C. SpiderTek Protocol 2015-1 v. 1.0

Procedure Description: Protocol to Extract Venom from Swabs

1. Break or cut off the cotton swab from the applicator stick and place the swab in a labeled 2ml centrifuge tube
2. Apply 250µl of 1x TBST to the swab in the tube
3. Vortex the swab+tube for a count of 5
4. Remove the swab (small forceps can be used to help) and add a spin column to the tube; place the swab in the top of the spin column
5. Centrifuge the swab/spin column at ≥10,000xg for 3min
6. Take out spin column and swab
7. Remove the solution in the bottom of the tube that had been excluded from swab during the centrifugation. Split the solution equally between two wells on the ELISA.

Procedure Description: ELISA Protocol to Detect Brown Recluse Spider Venom from Suspected Bite Sites **[Standard ELISA protocol along with optional signal amplification steps]**

Trapping Antibody. Make an antibody (Ab 58) working solution of 0.4µg/ml in 0.1M $NaHCO_3$, pH 9.5 and apply 0.1 ml to each well.

- Incubate the plate for 3 hours (extend to overnight if desired) at 4°C. prior to performing the next 'blocking' step.
- The trapped plates can be stored at 4°C for at least one week prior to use.

Block. Apply 0.2ml VWR BLOK™ Casein (cat # 786-196) in Tris-buffered saline (TBS) for at least 1hr.

Sample Incubation. *Add 0.05 ml of TBST-block (TBST with 1/10 dilution of block solution) to each well of the ELISA plate to keep the wells moist while adding samples.* Apply samples to the ELISA plate wells (see points below). Incubate the plate at 37°C for approximately 2 hours.

Notes:

- Split the samples between two or more wells if technical replication is desired.
- Adjust the samples with TBST to produce a common volume in each well (e.g. 0.1ml).
- Create a standard curve to include on each plate.
    - Standards are generated by 13 or 14 2-fold dilutions of whole venom. A useful standard curve is one in which the highest amount of venom

is 2.4ng (in 0.1ml) made up in TBST. The venom standards are added to wells containing 50µl of TBST-block (TBST diluted 10-fold with block).

- University of Missouri procedure: incubate the plate in a shaker incubator (e.g. STAT-FAX 2200) at 37°C for 2 hours.
    - Longer incubation periods can be performed at lower temperatures (e.g. 4°C overnight).

Wash. The plate is washed three times with 2X ELISA plate wash solution.

Secondary Antibody. Add 0.1ml of biotinylated Ab 56 that is at a concentration of 0.5µg/ml in 20mM Tris, pH 7.6. Allow it to bind for 20-30 minutes.

Wash. Wash the plate three (3) times with 2X ELISA plate wash
(see note below for signal amplification (recommended).

Detection Reagent. Add 100µl of Neutravidin-HRP (Pierce) at a 1:2000 dilution in 1% (w/v) bovine serum albumin (BSA) in 1xPBS with 0.05% tween-20. Incubate for 15 minutes.

This reagent can be made up in advance and kept for three days at 4°C

Wash. Wash the plate three (3) times with 2X ELISA plate wash.

Develop. Add 100µl of room temperature TMB. Incubate for 1 to 3 minutes or until sufficient color develops.

Terminate. Add 100µl 1M HCl, 0.6M sulfuric acid.

Amplification. After the addition of the secondary antibody, a modification of the protocol can be done if it is necessary to amplify the signal. This approach makes use of biotinylated tyramide. This approach is recommended.
The Modified protocol involves the following two steps:

- Add 100µl Neutravidin-HRP (Pierce) 1:2000 in 20mM Tris pH 7.6 for 15 minutes.
- Wash 3X with 2X ELISA plate wash.
- Add 100µl biotinylated tyramide (1:50) in 0.1M Borate – peroxide buffer; incubate for 20 minutes.
    - *Immediately prior to adding the biotinylated tyramide, include 0.6µl of 50% stabilized hydrogen peroxide to 10mls of 0.1M borate pH 8.5.*
- Wash 3X and continue with the next neutravidin-HRP step.

Definitions.

1x plate wash:	150 mM NaCl, 0.05% Tween-20
1xPBS	Phosphate-buffered saline (4mM Na2HPO4, pH7.4; 2.5mM KCl; 140mM NaCl)
TBS:	Tris-buffered saline (20mM Tris-HCl, pH8; 150 mM NaCl)
TBST:	TBS with 0.05% (v/v) Tween