

## A Sensitive Coupled Enzyme Assay for Measuring Kinase and ATPase Kinetics Using ADP-Specific Hexokinase

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**[Abstract]** Kinases and ATPases perform essential biological functions in metabolism and regulation. Activity of these enzymes is commonly measured by coupling ATP consumption to the synthesis of a detectable product. For most assay systems the ATP concentration during the reaction is unknown, compromising the precision of the assay.

Using the ADP-specific hexokinase (ADP-HK) from the thermophilic archaeon *Thermococcus litoralis* the protocol outlined here allows real time coupling of ATP consumption to downstream signal change enabling accurate kinetic measurements. ADP-HK phosphorylates glucose that is then used by glucose-6-phosphate dehydrogenase to reduce NAD<sup>+</sup> to NADH which can be measured at 340 nm. We have shown this assay to be sensitive to the detection of micromole quantities of ADP with no detectable background from ATP.

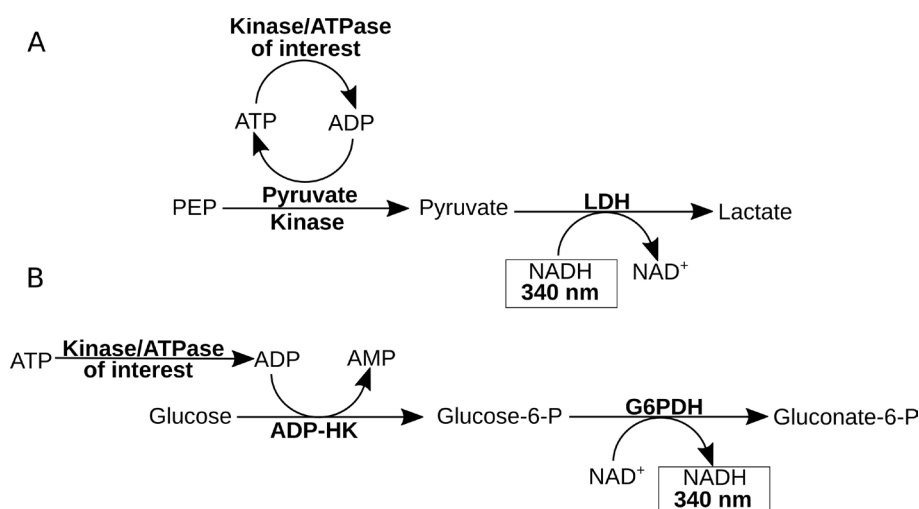
**Keywords:** Kinase assay, ATPase assay, Kinetics, ADP-specific hexokinase, Michaelis-Menten, Phosphoribulokinase, ATP, ADP

**[Background]** Kinases and ATPases can be measured by coupling the production of ADP to a spectrophotometrically detectable signal. Commercial vendors provide kits that detect the amount of ADP produced or ATP depleted by coupling them to the production of a fluorescent or bioluminescent signal (examples include ATP-Glo; Promega, ADP-sensor; Biovision). These kits are sensitive but are hard to apply to kinetic questions because they are end-point assays, measuring ADP amounts at a single time point. Furthermore, the black box nature of such kits make it difficult to determine if reaction mixtures are in the steady-state conditions required for Michaelis-Menten kinetics.

Continuous assays measure enzyme activity over time allowing the determination of enzyme rate in a single run. The classic continuous coupled-enzyme assay is the pyruvate kinase and lactate dehydrogenase system (Kornberg and Pricer, 1951). During the reaction, pyruvate kinase uses the ADP produced by the target enzyme to convert phosphoenolpyruvate to pyruvate and in the process regenerate ATP. Pyruvate is then used by lactate dehydrogenase to oxidize NADH to NAD<sup>+</sup> which is measured by the decrease of absorbance at 340 nm (Figure 1A). A drawback of this method is that in regenerating the ATP continuously, the ATP concentration at any given point is unknown which means that Michaelis-Menten kinetics for ATP cannot be calculated.

An optimal coupled reaction proceeds in a linear pathway so that the ADP produced is converted stoichiometrically into the component producing the signal. ADP-specific hexokinase (ADP-HK) from

*Thermococcus litoralis* uses ADP to phosphorylate glucose into glucose-6-phosphate, which can be used by glucose-6-phosphate dehydrogenase (G6PDH) to reduce  $\text{NAD}^+$  to NADH (Ito *et al.*, 2001; Sakuraba and Ohshima, 2002) (Figure 1B). ADP-HK is an ADP-specific enzyme from thermophilic archaea that is adapted to consume ADP instead of ATP, probably because of the higher thermostability of ADP over ATP. ADP-HK has previously been used in conjunction with G6PDH and diaphorase I to measure the abundance of dNDPs from cellular extracts with a colorimetric signal but has yet to be demonstrated in a continuous assay to measure kinetics (Kumagai *et al.*, 2014). Here, we have used the specificity of ADP-specific hexokinase to develop a method for assaying ADP production by kinases and ATPases.



**Figure 1. Schematic of two coupled enzyme assays to measure ATPase/kinase activity.** A. The traditional method for measuring ATPase/kinase activity uses pyruvate kinase to couple ADP production to the conversion of phosphoenolpyruvate (PEP) to pyruvate and then lactate dehydrogenase (LDH) to convert pyruvate to lactate—which simultaneously oxidizes NADH to  $\text{NAD}^+$  resulting in a decrease of absorbance at 340 nm. B. Our alternative method uses ADP-HK to couple ADP production to the phosphorylation of glucose which is then used by glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-phosphate to gluconate-6-phosphate—which simultaneously reduces  $\text{NAD}^+$  to NADH resulting in an increase of absorbance at 340 nm.

## Materials and Reagents

1. Proteosec 6-600 column (Generon)
2. *Thermococcus litoralis* gDNA (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Strain number: DSM-5473)
3. pRSET-A expression plasmid (Thermo Fisher)
4. Competent *E. coli* KRX (Promega)
5. ADP-HK expressed and purified from *E. coli*, stored at  $-80\text{ }^\circ\text{C}$
6. G6PDH from baker's yeast (Sigma-Aldrich, catalog number: G6378-100UN), stored at  $4\text{ }^\circ\text{C}$

7. D-Glucose, powder stored at room (Sigma-Aldrich, catalog number: G8270-100G), stored as powder at room temperature
8. NAD<sup>+</sup> (Sigma-Aldrich, catalog number: N1636-25MG), stored in aliquots of 50 mM reaction buffer (see Recipes) at -80 °C
9. ADP (Sigma-Aldrich, catalog number: A2754-100MG), stored in aliquots of 100 mM reaction buffer (see Recipes) at -80 °C
10. ATP (Sigma-Aldrich, catalog number: A2383-1G), stored in aliquots of 100 mM in reaction buffer (see Recipes) at -80 °C
11. Tris Base (Melford, catalog number: T60040-5000)
12. NaCl (Sigma-Aldrich, catalog number: S9888-1KG)
13. MgCl<sub>2</sub> (Sigma-Aldrich, catalog number: 208337-1KG)
14. Terrific Broth (Melford, catalogue number: 7758-11-4)
15. Rhamnose (Melford, catalogue number: 10030-85-0)
16. Ni-NTA resin (Generon NB-45-00042-100)
17. Imidazole (Sigma-Aldrich, catalogue number: I5513-100G)
18. BCA (bicinchoninic acid) (Thermo Fisher, catalogue number: 23225)
19. Reaction buffer (see Recipes)
20. ATPase/kinase (see Recipes)
21. 1,000 Units/ml G6PDH (see Recipes)
22. 50 mM NAD<sup>+</sup> (see Recipes)
23. 100 mM ADP (see Recipes)
24. 100 mM ATP (see Recipes)
25. 1 M Glucose (see Recipes)
26. 2x Assay reagent (see Recipes)

### **Equipment**

1. UV spectrophotometer (Shimadzu, model: UV-1601)
2. 100 µl quartz microcuvette (Hellma, model: 105-201-15-40)
3. Ti 45 Fixed-Angle Titanium Rotor (Beckman Coulter, model: Ti 45)
4. Centrifuge (Beckman Coulter, model: Allegra® J2-21)
5. Äkta Purifier (GE Healthcare)
6. -80 °C freezer

### **Software**

1. Graphing software (e.g., Origin Pro, OriginLab)

## Procedure

### A. Assay Reagent Formulation (Figure 2)

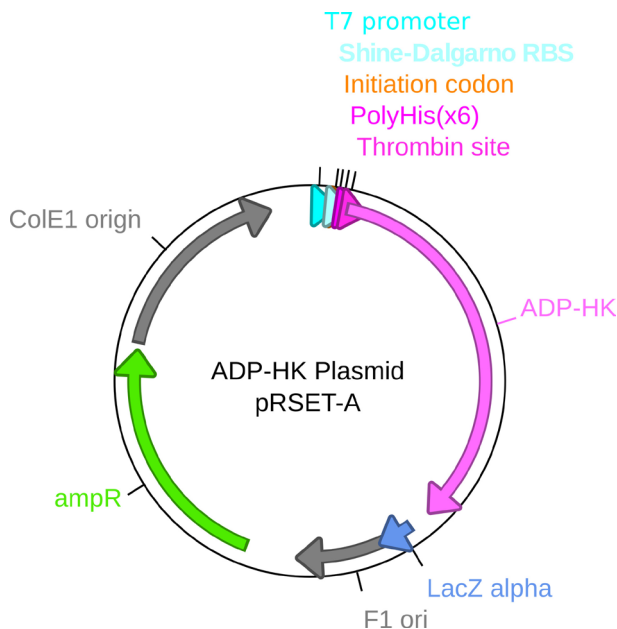
1. The coupled enzyme mix contains 25 µg/ml ADP-HK, 10 mM Glucose, 400 µM NAD<sup>+</sup> and 4 units/ml G6PDH.

*Notes:*

- a. *Glucose, G6PDH and NAD<sup>+</sup> are available commercially. Individual components are stored as stock solutions at -80 °C and mixed on the day.*
  - b. *For each measurement, an assay reagent containing ADP-HK, G6PDH, glucose and NAD<sup>+</sup> is prepared at 2x concentration (50 µg/ml ADP-HK, 20 mM Glucose, 800 µM NAD<sup>+</sup> and 8 units/ml G6PDH) and mixed in the cuvette with an equal volume of 2x concentration substrate. By mixing equal volumes i.e., 100 µl + 100 µl, fast mixing conditions are obtained and concentrations of the assay reagent and substrate are halved.*
  - c. *The detectable absorbance signal is produced by NADH which has an absorbance peak at 340 nm ( $A_{340}$ ) that is not present for NAD<sup>+</sup>. The extinction coefficient for NADH at 340 nm is 6,220 M<sup>-1</sup>cm<sup>-1</sup>.*
2. Clone ADP-HK from *T. litoralis* gDNA into an *E. coli* expression plasmid and purified by Ni-affinity purification via an N-terminal hexa-Histidine tag followed by size-exclusion chromatography.
  3. Produce the ADP-hexokinase in-house by purifying a recombinantly expressed with an N-terminal His-tagged construct.
    - a. Produce the plasmid by cloning the ADP-hexokinase from *Thermococcus litoralis* gDNA (DSM-5473, Deutsche Sammlung von Mikroorganismen und Zellkulturen) gene into a pRSET-A expression plasmid (Thermo Fisher).
    - b. For expression, transform the plasmid into chemically competent *E. coli* KRX (Promega) and grow in 1 L Terrific Broth until OD<sub>600</sub> ~0.6-0.8 and then induced with 0.1% (w/v) rhamnose for 15 h at 18 °C.
    - c. Harvest cells by centrifugation at 2,700 x g for 15 min, resuspend in 50 mM Tris, 150 mM NaCl, pH 7.9.
    - d. Cells are lysed by sonication (Bandelin Sonopuls Sonication) with 2 s on/off pulses for 5 min, followed by centrifugation for 1 h at 80,000 x g (Beckman Ti 45 rotor in an Allegra J2-21).
    - e. Mix the supernatant with 3 ml of Ni-NTA resin (Qiagen) and left on a turning rotor overnight at 4 °C.
    - f. Wash the resin three times by resuspending in 15 ml of 50 mM Tris, 150 mM NaCl, 30 mM imidazole, pH 7.9 followed by centrifugation at 1,000 x g for 5 min.
    - g. Elute the protein by washing three times with 15 ml of 50 mM Tris, 150 mM NaCl, 300 mM imidazole, pH 7.9.
    - h. Concentrate the eluted protein to 1 ml and further purify using size-exclusion chromatography using a Proteosec 6-600 column (Generon) and FPLC on an Äkta Purifier

(GE Healthcare).

- i. Concentrate the protein to 5 mg/ml with 20% (v/v) glycerol, dispense into 50  $\mu$ l aliquots and flash freeze in liquid nitrogen.



**Figure 2. ADP-HK expression plasmid map.** The expression plasmid for ADP-HK is a modified pRSET-A plasmid. The ADP-HK gene from *Thermococcus litoralis* (DSM5473) was cloned into the plasmid with a thrombin cleavable hexa-Histidine tag. The insert is under the control of an inducible T7 promoter and a strong ribosome binding site (Shine-Dalgarno RBS).

## B. Assay Procedure

*Note: The volumes stated are for twenty-five 200  $\mu$ l reactions, enough to perform controls and produce a 5-point Michaelis-Menten curve in triplicate.*

1. Prepare 2.5 ml of assay reagent containing 50  $\mu$ g/ml ADP-HK, 20 mM Glucose, 800  $\mu$ M NAD<sup>+</sup> and 8 Units G6PDH in reaction buffer.
2. Divide the assay reagent into two unequal parts:
  - a. 2 ml in which the kinase/ATPase being measured is added. It is recommended that the final concentration of enzyme is 1  $\mu$ M in the first instance. If steady-state conditions are not met, *i.e.*, the reaction is too fast or if the reaction is too slow to be measured, then the concentration of enzyme can be adjusted.
  - b. 0.5 ml in which assay reaction buffer 5  $\mu$ l of assay buffer is added.
3. Prepare ATP over the range of concentrations to be measured in reaction buffer. For each concentration prepare ATP at 2x concentration, *e.g.*, for a 10  $\mu$ M ATP measurement prepare the solution at 20  $\mu$ M. A recommended concentration range is from 0 mM ATP to 10 times above the expected enzyme  $K_M$ . For kinases with a second substrate, ensure this is present in large excess to achieve pseudo-Michaelis-Menten conditions (100-fold greater than  $K_M$ ).

4. For each measurement, quickly mix 100  $\mu\text{l}$  of assay reagent with 100  $\mu\text{l}$  of substrate within the quartz cuvette, aspirate 3 times to mix and start measuring the absorbance at  $A_{340}$  for 200 s. *Note: Adjust measurement time in accordance with the speed of the reaction so that curve fitting is accurate. Slower kinases may require longer measurement times.*
5. Perform positive and negative controls for each assay. Positive control; mixing 100  $\mu\text{M}$  ADP and assay reaction reagent and ensuring the increase at  $A_{340}$  is as expected, corresponding to a stoichiometric amount of NADH. Negative control; mixing 100  $\mu\text{M}$  ATP and assay reagent without the kinase/ATPase and ensuring there is no increase in  $A_{340}$ .
6. Measure activity starting at 0  $\mu\text{M}$  ATP then at increasing ATP concentrations. It is recommended to measure activity at at least 5 different ATP concentrations with the highest concentration at 10-fold above the expected  $K_M$ .

### Data analysis

1.  $A_{340}$  timecourses were recorded in triplicate and plotted using graphing software (here we have used the graphing software Origin Pro).
2. For Michaelis-Menten analysis, the steady-state rate ( $A_{340}/\text{s}$ ) were determined by plotting a straight line over the first 30 s of each plot and calculating the slope. The average rate with standard deviation (S.D.) was plotted for each concentration of ATP and fitted to a Michaelis-Menten equation [1] to calculate  $V_{\text{max}}$  and  $K_M$ .
3. The concentration of NADH, which is stoichiometric to the conversion of ATP to ADP, was calculated using the Beer-Lambert law [2].

$$[1] V_o = \frac{V_{\text{max}} \times [S]}{[S] + K_M}$$

$V_o$  is the initial rate,  $[S]$  the substrate concentration,  $V_{\text{max}}$  is the maximal velocity and  $K_M$  is the substrate concentration which produces that half-maximal velocity.

$$[2] [NADH] = \frac{A_{340}}{\epsilon \times l}$$

$[NADH]$  is the concentration of NADH,  $A_{340}$  is the UV absorbance at 340 nm,  $\epsilon$  is the extinction coefficient of NADH ( $6,220 \text{ M}^{-1}\text{cm}^{-1}$ ) and  $l$  is the cuvette pathlength (in this case 1 cm).

### **Statistics**

1. Mean and Standard Deviation

Each plotted rate was the mean rate ( $\bar{x}$ ), calculated by dividing the sum of rates ( $\sum x_i$ ) with the number of data points ( $n$ ), which in this case is 3. The standard deviation of the rate was estimated using the standard formula [3].

$$[3] SD = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

## 2. Error $K_M$ and $V_{max}$

The error for  $K_M$  and  $V_{max}$  are calculated by Origin Pro (OriginLab) software by determining the  $K_M$  and  $V_{max}$  of each individual data set and calculating the standard deviation.

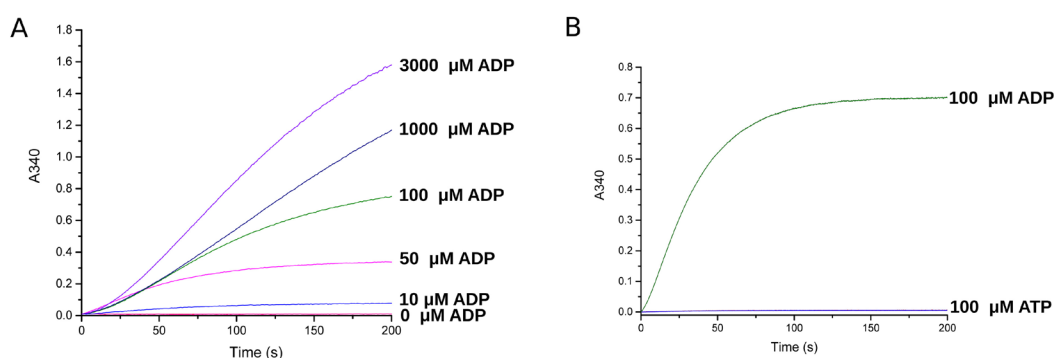
## Notes

### 1. Validation of sensitivity and specificity

The assay was tested for its range of sensitivity and specificity for ADP (Figure 3A). For each substrate concentration the  $A_{340}$  change was recorded in triplicate and the corresponding difference between the curve baseline and plateau was measured. The initial rate, an approximation of the steady-state rate, was determined by calculating the curve slope for the first 30 s of the reaction.

The assay is sensitive from low micromolar concentration of ADP to millimolar concentration. Recordings using 10  $\mu\text{M}$  to 3,000  $\mu\text{M}$  ADP showed that at increasing ADP concentrations the reaction rate increased such that at up to 3,000  $\mu\text{M}$  ADP, glucose and  $\text{NAD}^+$  were not rate limiting.

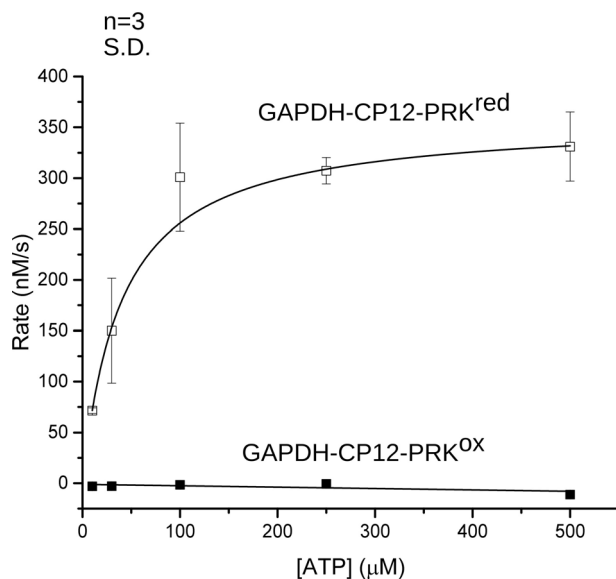
To determine ADP specificity over ATP, 100  $\mu\text{M}$  of each substrate was measured for the reaction rate and the total  $A_{340}$  change (Figure 3B). ATP produced no measurable activity with a total  $A_{340}$  change of  $0.006 \pm 0.000$  at an initial rate of  $0.002 \pm 0.000$   $A_{340}/\text{s}$ . Conversely, ADP produced a measurable reaction with a total  $A_{340}$  change of  $0.7 \pm 0.1$  at an initial rate of  $1.42 \pm 0.00$   $A_{340}/\text{s}$ . Therefore ADP produced 116 times higher final signal than ATP at 705 times faster rate. The  $A_{340}$  implied 113  $\mu\text{M}$  NADH, suggesting that the 100  $\mu\text{M}$  ADP produced NADH stoichiometrically in the assay, and that the reaction had gone to completion.



**Figure 3. Validation of ADP concentration dependence and specificity.** A.  $A_{340}$  recordings for increasing ADP concentrations. B. The  $A_{340}$  difference for ADP versus ATP. The absorbance was measured spectrophotometrically (Shimadzu, UV-1601) at 340 nm at 1 Hz and with a slit-length of 1 nm. Traces were plotted and analysed using Origin Pro software (OriginLab).

2. Michaelis-Menten kinetics of GAPDH-CP12-PRK

Michaelis-Menten curves of phosphoribulokinase (PRK) activity were measured for oxidized and reduced GAPDH-CP12-PRK to determine the redox dependent inactivation of the kinase (Figure 4) (McFarlane *et al.*, 2019). PRK is a sugar kinase that phosphorylates ribulose-5-phosphate to ribulose-1,5-bisphosphate using ATP. The GAPDH-CP12-PRK complex was purified to homogeneity and the activity of PRK was measured at increasing concentrations of ATP to determine the Michaelis-Menten constants of PRK within the GAPDH-CP12-PRK complex under oxidizing and reducing conditions.



**Figure 4. GAPDH-CP12-PRK Michaelis-Menten curves.** Michaelis-Menten fits of oxidized (ox) and reduced (red) GAPDH-CP12-PRK (McFarlane *et al.*, 2019).

Measuring Michaelis-Menten kinetics of the complex allowed detection of any changes of PRK activity in a substrate concentration dependent manner and the low background of the ADP-HK assay allowed accurate determination of the extent of inactivation. In the presence of excess Ru5P (2.5 mM), the rate of ADP production was measured at increasing ATP concentrations. Initial rates for each trace was measured by fitting a straight line over the initial 30 s. Rates were then plotted and fitted to a Michaelis-Menten equation.

Pre-formed oxidized complex showed zero measurable activity for all ATP concentrations and the reduced complex showed activity with a  $K_M$  of  $40 \pm 2 \mu\text{M}$  and and  $V_{\text{max}}$  of  $13.6 \pm 0.3 \text{ s}^{-1}$ . These values correspond to the fact that the oxidized complex is completely inactive for PRK activity and that the inactivation is independent of ATP concentration. When the complex is chemically reduced, phosphoribulokinase is activated and conforms to Michaelis-Menten kinetics by showing increased activity at increased ATP concentration until the maximal velocity in reached.



## Conclusion

The protocol described here demonstrates how the specificity of ADP-hexokinase from *T. litoralis* can be used in a coupled enzyme reaction to measure ATPase or kinase activities. The materials and equipment required for this assay are commonly accessible allowing for sensitive measurements to be obtained without purchasing expensive commercial kits. The assay produces a rapid and stoichiometric conversion of ADP to an absorbance signal that can be measured continuously and the high specificity and sensitivity ensures low background and therefore accurate kinetic measurements.

## Recipes

1. Reaction buffer
  - 50 mM Tris
  - 150 mM NaCl
  - 10 mM MgCl<sub>2</sub>
  - pH 7.9
  - Dissolve 6.02 g of Tris, 8.76 g of NaCl and 0.95 g of MgCl<sub>2</sub> in 1 L of ultrapure water
2. ATPase/kinase
  - Determine the ATPase/Kinase protein concentration for example using a Pierce BCA (bicinchoninic acid) assay (Thermo Fisher) and prepare the ATPase/Kinase in a stock concentration of 100 μM in the reaction buffer.
3. 1,000 Units/ml G6PDH
  - a. Dissolve lyophilized G6PDH powder in 100 μl of reaction buffer
  - b. Store at 4 °C and use within 1 month
4. 50 mM NAD<sup>+</sup>
  - a. Dissolve contents of a 25 mg vial in 753 μl of ultrapure water
  - b. Dispense into 80 μM aliquots and stored at -80 °C
5. 100 mM ADP
  - a. Dissolve 100 mg of ADP into 2.34 ml of ultrapure water
  - b. Dispense into 100 μl aliquots and store at -80 °C
6. 100 mM ATP
  - a. Dissolve 100 mg of ADP into 1.81 ml of ultrapure water
  - b. Dispense into 100 μl aliquots and store at -80 °C
7. 1 M Glucose
  - Dissolve 180 mg of D-glucose into 1 ml of reaction buffer on the day of the experiment
8. 2x Assay reagent (2.5 ml)
  - 50 μg/ml ADP-HK
  - 20 mM Glucose
  - 800 μM NAD<sup>+</sup>

8 Units G6PDH

Add 25  $\mu$ l of 5 mg/ml ADP-HK, 50  $\mu$ l of 1 M Glucose, 40  $\mu$ l of 50 mM NAD<sup>+</sup>, 20  $\mu$ l of 1,000 Units/ml G6PDH and 2.36 ml of buffered saline solution

*Note: The assay was developed under the conditions given, however it is likely that some variation can be tolerated. G6PDH is stable at a few hours at pH 4.0 (Souza et al., 2002), and ADP-HK is stable up to 100 °C (Kengen et al., 1995).*

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### **Competing interests**

The authors declare no competing interests.

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