biosensis® Human FAS Kit Protocol

Catalogue No: BEK-2014-2P

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I. Materials Provided

Kit Components	Unit Size/Volume
Lyophilised recombinant human FAS standard (Reagent A)	10ng/tube x 4
96 wells plate pre-coated with anti- human FAS antibody	2 plates
Sample diluent buffer: 5X concentration	12ml
Biotinylated anti- human FAS (dilution 1:100) (Reagent B)	260µl
Antibody diluent buffer: 5X concentration	4.8ml
Avidin-Biotin-Peroxidase Complex (ABC) (dilution 1:100)	260µl
ABC diluent buffer: 5X concentration	4.8ml
TMB colour developing agent	10ml x 2
TMB stop solution	20ml

II. Equipment Required but Not Supplied

1. Wash buffer (0.01M PBS or TBS). *See technical hints*
2. Multi-channel or repeating pipette.
3. Clean tubes and Eppendorf tubes.
4. Plate shaker.
5. Mechanical Vortex.
6. Microplate reader with 450nm filter.

III. Technical Hints

1. Spin all kit components to get liquid to the bottom of tubes before use.

2. Duplicate well assays are recommended for both standard and sample testing.

3. In order to avoid marginal effects of plate incubation due to temperature differences, it is suggested that the diluted ABC and TMB solutions be brought to room temperature (37°C) 30 min before use.

4. Recommended method for manual plate washing.

    - Emptying of wells on the plate:

Place the plate on the palm of the hand in a position that enables easy flicking movement using the wrist. Holding the plate over a sink, quickly invert the plate, whilst accelerating the arm downward toward the sink. Abruptly stop the downward acceleration to force the liquid from the wells into the sink. When done correctly the technique should prevent liquid from getting on to the fingers or on the outside of the strip wells or plate holder. *Note: Retain the upside down position of the plate to avoid any back flow into the wells.*

- Blotting the plate:
  Immediately blot the inverted plate by lightly tapping the plate 3-4 times on blotting paper.

- Washing:
  Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using technique described above. Repeat washing and flicking procedures thrice. *Note: **Avoid** touching the inside surface of the wells with the pipette tips.*

  Do not let the wells dry out at any time or enzymatic activity will be lost.

5. Preparation of Wash buffer: (not provided in the kit)

Preparation of 0.01M **TBS:** Add 1.2g Tris, 8.5g Nacl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H_2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Preparation of 0.01 M **PBS:** Add 8.5g sodium chloride, 1.4g Na_2HPO_4 and 0.2g NaH_2PO_4 to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

6. Preparation of Buffer Working Standards

The following buffers are provided at 5X concentration:

a) Sample diluent buffer

b) Antibody diluent buffer

c) ABC diluent buffer

Dilute each buffer in a 1:5 ratio in deionised water to get a working solution of each.

IV. Storage of Kit Components

This kit may be stored for up to 6 months at 2°C to 8°C from the date of shipment. Antibody Standards should be stored at -20°C or -80°C (recommended at –80°C) after reconstitution. Coated strips or reagents may be store for up to 1 month at 2°C to 8°C.

Return unused wells to the pack and reseal pack. *Note: the kit can be used within 8 months if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.*

V. Sample Preparation and Dilution

- **Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation, analyze immediately or aliquot and store at -20°C
- **Serum:** Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20°C.
- **Plasma:** Collect plasma using heparin, EDTA, citrate as an anticoagulant. Centrifuge for 15 min at 2-8°C at 1500 x g within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at 2-8°C at 10000 x g. Analyze immediately or aliquot and store samples at -20°C.

Note: The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve.

The following table can be used as a guideline for sample dilution. Dilute the sample using the provided diluent buffer. Mix the sample thoroughly with diluent buffer.

Target protein concentration	**Concentration range**	**Working dilution**	**Amount of sample diluent buffer to be added**
High target protein concentration	**(20-200ng/ml).**	1:100	Add 1µl sample into 99 µl sample diluent buffer
Medium target protein concentration	**(2-20ng/ml).**	1:10	Add 10 µl sample into 90 µl sample diluent buffer
Low target protein concentration	**(31.2-2000pg/ml).**	1:2	Add 50 µl sample to 50 µl sample diluent buffer

Very Low target protein concentration	**(≤31.2pg/ml).**	No dilution necessary	

VI. Reagent Preparation

1. Preparation of Standards

- Reconstitution of antibody standard (Reagent A) with the sample diluent provided:

(a) 10,000pg/ml of standard antibody solution: Add 1ml of sample diluent buffer into ***one*** tube of standard, mix thoroughly and keep the tube at room temperature for 10 min.

(b) 2000pg/ml of antibody standard solution: Add 0.2 ml of the above 10ng/ml standard solution into 0.8 ml sample diluent buffer and mix thoroughly.

(c) 1000pg/ml-31.2pg/ml of antibody standard solutions: Label 6 Eppendorf tubes with 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.2pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 2000pg/ml standard solution into 1st tube and mix. Continue performing serial dilutions.

- *Note: Antibody standard solution should be prepared no more than 2 hours prior to the experiment. The 10 ng/ml standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

2. Preparation of Biotinylated antibody working solution

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- Biotinylated antibody (Reagent B) should be diluted in 1:99 with the antibody diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than two hours prior to the experiment.*

3. Avidin-Biotin-Peroxidase Complex (ABC)

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- ABC should be diluted in 1:99 with the ABC diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than one hour prior to the experiment.*

VII. Assay Procedure

We recommend that standard solution dilutions and each samples are plated in duplicate.

1. Aliquot out the standards into each well of the pre-coated plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human sera, plasma, body fluids, tissue lysates or cell culture supernatants into each empty well. *Note: See "VII. Sample Preparation and Dilution Guideline" above for details.*

2. Seal the plate with parafilm and incubate the plate at 4°C overnight. *Note: we recommend users leave the plate on a shaker if possible.*

3. Remove the cover, discard the plate contents and blot the plate onto paper towels or other absorbent material. **DO NOT** let the wells completely dry at any time. Wash plate 5 times using wash buffer as described in the technical hints. *Note: See "V. Technical Hints - Recommended method for manual plate washing" above for details.*

4. Add 0.1ml of biotinylated antibody working solution into each well. Cover and incubate the plate at room temperature on a plate shaker for 2-3 hours. Wash as describe in the technical hints.

5. Add 0.1ml of prepared ABC working solution (*Note: See "VIII. Reagent Preparation"* above for details) into each well. Cover and incubate the plate at room temperature on a plate shaker for 1 hour. Wash as describe in technical hints allowing the wash buffer to remain in the wells for 1-2 minutes.

6. Warm TMB to room temperature. Add 90µl of prepared TMB color developing agent into each well and incubate plate at room temperature for 5-20min (shades of blue can be seen in the wells with the four most concentrated antibody standard solutions; the other wells show no obvious color).

7. Stop the reaction by adding 0.1ml of prepared TMB stop solution into each well. The color will change into yellow.

8. Immediately read the plate at 450nm with a microplate reader. *Note: Color will fade over time; hence, we recommend plate to be read within 30 min after adding the stop solution.*

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

VIII. Calculation of Results

(a) Manual Plate Reading:

(The relative $O.D._{450}$) = (the $O.D._{450}$ of each well) – (the $O.D._{450}$ of Zero well).

The standard curve can be plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding $O.D._{450}$ on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding $O.D._{450.}$ The greater the concentration of target protein in the sample, the higher the $O.D._{450.}$

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Determine concentration of target protein in unknown sample:

The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample.

(b) PC Interface Plate Reading

Enter the data into computer program curve fitting software. Existing spreadsheet software can perform a good fit with a linear regression analysis.