ab65342

Pyruvate Assay Kit
(Colorimetric/Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of pyruvate levels in various samples.

This product is for research use only and is not intended for diagnostic use.
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1. **BACKGROUND**

Pyruvate Assay Kit (Colorimetric/Fluorometric) (ab65342) provides a simple, direct and automation-ready procedure for measuring pyruvate concentration in various biological samples such as blood, cells, culture and fermentation media, etc. In the assay, pyruvate is oxidized by pyruvate oxidase via enzyme reactions to generate color ($\lambda = 570$ nm) and fluorescence (at Ex/Em = 535/587 nm).

Since the color or fluorescence intensity is proportional to pyruvate content, the pyruvate concentration can be accurately measured. Detection ranges of this kit are 40 µM – 200 µM (colorimetric assay) and 4 µM – 20 µM (fluorometric assay).

Pyruvate is a central molecule in metabolism through which sugars enter the citric acid cycle. Pyruvate can be converted to carbohydrates during gluconeogenesis or to fatty acids via acetyl CoA. High levels of pyruvate are associated with liver disease and genetic disorders. Pyruvate has also been used to stimulate metabolism leading to loss of body weight.
2. **ASSAY SUMMARY**

Sample preparation

↓

Standard curve preparation

↓

Add reaction mix and incubate at RT for 30 mins

↓

Measure optical density (OD = 570 nm) or fluorescence (Ex/Em = 535/587 nm)
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Pyruvate Probe (in DMSO)</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Pyruvate Enzyme Mix</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Pyruvate Standard (100 nmol/µL)</td>
<td>100 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

6. **MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD 570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: black plates (clear bottoms) for fluorometric assay; clear plates for colorimetric assay
- Orbital shaker
- Dounce homogenizer (if using cells or tissue)

If performing deproteinization step, additional reagents are required:

- Perchloric acid (PCA) 4M, ice cold
- Potassium hydroxide (KOH), 2M
- Ice
7. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Keep enzymes and heat labile components and samples on ice during the assay.

- Make sure all buffers and developing solutions are at room temperature before starting the experiment.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.

- Make sure you have the appropriate type of plate for the detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
9. **REAGENT PREPARATION**

- Briefly centrifuge small vials at low speed prior to opening.

  9.1 **Pyruvate Assay Buffer:**
  
  Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

  9.2 **Pyruvate Probe:**
  
  Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use. 
  
  **NOTE:** DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C. Store at -20°C protected from light and moisture. Once the probe is thawed, use with two months.

  9.3 **Pyruvate Enzyme Mix:**
  
  Reconstitute in 220 µL Pyruvate Assay Buffer. Aliquot enzyme mix so that you have enough to perform the desired number of assays. Store at -20°C. Use within two months.

  9.4 **Pyruvate Standard:**
  
  Ready to use as supplied. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 For the colorimetric assay:

10.1.1 Prepare 1 nmol/µL of Pyruvate Standard by adding 5 µL of the Pyruvate Standard to 495 µL of Pyruvate Assay Buffer.

10.1.2 Using 1 nmol/µL Pyruvate standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of 1 nmol/µL Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc Pyruvate in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0 nmol/well</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>2 nmol/well</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>4 nmol/well</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>6 nmol/well</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>8 nmol/well</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>10 nmol/well</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate reading (2 x 50 µL).
10.2  For the flurometric assay:

10.2.1 Prepare 1 nmol/µL of Pyruvate Standard by adding 5 µL of the Pyruvate Standard to 495 µL of Pyruvate Assay Buffer.

10.2.2 Prepare 0.1 nmol/µL Pyruvate Standard by diluting 10 µL of 1 nmol/µL Pyruvate Standard into 90 µL of Pyruvate Assay Buffer.

10.2.3 Using 0.1 nmol/µL standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of 0.1 nmol/µL Standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc Pyruvate in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>150</td>
<td>50</td>
<td>0 nmol/well</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>144</td>
<td>50</td>
<td>0.2 nmol/well</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>138</td>
<td>50</td>
<td>0.4 nmol/well</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>132</td>
<td>50</td>
<td>0.6 nmol/well</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>126</td>
<td>50</td>
<td>0.8 nmol/well</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>120</td>
<td>50</td>
<td>1 nmol/well</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

**NOTE:** If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.
11. **SAMPLE PREPARATION**

**General Sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 **Cell (adherent or suspension) samples:**

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10⁶ cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 500 µL (4X volumes) Pyruvate Assay Buffer.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.1.8 Perform deproteinization step as described in section 11.4.

**NOTE:** If cells are collected by trypsinization, it is essential to neutralize the trypsin with medium. Then all media must be removed and cells washed with PBS. Follow protocol from step 11.1.2.
11.2 **Tissue samples:**

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 500 – 1,000 µL (4X volumes) of Pyruvate Assay Buffer.

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.2.7 Keep on ice.

11.2.8 Perform deproteinization step as described in section 11.4.

11.3 **Plasma, Serum and Urine and other biological fluids:**

Can be tested directly by adding sample to the microplate wells. However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (1/2 – 1/5 – 1/10).

**NOTE:** We suggest using different volumes of sample to ensure readings are within the Standard Curve range.

11.4 **Deproteinization protocol**

**NOTE:** Endogenous Lactate Dehydrogenase (LDH) may degrade lactate. Samples containing LDH (such as culture medium or tissue lysate) should be kept at -80°C for storage. Alternatively perform deproteinization as described in this section.

11.4.1 Prepare sample as specified in the section 11.1 or 11.2. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.
11.4.2 Add PCA to a final concentration of 1M in the homogenate solution and vortex briefly to mix well. **NOTE: High protein concentration samples might need more PCA.**

11.4.3 Incubate samples on ice 5 min. Centrifuge samples at 13000 rpm 2 minutes in a cold centrifuge.

11.4.4 Transfer supernatant to a fresh tube.

11.4.5 Precipitate excess PCA by adding ice-cold 2 M KOH that equals 34% of the supernatant to your sample (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA. There may be some gas (CO₂) evolution so vent the sample tube.

11.4.6 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). If necessary, adjust the pH with 0.1 M KOH.

11.4.7 Centrifuge samples at 13000 rpm 15 minutes in a cold centrifuge.

11.4.8 Transfer supernatant to a clean tube, and keep on ice.

11.4.9 Samples are now deproteinized, neutralized and PCA has been removed. The samples may now be used directly for the relevant assays.

The deproteinized samples will be diluted from the original concentration. To calculate the dilution factor of your final sample, simply apply the following formula:

\[
\text{% original concentration} = \frac{\text{Initial sample volume}}{(\text{initial sample volume} + \text{vol PCA} + \text{vol KOH})} \times 100
\]
12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.
- Endogenous compounds may interfere with the reaction. To ensure accurate determination of pyruvate in the test samples, we recommend spiking samples with a known amount of pyruvate standard (6 nmol).

12.1 Set up Reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Background wells = 50 µL Pyruvate Assay Buffer.
- (Optional Sample Background Control) = 1 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer). Use this for samples with background.

12.2 Reaction Mix:

Prepare 50 µL of Reaction Mix for each reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Colorimetric Reaction Mix (µL)</th>
<th>Fluorometric Reaction Mix (µL)</th>
<th>Colorimetric Background control (µL)</th>
<th>Fluorometric Background control (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate Assay Buffer</td>
<td>46</td>
<td>47.6</td>
<td>48</td>
<td>49.6</td>
</tr>
<tr>
<td>Pyruvate Probe</td>
<td>2</td>
<td>0.4*</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*NOTE: The fluorometric assay is ~10 times more sensitive than the colorimetric assay. Use 0.4 µL of the probe per reaction to decrease the background reading and increase detection sensitivity.
Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a Master Mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

\[ X \mu L \text{ component } x (\text{Number samples + standards +1}). \]

12.3 Add 50 \mu L of the Reaction Mix to standard and samples wells.

12.4 Add 50 \mu L of the Background control mix to Sample Background control wells.

12.5 Mix and incubate at room temperature for 30 minutes protected from light.

12.6 Measure on a microplate reader.
- Colorimetric assay: measure OD 570nm.
- Fluorometric assay measure Ex/Em = 535/587 nm.
13. **CALCULATIONS**

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of pyruvate.

13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Extrapolate sample readings from the standard curve plotted using the following equation:

\[ P_y = \left( \frac{\text{Corrected absorbance} - (y - \text{intercept})}{\text{Slope}} \right) \]

13.6 Concentration of pyruvate in the test samples is calculated as:

\[ \text{Pyruvate Concentration} = \left( \frac{P_y}{S_v} \right) \times D \]

Where:

- \( P_y \) = Amount of pyruvate (nmol) of sample well (concentration).
- \( S_v \) = Sample volume (µL) added to the reaction well.
- \( D \) = Sample dilution factor.
- Pyruvate molecular weight: 88.08 g/mol.
13.6 For spiked samples, correct for any sample interference by subtracting the sample reading from spiked sample reading.

13.7 For spiked samples, the concentration of Pyruvate in sample well is calculated as:

\[
Pyruvate = \left( \frac{ODs\ cor}{(ODs + Ts\ cor) - (ODs\ cor)} \right) \times Pyr\ Spike\ (pmol)
\]

Where:
- ODs cor = OD sample corrected.
- ODs = OD sample.
- Ts = Pyruvate amount from standard curve corrected.
14. **TYPICAL DATA**

**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

![Graph](image1.png)

**Figure 1**: Typical pyruvate standard calibration curve using colorimetric reading.

![Graph](image2.png)

**Figure 2**: Typical pyruvate standard calibration curve using fluorometric reading.
Figure 3: Pyruvate measured fluorometrically in mouse tissue lysates showing quantity (nmol) per mg protein of tested sample.

Figure 4: Pyruvate measured colorimetrically in mouse tissue lysates showing quantity (nmol) per mg protein of tested sample.
Figure 5: Pyruvate measured fluorometrically in biological fluids showing quantity (nmol) per mln of tested cells.
15. **QUICK ASSAY PROCEDURE**

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare standard, Pyruvate probe and enzyme mix (aliquot if necessary); get equipment ready
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Prepare Reaction Mix (Number samples + standards + 1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Colorimetric Reaction Mix (µL)</th>
<th>Fluorometric Reaction Mix (µL)</th>
<th>Colorimetric Background control (µL)</th>
<th>Fluorometric Background control (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate Assay Buffer</td>
<td>46</td>
<td>47.6</td>
<td>48</td>
<td>49.6</td>
</tr>
<tr>
<td>Pyruvate Probe</td>
<td>2</td>
<td>0.4*</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- Set up plate for standard (50 µL), samples (50 µL) and background control (50 µL).
- Add 50 µL Reaction Mix to each well.
- Incubate plate at RT 30 min protected from light.
- Measure plate at OD570 nm for colorimetric assay or Ex/Em= 535/590 nm for fluorometric assay.
## TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of inappropriate plate for reader</td>
<td>Colorimetry: Clear plates Fluorescence: Black plates (clear bottom)</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples not deproteinized (if indicated on protocol)</td>
<td>Use PCA precipitation protocol for deproteinization</td>
</tr>
<tr>
<td></td>
<td>Cells/tissue samples not homogenized completely</td>
<td>Use Dounce homogenizer (increase number of strokes); observe for lysis under microscope</td>
</tr>
<tr>
<td></td>
<td>Samples used after multiple free/thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td>Lower/Higher readings in samples and Standards</td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td></td>
<td>Allowing reagents to sit for extended times on ice</td>
<td>Always thaw and prepare fresh reaction mix before use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
</tbody>
</table>
## RESOURCES

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard readings do not follow a linear pattern</td>
<td>Pipetting errors in standard or reaction mix</td>
<td>Avoid pipetting small volumes and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td></td>
<td>Standard stock is at incorrect concentration</td>
<td>Always refer to dilutions on protocol</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so as to be in the linear range</td>
</tr>
</tbody>
</table>
17. FAQs

The OD values are very low. What could be the problem?
The most common reason for low OD values is that the Assay buffer was cold, which led to a slow reaction.

Is it possible that the pyruvate in the sample is degraded with time?
Yes, pyruvate is lost fairly rapidly and can get used up by cellular enzymes. It is essential to deproteinize the samples as soon as possible.

Should the samples be deproteinized and stored?
Yes, it is recommended that cell/tissue lysates or media is deproteinized and then stored at -80°C if needed.

How much pyruvate is there in serum versus whole blood?
Typically, normal human serum has a pyruvate concentration in the range of 60-150 µM and normal human blood has a pyruvate concentration in the range of 35-100 µM.

What kind of interfering substances can affect the assay results?
Undiluted deproteinized samples show values above the expected/reported range.
Compounds such as lactate and α-keto acids in the samples are known to have potential to interfere with the measurement of pyruvate, particularly, 2-Oxobutyrate and oxaloacetate. This could explain why the undiluted deproteinized samples suffer from overestimation.
18. INTERFERENCES