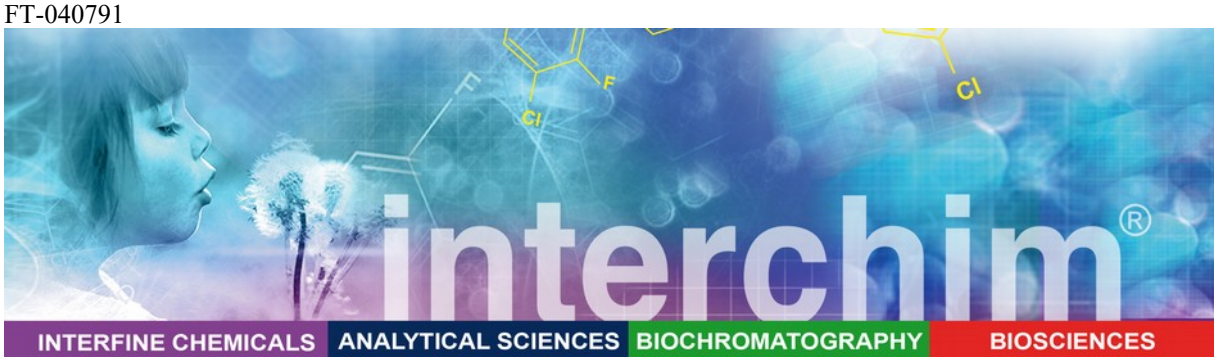
FT-040791

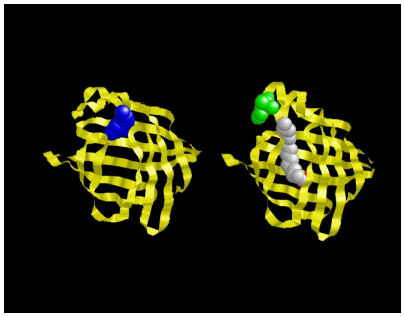



# ADIFAB/2 free fatty acid probes

*A great probe to assay aqueous free fatty acids in as little as one minute in a variety of biochemical and clinical applications including: determination of lipase activity, fatty acid binding to membranes and proteins, and serum unbound free fatty acid levels*

## Product Information

| | |
|---|---|
| **Name :** | **ADIFAB unbound fatty acid indicator** |
| **Catalog Number :** | FP-040791, 200µg FP-040792, 1mg |
| **Absorption / Emission :** | $\lambda_{exc}\backslash\lambda_{em}$ (fatty acid free.) = 386 / 432nm<br>$\lambda_{exc}\backslash\lambda_{em}$ (with fatty acid) = 386 / 505nm |
| **EC** ($M^{-1}$ $cm^{-1}$) **:** | |



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| | |
|---|---|
| **Name :** | **ADIFAB2 unbound fatty acid indicator** |
| **Catalog Number :** | FP-BB6681 200µg P-BB6682, 1mg |
| **Absorption / Emission :** | $\lambda_{exc}\backslash\lambda_{em}$ (fatty acid free) = 375 / 550nm<br>$\lambda_{exc}\backslash\lambda_{em}$ (with fatty acid) = 375 / 447nm |
| **EC** ($M^{-1}$ $cm^{-1}$) **:** | |

Kits include the indicator and, at no additional cost, a storage buffer, a measuring buffer and 0.25 ml of an unbound free fatty acid standard for measurement calibrations.

**Storage:** –20°C (J.) Protect from light and moisture

**Related products:**
I-FABP (recombinant rat intestinal fatty acid binding protein.)
FFA Standards (calibrated aqueous solutions of fatty acids complexed with bovine serum albumin)

## Introduction

ADIFAB (Acrylodan labeled Intestinal Fatty Acid Binding Protein) is a fluorescent molecule that enables to assay the aqueous concentration of free fatty acids, also known as unbound free fatty acids.
ADIFAB2 is a high affinity version of our original ADIFAB assay and provides greater sensitivity for low concentrations of unbound free fatty acids. ADIFAB2 is recommended for serum or plasma measurements.

The assay can be used in a variety of biochemical applications including the determination of lipase activity, fatty acid binding to membranes and proteins, and serum unbound free fatty acid levels, as well as clinical applications (fat status, ischemia).



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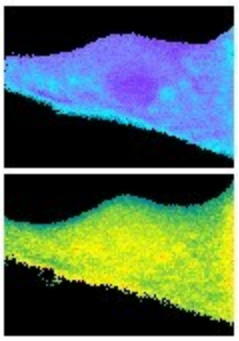
## Application Examples

- **Assay Methods**

You can determine unbound free fatty acid levels in an aqueous solution with a simple, one step procedure that completes in approximately one minute: add a small amount of the ADIFAB reagent to a solution suspected of containing unbound free fatty acid and measure the resulting fluorescence. In the absence of unbound free fatty acids, the ADIFAB probe fluoresces in the blue at 432 nm. In the presence of unbound free fatty acids, the emission shifts to the green with a peak at 505 nm. The ratio of fluorescence at 505 nm and 432 nm allows the concentration of unbound free fatty acids to be determined without further calibration.

Although the assay method for ADIFAB2 is similar to that for ADIFAB, the excitation and emission wavelengths are different. The excitation value for ADIFAB2 is 375 nm and the emission wavelengths are 550 and 447nm.



Intracellular FFAu levels measured with ADIFAB micro-injected into fat cells

- **Enzyme Activity**

Unbound free fatty acid measurements can be used to monitor continuously the activity of enzymes for which fatty acids are a reactant or product. Because of ADIFAB2's greater affinities, we would expect the sensitivity for lipase activity of ADIFAB2 to be less than or equal to ADIFAB. The assay does not require labeled (radioactive or fluorescent) substrates and can be used under physiologic conditions. ADIFAB2, therefore, is well-suited to evaluate the efficacy of molecules designed to modify the enzymatic activities.

- **Serum Unbound Free Fatty Acid Measurements**

Elevated levels of unbound free fatty acids are associated with a number of disease states, including ischemia, diabetes, and cancer. Unbound, rather than total free fatty acid levels, provide the most sensitive indicator of the pathologic state. Among the reasons for this sensitivity are: Unbound free fatty acids interact directly with cells, and the level of unbound free fatty acids increases exponentially with a linear rise in total free fatty acids.

- **Fat Status**

Unbound free fatty acid levels provide a convenient way to measure autolipolysis in fats and oils and can therefore be used to evaluate the quality of edible oils, for example to distinguish extra virgin from ungraded olive oil.

- **Clinical applications - Ishemia**

ADIFAB is the only way to measure blood levels of unbound free fatty acids (FFAu), possibly the most sensitive indicator of ischemia (the largest cause of death in industrialized countries).
Diagnosing cardiovascular ischemia is considered to be one of he "Holy Frails" of cardiovascular medicine. As example study, in all patients undergoing balloon angioplasty, a model for ischemia, FFAu levels were found risen on average 14fold above normal, within 30min of the balloon inflation. FFAu measurements was a much more sensitive indicator of ischemia that EGC that showed level change in only half patients.
The problem: “What you really want to know is how to identify the person who has acute coronary syndrome or unstable angina in the absence of necrosis. Quite simply, markers of necrosis - which are the current state of medical practice - develop too late for use on most of the patients we are seeing.” (Robert Jesse, M.D., Ph.D., IVD Technology, Sept 1999.)
Problem Solved with ADIFAB: Our results show that plasma FFAu are an extremely sensitive indicator of the ischemia that precedes necrosis. Until the invention of ADIFAB there was no way to measure unbound FFA. Not only has ADIFAB solved this problem, but it can provide within seconds accurate measurements of plasma FFAu using only a drop of blood.

Other possible applications include fatty acid metabolism, monitoring cardiovascuar diseases treatment, cancer, diabetes and obesity.



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# Directions for use

## Handling and Storage

ADIFAB and ADIFAB2 are shipped on blue ice, and should be stored frozen (at –70°C if possible) upon receipt, protected from light. Buffer can kept t +4°C. Standard should be frozen at –20°C.
After ADIFAB/2 is brought up in a solution, it should be refrigerated.

FFAu standard are shipped , and should be frozen at –20C.

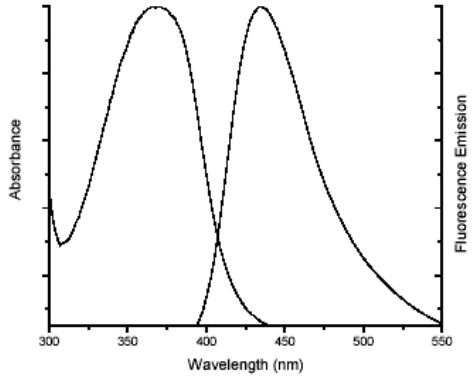
## Protocol – Preparation (r)

**1/** Preparation of **ADIFAB stock solution**:
Prepare storage buffer:
-for ADIFAB: 50 mM TRIS, 1 mM EDTA, and 0.05% sodium azide. The pH at room temperature is 8.0 ± 0.1.
-for ADIFAB2: 20 mM HEPES, 140 mM NaCl, 5 mM KCL, 1 mM $Na_2HPO_4$, 1 mM EDTA and 0.05% sodium azide. The pH at room temperature is 7.4 ± 0.1.
Add storage buffer to the vial of lyophilized ADIFAB powder to give a final concentration of ~100 µM. The approximate molecular weight of ADIFAB is 15000 g/mole; therefore, for every 200 µg ADIFAB, add 133 µL storage buffer and for 1 mg ADIFAB, add 667 µL.
Once storage buffer is added to ADIFAB, store at 4°C. The product is stable at 4°C for >3 months.

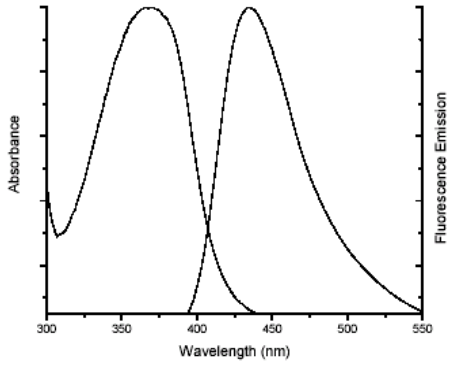
**2/Measuring conditions - buffers**
Prepare measuring buffer:
-for ADIFAB: 50 mM TRIS, 1 mM EDTA, and 0.05% sodium azide. The pH at room temperature is 8.0 ± 0.1.
20 mM HEPES, 140 mM NaCl, 5 mM KCL, 1 mM $Na_2HPO_4$, 1 mM EDTA and 0.05% sodium. The pH at room temperature equals 7.4 ± 0.1.
-for ADIFAB2: 20 mM HEPES, 140 mM NaCl, 5 mM KCl, and 1 mM Na2HPO4. The pH at room temperature equals 7.4 ± 0.1.
The measuring buffer consists of 20 mM HEPES, 140 mM NaCl, 5 mM KCl, and 1 mM $Na_2HPO_4$. Store the measuring buffer at 4ºC. The recommended concentration to be used in the measuring buffer is approximately 0.2 µM (ADIFAB) or 0.5 MM (ADIFAB2). The concentration can be increased or decreased depending on the efficiency of the fluorometer used.

**3/Measuring conditions - Cuvettes**:
Cuvettes made of glass or quartz are recommended for the measurement of ADIFAB/2. These cuvettes must be very clean and all traces of soap rinsed away. A final rinse of ethanol and then drying under a nitrogen stream is recommended. Disposable cuvettes can also be used. Plastic cuvettes made of polystyrene from Sarstedt (cat# 67.741) have been found to work well with the ADIFAB/2 probe. However acrylic cuvettes have been found to leach a substance that reacts with the probe. Cuvettes made of other materials can be easily tested by determining if the ADIFAB/2 ratio changes over time.

Absorption and emission spectra (excited at 386 nm) of ADIFAB in HEPES measuring buffer.




Absorption and emission spectra (excited at 386 nm) of ADIFAB in HEPES measuring buffer.




4/**FFAu standard** preparation and use



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**a.** FFAu Standards are calibrated solutions of fatty acids complexed with 600 µM bovine serum albumin. The fatty acid to BSA molar ratios of the standards range from 5:1 to 6:1, for more detailed lot information contact Interchim.

**b.** To measure the free fatty acid concentration of the FFAu Standards with ADIFAB add a small amount of the FFAu Standard (we recommend adding 1% by volume) to a cuvette of measuring buffer. Measure the fluorescence intensity of this blank cuvette according to the wavelengths given for the ADIFAB. Add ADIFAB to the cuvette (we recommend a concentration of ~0.2 µM) and again measure the intensities at the appropriate wavelengths. To use these intensities to calculate the ratio (R) value and the free fatty acid concentration refer to the ADIFAB Information Sheet.

**c.** To measure the free fatty acid concentration with ADIFAB2 follow the protocol above but substitute ADIFAB with ~0.5 µM ADIFAB2 and use the wavelengths of ADIFAB2.

## Protocol of use – Determining the Concentration of Fatty Acid in an Aqueous Solution (r)

- **Synopsis**

Measuring the concentration of an aqueous fatty acid solution is easy with ADIFAB/2—simply add an aliquot of the solution of interest to a cuvette containing ADIFAB and buffer and measure the fluorescence intensities at 505 and 432 nm (upon excitation at 386 nm) for ADIFAB, or at 550 and 457 nm (upon excitation at 375 nm) for ADIFAB2.

- **Procedure**

For details on measuring the ADIFAB ratio and calculating [FFA] and [$ADIFAB_{bound}$] see section 'Determining the ADIFAB Ratio'. Begin by measuring the ADIFAB ratio without FA present (R0) in a cuvette containing measuring buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4). Add an aliquot of the fatty acid solution to the cuvette and measure R. The total fatty acid concentration in the cuvette is then [FFA] plus [$ADIFAB_{bound}$] corrected for the fatty acid bound to the cuvette walls (Table 1). The concentration of the original fatty acid solution is the cuvette concentration multiplied by the dilution factor.

Table 1. Degree of FA binding to glass cuvettes
Note: To quantify fatty acid binding to cuvettes of other materials see Determining FA Wall Binding

| | % wall binding | | | |
|---|---|---|---|---|
| Fatty Acid | 10 | 30 | 37 | 50 |
| Laurate (12:0) | | | 1.5 | |
| Myristate (14:0) | | | 8.5 | |
| Palmitate (16:0) | 7 | 20 | 22.3 | 30 |
| Palmitoleate (16:1) 9 cis | | | 8.1 | |
| Stearate (18:0) | | | >50 | |
| Oleate (18:1) 9 cis | 18 | 20 | 21 | 29 |
| Elaidate (18:1) 9 trans | | | 21 | |
| Petroselinate (18:1) 6 cis | | | 21 | |
| Vaccenate (18:1) 11 cis | | | 21 | |
| Linoleate (18:2) 9,12 cis | 15 | 15 | 15 | 15 |
| Linolenate (18:3) 9, 12, 15 cis | 3 | 2 | 2 | 3 |
| Arachidonate (20:4) 5, 8, 11, 14 cis | 14 | 14 | 14 | 14 |

- **Example** with ADIFAB

ADIFAB was added to a cuvette containing 1.5 ml buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4 and 37°C), so that the final ADIFAB concentration was 0.2 µM. The $R_0$ value was measured and found to be 0.300. Next, 0.005 ml of an oleate stock solution was added to the cuvette. The R value was measured and found to be 0.420. Using Eq. (1), Eq. (2) and a $K_d$ of 0.28 from Determining the ADIFAB ratio, [FFA] = 59 nM and [ADIFAB2bound] = 35 nM. Under these conditions about 21% of the sodium oleate binds to the cuvette walls (Table 1) so the total amount of FA added to the cuvette was (59 +35)/0.79 = 119 nM. Thus the stock concentration was 119 nM • 1.5 ml/0.005 ml = 35.7 µM.

- **Example** with ADIFAB2

ADIFAB2 was added to a cuvette containing 1.5 ml buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4 and 22°C), so that the final ADIFAB2 concentration was 0.5 µM. The $R_0$ value was



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measured and found to be 0.085. Next, 0.005 ml of an oleate stock solution was added to the cuvette. The R value was measured and found to be 0.300. Using the equations and constants found in Determining the ADIFAB2 Ratio, [FFA] = 74 nM and [ADIFAB2$_{bound}$] = 350 nM. Under these conditions about 19% of the sodium oleate was bound to the cuvette walls (Table 1) so the total amount of FA added to the cuvette was (74 +350)/0.81 = 523 nM. Thus the stock concentration was 523 nM • 1.5 ml/0.005 ml = 157 µM.

## Protocol of use – Determining the Unbound Free Fatty Acid Concentration in Serum Samples(r)

The protocol is recommended and given with ADIFAB2. ADIFAB may be used with small modifications, but it is less sensitive and susceptible to interferences (i.e. with samples containing hemoglobin that preferentially absorbs the 432 nm fluorescence intensity of ADIFAB, giving erroneously high FFA values).

- **Synopsis**

ADIFAB2 can be used to determine the unbound free fatty acid (FFA) concentration in serum by measuring the value of the ADIFAB2 ratio with and without serum present.

- **Procedure**

For details on measuring the ADIFAB2 ratio and calculating [FFA] see section 'Determining the ADIFAB2 Ratio'. To determine $R_0$, add 0.5 µM ADIFAB2 and 6 µM fatty acid free bovine serum albumin (BSA) to a cuvette containing buffer, and measure the fluorescence ratio (550/457 upon excitation at 375 nm). To measure the R value of a serum sample, add 0.5 µM ADIFAB2 and 1% serum (by volume) to a separate cuvette containing buffer and measure 550/457. The 100 fold dilution of the serum yields an albumin concentration of ~6 µM, the same as used to determine R0. This dilution does not effect [FFA] since [FFA] is buffered by the [FA]$_{total}$:albumin ratio. To calculate [FFA] substitute R and $R_0$ into Eq. (1):

$$[FFA] = K_d \bullet Q \bullet \frac{(R - R_0)}{(R_{max} - R)} \quad (1)$$

For serum at 22°C, $K_d$ = 45.5 nM, Q = 5 and $R_{max}$ = 0.925.

**Notes**

- Because the difference between R and $R_0$ is very small (less than 0.01 for serum from a healthy donor), to insure accuracy, average at least 5 measurements of R and $R_0$ (which can be done automatically on most fluorometers).
- When measuring multiple serum samples, we advise taking 2 $R_0$ measurements, 8-10 serum sample measurements, 2 $R_0$, 8-10 serum samples, etc.

- **Example**

6 µM BSA was added to a cuvette containing 1.5 ml buffer (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4 and 22°C) and blank intensities at 457 and 550 nm (upon excitation at 375 nm) were measured. 0.5 µM ADIFAB2 was added to the cuvette, and after gently mixing the solution, the $R_0$ value was measured and found to be 0.0870. To another cuvette, 15 µl of a serum sample were added and blank intensities were measured. The R value was measured and found to be 0.0950 after 0.5 µM ADIFAB2 was added. Using Eq. (1) and the constants above, the FFA concentration was calculated to be 2.2 nM.

## Protocol of use – Determining the Fatty Acid Binding Affinity of a Protein (r)

ADIFAB can be used to determine FA binding constants to any unlabeled protein by monitoring free fatty acid levels as a solution of ADIFAB and protein is titrated with fatty acid. The protocol can be adapted for ADIFAB2, substituting 0.2µM by 0.5µM, and ADIFAB 386/432&550nm abs/emission values by 375/550&447nm.

- **Procedure**

For details on measuring the ADIFAB ratio and calculating [FFA] and [ADIFAB$_{bound}$] see section 'Determining the ADIFAB Ratio'. To determine $R_0$, measure the fluorescence ratio (505/432) of a cuvette containing ADIFAB, the protein of interest and measuring buffer (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4). Titrate the solution with small fatty acid aliquots of known concentration and measure the R value after each addition—be sure to wait at least 5-10 minutes for equilibrium before measuring R. For each R measured, calculate the amount of fatty acid bound to the protein using Eq. (1):

$$[FA]_{bound} = [FA]_{total} - [FFA] - [ADIFAB]_{bound} \quad (1)$$

where [FA]$_{total}$ is the total fatty acid concentration in the cuvette after each addition,
[FFA] is the free fatty acid concentration and



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$[ADIFAB_{bound}]$ is the concentration of fatty acid bound to ADIFAB.

**Note:** to accurately report $[FA]_{total}$l, measure the concentration of the fatty acid stock according to section 'Determining the Concentration of Fatty Acid in an Aqueous Solution'). In the case of single-affinity binding sites, analyze the data using the Scatchard method, Eq. (2):

$$\frac{\frac{[FA]_{bound}}{[Protein]_{total}}}{[FFA]} = \frac{1}{K_d'} \bullet \frac{[FA]_{bound}}{[Protein]_{total}} + \frac{n}{K_d'} \quad (2)$$

where $[Protein]_{total}$ is the concentration of binding protein added, n is the number of fatty acid binding sites per protein monomer, and $K_{d'}$ is the binding affinity of the fatty acid to the protein.

Plotting the data as $[FA]_{bound}/[Protein]_{total}/[FFA]$ vs. $[FA]_{bound}/[Protein]_{total}$ yields a straight line with a slope equal to $-1/K_{d'}$ and a x-axis intercept equal to *n*. For multiple binding sites of different affinities, the Scatchard plot is nonlinear.

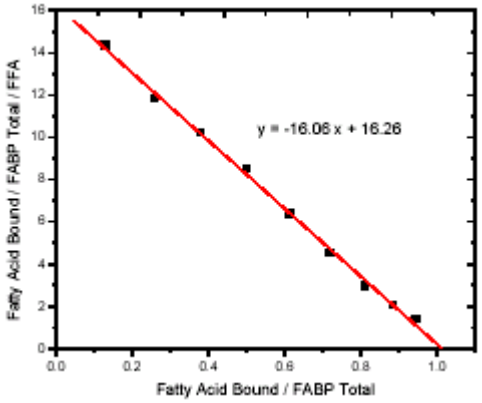
- **Example**

In order to determine the affinity of oleic acid to murine adipocyte fatty acid binding protein (mAFABP), 4.0 µM of AFABP and 0.2 µM ADIFAB were added to a cuvette containing 1.5 ml buffer (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4 and 37°C). The R0 value was measured and found to be 0.2671. Sodium oleate was added so that the OA concentration in the cuvette was 0.53 µM, and after waiting 10 minutes for equilibrium, the R value was measured and found to be 0.2855.
Additional aliquots of sodium oleate were added and after each addition R values were measured. The results and analysis of these measurements are listed in Table 1. A Scatchard plot (Fig.1) of this data (column G vs. column F) resulted in a straight line with a slope of –16.06 ($-1/K_{d'}$) and an x-intercept of 1.01 (n). Therefore the oleic acid binding affinity of mAFABP is 62 nM at a single binding site.

Table 1.
Analysis of ADIFAB measurements to determine the affinity of oleic acid and murine adipocyte FABP. All concentrations in µM.

| *Column A* | *Column B* | *Column C* | *Column D* | *Column E* | *Column F* | *Column G* |
|---|---|---|---|---|---|---|
| [FA] Added | Measured R Value | [FFA] | $[ADIFAB]_{bound}$ | $[FA]_{bound}$ | $\frac{[FA]_{bound}}{[mAFABP]_{total}}$ | $\frac{\frac{[FA]_{bound}}{[mAFABP]_{total}}}{[FFA]}$ |
| 0 | 0.2671 | 0 | | | | |
| 0.053 | 0.2855 | 8.96E-03 | 6.20E-03 | 0.515 | 0.129 | 14.368 |
| 1.06 | 0.3113 | 2.16E-02 | 1.43E-02 | 1.024 | 0.256 | 11.870 |
| 1.57 | 0.3424 | 3.68E-02 | 2.33E-02 | 1.510 | 0.377 | 10.244 |
| 2.09 | 0.3865 | 5.87E-02 | 3.46E-02 | 1.997 | 0.499 | 8.510 |
| 2.6 | 0.4612 | 9.60E-02 | 5.11E-02 | 2.453 | 0.613 | 6.387 |
| 3.1 | 0.5846 | 1.59E-01 | 7.24E-02 | 2.869 | 0.717 | 4.516 |
| 3.6 | 0.7977 | 2.71E-01 | 9.83E-02 | 3.231 | 0.808 | 2.984 |
| 4.09 | 1.09 | 4.32E-01 | 1.21E-01 | 3.537 | 0.884 | 2.049 |
| 4.58 | 1.477 | 6.59E-01 | 1.40E-01 | 3.781 | 0.945 | 1.434 |

Figure 1:
Scatchard plot of OA binding to mAFABP.




## Method – Determining Fatty Acid / Membrane Partition Coefficients [r]

ADIFAB can be used to determine the partition coefficient of a fatty acid between a membrane phase and aqueous solution. Simply add fatty acid to a cuvette containing ADIFAB and a membrane and measure the fluorescence ratio (505/432 upon excitation at 386 nm). The protocol can be adapted for ADIFAB2, substituting substituting ADIFAB 386/432&550nm abs/emission values by 375/550&447nm.

- **Procedure**

For details on measuring the ADIFAB ratio and calculating [FFA] see Determining the ADIFAB Ratio. To determine $R_0$, add ADIFAB and a membrane of known lipid concentration to a cuvette containing measuring

buffer (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4), and measure the fluorescence ratio (505/432 nm).
Titrate the solution with fatty acid aliquots of known concentration and measure the R value after each addition —be sure to wait at least 5-10 minutes for equilibrium before measuring R. For each R measured, calculate the partition coefficient, $K_p$, using Eq. (1):

$$K_p = \frac{\frac{V_a}{V_m} \bullet ([FA]_{total} - [FFA])}{[FFA]} \quad (1)$$

where $[FA]_{total}$ is the total fatty acid concentration in the cuvette after each addition,
[FFA] is the free fatty acid concentration, and
$V_a$ and $V_m$ are the volumes of the aqueous and membrane phases, respectively.

**Note**: to accurately report $[FA]_{total}$, measure the concentration of the fatty acid stock according to section 'Determining the Concentration of Fatty Acid in an Aqueous Solution'.
With sufficient membrane present, no correction for wall binding to the cuvette walls is necessary because for typical conditions >95% of the fatty acid will be bound to the membrane; very little will be free, bound to ADIFAB or bound to the walls.

- **Example**

In a cuvette containing 2 ml buffer (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4 and 37°C), 100 µM egg phosphatidylcholine vesicles (EPC) and 0.2 µM ADIFAB, the $R_0$ was measured and found to be 0.279. 3 µM of sodium palmitate was added, and after waiting 10 minutes for equilibrium, the R value was measured and found to be 0.335. Using the R value to determine that [FFA] = 88.6 nM and substituting $V_a/V_m$ = 10000 and $[FA]_{total}$ = 3 µM into Eq. (1), Kp was calculated to be $3.29x10^5$. Additional aliquots of sodium palmitate were added and after each addition R values were measured and $K_p$ values were calculated. The average value of $K_p$ for the titration was $3.47x10^5$. The complete set of R, [FFA] and $K_p$ values are listed in Table 1.

Table 1. Titration data from palmitate and EPC vesicles $K_p$ determination.

| $[FA]_{total}$ (uM) | Measured R Value | [FFA] (nM) | $K_p$ x $10^{-5}$ |
|---|---|---|---|
| 0 | 0.279 | 0 | |
| 3 | 0.427 | 89 | 3.29 |
| 5 | 0.516 | 143 | 3.40 |
| 7 | 0.606 | 199 | 3.42 |
| 10 | 0.718 | 270 | 3.60 |
| 15 | 0.920 | 402 | 3.63 |
| 20 | 1.150 | 558 | 3.48 |
| 25 | 1.345 | 696 | 3.49 |
| | | average = | 3.47 |

## Method – Determining the ADIFAB Ratio (r)

- **Synopsis**

In the absence of unbound free fatty acids, the ADIFAB probe fluoresces in the blue at 432 nm. In the presence of unbound free fatty acids, the emission shifts to the green with a peak at 505 nm. The ratio of fluorescence at 505 nm and 432 nm allows the concentration of unbound free fatty acids to be determined. This protocol outlines how to calculate and use the ADIFAB ratio (R). The method works for ADIFAB2, substituting ADIFAB 386/432&550nm abs/emission values by 375/550&447nm.

- **Procedure**

$R_0$ is the ADIFAB ratio without fatty acid present. To determine $R_0$, excite a cuvette containing measuring buffer only (20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4) at 386 nm and measure the fluorescence intensities at 505 and 432 nm. These are the blank intensities. Then add 0.2 µM ADIFAB to the cuvette, mix gently *avoiding bubble formation*, and measure the emission at 505 and 432 nm. Substitute the intensities into the $R_0$ expression:

$$R_0 = \frac{I^{\circ}_{505} - I^{blank}_{505}}{I^{\circ}_{432} - I^{blank}_{432}}$$

To measure R, add an aliquot of a solution containing fatty acid to the cuvette and mix.
Measure the intensities at 505 and 432 nm and substitute them into the R expression below. If the fatty acid solution contributes significant scattering or fluorescence, remeasure the blank intensities with fatty acid.

$$R = \frac{I_{505} - I^{blank}_{505}}{I_{432} - I^{blank}_{432}}$$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Substitute $R_0$ and R into Eq. (1), along with the appropriate dissociation constant, $K_d$, from Table 1 to determine the free fatty acid (FFA) concentration. The equation uses ADIFAB values Q=19.5 and $R_{max}$=11.5. For ADIFAB2, please refer to table 2 to set proper Q and $R_{max}$ values.

$$[FFA] = K_d \bullet 19.5 \bullet \frac{(R-R_o)}{(11.5-R)} \quad (1)$$

Kd refers to the ADIFAB (or ADIFAB2) dissociation constant.
$R_{max}$ refers to the ADIFAB ratio or ADIFAB2 (550/457) in the completely bound state.
Q refers to the intensity of ADIFAB (or ADIFAB2) at 4325 (or 457) nm in the unbound state (no fatty acid present) divided by the intensity at 432(or 457) nm in the bound state (completely saturated with fatty acid),

To determine the fatty acid concentration bound to ADIFAB, use Eq. (2). The equation uses ADIFAB values Q=19.5 and $R_{max}$=11.5. Please refer to table 2 for ADIFAB2 to set proper values.

$$[ADIFAB_{bound}] = \frac{[ADIFAB_{total}] \bullet 19.5 \bullet (R-R_o)}{11.5-R+19.5 \bullet (R-R_o)} \quad (2)$$

Table 1. $K_d$ values for fatty acid binding to ADIFAB (in µM).

All $K_d$ values measured in HEPES measuring buffer: 20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4. To determine constants in an alternative buffer or at a different temperature see section 'Determining ADIFAB Fatty Acid Dissociation Constants: Kd '

| Temperature (°C) | Laurate | Myristate | Palmitate | Oleate | Linoleate | Linolenate | Arachidonate |
|---|---|---|---|---|---|---|---|
| 5 | 12.3 | 2.54 | 0.20 | 0.17 | 0.48 | 1.31 | 0.72 |
| 10 | 12.5 | 2.67 | 0.22 | 0.19 | 0.54 | 1.47 | 0.82 |
| 15 | 12.8 | 2.80 | 0.24 | 0.20 | 0.60 | 1.63 | 0.94 |
| 20 | 13.1 | 2.93 | 0.26 | 0.22 | 0.67 | 1.81 | 1.07 |
| 25 | 13.4 | 3.07 | 0.28 | 0.23 | 0.74 | 2.00 | 1.22 |
| 30 | 13.6 | 3.21 | 0.31 | 0.25 | 0.82 | 2.21 | 1.38 |
| 35 | 13.8 | 3.30 | 0.33 | 0.27 | 0.90 | 2.42 | 1.55 |
| 37 | 14.0 | 3.40 | 0.34 | 0.28 | 0.94 | 2.51 | 1.62 |
| 40 | 14.2 | 3.48 | 0.36 | 0.29 | 0.99 | 2.65 | 1.73 |
| 45 | 14.4 | 3.62 | 0.39 | 0.31 | 1.09 | 2.90 | 1.94 |
| 50 | 14.7 | 3.77 | 0.41 | 0.33 | 1.18 | 3.15 | 2.16 |

Table 2: Fluorometric constants for fatty acid binding to ADIFAB2 at 22°C.

| Fatty Acid | Q | $K_d$ (nM) | $R_{max}$ |
|---|---|---|---|
| Palmitate (16:0) | 6.6 | 21.4 | 1.16 |
| Stearate (18:0) | 6.6 | 9.3 | 1.19 |
| Oleate (18:1) 9 cis | 5 | 32 | 0.762 |
| Linoleate (18:2) 9,12 cis | 4 | 101 | 0.727 |
| Linolenate (18:3) 9,12,15 cis | 5 | 237 | 0.798 |
| Arachidonate (20:4) | 5 | 167 | 0.912 |

All values measured in HEPES measuring buffer: 20 mM HEPES, 140 nM NaCl, 5 mM KCl, 1 mM $Na_2HPO_4$, at pH 7.4. To determine constants in an alternative buffer or at a different temperature see section 'Determining ADIFAB2 Fatty Acid Binding Constants'

## Method – Determining ADIFAB Fatty Acid Dissociation Constants: Kd (r)

This protocol outlines how to calibrate ADIFAB for a particular fatty acid in order to determine the binding constant Kd. ADIFAB Kd values for some common fatty acids are listed in section 'Determining the ADIFAB Ratio'.

- **Procedure**

For details on measuring the ADIFAB ratio and calculating [FFA] see Determining the ADIFAB Ratio. To determine $R_0$, add 0.2 µM ADIFAB to a cuvette containing buffer, and measure the fluorescence ratio (505/432 upon excitation at 386 nm). Titrate the cuvette with known concentrations of FA (to measure the concentration of the FA stock see section 'Determining the Concentration of Fatty Acid in an Aqueous Solution') and measure R after each addition—be sure to allow 5 – 10 minutes for equilibrium before measuring R. Continue the titration until R decreases or no longer significantly changes with additional fatty acid aliquots. Plot R vs. [FA] and fit this titration curve with Eq. (1) by the method of least squares:

$$R = R_0 + \frac{(R_{max}-R_0) \bullet (Q \bullet \sqrt{FA^2+2 \bullet FA \bullet (K_d-AD)+K_d^2+AD \bullet (2 \bullet K_d+AD)}+FA \bullet (Q-2)-Q \bullet (K_d+AD))}{2 \bullet (FA \bullet (Q-1)+K_d \bullet Q^2-Q \bullet (K_d+AD))} \quad (1)$$

or, written linearly for ease of plugging into a fitting program:

R = $R_o$-(($R_o$-Rm)*(Q*(FA^2+2*FA*(Kd-AD)+$K_d$^2+AD*(2*$K_d$+AD))^0.5+FA*(Q-2)-Q*($K_d$+AD))/
(2*(FA*(Q-1)+$K_d$*Q^2-Q*($K_d$+AD)))) (1)

where: R = measured ADIFAB ratio (505/432 upon excitation at 386 nm)—from titration data
$R_0$ = ADIFAB ratio in the completely unbound state (with no FA present)—from titration data
$R_{max}$ = ADIFAB ratio in the completely bound state (saturated with FA)—hold $R_{max}$ constant at 11.5
Q = intensity of ADIFAB at 432 nm in the unbound state (no fatty acid present) divided by the intensity at 432 nm in the bound state (completely saturated with fatty acid0— hold Q constant at 19.5



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FA = total fatty acid concentration—from titration data, correct for wall binding (see section 'Determining Wall Binding'
Kd = ADIFAB dissociation constant—allow $K_d$ to vary
AD = ADIFAB concentration—hold constant at 0.2 µM

**Notes**

- Kd is dependent on buffer conditions—changes in pH, temperature and ionic strength will alter Kd.
- Rmax and Q cannot be determined from this experiment because fatty acid aggregates at concentrations lower than ADIFAB saturation. When the fatty acid begins to aggregate, R will no longer increase with FA, even though ADIFAB is not fully saturated, because ADIFAB binds only monomeric fatty acids. Values of Rmax = 11.5 and Q = 19.5 have been calculated numerically from titration data, and we recommend that these values be used in all ADIFAB data analysis.

Example experiment in preparation.

## References


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- **Richieri G**. *et al*., Interactions of Long-chain Fatty Acids and Albumin: Determination of Free Fatty Acid Levels Using the Fluorescent Probe ADIFAB, *Biochemistry*, 32, 7574-7580 (1993) Article


## Other information

- **Characteristics of ADIFAB/ADIBAF2**

Accuracy: to greater than 1nM depending on type of FA
Purity : Gel electrophoresis > 95%
Form: Lyophilized powder
Weight: 15000 Da

- **Legal information**

ADIFAB: Patent Number US 5,470,714 : EU 0457901
ADIFAB2: European Patent 0457901 US 5,470,714
Safety: ADIFAB/2 do not contain any hazardous components above 1% or any carcinogens above 0.1% as defined in 29 CFR 1910.1200, the OSHA Hazard Communication Standard.

## Other information

- **Related products**

- Biochemistry assays kits
(Glycerol, HDL, Triglycerides...)
- Beta-py-C10-HPM, FP-31900A
- Beta-py-C10-PG, FP-73498A

- **Ordering information**

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