Instructions for Preparation and Use of ADIFAB

UPON ARRIVAL IMMEDIATELY:

- FREEZE (at -20 or -70°C if possible) LYOPHOLIZED ADIFAB
  - When you reconstitute in solution do not freeze ADIFAB (keep refrigerated)
- FREEZE (at -20°C) OA:BSA STANDARD(S). **DO NOT USE A FROST FREE FREEZER**
- REFRIGERATE (at 4°C) STORAGE AND MEASURING BUFFERS

1. **Cuvettes:** Cuvettes made of glass or quartz are recommended for the measurement of ADIFAB. 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. **Plastic cuvettes made of polystyrene from Sarstedt (cat# 67.754) have been found to work well. 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 ratio changes over time. If using plates, our experience has been that black Nalgene plates (96 well catalog # 237108 & 384 well catalog # 262260) perform the best. Observe Protocol for Measuring FFA with ADIFAB on 96-Well Plate for more information about measuring on a plate.

2. **Instrumentation:** Review the information on the Information Sheet for Instrument specific information on the Ro measurement and the Rmax constant. The excitation wavelength is 386 nm and the two emission wavelengths are 505 and 432nm

3. **Preparation of ADIFAB stock solution**: Add Storage Buffer to the vial of lyophilized ADIFAB powder to give a final concentration of ~100 μM (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 (Don't freeze).** Storage Buffer consists of the Measuring Buffer (described below) with 1 mM EDTA, and 0.05% sodium azide. The pH at room temperature is 7.4 ± 0.1

4. **Measuring Buffer**: The Measuring Buffer consists of 50 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4. The pH at room temperature is 7.4 ± 0.1. Store the Measuring Buffer at 4ºC.

5. **BSA Stock Solution:** Make a small amount of stock solution (600μM) of Bovine Serum Albumin (BSA) with some of the included measuring buffer (HEPES). **Only use Fatty Acid Free BSA. We have had good experiences with Sigma catalog # A6003.**

ADIFAB is intended for research use only. For additional information please contact FFA Sciences at (858)-455-3776.

6. Measurements:

(a) Ro's: Ro's are essential to the calculations for determining the unbound FFA concentration. The Ro is the ratio value for the probe with no unbound free fatty acid present. To remove any unbound FFA and possibly other hydrophobic compounds (potential interferents), we add fatty acid free albumin (typically BSA) to the measuring buffer. Multiple Ro values should be measured and averaged.

To measure Ro, first measure the background fluorescence (the Blank) for the buffer/albumin solution. Then add ADIFAB and take a second measurement**. This procedure of measuring the fluorescence of the sample before and after probe addition is carried out for all measurements.**

Start by adding 1.425ml of Hepes Measuring Buffer to a cuvette. Then add 75 μL of 600 μM BSA, mix and take the "Blank" measurement. Next add 7.5 μL of 100 μM ADIFAB to the same cuvette and take the Ro measurement. In general, the recommended concentration of ADIFAB to be used is 0.5 μM. This concentration can be increased or decreased to better suit the sample and the measurement conditions. For example, more or less probe may be needed depending on the efficiency of the fluorometer used. Generally, it is best to use the lowest concentration of probe you need to get reliable intensity values.

Ro will be calculated by subtracting the intensity of the Blank from the Sample at the two wavelengths as shown in the figure just below.

$$\mathrm{Ro} = \frac{\mathrm{I}_{505}^{\mathrm{o}} - \mathrm{I}_{505}^{\mathrm{blank}}}{\mathrm{I}_{432}^{\mathrm{o}} - \mathrm{I}_{432}^{\mathrm{blank}}}$$

(b) OA:BSA Standards: Use the included standard to check your instrument setup and your measuring procedure. The standard is an oleate (OA)-BSA complex with a BSA concentration of approximately 600 μM and an unbound OA concentration of approximately 50nM. Because the OA-albumin complex acts as a reservoir for OA, the unbound OA concentration is buffered and limited dilution of the complex will not significantly alter the measured OA concentration
To measure the complex, add 1.425mL Measuring Buffer and 75 μL OA-BSA complex to a cuvette.

Mix and take a Blank reading (no ADIFAB) at the two wavelengths specified on the Information Sheet. Next, add ADIFAB to the cuvette at the recommended final concentration (0.5uM), mix and measure the fluorescence. As with the Ro measurement, determine the R value for the complex. Then apply equations included in Information Sheet to determine free fatty acid concentration.

R Value:
$$\mathrm{R} = \frac{\mathrm{I}_{505} - \mathrm{I}_{505}^{\mathrm{blank}}}{\mathrm{I}_{432} - \mathrm{I}_{432}^{\mathrm{blank}}}$$

$$[\mathrm{FFAu}] = \mathrm{K_d} \bullet 19.5 \bullet \frac{(\mathrm{R} - \mathrm{R_o})}{(R\max - \mathrm{R})}$$

If the OA concentration you obtain is similar to the concentration noted on the standard vial then you are making the measurements correctly and can proceed. If not, check your instrument.

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(c) <u>Your Samples:</u> Sample measurements and unbound FFA calculations follow the procedure described above for measuring the OA standard. The optimal sample concentration for measurement depends on the sample.

Two major considerations regarding sample dilution are 1) whether the sample unbound FFA concentration is sufficiently buffered against dilution and 2) whether the sample contains any compounds that cause excitation or emission inner filter. Of course, if the sample unbound FFA concentration is not well buffered, the sample dilution factor should be minimized. If the sample has inner filter issues, the sample dilution should be maximized, because the unbound FFA concentration should be buffered (i.e. will remain constant) while the interfering substance concentrations should not be buffered (i.e. will decrease).

When using 96 Well Plates: *We highly recommend black round bottom Nunc plates. These plates have been found to perform the best. 96 & 384 well – catalog # 237108 & catalog # 262260*