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We use this protocol and it's working

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Lactate Concentration assay (LDH method)

Forked from Glucose Concentration assay (Hexokinase/G6PDH method)

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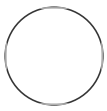

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ABSTRACT

The assay I describe measures lactate concentration in a sample. The principle of the assay is to convert lactate in a sample and excess NAD^+ in the buffer to pyruvate and NADH with lactic dehydrogenase. Pyruvate is trapped by hydrazine in the buffer to form pyruvate hydrazone, effectively decreasing concentrations of free pyruvate and driving forward lactate consumption. NADH can be observed on a microplate reader because it absorbs light at 340 nm. I adapted this assay for use in a microplate reader from "A flexible system of enzymatic analysis" by Oliver Lowry and Janet Passoneau (Lactate method II). It is reliable, inexpensive, and diagnostically useful for calculating cellular lactate production rates.

IMAGE ATTRIBUTION

Image made using Biorender

GUIDELINES

- Advice corresponding to individual steps is placed in the step's "notes" section
- There are alternatives to some of the materials used herein. If you prefer to measure fluorescence, your plate does not need a clear bottom.

MATERIALS

Consumables

- 96-well microplate (Corning, 3603) - black walled for fluorescence, clear bottom for absorbance
- 20 and 200 uL pipette tips
- (optional) Reagent reservoir

Reagents

- sodium lactate (for standards; Sigma, N7004)
- glycine (Sigma, G7126)
- hydrazine (Sigma, 216046)
- NaOH pellets (Fisher, S318-1)
- NAD^+ (Sigma, N7004)
- lactate dehydrogenase (Sigma, L2500)
- dH_2O

Equipment

- Pipette (200 and 20 uL capacity should suffice for this assay)
- (optional) multichannel pipette (200uL capacity)
- Plate reader that can determine absorbance at 340 nm or fluorescence (ex/em of 340/460) (for us, a BioTek Synergy 4)
- A balance

BEFORE START INSTRUCTIONS

- Read the instructions! That's it I think...

Generate lactate standards

1 Make a lactate stock solution (for me, normally 1 M), and dilute to make standards. My typical standards are 0-20 mM (20, 16, 12, 8, 6, 4, 2, 1, 0.5, 0 mM). The composition of standards may of course be adapted to your needs.

Note:

- The range of the standard curve must exceed the range of the samples you will use. Otherwise the data may not be as interpretable as it appears.
- If you plan to increase the concentration of the samples/standards you use, I recommend lowering the volume of standards and samples in the assay.

- As you decrease sample/standard concentration, I recommend increasing the volume of standards and samples in the assay.

Prepare Glycine-Hydrazine buffer

2 This buffer will serve as the solvent for many future assays

For 500 mL of buffer (enough for many lactate assays):

Component	Amount
Glycine	32.53 g
Hydrazine	22.52 g
dH_2O	500 mL

Use a magnetic stir bar to dissolve buffer components
Adjust pH to 9.2 with NaOH pellets

Note:
- Final concentrations: 0.6 M glycine and 0.5 M hydrazine
- The glycine-hydrazine stock solution can be kept at room temperature

Prepare lactate assay buffer

3 For 50 mL of assay buffer (enough for ~2.5 96 well plates, can be scaled). I usually add the following components

Stock solutions	Volume used
Glycine-hydrazine buffer	16.6 mL
dH_2O	33.3 mL
Add solid:	
NAD+	83 mg
lactate dehydrogenase	400 U

Once all components are in the buffer, mix by inversion until all components are dissolved and uniformly distributed. The final assay buffer composition is 200 mM glycine, 166 mM hydrazine, 2.5 mM NAD+, 8 U/mL lactate dehydrogenase.

Note:
- I have used less lactate dehydrogenase with much success. The concentration of LDH in the assay may be halved at least, but keep in mind that this will increase the amount of time needed to complete the assay.
- Now is also a good time to pre-warm your spectrophotometer to 37 degrees C. If your

spectrophotometer does not have a warming function, that is ok, but as a consequence reaction times may take longer to reach completion.

- Once made, the assay buffer works for at least a week. Over time the buffer will gain a yellow hue, which should result from auto-reduction of NAD^+ to NADH. If there is a visible hue, it would be best not to use the buffer, and make a new stock instead.
- I have used lactic dehydrogenase from multiple sources and companies. All of them have worked, so if supply is short, consider ordering the enzyme from another source.

Add samples and standards to plate

4 Add samples and standards to the microplate. 2-10 uL for samples/standard.

Note:

- Samples and standards should be added at least in duplicate
- The volume of samples and standards is best kept consistant, as this simplifies the downstream calculations
- 2-5 uL at ~1-20 mM lactate should yield good data.

5 Add 195 uL assay buffer to the plate.

Note:

- Ensure that the addition of assay buffer follows the addition of standards and samples.
- The addition of a larger volume helps to mix the samples with the buffer.

Read plate

6 Insert plate with samples, standards, and assay buffer into the pre-warmed spectrophotometer *(if it has not been pre-warmed or cannot warm up, the assay can still proceed but may do so more slowly).*

7 Read absorbance at 340 nm or fluorescence (ex:340, em:460) to determine NADH levels in samples and standards. Read multiple times over ~60-90 minutes, or until the reaction has stopped. The reaction stops when absorbance or fluorescence no longer increases over time.

Note:

- Absorbance values approximately >2 are normally outside the dynamic range of quantitation that you should trust. You may attempt to salvage the sample by diluting it in assay buffer, re-reading, and compensating for the dilution factor in your calculations. This may not work, in which case the next time you perform the assay, attempt a sample dilution series to determine the concentrations that lie within your standard curve and within the range of accurate quantitation by your spectrophotometer.

Analyze data

8 Once absorbance at 340 nm or fluorescence has reached a steady-state, export data into a spreadsheet. The standard curve should fit a linear function well ($R^2 \geq 0.99$). If this is the case, use this linear function to determine sample concentration.

An example spreadsheet is attached to help demonstrate data analysis.

lactate_assay_example2.xlsx