

## ADIFAB Free Fatty Acid Indicator (A3880)

### Quick Facts

#### Storage upon receipt:

- $\leq -20^{\circ}\text{C}$
- Desiccate

**Notes:** Glassware and reagents must be free of detergents. Do not use or store in plastic containers.

Detection of FFA by ADIFAB is based on a change in the position of the acrylodan fluorophore relative to the nonpolar binding pocket of the protein when it becomes occupied by a fatty acid (FA). The resulting red shift of the fluorescence spectrum is illustrated by data from a titration of ADIFAB with *cis*-9-octadecenoic (oleic) acid shown in Figure 1.

The spectral shift of ADIFAB allows determination of FFA concentrations from the **ratio** of the fluorescence intensities of bound and unbound indicator measured at about 505 nm and 432 nm respectively, in a manner analogous to dual wavelength calcium indicators.<sup>3,5</sup>

### Materials

#### Storage of Solid

Store lyophilized powder desiccated in freezer ( $\leq -20^{\circ}\text{C}$ ). The expected shelf life is one year under these conditions.

#### Glassware

**Due to the sensitivity of ADIFAB, it is most important that all glassware and reagents that the indicator comes into contact with are free of traces of detergent. Washing glassware and cuvettes with methanol, followed by thorough drying (ADIFAB fluorescence is also sensitive to methanol) is recommended.**

#### Plastics

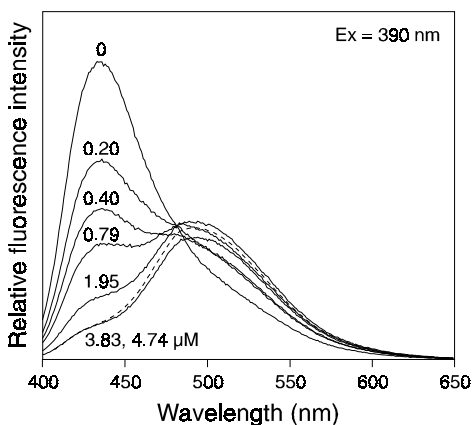
It has been observed that materials leached from some types of plastic containers bind to ADIFAB and degrade its response. **For this reason, storage in glass containers and use of glass or quartz cuvettes is generally recommended.** Plastic containers should only be used if it has been positively confirmed that the spectral properties of the indicator remain stable. Polystyrene cuvettes have been found to give satisfactory results.

#### Preparation of Solutions

The exact nature and concentration of buffer solutions used with ADIFAB do not appear to be critical. Significant experimental variables are the pH and the presence of calcium, as discussed below (see steps 1.3 and 2.1). For calcium-free measurements, include 1 mM EGTA in the buffer solution. A suitable stock solution can be prepared by dissolving one sale unit of ADIFAB (200  $\mu\text{g}$ ) in 1.0 mL of buffer containing antimicrobial additives (e.g. 50 mM Tris, 1 mM EGTA, 0.05% azide, pH 8.0). The nominal concentration of this ADIFAB stock solution is about  $1.3 \times 10^{-5}$  M (13  $\mu\text{M}$ ) based on the assumed molecular weight of 15,350. It should be stable for up to 3 months stored in a refrigerator at  $4^{\circ}\text{C}$ . Submicromolar ADIFAB concentrations (typically 0.2  $\mu\text{M}$ ) are recommended for FFA concentration measurements. Sufficient 0.2  $\mu\text{M}$  working solution for use in a standard 10 mm  $\times$  10 mm cuvette can be prepared by addition of 38  $\mu\text{L}$ .

### Introduction

ADIFAB (AcryloDated Intestinal Fatty Acid Binding protein) is a fluorescent indicator for measurement of free fatty acid (FFA) concentrations in the range 1 nM to  $>20$   $\mu\text{M}$ . As denoted by the name, the indicator is a conjugate of the polarity-sensitive fluorescent probe acrylodan<sup>1</sup> and intestinal fatty acid binding protein (I-FABP), a low molecular weight (15 kD) protein with high binding affinity for FFA.<sup>2</sup> The stoichiometry of the conjugate is approximately 1 mol:mol (fluorophore:protein). ADIFAB has been developed by Dr. Alan Kleinfeld and co-workers at the Medical Biology Institute (La Jolla, CA).<sup>3,4</sup>



**Figure 1.** Fluorescence-monitored titration of ADIFAB with *cis*-9-octadecenoic (oleic) acid at  $25^{\circ}\text{C}$ . The indicator concentration is 0.2  $\mu\text{M}$  in 10 mM Tris/HCl, 0.15 M NaCl, 1 mM EGTA, pH 8.0. Oleic acid (sodium salt, Sigma Chemical Co., O-3880) was added from a 102  $\mu\text{M}$  stock solution in 0.01 M NaOH. The total oleic acid concentration ( $\mu\text{M}$ ) is noted adjacent to the corresponding data trace. The spectrum representing the highest concentration (4.74  $\mu\text{M}$ ) is plotted with a dashed line (---). A background spectrum (buffer only) was subtracted from all traces.

of the above stock solution to 2.5 mL of aqueous buffer. The working solution should be used immediately after preparation.

## Experimental Application

### Absorption

The long-wavelength absorption maximum of ADIFAB (due to the fluorophore alone) in aqueous buffer (pH 7–8) is at about 365 nm ( $\epsilon_{365\text{ nm}} = 10,600\text{ cm}^{-1}\text{M}^{-1}$ ). The protein concentration can be estimated from  $\epsilon_{280\text{ nm}} = 16,900\text{ cm}^{-1}\text{M}^{-1}$  after subtraction of the absorbance due to the fluorophore ( $\epsilon_{280\text{ nm}} \sim 5400\text{ cm}^{-1}\text{M}^{-1}$ ). From these data, the fluorophore:protein molar ratio (F:P) can be determined. The F:P ratio should be close to 1.

### Fluorescence

#### 1.1 Excitation

For dual wavelength ratio measurements (Figure 1) using ADIFAB, fluorescence excitation at about 390 nm is recommended to enhance the signal from the bound indicator (which has substantially weaker fluorescence than the unbound form).

#### 1.2 Background Subtraction

Subtraction of background signals is important due to the fairly low concentrations (0.2  $\mu\text{M}$ ) at which ADIFAB is typically used. With excitation at 390 nm, background signals due to water Raman scattering appear at about 449 nm. These can readily be observed as a spike in the spectral profile, particularly in the spectrum of FA-bound ADIFAB.

#### 1.3 Initial Intensity Ratio

The ratio (R) of ADIFAB fluorescence intensities at 505 nm/432 nm is used to determine FFA concentrations (see step 2.1). The value of R in the absence of FFA ( $R_o$ ) should be approximately 0.2 to 0.3 at pH 8.0 ( $R_o$  increases with decreasing pH). Observed  $R_o$  values may vary due to instrumental spectral sensitivity factors.  $R_o$  should exhibit little or no change on addition of a molar excess of fatty acid free bovine serum albumin (BSA).  $R_o$  is not temperature dependent under normal working conditions (20 to 37°C).

### Fatty Acid Binding Measurements

#### 2.1 Ratio Mode Measurements

For ratio mode measurements using ADIFAB, the FFA concentration can be obtained from the following expression:<sup>3</sup>

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

where R represents the fluorescence intensity ratio ( $F_{505\text{ nm}}/F_{432\text{ nm}}$ ) and the subscripts o and max refer to the fully unbound and bound forms of the indicator respectively. Q is the ratio  $F_o/F_{\text{max}}$  at 432 nm and  $K_d$  is the dissociation constant for the FFA species in question (see step 2.3). It is most important to note that the values of  $R_{\text{max}}$  and  $F_{\text{max}}$  (and hence Q) cannot be obtained directly from experimental data. This is because of fatty acid micelle formation. ADIFAB is only capable of binding monomeric fatty acids, and consequently when the FFA concentration rises above the critical micelle concentration (cmc), the value of R ceases to change even though the indicator is not fully saturated. For oleic acid (Figure 1), the cmc is about 6  $\mu\text{M}$  (determined using ADIFAB<sup>3</sup>).

Saturation values of  $R_{\text{max}} = 11.5$  and  $Q = 19.5$  have been derived from numerical analysis of FFA titration data.<sup>3</sup> We recommend that these values be used where applicable in all ADIFAB data analysis. As they are indicator constants, these values are therefore the same for all FFA. Note that because fatty acid critical micelle concentrations are highly  $\text{Ca}^{2+}$  dependent, the highest observable R value is much lower in the presence of this cation.

#### 2.2 Single Wavelength Measurements

ADIFAB may also be used in single wavelength mode using the appropriate form of equation (1):

$$[\text{FFA}] = K_d \frac{(F - F_o)}{(F_{\text{max}} - F)} \quad (2)$$

Obviously, the 432 nm signal from the unbound indicator is preferable for this purpose due to its much larger dynamic range (Figure 1). Note that, as in the case of ratio measurements, the signal corresponding to full indicator saturation ( $F_{\text{max}}$ ) cannot be directly measured. For measurements at 432 nm,  $F_{\text{max}} = F_o/19.5$  (see step 2.1).

#### 2.3 Calibration

Calibration of ADIFAB under specific conditions may be accomplished by titration of the indicator with known amounts of FFA. FFA stock solutions should be 100% aqueous; i.e. use of methanol or ethanol to dissolve the FFA should be avoided, as these organic solvents alter the response of the indicator. Due to the low aqueous solubility of the free acid form, FA sodium salts should be used to prepare stock solutions. The sodium salt may be either obtained commercially or by dissolving the free acid in strong base (for example a 4 mM solution of linoleic acid can be obtained by dispersing 10  $\mu\text{L}$  of liquid free acid in 8 mL of 0.01 M NaOH). In order to determine the FA dissociation constant ( $K_d$ ) it is necessary to evaluate the FFA concentration at each titration step. The value of [FFA] must be calculated by subtraction of the bound FA concentration (derived from the spectral response of the indicator) from the total added FA concentration. This procedure is necessary because there is no practical means of buffering the FFA concentration and because the concentration of the indicator for most practical applications is comparable to  $K_d$ . For ratio data, the following expression can be used to obtain [FFA] values:

$$[\text{FFA}] = [\text{FA}]_T - \left( [\text{ADIFAB}]_T \frac{(Q f_H)}{(1 + Q f_H)} \right) \quad (3)$$

$$\text{where } f_H = \frac{(R - R_o)}{(R_{\text{max}} - R)} \text{ as in (1)}$$

and the subscripts T denote the total concentration of the species concerned. Note that this expression involves the implicit assumption that all the ADIFAB present is available for binding fatty acids with a 1:1 stoichiometry. For single wavelength measurements (see step 2.2), the following expression can be used instead of (3):

$$[\text{FFA}] = [\text{FA}]_T - \left( [\text{ADIFAB}]_T \frac{(F - F_o)}{(F_{\text{max}} - F_o)} \right) \quad (4)$$

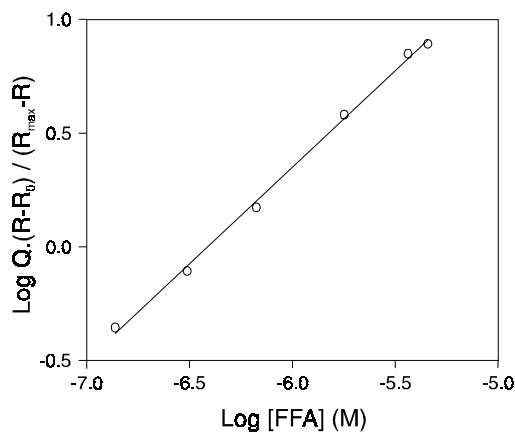
The following  $K_d$  values for ADIFAB at pH 7.4 and 37°C have been determined by nonlinear least squares fitting of a binding hyperbola to FFA titration data.<sup>3</sup> Note that  $K_d$  is not  $\text{Ca}^{2+}$  dependent.

Oleic ( <i>cis</i> -9-octadecenoic) Acid	0.28 $\mu\text{M}$
Palmitic (hexadecanoic) Acid	0.32 $\mu\text{M}$
Linoleic ( <i>cis</i> , <i>cis</i> -9,12-octadecadienoic) Acid	0.97 $\mu\text{M}$
Arachidonic (5,8,11,14-eicosatetraenoic) Acid	1.63 $\mu\text{M}$

A ratio mode calibration of ADIFAB using the data from Figure 1 linearized according to a logarithmic form of equation (1) (Hill plot) is shown in Figure 2.

## 2.4 Binding Kinetics

Stopped-flow fluorescence measurements indicate that binding and release of FFA to and from ADIFAB is rapid ( $>1 \text{ s}^{-1}$  at 20°C). The indicator is therefore suitable for real time monitoring of enzymatic reactions (e.g. phospholipase  $\text{A}_2$  hydrolysis) that release FFA.



**Figure 2.** Calibration plot of oleic acid titration of ADIFAB at 25°C (data from Figure 1). The value of  $K_d$  (obtained from the x-intercept) is 0.39  $\mu\text{M}$ . The slope of the linear regression fit line is 0.85 (theoretically expected value is 1). Fluorescence intensities were read at 500 nm and 435 nm after background subtraction. [FFA] was estimated according to equation (3). Values of  $R_{\text{max}}$  and  $Q$  were assumed to be 11.5 and 19.5 respectively (see step 2.1).

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## References

1. J Biol Chem 258, 7541–7544 (1983);
2. J Mol Biol 208, 327–339 (1989);
3. J Biol Chem 267, 23495–23501 (1992);
4. Mol Cell Biochem 192, 77–85 (1999);
5. J Biol Chem 260, 3440–3450 (1985).

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## Product List

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Cat #	Product Name	Unit Size
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