

Non-Alcoholic Fatty Liver Disease (NAFLD) Detection Kit

07/17

(Catalog K489-100; 100 assays; Store at -20°C)

I. Introduction:

Non-Alcoholic Fatty Liver Disease (NAFLD; also known as Hepatosteatorosis) is a disease caused by the accumulation of lipids in liver cells that is not related to alcohol usage. In order to reduce the use of invasive liver biopsies for the diagnosis of NAFLD progression, various biomarkers for hepatosteatorosis has been evaluated. Recent studies on searching possible metabolic biomarkers for NAFLD, it was shown alanine to pyruvate ratio is significantly increased in rats that developed NAFLD. The result closely correlates with the increased activities of alanine aminotransferase, which is an established biomarker for liver damage. Thus, the measurement of alanine/pyruvate ratio in serum or liver could be a promising marker to detect NAFLD. BioVision's NAFLD detection kit determines the alanine to pyruvate ratio in two independent enzymatic reactions. The difference between these two measurements gives the actual amount of alanine and pyruvate present in the samples. This kit provides a simple, fast and high throughput adaptable assay to measure the ratio of alanine and pyruvate with in various biological samples in both colorimetric (O.D. 570 nm) with detection range of alanine and pyruvate 2-10 nmol and fluorometric mode (Ex/Em = 535/587nm) with detection range 0.2-1 nmol.



II. Applications:

- Analysis of alanine and pyruvate in NAFLD
- Measurement of alanine/pyruvate ratio in serum and liver samples

III. Sample Type:

- Rat liver
- Human serum

IV. Kit Contents:

| Components | K489-100 | Cap Code | Part Number |
|-------------------------------|----------|----------|-------------|
| NAFLD Assay buffer | 25 ml | WM | K489-100-1 |
| NAFLD Substrate | 1 vial | Blue | K489-100-2 |
| Probe (in DMSO) | 220 µl | Red | K489-100-3 |
| Development Enzyme Mix | 1 vial | Green | K489-100-4 |
| Alanine Converting Enzyme Mix | 1 vial | Purple | K489-100-5 |
| Sulfosalicylic acid (SSA) | 1 bottle | WM | K489-100-6 |
| Pyruvate Standard (100 mM) | 100 µl | Yellow | K489-100-7 |
| Alanine Standard (10 µmol) | 1 vial | Orange | K489-100-8 |

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer (ELISA reader)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **NAFLD Assay Buffer:** Warm to room temperature before use. Store at 4°C or -20°C.
- **NAFLD Substrate:** Reconstitute with 220 µl dH₂O. Store at -20°C. Keep on ice while in use. Use within two months.
- **Probe (in DMSO):** Ready to use as supplied. Warm to room temperature before use. Store at -20°C. Avoid from light.
- **Development Enzyme Mix:** Reconstitute with 220 µl NAFLD Assay Buffer. Pipette up and down to dissolve. Aliquot and store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use. Use within two months.
- **Alanine Converting Enzyme Mix:** Reconstitute with 880 µl NAFLD Assay Buffer. Pipette up and down to dissolve. Aliquot and store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use. Use within two months.
- **Sulfosalicylic acid (SSA):** Add 3.9 mL dH₂O to make a **25% SSA** solution. Keep on ice while in use. Store at 4°C.
- **Pyruvate Standard:** Ready to use as supplied. Keep on ice while in use. Store at -20°C.
- **Alanine Standard:** Dissolve in 100 µl dH₂O to generate 100 mM (100 nmol/µl) Alanine standard solution. Keep cold while in use. Store at -20°C.

VII. NAFLD Detection Assay Protocol:

1. Sample Preparation: For tissue samples: Rapidly homogenize tissue (~25 mg) with 84 µl ice cold NAFLD Assay Buffer, and keep on ice for 10 min. Add 16 µl of the 25% SSA solution to precipitate the protein in the samples, mix well by vortexing and keep on ice for 5 min. Centrifuge at 16,000 x g for 10 min at 4°C to remove the precipitated proteins and collect the supernatant. We recommend using samples within 8 hours of the deproteinization. Dilute the samples 5X by adding 20 µl of the deproteinized samples into 80 µl of NAFLD Assay buffer. For every sample, prepare two sets of duplicates. (A) For pyruvate: add 5-20 µl into two wells. Label as "Pyruvate" and "background control". (B) For alanine: Further dilute the samples by 5X. Add 5-20 µl into two wells. Label as "Alanine and background control". **For serum samples:** Centrifuge at 16,000 x g for 5 min to remove insoluble material. Add 32 µl of 25% SSA to 168 µl of serum, vortex and keep on ice for 5 min. Centrifuge at 16,000 x g for 10 min and collect the supernatant. (A) For pyruvate:

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add 2-6 µl into two wells. Label as "Pyruvate" and "Pyruvate background control". (B) For alanine: Further dilute the samples by 5X. Add 2-6 µl into two wells. Label as "Alanine" and "Alanine background control". Bring the volume to 50 µl with the NAFLD Assay buffer.

Notes:

- For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.
- Due to the presence of LDH and ALT in serum and liver, serum or tissue samples should be kept at -80°C before lysis and deproteinization to prevent the conversion of pyruvate to lactate and alanine.

2. Standard Curve Preparation*:

Colorimetric assay: Dilute both pyruvate standard and alanine standard to 1 nmol/µl by adding 10 µl of the respective standards to 990 µl of dH₂O to generate the 1 mM standard solutions. Mix well. Add 0, 2, 4, 8, 10 µl of the 1 mM standards into a 96-well plate to generate 0, 2, 4, 6, 8, 10 nmol/well. Bring the volume to 50 µl with the NAFLD Assay Buffer.

Fluorometric assay: Make the 1 mM standard solutions according to the colorimetric assay. Take 100 µl of the 1 mM standard solution and add to 900 µl of dH₂O to make 0.1 mM standard solutions. Add 0, 2, 4, 8, 10 µl of the 0.1 mM standard solutions to a 96-well plate to generate 0, 0.2, 0.4, 0.6, 0.8, 1.0 nmol/well standards. Bring the volume to 50 µl with NAFLD Assay buffer.

*Prepare fresh diluted standard solutions. Do not store the diluted stocks.

3. Reaction Mix: Mix enough reagents for the number of assays to be performed. For each well, prepare 50 µl Mix containing:

For Colorimetric Assay:

| | Alanine Mix | Pyruvate Mix | Background Mix |
|-------------------------------|-------------|--------------|----------------|
| NAFLD Assay Buffer | 36 µl | 44 µl | 46 µl |
| NAFLD Substrate | 2 µl | 2 µl | 2 µl |
| Probe | 2 µl | 2 µl | 2 µl |
| Development Enzyme Mix | 2 µl | 2 µl | ---- |
| Alanine Converting Enzyme Mix | 8 µl | ---- | ---- |

For Fluorometric assay:

| | Alanine Mix | Pyruvate Mix | Background Mix |
|-------------------------------|-------------|--------------|----------------|
| NAFLD Assay Buffer | 37.6 µl | 45.6 µl | 47.6 µl |
| NAFLD Substrate | 2 µl | 2 µl | 2 µl |
| Probe | 0.4 µl | 0.4 µl | 0.4 µl |
| Development Enzyme Mix | 2 µl | 2 µl | ---- |
| Alanine Converting Enzyme Mix | 8 µl | ---- | ---- |

Mix and add 50 µl of the Alanine Reaction Mix to each well containing the Alanine Standards and Alanine test samples. Add 50 µl of the Pyruvate Reaction Mix to each well containing the Pyruvate Standards and Pyruvate test samples. Add 50 µl of Background mix to both the Pyruvate background control and Alanine background control. Mix well.

- Measurement:** Incubate at 37°C for 30 min. Protect from light. Measure OD at 570 nm or Fluorescence (Ex/Em = 535/587 nm) in a microplate reader.
- Calculation:** Subtract 0 Alanine Standard reading from all alanine readings and subtract 0 Pyruvate Standard reading from all pyruvate readings. If background controls samples are significant, correct samples using background control readings. Plot the pyruvate and alanine standard curves respectively and set the intercept of the standard curves to 0.

$$\text{Alanine: Pyruvate Ratio} = \frac{\left(\frac{R_a * D_a}{V_a} - \frac{R_p * D_p}{V_p}\right) * S_p}{\frac{R_p * D_p}{V_p} * S_a}$$

Alanine Readings

- R_a = Corrected sample reading from alanine reaction mix
- D_a = Dilution factor used for the alanine sample
- V_a = Sample volume added into the reaction well for alanine reaction (µl)
- S_a = Slope from alanine standard curve (RFU/nmol)

Pyruvate Readings

- R_p = Blanked sample reading from pyruvate reaction mix
- D_p = Dilution factor used for the pyruvate sample
- V_p = Sample volume added into the reaction well for pyruvate reaction (µl)
- S_p = Slope from pyruvate standard curve (RFU/nmol)

1:1 alanine/pyruvate ratio = 1 mole of alanine per mole of pyruvate present in the biological samples
= 1.01 gram of alanine per gram of pyruvate present in the biological samples

*Moon, C.M. et al., 2017, Biochemical and Biophysical research Communications, Vol. 482, Issue1, provides a more detailed explanation on the correlation between Alanine:Pyruvate ratio with NAFLD.

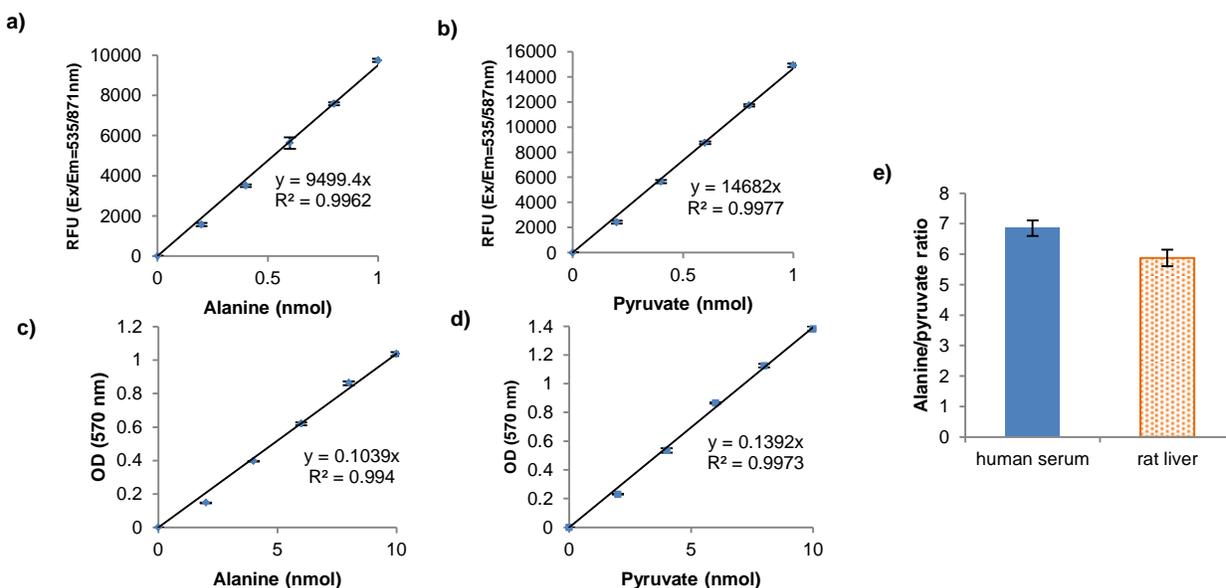


Figure: Fluorometric Standard Curves. (a) Alanine. (b) Pyruvate. Colorimetric Standard Curves. (c) Alanine. (d) Pyruvate. (e) Alanine/pyruvate ratio in deproteinized human serum (Pyruvate, DF= 1, 6 μ l; Alanine, DF= 5, 6 μ l) and rat liver (DF= 5; Pyruvate, 10 μ l Alanine, 2 μ l) measured under fluorometric protocols. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Alanine Colorimetric/Fluorometric Assay kit (K652)

Pyruvate Colorimetric/Fluorometric Assay Kit (K609)

Aspartate Aminotransferase Activity Colorimetric Assay kit (K753)

Lactate Colorimetric/Fluorometric Assay Kit (K607)

α -Ketoglutarate Assay Kit (K677)

Alanine Aminotransferase Activity Assay kit (K752)

PicoProbe™ Lactate Dehydrogenase Activity Assay kit (K730)

Lactate Dehydrogenase Activity Colorimetric Assay kit (K726)

Glucose Colorimetric/Fluorometric Assay kit (K606)

Malate Colorimetric Assay Kit (K637)

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