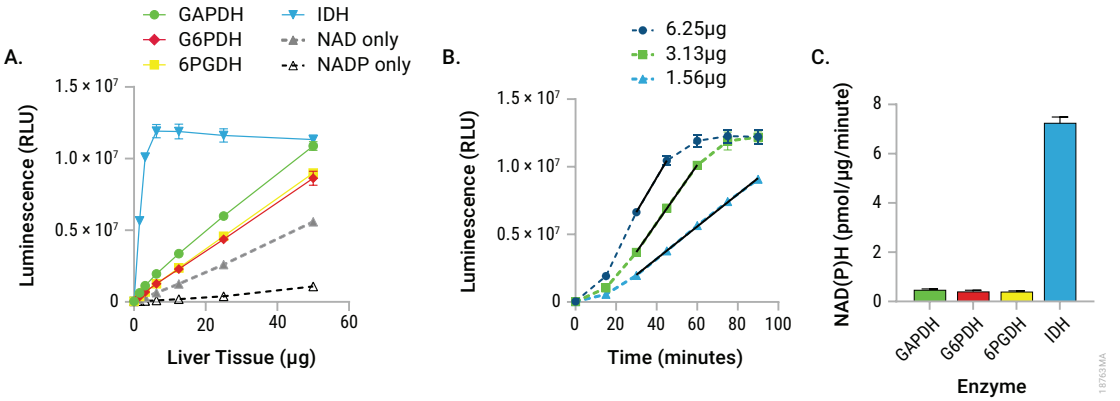

Figure 4. Optimizing dehydrogenase assay in mouse liver tissue. A sample (10mg/ml) of frozen mouse liver tissue was homogenized in storage buffer for 20–30 seconds using a mechanical homogenizer (e.g., Tissue-Tearor™, BioSpec Cat.# 985370-07). Samples were then diluted in storage buffer to 1mg/ml and diluted twofold in the same buffer, including a no-tissue control. Dehydrogenase detection reagent was prepared per Section 4.A for each dehydrogenase and 50µl was added to sample wells. No-substrate controls (NAD only and NADP only) were included to determine background from nonspecific enzyme activity in mouse liver homogenates. Luminescence was read every 15 minutes for 90 minutes. After 60 minutes, relative light units (RLU) were assessed for all dehydrogenases at various tissue amounts (**Panel A**). Linearity was assessed over time for IDH (**Panel B**), indicating that assay linear range depends on the amount of sample and time. The rate of NAD(P)H production from 45–60 minutes was calculated after subtracting the no-substrate control signal using the NAD(P)H positive controls for each dehydrogenase (**Panel C**). Data represent the average of three replicates with error bars indicating one standard deviation.

To determine optimal conditions for the dehydrogenase of interest, we recommend performing a titration of tissue to determine specific dehydrogenase activity and nonspecific assay background, and measuring luminescence over time. As shown in Figure 4, Panel A, nonspecific assay background increased with increasing tissue amount. Furthermore, higher background was observed for NAD-only wells compared to NADP only controls, indicating higher activity of endogenous NAD-dependent dehydrogenases in these samples. More importantly, for all dehydrogenases tested, the signal measured in the presence of dehydrogenase specific substrate was higher than the no-substrate controls. At 60 minutes, for G6PDH, GAPDH and 6PGDH luminescence signal remained within the linear range of the assay with 50µg of liver tissue. IDH demonstrated the highest activity of the four dehydrogenases tested with approximately 7pmol of NADPH produced per microgram of tissue per minute. To measure within the linear range for IDH, signal from less tissue (1.56–6.25µg) was used.

6. Appendix

6.A. Temperature and Reagent Compatibility

The intensity and stability of the luminescent signal is temperature sensitive. For consistent results, equilibrate the reagents and samples to room temperature before using.

Avoid the presence of DTT and other reducing agents in the samples to be tested. Reducing agents will react with the Reductase Substrate and increase background.

6.B. Plates and Equipment

Most standard plate readers are designed to measure luminescence and are suitable for this assay. Some instruments do not require gain adjustment while others might require optimizing the gain settings to achieve sensitivity and dynamic range. An integration time of 0.25–1 second per well should serve as guidance. For exact instrument settings consult the instrument manual.

Use opaque, white multiwell plates that are compatible with your luminometer (e.g., Corning® Costar® 96-well plates, Cat.# 3917, Costar® 384-well plates, Cat.# 3570). For cultured cells, white-walled clear bottom tissue culture plates (e.g., Corning® 96-well plates, Cat.# 3903) are acceptable. Luminescent metabolite assays are well suited for miniaturization. When samples are limited, consider using 96-half area (Corning® Cat.# 3696), 384-well (Costar® Cat.# 3570) or 384-low volume (Corning® Cat.# 4512) plates. We do not recommend black or clear plates. Light signal is diminished in black plates and increased well-to-well cross talk is observed in clear plates.

Note: The RLU values shown in the figures of this technical manual vary depending on the plates and luminometers used to generate data. Although relative luminescence output will vary with different instruments, this variation does not affect assay performance.

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6.C. References

1. Zhou, W. *et al.* (2014) Self-immolative bioluminogenic quinone luciferins for NAD(P)H assays and reducing capacity-based cell viability assays. *Chembiochem.* **15**, 670–5.
2. Vidugiriene, J. *et al.* (2014) Bioluminescent cell-based NAD(P)/NAD(P)H assays for rapid dinucleotide measurement and inhibitor screening. *Assay Drug Dev.Technol.* **12**, 514–26.
3. Ghergurovich, J.M. *et al.* (2020) A small molecule G6PD inhibitor reveals immune dependence on pentose phosphate pathway. *Nat. Chem. Biol.* **16**, 731–9.

6.D. Related Products

Energy Metabolism Assays

Product	Size	Cat.#
BCAA-Glo™ Assay	5ml	JE9300
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Glycogen-Glo™ Assay	5ml	J5051
Lactate-Glo™ Assay	5ml	J5021
Malate-Glo™ Assay	5ml	JE9100
Metabolite-Glo™ Detection System	5ml	J9030
Pyruvate-Glo™ Assay	5ml	J4051
Triglyceride-Glo™ Assay	5ml	J3160

Additional sizes available.

Oxidative Stress Assays

Product	Size	Cat.#
GSH/GSSG-Glo™ Assay	10ml	V6611
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
ROS-Glo™ H2O2 Assay	10ml	G8820

Additional sizes available.

Cell Viability, Cytotoxicity and Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay System	2.5ml	G8090
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
RealTime-Glo™ MT Cell Viability Assay	100 assays	G9711

Additional sizes available.

[a]U.S. Pat. Nos. 9,273,343 and 9,951,372, European Pat. No. 2751089, Japanese Pat. No. 6067019 and other patents pending.