ab105134

Alanine Transaminase Activity Assay kit (Colorimetric/Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Alanine Transaminase (ALT) activity in various samples.

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

Alanine Transaminase Activity Assay Kit (Colorimetric/Fluorometric) (ab105134) is a kit where ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate, the products of this reversible transamination reaction being pyruvate and glutamate. The pyruvate is detected in a reaction that concomitantly converts a nearly colorless probe to both color ($\lambda_{max} = 570$ nm) and fluorescence (Ex/Em = 535/587 nm).

Alanine Transaminase Activity Assay Kit (ab105134) provides a rapid, simple, sensitive, and reliable test suitable for high throughput activity assay of ALT with a detection limit of 10 mU per well.

Alanine transaminase (ALT) is a transaminase enzyme. It is also called serum glutamic pyruvic transaminase (SGPT) or alanine aminotransferase. (ALAT) is found in serum and in various bodily tissues, but is usually associated with the liver. It catalyzes the reaction:

$$\alpha\text{-ketoglutarate} + \text{alanine} \rightleftharpoons \text{glutamate} + \text{pyruvate}$$

It is commonly measured clinically as a part of a diagnostic liver function test to determine liver health. Diagnostically, it is almost always measured in units/liter (U/L).
2. **ASSAY SUMMARY**

- Standard curve preparation
- Sample preparation
- Add reaction mix
- Measure optical density (OD570 nm) or fluorescence (Ex/Em = 535/587 nm) in a kinetic mode at 37°C 60 minutes

*For kinetic mode detection, incubation time given in this summary is for guidance only.*
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>ALT Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>OxiRed Probe (in DMSO)</td>
<td>200 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ALT Enzyme Mix (Lyophilized)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>ALT Substrate (Lyophilized)</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>
<tr>
<td>ALT Positive Control (Lyophilized)</td>
<td>1 vial</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:

- MilliQ water or other type of double distilled water (ddH$_2$O)
- Cold PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (clear bottoms) for fluorometric assay
- Orbital shaker
- Heat bock or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)
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 **ALT Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **OxiRed 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. Aliquot probe so that you have enough volume to perform the desired number of tests. Store at -20°C protected from light. Once the probe is thawed, use with two months.

9.3 **ALT Enzyme Mix:**

Reconstitute with 220 µL ddH$_2$O. Aliquot enzyme mix so that you have enough volume to perform the desired number of tests. Store at -20°C. Use within two months. Keep on ice while in use.

9.4 **ALT Substrate:**

Reconstitute with 1.1 mL Assay Buffer. Aliquot substrate so that you have enough volume to perform the desired number of tests. Store at -20°C. Keep on ice while in use.

9.5 **Pyruvate Standard:**

Ready to use as supplied. Aliquot standard so that you have enough to perform the desired number of tests. Store at -20°C. Keep on ice while in use.

9.6 **ALT Positive Control:**

Reconstitute with 100 µL ddH$_2$O. Aliquot positive control so that you have enough volume to perform the desired number of tests. Store at -20°C. Keep on ice while in use.
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 a 1 nmol/µL Pyruvate Standard by diluting 5 µL Pyruvate Standard in 495 µL of ALT Assay Buffer.

10.1.2 Using 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 standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End [alanine transaminase] in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>60</td>
<td>20</td>
<td>0 nmol/well</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>54</td>
<td>20</td>
<td>2 nmol/well</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>48</td>
<td>20</td>
<td>4 nmol/well</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>42</td>
<td>20</td>
<td>6 nmol/well</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>36</td>
<td>20</td>
<td>8 nmol/well</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>30</td>
<td>20</td>
<td>10 nmol/well</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate reading (2 x 20 µL).
For the fluorometric assay:

10.2.1 Prepare a 1 nmol/µL Pyruvate Standard by diluting 5 µL Pyruvate Standard in 495 µL of ALT Assay Buffer.

10.2.2 Prepare a 0.1 nmol/µL Pyruvate Standard by diluting 100 µL 1nmol/µL Pyruvate Standard in 900 µL of ALT Assay Buffer.

10.2.3 Using 0.1 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 standard (µL)</th>
<th>Assay Buffer (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End [alanine transaminase] in well</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>60</td>
<td>20</td>
<td>0 nmol/well</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>54</td>
<td>20</td>
<td>0.2 nmol/well</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>48</td>
<td>20</td>
<td>0.4 nmol/well</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>42</td>
<td>20</td>
<td>0.6 nmol/well</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>36</td>
<td>20</td>
<td>0.8 nmol/well</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>30</td>
<td>20</td>
<td>1.0 nmol/well</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate reading (2 x 20 µL).
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 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 = 1 x 10⁶ cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 200 µL ice cold ALT 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.

11.2 **Tissue samples:**

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 50 mg tissue).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in ~ 200 µL ice cold ALT 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.

11.3 **Serum and urine samples:**

Serum samples can be directly 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.
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.

12.1 **Set up Reaction wells:**
- Standard wells = 20 µL Standard dilutions.
- Sample wells = 2 – 20 µL samples (adjust volume to 20 µL/well with ALT Assay Buffer).
- (OPTIONAL) Positive control = 5 – 10 µL Positive control (adjust volume to 20 µL/well with ALT Buffer).

12.2 **Reaction Mix:**
Prepare 100 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>
</tr>
</thead>
<tbody>
<tr>
<td>ALT Assay Buffer</td>
<td>86</td>
<td>87.6</td>
</tr>
<tr>
<td>OxiRed Probe*</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>ALT Enzyme Mix</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ALT Substrate</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

**NOTE:** *For fluorometric reading, using 0.4µL/well of the OxiRed probe decreases the background readings, therefore increasing 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:

\[ X \text{ µL component} \times (\text{Number of samples} + \text{standards} + 1) \]

12.3 Add 100 µL of Reaction Mix into each well of standard, samples and positive controls if using.

12.4 Measure output on a microplate reader in a kinetic mode after 10 minutes, every 2 – 3 minutes, for at least 60 minutes at 37°C protected from light.
- Colorimetric assay: measure OD570 nm.
- Fluorometric assay: measure Ex/Em = 535/587nm.

There is an initial lag phase of 10 minutes that can lead to underestimation of the ALT activity. We recommend measuring activity after the initial 10 min incubation.

**NOTE:** Sample incubation time can vary depending on ALT activity in the samples. We recommend measuring absorbance/fluorescence in kinetic mode and then choosing two time points (T₁ and T₂) after the initial lag phase, during the linear range.

RFU/OD value at T₂ should not exceed the highest OD in the standard curve. For standard curve, do not subtract A₁ from A₂ reading.
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 Activity of ALT is calculated as:

\[ \frac{\Delta A_{570\text{nm}}}{\Delta \text{RFU}_{535/575\text{nm}}} = A_2 - A_1 \]

Where:
- A1 is the sample reading at time T1
- A2 is the sample reading at time T2

13.6 Use the \( \Delta A_{570\text{nm}}/ \Delta \text{RFU}_{535/575\text{nm}} \) to obtain B nmol of Pyruvate generated by ALT during the reaction time \( (\Delta T = T_2 – T_1) \).

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

\[ ALT \text{ Activity} = \left( \frac{B}{\Delta T \times V} \right) \times D \]

ALT activity= nmol/min/mL =mU/mL
DATA ANALYSIS

Where:
B = Amount of pyruvate from Pyruvate Standard Curve.
\( \Delta T \) = reaction time (min).
V = original sample volume added into the reaction well (in mL).
D = sample dilution factor.

ALT molecular weight: 54.47 g/mol.

Unit Definition:
1 Unit ALT = amount of ALT which generates 1.0 \( \mu \)mol of Pyruvate per min at 37°C.
14. TYPICAL DATA

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

Figure 1: Typical Pyruvate standard calibration curve using colorimetric reading.

Figure 2: Typical Pyruvate standard calibration curve using fluorometric reading.
Figure 3: Measurement of alanine transferase activity in Positive control.

Figure 4: Measurement of alanine transferase activity in HepG2 lysate and liver lysate following kit protocol.
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.

- Thaw OxiRed probe, solubilize enzyme mix and ALT Substrate (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, make up to 20 µL with Assay Buffer).
- Set up a plate for standard (20 µL); samples (20 µL) and positive control if using (20 µL).
- Prepare ALT Reaction Mix (100 µL/well) (Number samples + standards + 1).

<table>
<thead>
<tr>
<th>Component</th>
<th>Colorimetric Reaction Mix (µL)</th>
<th>Fluorometric Reaction Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT Assay Buffer</td>
<td>86</td>
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<tr>
<td>OxiRed Probe*</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>ALT Enzyme Mix</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>ALT Substrate</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

- Add 100 µL of ALT Reaction Mix into standard, samples and positive control wells (if using). Mix well.
- Incubate plate at 37°C during 30 minutes and read absorbance at OD=570 nm or fluorescence at Ex/Em= 535/587 nm in a kinetic mode.
# 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 a different 96-well plate</td>
<td>Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate</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</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>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>------------------------------------------------</td>
<td>----------------------------------------------------------------</td>
</tr>
<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 (&lt; 5 µL) 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 described in the 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 it is within the linear range</td>
</tr>
</tbody>
</table>
17. FAQ

At what time points should the standards and samples be read?
OD measurement must be done at an initial time point T1 (T1 > 10min) then again at T2 after incubating the reaction at 37 °C for 60 min (or longer if the ALT activity is low). T2 should be chosen such that the OD at this time T2 is within the OD for the highest standard. The exact time points will need to be optimized based on the level of activity in your samples. These times provided on the datasheet are only guidelines. The standards are read at endpoint (highest timepoint for the samples).

Why can't this assay be done at endpoint only?
Enzyme activity is a rate of change with respect to time. Enzyme activity varies with time and hence to calculate rate accurately, change in OD over a time difference is to be measured. Doing an endpoint assay is only a rough estimate. The data should be analyzed within the linear increase regime of a Michaelis-Menten curve.

Why does T1 have to be > 10 minutes?
The 2 – 3 minute measurement is within the lag phase as is shown by the grey shaded area on the positive control data plot (see figure 3). Taking a measurement within this time is not recommended and can definitely result in major underestimation of the ALT activity. We recommend making the initial measurement at 10 minutes and no earlier than that to avoid being in the lag phase.

The kit recommends adding up to 20 µL of sample to 100 µL of mastermix, however we are having trouble with our samples having low levels of ALT. Would it be possible to add a greater amount of sample to mastermix e.g. 50 µL of sample to 100 µL?
If there is less ALT in your sample, we suggest you run the fluorometric version of the assay using 20 µL sample but lowering the probe to
0.4 µL. The fluorometric method is 10 – 100 times more sensitive and should work for any samples with low ALT activity.

**Can this kit work with rat or pig samples?**
Our kits can work with a variety of mammalian samples including rat and pig. You might have to try a few different volumes to make sure the readings are within the linear range of the std. curve. This kit has a detection limit of 10 mU of enzyme activity per well.

**Can ALT be measured from serum or cell culture media? What type of cells are used normally to test ALT activity?**
Typically liver cells or liver-derived cell lines (HepG2 cells, for example) are commonly used. Liver cells release these enzymes when there is damage to the cells and hence in such situations can be measured in the culture medium or serum. Normally, levels in the serum is low. The normal range of values for ALT (SGPT) ranges from 7 – 56 units per liter of human serum.

**Can RIPA buffer be used to prepare samples for this kit?**
For any enzyme assay, we do not recommend RIPA buffer since it contains SDS and this can denature proteins and affect enzyme activity. We have tested and recommend using our assay buffer provided in the kit for best results.
18. **INTERFERENCES**

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- RIPA buffer – contains SDS which can denature proteins and affect enzyme activity.
19. **NOTES**