Obesity Biomarker Array:

FLUORESCENCE ASSAY PROTOCOL

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I. Introduction and Protocol Overview

The ability to efficiently profile a diverse set of proteins and peptides has valuable applications. It is well known that the amount of proteins or peptides in any biological sample can be quantified using a specific antibody in an immunoassay. Many molecular markers may be efficiently screened because these antibodies can be tested in parallel. The comparison of biomarker profiles is essential for the better understanding of pathophysiology as well as the development of improved clinical diagnostics. In a step toward such capability, this biomarker kit, based on Phoenix Pharmaceuticals' proprietary antibody technology, is able to quickly screen protein or peptide levels in complex biological fluids.

Obesity is considered as a type of metabolic disease. In response to environmental stimulation, adipocytes release several hormones such as adiponectin, IL-6, leptin, resistin, and TNF-alpha. In addition, adipocytes have been proposed to play an important role in regulating body weight and other metabolic functions. Elevated levels of resistin and TNF-alpha combined with decreased levels of adiponectin secretion may have implications for the development of type-2 diabetes and cardiovascular disease. Leptin is an important component in long-term regulation of body weight. Recent studies with obese and non-obese subjects have demonstrated a strong positive correlation between serum leptin, TNF-alpha, and IL-6 concentrations with body fat proportion. For the parallel detection of the above endogenous hormones and others, Phoenix Pharmaceuticals, Inc. has developed the Obesity Peptide Biomarker Array. Custom-designed antibodies are immobilized on the nitrocellulose-coated slide. The results from this microarray can semi-quantitatively indicate the concentration of obesity-related hormones from serum/plasma, adipocytes, or conditioned medium.

This kit contains two two-pad glass slide. Each pad has been spotted with about 47 different capture antibodies that detect their corresponding antigen in the sample. First, proteins/peptides in the test sample are biotinylated and purified. Following purification, the nonspecific binding sites of the capture antibodies are blocked with blocking solution. Biotinylated antigens in the sample can then bind to the capture antibodies immobilized on the nitrocellulose pads. Streptavidin-DY647 (SA-DY647) is added and will subsequently emit the fluorescence after excitation by a light source at wavelength 635nm. The fluorescent image is captured by a microarray scanner. Spot intensity is directly proportional to the amount of antigens in the test sample. By using appropriate normalization methods shown in this protocol, it is possible to obtain the biomarker profile in a consistent and reliable manner from a variety of samples. We recommend to use one of the slide to probe the difference of two samples on microarray assay for the first time user. If the results of global intensity of spots looks equal on both pads of slide, therefore, such results can be repeated again by using another slide. Thus, this kit has provided with one slide chamber and two identical slides for the assay. A direct comparison between the probed specific spot intensity of samples will provide the biomarker profile in question.

II. Assay Conditions

All assay procedures are to be done at room temperature (20–23°C). Plasma, serum, culture media, tissue homogenate, CSF, urine, or any other biological fluids can be assayed as long as the level of antigens in the sample are high enough for the sensitivity of this kit to detect.

CAUTION: Phoenix Pharmaceuticals, Inc. guarantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for its particular use.

III. Kit Contents & Storage Conditions

Contents	Quantity	Storage
Obesity Biomarker Chip	2 Glass Slides	4°C
1X Assay Buffer	100ml	4°C
1X Blocking Solution	20ml	4°C
HABA/Avidin Mixture	1 Vial	4°C
NHS-Biotin	1 Vial	-20°C
Biotinylation Buffer	2ml	4°C
Streptavidin-DY647	5µl	4°C in the dark
DY547 Anti-Rabbit Mouse Antibody	15µl	4°C in the dark
Acetate Plate Sealer (APS)	5 Pieces	4°C
Slide Chamber	1 Piece	4°C
Free-Biotin Captured magnetic Beads	2 tubes 200µl beads	4°C
20X PBS	5ml	4°C
1X Tris-HCl buffer	0.5ml	4°C
BCA Protein Assay Reagent A	39.2ml	Room Temperature
BCA Protein Assay Reagent B	0.8ml	Room Temperature
BSA Standard for BCA	1ml	Room Temperature
Biotinylated LEAP-2	1 Vial	4°C
Program Disc	1 CD	Room Temperature
Magnets	2 Pieces	Room Temperature

IV. Additional Materials REQUIRED

The following materials are CRUCIAL in order to perform the assay:

- Microarray FAST Frame® (Phoenix Pharmaceuticals – Catalogue No. F-004) or Chip Clip® (Phoenix Pharmaceuticals – Catalogue No. F-001) to secure chamber onto glass slide

- Fluorescence microarray scanner capable of exciting the dye at range of 635 to 652nm and to quantitatively measure the fluorescence at wavelength 670nm for DY647. For checking the spotted antibodies, it also requires microarray scanner capable of exciting the dye at range of 540 to 553nm and to quantitatively measure the fluorescence at wavelength 570nm for DY547

- Data Analysis Software

V. Reagent Preparation

1. **1X PBS:** Add 10ml of 20X PBS into 95ml of distilled water (total volume 100ml)

2. **Pre-measured HABA/Avidin Mixture:** Centrifuge tube. Add 1mL of 1X PBS to the HABA/Avidin mixture and vortex. Place on orbital shaker for 15 minutes at 250rpm to dissolve completely. Dilute this 1mL mixture with 1X PBS to a total volume of 4.8mL and vortex. The reconstituted solution is stable for only one day.
3. **Biotinylated LEAP-2:** Centrifuge tube. Add 24µl of 1X PBS to tube. Place on table for 5 minutes. Vortex and spin before use at the following **Step 2** section 5.

VI. Obesity Biomarker Array Protocol

Use both pads on the slide when comparing biomarker profiles of multiple samples. Each captured antibody is on a specific position on the pad and will only bind to their specific antigen; they will not cross react with other antigens. The first three procedures, indicated with (A), (B), and (C), have been written for one sample only (e.g. Sample X) for simplification. If your experiment is designed to run more samples, do these procedures at the same time.
(For an illustrative flow chart, please refer to assay diagram on page 13)

(Note: Biotinylation of Samples and Free-biotin separation by beads should be completed early in the afternoon so that you will have enough time to do BCA Assay, HABA Assay, and loading of samples later.)

(A) Sample Preparation and Biotinylation

Step 1: Sample Preparation

One of the following methods can be used to prepare the sample before biotinylation

A-1-1. Extracts from Tissue

1. Transfer frozen tissue on to ice.
2. Add enough Extraction Buffer to cover the tissue in a homogenizing tube.
3. Homogenize the tissue on ice.
4. Transfer the homogenate to a centrifugation tube (4°C).
5. Centrifuge the homogenate at 10,000 x g for 30 minutes at 4°C.
6. Transfer the supernatant to a clean tube.
7. Measure protein concentration using BCA protein assay kit.
8. Proceed immediately to Step 2, sample biotinylation, or store the samples at -80°C.

A-1-2. Extracts from Cells

1. Harvest cells by centrifugation.
2. Wash the cells with 1X PBS and spin at 4°C. Aspirate the supernatant.
3. Wash the cells twice with cold 1X PBS and spin at 4°C to completely remove serum proteins.
4. Keep cell pellet on ice for 5 minutes.
5. Add Extraction Buffer to the cell pellet and mix thoroughly by pipetting.
6. Incubate the sample on ice (4°C) for 10 minutes with occasional mixing.
7. Centrifuge the sample at 10,000 x g for 30 minutes at 4°C or in a microcentrifuge at highest speed for 10 minutes at 4°C.
8. Transfer the supernatant to a clean tube.
9. Measure protein concentration using BCA protein assay kit.
10. Proceed immediately to Step 2, sample biotinylation, or store the samples at -80°C.

A-1-3. Plasma, Serum or other bodily fluids

1. Centrifuge the sample at 10,000 x g for 10 minutes or microcentrifuge at highest speed for 10 minutes at 4°C.
2. Transfer the supernatant to a clean tube.
3. Measure protein concentration using BCA protein assay kit.
4. Proceed immediately to Step 2, sample biotinylation, or store the samples at -80°C.

Step 2: Sample Biotinylation

1. **10mM Biotin Stock Solution:** Dissolve sulfo-NHS-biotin powder in 20µl of DMSO. To prevent hydrolysis of sulfo-NHS-biotin in tube, this solution MUST be freshly prepared.
2. After thawing frozen sample, centrifuge at 5000g for 10 minutes to separate particulate matter. In a new 1.5ml eppendorf tube, mix 240µl H_2O, 50µl biotinylation buffer, 100µl test

3. Quench the biotinylation reaction by adding 50µl Tris-HCl buffer (20mM Tris HCl) to each reaction and incubate at room temperature for 5 minutes.
4. Label sample, i.e., for two samples "X" and "Y"
5. Pipette 10µl of dissolved biotinylated LEAP-2 to spike into sample "X" and "Y" before moving onto the next step (separation of free biotin from labeled peptides/proteins).

(B) Separation of free biotin from labeled peptides/proteins

1. Place the free-biotin-captured magnetic bead on top of magnet. Let free-biotin-captured magnetic bead beads (200 µl brown and white colors) settle to the bottom of each tube.
2. The buffer solution above the beads needs to be removed to minimize the effect of dilution. Add 120µl of labeled protein mixture to the beads. Lay tubes on a rocker plate for 30 minutes. *All your samples with beads are to be incubated for exactly 30 minutes.*
4. Let beads settle to bottom again by magnet. Transfer as much of the supernatant (labeled protein mixture) you can get into another tube without taking any of the white beads. This tube will have some magnetic beads (brown color).
5. Use the magnet to draw all the magnetic beads to one spot in tube.
6. Without drawing any of the magnetic beads, transfer the supernatant plasma to another tube. Label the tube "XB".
7. If you did draw some of the magnetic beads, repeat steps 5 and 6.

(C) BCA Assay

1. **Preparation of Diluted Albumin (BSA) Standards**

Vial	Volume of 1X PBS	Volume and Source of BSA	Final BSA Concentration
A	0	300µl of Stock	2000µg/ml
B	100µl	300µl of Stock	1500µg/ml
C	300µl	300µl of Stock	1000µg/ml
D	125µl	75µl of Stock	750µg/ml
E	325µl	325µl of vial **C** dilution	500µg/ml
F	300µl	100µl of vial **C** dilution	250µg/ml
G	700µl	100µl of vial **C** dilution	125µg/ml
H	780µl	20µl of vial **C** dilution	25µg/ml
I	400µl	0	0µg/ml = Blank

2. **Working reagent:** Transfer all of BCA Protein Assay Reagent B into BCA Protein Assay Reagent A and vortex (total volume 40ml).
3. Label 9 test tubes for standards and 2 test tubes for samples.
4. Pipette 970µl of working reagent into test tubes for standards.
5. Add 30µl of vials A-I, respectively into each test tube.
6. Pipette 997µl of working reagent into 2 test tubes.
7. Add 3µl of each sample (XB and YB) into their respective sample test tubes.
8. Vortex all test tubes and incubate at room temperature for 2 hours.

9. After incubation, read absorbance (2x) at 562nm for all test tubes and record results.

(D) HABA Assay

The HABA assay is used to quantify the amount of biotin-labeled proteins in each sample. The unpurified biotinylated sample may be used as a positive control, to verify assay performance. The same volume of 1X PBS is used as a negative reference control for calculation.

1. For cuvette assay: Add 0.5ml of HABA/Avidin mixture (HABA ~0.3mM) into 2 cuvettes for samples and another cuvette for PBS reference.
   For 96-well plate assay: Add 200μl of HABA/Avidin mixture (HABA ~0.3mM) to 2 wells for each sample and another 2 wells for PBS reference.

2. For cuvette assay: transfer 50μl of XB retenate into one cuvette. Mix by pipetting. Repeat for sample YB and for PBS reference. Place cuvettes on an orbital shaker for 5 minutes to mix. Measure the absorbance at 500nm.
   For 96-well plate assay: transfer 22μl of XB retenate into two wells. Mix by pipetting. Repeat for sample YB and for PBS reference. Read the absorbance at 500nm until the values remain constant, normally it may take a minute or two, but should not exceed 3 minutes.

(E) Calculations

Calculations for (1) BCA Assay, (2) HABA Assay, and (3) Amount of protein needed are included on a CD disc.

(1) Calculation of Protein Concentration from BCA Assay

Using excel or similar programs, create a standard curve using the absorbance values on the "Y-axs" and its corresponding BSA standard concentration on the "X-axis".

Protein concentration of sample (mg/ml)

= (OD562 – intercept)/slope x (dilution factor/1000) = Calc #*1*

*OD562: average absorbance value from each sample test tube (i.e., XB or YB)
*intercept and slope: from standard curve
*dilution factor: volume of standard used/volume of sample used (i.e., for XB: 30μl/3μl = 10)

(2) Calculation of Moles of Biotin/Mole of Protein

Note: An automatic HABA Calculator is available at the Pierce web site (www.piercenet.com/HABA) to perform these calculations.

These calculations are based on the Beer Lambert Law (Beer's Law): $\mathbf{A_\lambda = \varepsilon_\lambda bC}$, where

A is the absorbance of the sample at a particular wavelength (**λ**). The wavelength for HABA assay is 500nm. There are no units for absorbance.

ε is the absorptivity or extinction coefficient at that wavelength (**λ**). For HABA/avidin samples at 500nm, pH 7.0, the extinction coefficient is 34,000 ml/(M^{-1} cm^{-1}).

b is the cell path length in centimeters (cm). A 10mm square cuvette has a path length of 1.0cm. A 96-well plate has a path length of 0.5cm

C is the concentration of the sample expressed in mmoles/ml.

The following values are needed to calculate the number of moles of biotin per mole of protein/sample:

- Concentration of the protein/sample used expressed as mg/ml (from BCA Assay)
- Molecular weight (MW) of the protein/sample used expressed as Daltons (60,000 Daltons)
- Absorbance at 500nm for HABA/Avidin solution (**A_{500} HABA/avidin**)
- Absorbance at 500nm for HABA/Avidin/Biotin sample (**A_{500} HABA/avidin/biotin**)
- Dilution factor - dilution of the sample after it was added into the HABA/Avidin mixture.

Note: It is necessary to account for the dilution of biotin-labeled sample with HABA/Avidin solution in the calculation. After the addition of biotinylated sample to HABA/Avidin mixture, 90% of the solution is HABA/avidin and 10% is sample. Therefore, a multiplier of 10 is used in the calculation.

1. Calc #2=biotinylated sample concentration in mmoles/ml:

$$\text{mmoles biotinylated protein per ml} = \frac{\text{protein concentration (mg/ml)}}{\text{60,000 Daltons}} = \text{Calc \#2}$$

2. Calc #3 = difference in absorbance at 500nm between PBS reference and sample:
   ΔA_{500} = (A_{500} of HABA/avidin/PBS) – (A_{500} of HABA/avidin/biotin sample) = Calc #3

3. Calc #4 = concentration of biotin in mmoles/ml:
   (mmoles Biotin) / (ml reaction mixture) = ΔA_{500} / (34,000 × b) = Calc #4
   *cuvette = Calc #3 / (34,000 × 1) = Calc *#4*
   or
   *96-well plate = Calc #3 / (34,000 × 0.5) = Calc *#4*
4. Calc #5 = mmoles of biotin per mmole of protein:
   (mmoles Biotin) / (mmole protein) = [(mmoles Biotin per ml) x (dilution factor)] / (mmoles protein per ml) = Calc #4 ×10 / Calc #2 = Calc *#5*

(3) Calculation for The Amount of Protein Needed for Microarray Assay

With the protein concentration and the amount of biotin per mmole of protein, you can normalize the volume of biotinylated samples to be added into the microarray wells. The following calculation is suggested:

Amount of protein needed = 10 / (protein concentration in mg/ml x mmoles of biotin per mmole of protein) = 10 / (Calc #1 x Calc #5)

For example: biotinylated sample X has 2.11 biotin molecules per total protein and biotinylated sample Y has 4.5 biotin molecules per total protein. Protein concentration for sample X is 1.37mg/ml and sample Y is 1.07mg/ml. The calculation for sample X: 10 / (2.11 × 1.37) = 3.5μl, and for Sample Y: 10 / (4.5 x 1.07) = 2.1μl. Therefore, 3.5μl of sample X and 2.1μl of sample Y are used. Do not overload the biotinylated protein in the microarray assay. Protein overload will result in increase background reactivity and a decrease of the specific signals.

(F) Microarray Assay

Thoroughly read this protocol before performing assay. Allow all reagents to come to room temperature (20-30°C) prior to the start of the assay.

Assembly

1. Remove antibody coated glass slide from plastic container.
   Do not touch the nitrocellulose membrane on the slide while performing any of these procedures.
2. After matching each pad on the glass slide with the wells of the chamber, insert the assembly into Chip Clip® or Fast Frame® (the rest of the protocol will only refer to Fast Frame®, but Chip Clip® can also be used). Repeat for all slides used.

Blocking

3. Add 600μl of Blocking solution to each well and incubate on a rocker plate at 22rpm for 15 minutes. Drain the wells by flicking the Fast Frame® upside-down over an appropriate sink.
4. Add another 600μl of Blocking solution to incubate for 30 minutes on a rocker plate at 22rpm. Remove Blocking solution (as described at the end of step 3).
   When adding any solution to the slide, be sure to add very slowly and gently to prevent damage to the membrane and spotted antibodies.

Wash

5. Add 600μl of 1X Assay Buffer to each well and incubate on a rocker plate at 22rpm for 3 minutes. Then, drain the wells by flicking the Fast Frame®upside-down over an appropriate

sink. Repeat this wash one more time.

Sample and Standard Incubation

6. Add an appropriate amount of Sample X (as determined by your calculations) to 1X Assay Buffer to give a total volume of 0.4ml. Vortex and centrifuge. Transfer into the well that is away from the barcode.
7. Add an appropriate amount of Sample Y (as determined by your calculations) to 1X Assay Buffer to give a total volume of 0.4ml. Vortex and centrifuge. Transfer into the other well that is closest to the barcode.

Use half of APS to cover each well on top of the frame. Cover both wells with APS. Incubate of 3 hours at room temperature on a rocker plate at 22rpm. When use Fast Frame® for slide incubation, use 96 well lid to cover the top of Fast Frame® to prevent the evaporation of sample. Alternatively, the slide may be incubated for overnight (16 hours) at room temperature; because better results might be obtained from overnight incubation, especially for those peptides whose concentration is low. In order to prevent the slide from drying out, place the glass slide assembly on top of a wet paper towel and seal it in a resealable sandwich bag.

Wash

8. Following sample incubation, remove and discard the samples from each well with a fresh pipette tip. Do not mix the contents of the wells.
9. Wash the wells by adding 600µl of 1X Assay Buffer, and incubate for 4 minutes on a rocker plate at 22rpm. After 4 minutes, drain the wells by flicking the Chip Clip® upside-down over an appropriate sink. Repeat 5 times (total 6 washes).

SA-DY647 and DY547 Anti-Rabbit Antibody Incubation

10. Centrifuge SA-DY647 vial (5µl) and transfer 1µl SA-DY647 in 12ml Assay Buffer solution to make SA-DY647-Assay Buffer solution.

To quantify the immobilized antibody, centrifuge DY547 Anti-Rabbit Mouse Antibody vial (15µl) and dilute 3µl of DY547 Antibody into the above 12ml SA-DY647-Assay Buffer solution. All sample incubations should be done using the same diluted solution.

Pipette 400µl of the above diluted SA-DY647 with DY547 Anti-Rabbit Mouse Antibody into each well. Protect from light and incubate for 45 minutes on a rocker plate at 22rpm.

Wash

11. Wash 6 times with Assay Buffer (as in step 9). For each wash need to protect from light.

Detection

12. Prepare 40ml of distilled water in a 50ml centrifuge tube. Immerse the slide in distilled water and invert several times. Remove the slide and place face-up inside a drawer to protect from light. Allow the slide to air dry for a few minutes. Once the nitrocellulose-coated region is dry, it will appear white. The slide is scanned by a fluorescent microarray scanner.

    Note: Store slide in a dark, dust-free environment until scanned. Dry slides are stable for one month when stored in a refrigerator.

13. For optimal results, we recommend using GenePix scanner from Axon Instruments. If your scanner is a confocal scanner with focal depth adjustment capabilities, the focal depth should be optimized. The nitrocellulose pad is approximately 11µm above the glass slide. This can usually be accomplished by running a focal-line scan on the imager. The Obesity Biomarker Array requires a photomultiplier tube (PMT) setting around 300 to 800 to obtain the best results. The nitrocellulose background fluorescence should be keep as low as possible and signal intensities at each spot should be as high as possible, but not exceed the maximum signal (65,535 units in a 16-bit imaging system), except for the position control spots. This method has been validated using the GenePix 4100A scanner with GenePix Pro 6.0 both from Axon Instruments.

Data Analysis

Each antibody is arrayed in duplicates to provide increased reliability. Using a compatible imaging system's software, determine the signal intensity for each spot. Then, determine the specific signal of each spot. The specific signal is determined by subtracting the average intensity of the background from the intensity of each spot. The fluorescent signal intensities from the spots on the array correspond to the relative amount of labeled proteins captured from the samples applied to the array. These fluorescent signal intensities are used to calculate a ratio between the two samples. The calculated ratio indicates the abundance of a protein/peptide in one sample relative to other. The accuracy of the measurement of antigen captured by the antibody are dependent on factors such as (1) the degree of biotinylation of peptides/proteins and (2) the amount of antibody applied to each slide. If the samples applied to the slide are from the same lot, we suggest that the intensity of each spot be divided with the average intensity of the positive controls A and Fibronectin control (position 48) in their well in order to normalize the variation between degrees of biotinylation. A ratio of signal intensity of either >1.5 or <0.8 indicates a significant difference in the level of labeled protein/peptide between the two samples. We have verified no significant variation of our antibodies immobilized on each pad of slides from the same lot number. However, if the sample comparisons are done using slides from different lot number, there is one more normalization factor to take into account.

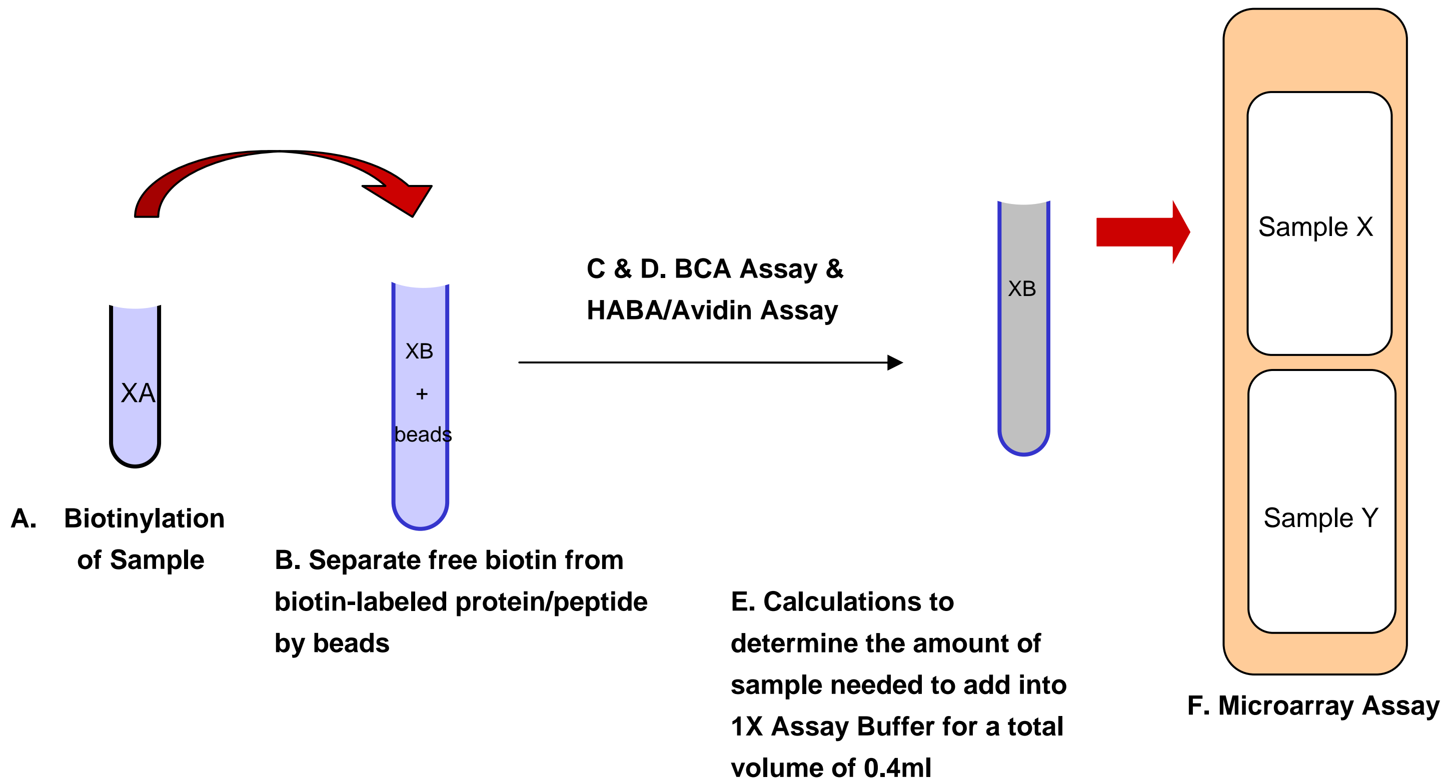
There is potential for some variation in the amount of a few antibodies spotted on slides from different lot numbers. DY547 helps to quantify and normalize the signals from different batches of slides. For the comparison of multiple samples, we used two normalization factors based on the results that contain positive controls for biotinylation efficiencies normalization, and a second color detection system to quantify the antibody. The calculation will be the following:

$$\text{Calculated antibody ratio} = \frac{\text{Original signal intensity of DY547 in specific antibody – slide \#1}}{\text{Signal intensity of DY547 at same antibody location – slide \#2}}$$

$$\text{Antigen signals} = \frac{\text{Original DY647 signal intensity in original well}}{\text{Average of positive control signal DY647 intensity in original well x}}$$

calculated antibody ratio

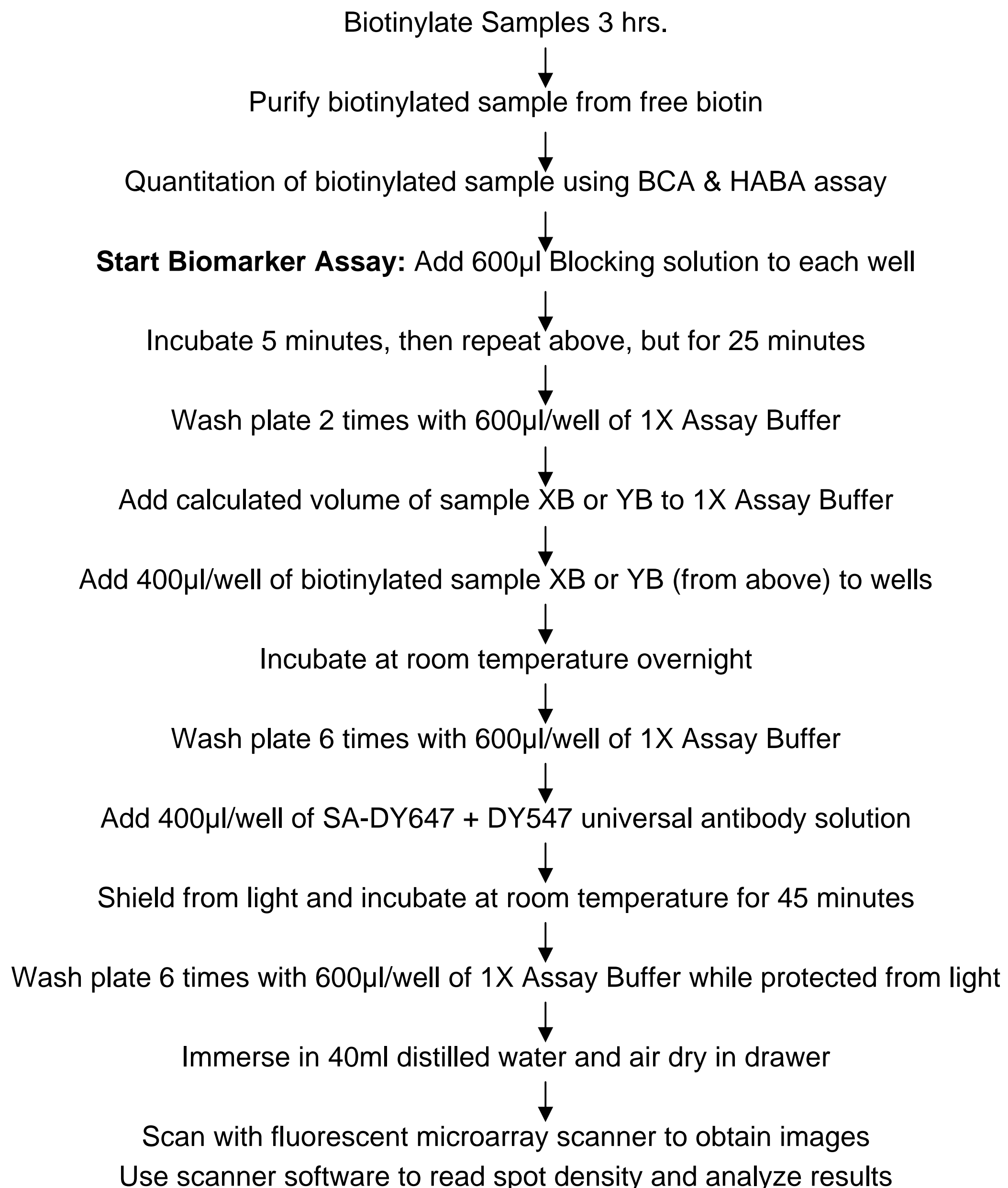
VII. Assay Diagram



In order to accurately quantify the amount of each ANALYTES in each sample, it is important to adjust the amount of sample applied to the upper and lower pad in the slide. Quantify the fluorescence intensity from the average of IgG positive control, spiked biotinylated LEAP-2 control, and fibronectin control to approximately equal intensity to know whether the amount of sample applied to the upper pad is similar to the amount of sample applied to the lower pad. (Please see Example 2.)

In case you find the average of controls in the upper and lower chambers have more than two times differences, you need to add more sample to the lower intensity pad and further incubate the sample for another 2 hours and then, fluorescence dye for another 45 minutes. If you have any technical questions, please email to: Robert@phoenixpeptide.com

VIII. Summary of Assay Protocol



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