

Version 1 Last updated 5 June 2020

# ab273320

## Non-Alcoholic Fatty Liver Disease (NAFLD) Detection Kit

View Kit datasheet: <https://www.abcam.com/ab273320>  
(use <https://www.abcam.cn/ab273320> for china, or  
<https://www.abcam.co.jp/ab273320> for Japan)

For the measurement of alanine/pyruvate ratio in serum and liver samples.

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

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## 1. Overview

Non-Alcoholic Fatty Liver Disease (NAFLD) Detection Kit (ab273320) 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.

## 2. Protocol Summary

Prepare samples as directed



Prepare all reagents as directed



Prepare colorimetric or fluorometric standards as required



Add Standard, Alanine Sample, Alanine Background Control, Pyruvate Sample and Pyruvate Background Control to appropriate wells and adjust volume to 50  $\mu$ L



Add Alanine Reaction Mix to Alanine Standards and Alanine test samples. Add Pyruvate Reaction Mix Pyruvate Standards and Pyruvate test samples. Add Background mix to Pyruvate background control and Alanine background control (50  $\mu$ L)



Mix and incubate at 37°C for 30 mins protected from light



Measure OD at 570 nm 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.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

### 4. Storage and Stability

**Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 6 months from receipt.**

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

Aliquot components in working volumes before storing at the recommended temperature.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- 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.

## 6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
NAFLD Assay Buffer	25 mL	-20°C	+4°C or -20°C
NAFLD Substrate (Lyophilized)	1 vial	-20°C	-20°C
Probe (in DMSO)	220 µL	-20°C	-20°C
Development Enzyme Mix (Lyophilized)	1 vial	-20°C	-20°C
Alanine Converting Enzyme Mix (Lyophilized)	1 vial	-20°C	-20°C
Sulfosalicylic acid (SSA) (Lyophilized)	1 bottle	-20°C	+4°C
Pyruvate Standard (100 mM)	100 µL	-20°C	-20°C
Alanine Standard (10 µmol) (Lyophilized)	1 vial	-20°C	-20°C

## 7. Materials Required, Not Supplied

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

- Multi-well spectrophotometer capable of measuring OD at 570 nm or Fluorescence (Ex/Em = 535/587 nm)
- Clear 96-well plate with flat bottom
- Dounce homogenizer

## 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.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

## 9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

### 9.1 **NAFLD Assay Buffer:**

Ready to use as supplied. Store at 4°C or -20°C. Bring to room temperature before use.

### 9.2 **NAFLD Substrate:**

Reconstitute with 220 µL dH<sub>2</sub>O. Store at -20°C. Keep on ice while in use. Use within two months.

### 9.3 **Probe (in DMSO):**

Ready to use as supplied. Warm to room temperature before use. Store at -20°C. Avoid from light.

### 9.4 **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.

### 9.5 **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.

### 9.6 **Sulfosalicylic acid (SSA):**

Add 3.9 mL dH<sub>2</sub>O to make a 25% SSA solution. Keep on ice while in use. Store at 4°C.

### 9.7 **Pyruvate Standard:**

Ready to use as supplied. Keep on ice while in use. Store at -20°C.

### 9.8 **Alanine Standard:**

Dissolve in 100 µL dH<sub>2</sub>O to generate 100 mM (100 nmol/µL) Alanine standard solution. Keep cold while in use. Store at -20°C.

## 10. Sample Preparation

### 10.1 Tissues Samples:

- 10.1.1 Rapidly homogenize tissue (~25 mg) with 84  $\mu$ L ice cold NADFLD Assay Buffer and keep on ice for 10 minutes.
- 10.1.2 Add 16  $\mu$ L of the 25% SSA solution to precipitate the protein in the samples, mix well by vortexing and keep on ice for 5 minutes.
- 10.1.3 Centrifuge at 16,000 x  $g$  for 10 minutes at 4°C to remove the precipitated proteins and collect the supernatant.
- 10.1.4 We recommend using samples within 8 hours of the deproteinization.
- 10.1.5 Dilute the samples 5X by adding 20  $\mu$ L of the deproteinized samples into 80  $\mu$ L of NADFLD Assay buffer. For every sample, prepare two sets of duplicates. (A) For pyruvate: add 5-20  $\mu$ L into two wells. Label as 'Pyruvate' and 'background control'. (B) For alanine: Further dilute the samples by 5X. Add 5-20  $\mu$ L into two wells. Label as 'Alanine' and 'background control'.

### 10.2 Serum Samples:

- 10.2.1 Centrifuge at 16,000 x  $g$  for 5 minutes to remove insoluble material.
- 10.2.2 Add 32  $\mu$ L of 25% SSA to 168  $\mu$ L of serum, vortex and keep on ice for 5 minutes.
- 10.2.3 Centrifuge at 16,000 x  $g$  for 10 minutes and collect the supernatant.

**Δ Note:** 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.

## 11. Standard Curve

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

### 11.1 Colorimetric Assay:

- 11.1.1 Prepare a working 1 mM solution of both pyruvate standard and alanine standard by diluting 10  $\mu\text{L}$  of the respective standards with 990  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . Mix well.
- 11.1.2 Add 0, 2, 4, 6, 8 and 10  $\mu\text{L}$  of the working 1 mM standards into a series of wells in a 96-well plate to obtain 0, 2, 4, 6, 8, 10 nmol/well.
- 11.1.3 Adjust the volume of each well to 50  $\mu\text{L}$  with NAFLD Assay Buffer.

Standard #	1 mM Alanine or Pyruvate Standard ( $\mu\text{L}$ )	NAFLD Assay Buffer ( $\mu\text{L}$ )	Alanine or Pyruvate Standard (nmol/well)
1	10	40	10
2	8	42	8
3	6	44	6
4	4	46	4
5	2	48	2
6	0	50	0

### 11.2 Fluorometric Assay:

- 11.2.1 Prepare a working 1 mM solution of both pyruvate standard and alanine standard by diluting 10  $\mu\text{L}$  of the respective standards with 990  $\mu\text{L}$  of  $\text{dH}_2\text{O}$ . Mix well.
- 11.2.2 Take 100  $\mu\text{L}$  of the 1 mM standard solution and add to 900  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  to make 0.1 mM standard solutions.
- 11.2.3 Add 0, 2, 4, 6, 8 and 10  $\mu\text{L}$  of the working 0.1 mM standards into a series of wells in a 96-well plate to obtain 0, 0.2, 0.4, 0.6, 0.8, 1.0 nmol/well.
- 11.2.4 Adjust the volume of each well to 50  $\mu\text{L}$  with NAFLD Assay Buffer.

Standard #	0.1 mM Alanine or Pyruvate Standard (μL)	NAFLD Assay Buffer (μL)	Alanine or Pyruvate Standard (nmol/well)
1	10	40	1.0
2	8	42	0.8
3	6	44	0.6
4	4	46	0.4
5	2	48	0.2
6	0	50	0

## 12. Assay Procedure

- Thaw all reagents thoroughly and mix gently.
  - For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.
- 12.1 For **tissue** samples, 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'.
  - 12.2 For **serum** samples, prepare two sets of duplicates. (A) For pyruvate: 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'.
  - 12.3 Adjust the volume of Sample to 50 μL/well with NAFLD Assay Buffer. Mix well.
  - 12.4 **Reaction Mix:** Prepare enough reagents for the number of assays to be performed. For each well, prepare 50 μL of Reaction Mix. Mix well.

### For Colorimetric Assay:

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

### For Fluorometric Assay:

Component	Alanine Mix (µl)	Pyruvate Mix (µl)	Background Mix (µl)
NAFLD Assay Buffer	37.6	45.6	47.66
NAFLD Substrate	2	2	2
Probe	0.4	0.4	0.4
Development Enzyme Mix	2	2	---
Alanine Converting Enzyme Mix	8	---	---

- 12.5 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.
- 12.6 Add 50 µL of Background mix to both the Pyruvate background control and Alanine background control. Mix well.
- 12.7 Incubate the plate for 30 minutes at 37°C. Protect from light.
- 12.8 Measure OD at 570 nm or Fluorescence (Ex/Em = 535/587 nm) in a microplate reader.

## 13. Calculations

- 13.1 Subtract 0 Alanine Standard reading from all alanine readings and subtract 0 Pyruvate Standard reading from all pyruvate readings.
- 13.2 If background controls samples are significant, correct samples using background control readings.
- 13.3 Plot the pyruvate and alanine standard curves respectively and set the intercept of the standard curves to 0.

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

### 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 ( $\mu\text{L}$ )

$S_a$  = 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 v sample

$V_p$  = Sample volume added into the reaction well for b reaction ( $\mu\text{L}$ )

$S_p$  = 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

## 14. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

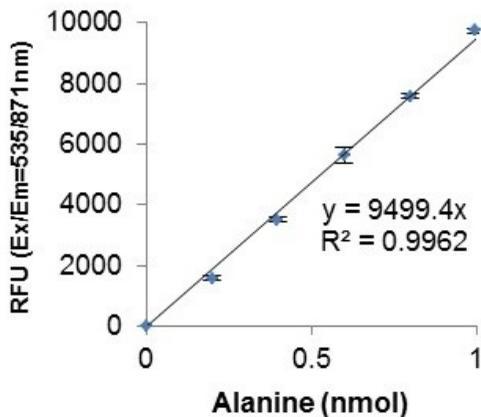


Figure 1. Fluorometric standard curve – Alanine.

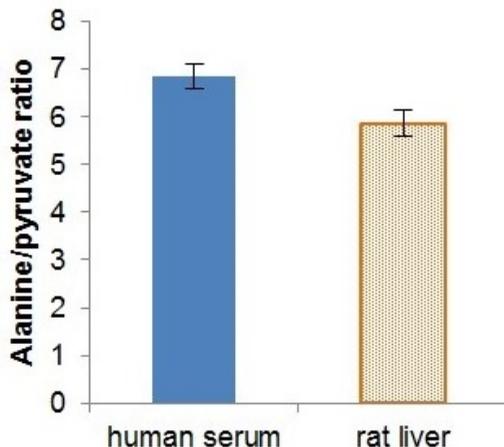


Figure 2. Alanine/pyruvate ratio in deproteinized human serum (Pyruvate, DF= 1, 6  $\mu$ L; Alanine, DF= 5, 6  $\mu$ L) and rat liver (DF= 5; Pyruvate, 10  $\mu$ L; Alanine, 2  $\mu$ L) measured under fluorometric protocols. Assays were performed following the kit protocol.

## 15. FAQ / Troubleshooting

General troubleshooting points are found at [www.abcam.com/assaykitguidelines](http://www.abcam.com/assaykitguidelines).

## 16. Notes

## Technical Support

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