Video Article

Administering and Detecting Protein Marks on Arthropods for Dispersal Research

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Abstract

Monitoring arthropod movement is often required to better understand associated population dynamics, dispersal patterns, host plant preferences, and other ecological interactions. Arthropods are usually tracked in nature by tagging them with a unique mark and then re-collecting them over time and space to determine their dispersal capabilities. In addition to actual physical tags, such as colored dust or paint, various types of proteins have proven very effective for marking arthropods for ecological research. Proteins can be administered internally and/or externally. The proteins can then be detected on recaptured arthropods with a protein-specific enzyme-linked immunosorbent assay (ELISA). Here we describe protocols for externally and internally tagging arthropods with protein. Two simple experimental examples are demonstrated: (1) an internal protein mark introduced to an insect by providing a protein-enriched diet and (2) an external protein mark topically applied to an insect using a medical nebulizer. We then relate a step-by-step guide of the sandwich and indirect ELISA methods used to detect protein marks on the insects. In this demonstration, various aspects of the acquisition and detection of protein markers on arthropods for mark-release-recapture, mark-capture, and self-mark-capture types of research are discussed, along with the various ways that the immunomarking procedure has been adapted to suit a wide variety of research objectives.

Video Link

The video component of this article can be found at https://www.jove.com/video/53693/

Introduction

Tracking the movement of arthropod pests, natural enemies (parasitoids and predators), and pollinators in nature is essential for better understanding how to improve ecosystem services. The key component for most types of dispersal research is having a reliable method to tag the arthropod(s) of interest. A variety of materials (*e.g.,* paints, dyes, colored dusts, tags, rare elements, proteins) have been used to mark arthropods to assess their population dynamics, dispersal capabilities, feeding behaviors, and other ecological interactions[1,2].

The appropriateness of a marker used for any given dispersal research will be dependent on the type of study being conducted. There are three broad categorizations for marking arthropods: (1) mark-release-recapture (MRR), (2) mark-capture, and (3) self-mark-capture. For mark-release-recapture research, the investigator typically marks the arthropods collectively in the laboratory and releases them at a central point in the field. The arthropods are then recaptured at various spatial and temporal intervals using different collection devices (*e.g.*, sweep net, vacuum, sticky trap)[3,4,5]. The recaptured specimens are then examined for the specific mark to distinguish released from native individuals. For mark-capture research, the investigator usually applies the mark directly in the field using spray equipment (*e.g.*, backpack sprayer, boom and nozzle sprayer). The best markers for mark-capture research are inexpensive and easily applied to the arthropod's habitat. For self-mark-capture research, the investigator usually applies marks to an arthropod bait[6,7] or nest entrance[8]. In turn, the arthropod marks itself internally by devouring the marked bait or externally by "brushing" up against the mark as it exits the nest.

As mentioned above, many types of markers have been used to tag a variety of arthropod species. However, very few are useful for all three of these dispersal research categories. The development of the protein immunomarking procedure was a major breakthrough for marking insects. Immunomarking puts a protein label on arthropods either internally or externally which, in turn, is detected by an anti-protein specific enzyme-linked immunosorbent assay (ELISA). The first such protein markers used were rabbit immunoglobulin (IgG) and chicken IgG/IgY[9,10]. They proved to be very effective marks for MRR and self-mark-capture research (see discussion). Unfortunately, IgG/IgY proteins are costly and are therefore not practical for mark-capture research and most types of self-mark-capture research. Subsequently, second-generation protein detection ELISAs were developed for proteins contained in chicken egg whites (albumin), cow's milk (casein) and soy milk (trypsin inhibitor protein). Each assay is highly sensitive, specific, and, most importantly, uses proteins that are much less expensive than the IgG/IgY proteins[11]. These proteins have proven effective for MRR, mark-capture, and self-mark-capture research (see discussion).

In this article, we describe and demonstrate how to conduct protein mark laboratory retention studies. Such studies are the first phase of research needed for any type of field dispersal study. Specifically, it is critical that investigators know how long the mark will be retained on the targeted arthropod species prior to embarking on field dispersal studies. Here, we describe and demonstrate how to internally and externally

mark insects for MRR, mark-capture, and self-mark-capture type field studies. We then demonstrate how to detect the presence of the marks with indirect and sandwich ELISAs.

Protocol

1. Internal Mark, Retention, and Detection Procedure

1. Internal marking procedure
    1. Collect insects of interest (*n* ≈ 100 individuals) from a laboratory colony reared on an artificial diet or from the field and divide into two clean rearing containers.
    2. Place a regular 20 ml diet packet (unmarked negative control treatment) into one of the containers. Supplement a second 20 ml artificial diet packet with 1.0 ml of a 1.0 mg/ml chicken IgG/IgY solution, mix thoroughly, and place it in the other container.
    Note: If the insect of interest does not have an artificial diet, the mark can be placed on or in whatever diet they are normally sustained on (*e.g.*, green bean, moth eggs, water, sugar, honey).
    3. Allow the insects to resume their feeding activity for 24 hr.

2. Internal mark retention procedure
    1. Remove the diet packets from each container and supply the insects with another food source to sustain them over the duration of the experiment (*e.g.*, green beans).
    2. Collect a cohort (*n* = 20) of insects daily (or at any desired time interval) from each treatment and freeze immediately at -20 °C.

3. Sandwich ELISA procedure
    1. Add 50 µl of rabbit anti-chicken IgY primary antibody diluted 1:500 in Tris buffered saline (TBS) to each well of a clean ELISA microplate and incubate for a minimum of 1 hr at RT (note: the microplates can also be incubated O/N at 4 °C).
    2. Discard the antibody from the plate and block each well with 300 µl of a 1.0% non-fat dry milk solution for 30 min.
    3. While waiting for the previous incubation steps, place frozen insects individually in 1.6 ml microcentrifuge tubes with 1.0 ml of TBS.
    4. Grind each insect with a clean pestle until thoroughly homogenized and add a 100 µl of each sample to an individual well of the ELISA plate. Allow the samples to incubate at RT for 1 hr.
    5. Add 100 µl of negative (TBS only) and positive (egg whites in TBS) controls to wells in the first column of each ELISA plate. Also, add 100 µl of negative insect controls (unmarked insects) to the 8 wells in the last column of each plate (**Figure 1**). Allow the control samples to incubate at RT for 1 hr. Note that the template provided in **Figure 1** is the template we use for our standardized assays. The placement of the samples in the wells is up to the discretion of the researcher.
    6. Wash each well three times with phosphate buffered saline (PBS)-tween and then add 50 µl of rabbit anti-chicken IgG/IgY conjugated with horseradish peroxidase diluted 1:10,000 in 1% milk solution and incubate for 1 hr at RT.
    7. Wash each well three times with PBS-tween and add 50 µl of tetramethylbenzidine (TMB) substrate to each well.
    8. After 10 min, read the wells with a microplate spectrophotometer set at a wavelength of 650 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	TBS	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33	Sample 41	Sample 49	Sample 57	Sample 65	Sample 73	Neg Ctrl 1
B	TBS	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42	Sample 50	Sample 58	Sample 66	Sample 74	Neg Ctrl 2
C	TBS	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43	Sample 51	Sample 59	Sample 67	Sample 75	Neg Ctrl 3
D	TBS	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44	Sample 52	Sample 60	Sample 68	Sample 76	Neg Ctrl 4
E	TBS	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45	Sample 53	Sample 61	Sample 69	Sample 77	Neg Ctrl 5
F	TBS	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38	Sample 46	Sample 54	Sample 62	Sample 70	Sample 78	Neg Ctrl 6
G	TBS	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39	Sample 47	Sample 55	Sample 63	Sample 71	Sample 79	Neg Ctrl 7
H	Pos Ctrl	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40	Sample 48	Sample 56	Sample 64	Sample 72	Sample 80	Neg Ctrl 8

Figure 1. A schematic diagram showing the standardized well designations used for a typical 96 well ELISA microplate. Each plate contains 7 wells dedicated to Tris-buffered saline negative controls (TBS), a well dedicated to a positive protein control (Pos Ctrl), 80 individual wells dedicated to recaptured insects (Sample 1…80), and 8 wells dedicated to unmarked (negative) insect controls (Neg Ctrl 1…8). Please click here to view a larger version of this figure.

2. External Mark, Retention, and Detection Procedure

1. External marking procedure
    1. Collect insects of interest (*n* ≈ 100 individuals) from a laboratory colony or from the field and place into two 1.0 L plastic containers with a 2.5 cm diameter hole punched out of the side of each container.
    2. Spray the insects in the first container with 1.0 ml of dH_2O (negative control treatment) using a medical nebulizer. Spray the insects in the second container with 1.0 ml of a 5.0% solution of chicken egg whites using a medical nebulizer.
    3. Attach the hose of the nebulizer to a standard laboratory air outlet and then insert the mouth of the nebulizer into the 2.5 cm hole of the container. Then, turn on the air outlet gradually until the nebulizer produces a vapor.

4. Continue spraying (marking) until the egg whites solution has been dispensed (approx. 2-5 min).
5. Remove the nebulizer and place a cork into the hole of the plastic container. Allow the insects to stand at RT until the solution has dried completely.

2. External mark retention procedure
    1. Place each treatment of the dry insects into a separate clean rearing container to avoid further exposure to the mark.
    2. Add food and water to the clean containers to sustain the insects over the duration of the study.
    3. Collect a cohort (*n* = 20) of insects from each treatment daily and freeze immediately at -20 °C.

3. Indirect ELISA procedure
    1. Place the frozen insects individually into 1.6 ml microcentrifuge tubes and add 1.0 ml of TBS.
    2. Soak the samples for 1 hr on an orbital shaker set at 120 rpm.
    3. Add negative and positive controls as described in Step 1.3.5.
    4. After soaking, pipette a 100 µl aliquot from each sample into one of the 80 designated sample wells on a new 96 well ELISA plate as shown in **Figure 1**. Allow the samples to incubate for 1 hr at RT (note: the microplates can also be incubated O/N at 4 °C).
    5. Wash the wells three times with PBS-tween and then add 300 µl of phosphate buffered saline containing 1% bovine serum albumin (PBS-BSA) to each well.
    6. After 30 min at RT wash twice with PBS-tween.
    7. Add 50 µl of rabbit anti-egg albumin primary antibody diluted 1:8,000 in PBS-BSA-Silwet (0.05%) to each well and incubate for 1 hr at RT.
    8. Wash each well three times with PBS-tween and then add 50 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:2,000 in PBS-BSA-Silwet (0.05%) for 1 hr at RT.
    9. Wash each well three times with PBS-tween and add 50 µl of TMB substrate to each well.
    10. After 10 min, read the wells with a microplate spectrophotometer set at a wavelength of 650 nm.

3. Data Analysis

1. Calculate the mean absorbance value of the 8 negative control insects on each ELISA plate and then calculate the standard deviation based on the pooled negative controls from all of the ELISA plates of a given study[12].
2. Add six times the pooled standard deviation to the mean obtained for each ELISA plate to obtain an ELISA positive threshold value. Any insect sample yielding an absorbance value equal to or greater than this value is deemed to be positive for the presence of the protein mark.

Representative Results

Internal marking:

The results of the internal mark retention test are shown in **Figure 2A**. The calculated ELISA critical threshold value was 0.054. Overall (all four sample dates combined), the insects treated without protein yielded consistently low ELISA values ($\bar{x}$ = 0.038 ± 0.002, *n* = 80). Conversely, all of the insects fed the protein enriched diet yielded consistently strong ELISA values ($\bar{x}$ = 0.475 ± 0.221, *n* = 80).

External marking:

The results of the external mark retention test are shown in **Figure 2B**. The calculated ELISA critical threshold value was 0.082. Overall, the insects topically marked with only water yielded consistently low ELISA values ($\bar{x}$ = 0.048 ± 0.007, *n* = 80). Conversely, all of the insects topically marked with the egg whites solution yielded consistently strong ELISA values ($\bar{x}$= 0.746 ± 0.232, *n* = 80).

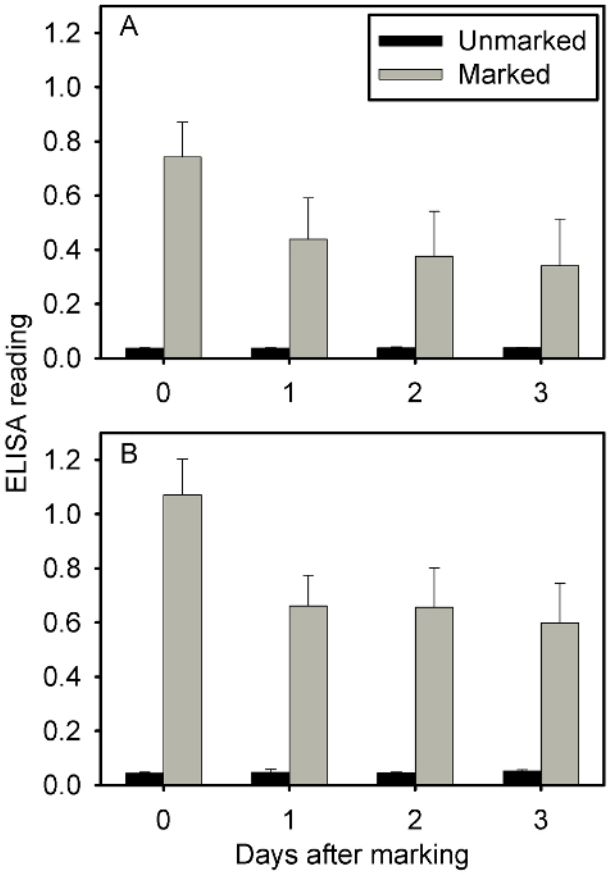

Figure 2. Mean (± SD) ELISA optical density values of (A) insects marked internally with chicken IgY and (B) insects marked externally with chicken egg whites (albumin). Black bars are the mean ± SD ELISA optical density values yielded by unmarked insects. Insect sample size for each daily protein treatment was 20 individuals. Please click here to view a larger version of this figure.

Discussion

The arthropod protein immunomarking procedure was first described almost a quarter of a century ago[9]. Since then, the procedure has been adapted to study the dispersal patterns of a wide variety of arthropods using both internally and externally administered IgG/IgYs. These proteins have proven steadfast markers for the wide variety of insect species tested thus far. However, as mentioned above, the major limitation for using IgG/IgYs is that they are very expensive. Consequently, IgG/IgYs are only useful for MRR and self-marking type studies where large batches of insects can be marked in a small space or the marks can be incorporated into a diet or bait. Here, we provided a simple demonstration of how an internal mark can be administered by feeding the target insect an artificial diet enriched with chicken IgG/IgY. In turn, the IgG/IgY was detected using a sandwich ELISA. This system might be especially advantageous to researchers that already use a diet that contains chicken eggs since the assay will naturally detect the IgG/IgY found in eggs. In addition, small amounts of IgG/IgY protein can be administered topically to insects using the medical nebulizer described above (**Figure 3A**). A nebulizer was first used to mass mark very delicate and tiny parasitoids with rabbit IgG for MRR type research[3]. Since then, other methods have been used to administer IgG/IgY marks to arthropods for dispersal research. For example, a perfume atomizer has been used to apply IgG/IgY marks on other parasitoid species[13,14]. Others have internally marked various insects by feeding them (*i.e.*, self-marking) rabbit IgG labeled honey[14,15] or sugar water[16] (**Figure 3B**). Finally, Baker *et al.*[7] fed cardboard to termites that was impregnated with rabbit IgG (**Figure 3C**). These termites readily self-marked as they ingested the baited foodstuffs.

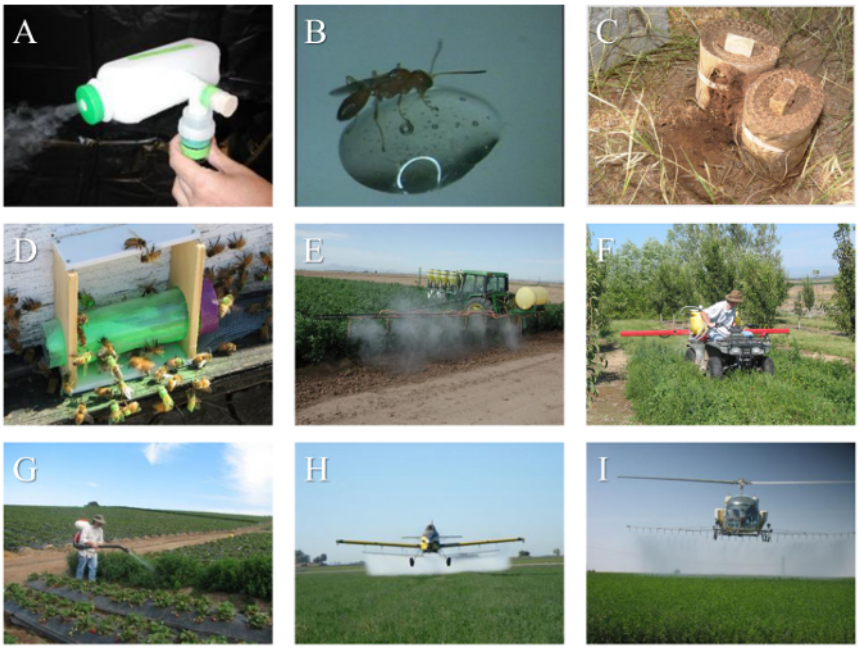

Figure 3. Various methods used to administer a protein mark: (**A**) delicate parasitoids being marked with chicken IgY using a medical nebulizer, (**B**) a parasitoid feeding on rabbit IgG-enriched honey solution, (**C**) termites feeding on a rabbit IgG-enriched cardboard bait, (**D**) honey bees self-marking with dry milk as they go through a dusting device placed at the entrance of a hive, (**E**) native insects being marked with milk in a cotton field using a conventional tractor spray rig, (**F**) native insects being marked with chicken egg whites with a boom and nozzle spray device mounted onto an ATV, (**G**) native insects in a strip of alfalfa being marked with chicken egg whites with a backpack spray device, and (**H**, **I**) native insects in alfalfa being marked with chicken egg whites using aerial applicators. Please click here to view a larger version of this figure.

IgG/IgY proteins have not just been used for studying insect dispersal. They have also been used for tracking trophic links in the arthropod food chain. Hagler and Durand[17] first proposed the concept of marking prey with IgG/IgY and, in turn, conducting an IgG/IgY-specific sandwich gut content ELISA on predators that consumed the marked prey. This technique has recently gained popularity among gut analysis researchers because it is less tedious and costly than conducting prey-specific ELISA or polymerase chain reaction (PCR) type molecular gut content analyses (see Hagler[18] and Fournier *et al.*[19] for reviews of the pros and cons of the various gut assay techniques). Within the past decade, the prey immunomarking technique has been used to identify feeding activity of cotton predators on rabbit IgG-marked pests[18,20,21], trophallaxis and feeding relationships in termite colonies using a rabbit IgG-treated paper food source[22], granivory of an arthropod assemblage on invasive dandelion weed seeds marked with rabbit IgG[23], stink bug predation rates on IgG-marked tomato hornworms collected in plots subjected to various semiochemical treatments[24], and predator scavenging activity on IgG/IgY-marked carrion[25].

A second generation of protein detection indirect ELISAs were developed almost a decade ago. These ELISAs were designed specifically to detect proteins contained in "off-the-shelf" food products, such as egg albumin protein in chicken egg whites, soy trypsin inhibitor protein in soy milk, and casein protein in bovine milk. The second-generation protein immunomarkers have proven useful for MRR, mark-capture and self-mark type research. For instance, Irvin *et al.*[26] showed that it was feasible to topically mark delicate parasitoids with these off-the-shelf proteins. Moreover, Hagler *et al.*[8,27] showed that honey bees can self-mark with egg white and bovine milk dry powders as they exit through a protein dispenser placed at the entrance of a hive (**Figure 3D**). Perhaps the most creative use of the protein marking involved targeting cow pats with egg whites using a jet sprayer[28]. In turn, face fly adults acquired a self-mark as they emerged from their pupal stage and exited the cow pat.

The most important characteristic of the second-generation protein immunomarkers is that they are readily available in mass quantities and at a fraction of the cost of IgG/IgY proteins[11]. This feature has revolutionized the way area-wide mark-capture type research is conducted. Specifically, investigators now have a reliable method to rapidly and uniformly tag arthropods over vast areas in the field using a wide variety of conventional spray rig equipment (**Figure 3E**). For instance, pest insects have been marked directly in orchards and fields using portable hand gun type sprayers[29,30], tractor-driven airblast sprayers[31,32], and skid sprayers[33]. Horton *et al.*[34] used an electric spray device mounted on an all-terrain vehicle to pinpoint applications of egg whites to insects occupying cover crops embedded in a pear orchard (**Figure 3F**). Similarly, Swezey *et al.*[5,35] used a backpack sprayer to apply precise applications of chicken egg whites to rows of an alfalfa trap crop (a preferred host plant) embedded in an organically grown strawberry field to mark a pest and its natural enemies (**Figure 3G**). The results from that research showed that the pest and natural enemies were retained by the alfalfa trap crop and rarely dispersed into the highly valued strawberries. Perhaps the most extreme application of a protein immunomarker was through the use of an aerial helicopter and airplane crop dusters. In that study, Sivakoff *et al.*[36] applied a broadcast application of bovine milk and egg white to 29 and 16 ha of blooming alfalfa, respectively (**Figure 3H, I**). The aim of that study was to mark the resident arthropods in the alfalfa, and then, after the alfalfa was harvested by mowing (sparking a dispersal event), to track the dispersal patterns of the arthropods into adjacent cotton fields (a crop susceptible to the pest species).

Some users of the second-generation mark-capture procedure have modified the original procedure somewhat to meet their specific research needs. The body type and size of the targeted insect, along with its behavioral characteristics, will dictate the volume and concentration of mark needed and how the mark is administered[37]. Moreover, the method used to recapture insects in the field will be contingent on these factors. To date, almost every method used (*e.g.*, sticky traps, sweep nets, vacuums) to collect insects has been used successfully to collect marked

insects[3,5,8,11,29,31,32,35,38]. However, due to the extreme sensitivities of the protein detection ELISAs, we emphasize that great care must be taken to ensure that unmarked specimens do not become contaminated during the collection or sorting processes[38].

The original study[11] that described the second generation of protein marking systems (*i.e.*, egg albumin, milk casein, and soy trypsin inhibitor) reported factors affecting assay sensitivity may vary among the types of marker. Specifically, they showed that different water sources, additives (surfactants), and even the type of ELISA plate used affected (either positively or negatively) the assay. Moreover, that study and subsequent studies[37,39,40] showed that the three protein mark detection assays vary in efficacy. The egg white marker was retained longer than the milk marker, which was retained longer than soy milk marker. These results highlight the importance of testing protein mark retention on any given insect prior to embarking on field research. Also, these second-generation indirect ELISAs sometimes show a low level of background noise with certain types of insects (*e.g.*, herbivores) (pers. obs). This background noise can be reduced by adding 100 µl of hydrogen peroxide to each well of the ELISA plate for 30 min between Step 2.3.4 and Step 2.3.5 in the protocol above (unpubl. data).

It should be noted that the indirect ELISAs used to detect the second-generation protein marks are very effective at detecting the protein on insects soaked in the sample buffer. However, the indirect ELISAs are not as effective as the sandwich ELISA at detecting internal protein marks or prey proteins on insects. Studies have shown that indirect ELISAs are not as efficient as sandwich ELISAs at detecting protein in homogenized insect samples[34,41].

An important factor to consider with any ELISA is that the procedure often shows plate-to-plate (attributed mainly to day-to-day effects of running an assay) variability[42,43]. Therefore, it is essential that every ELISA plate includes: (1) one or more TBS only negative controls, (2) one or more TBS + pure protein positive controls, and (3) at least eight negative insect controls (*i.e.*, individuals known not to contain a protein mark). The TBS only, TBS + protein, and insect negative controls assure that the buffer is not a source of error, the reagents are working properly, and that there are not any proteins in the insect that cross react with the ELISA reagents, respectively. Moreover, the insect negative controls are essential for calculating ELISA threshold values (see below). The plate design shown in this demonstration included 7 TBS blanks, 1 protein + TBS positive control (in the first column) and eight negative controls (in the last column) on each ELISA plate (**Figure 1**).

Another important factor to consider when handling ELISA data is to determine a threshold value to score an insect for the presence or absence of a protein mark. The method originally proposed by Stimmann[44] for scoring rubidium-marked insects was defined as the mean level of marker in a pool of unmarked insects plus three times the standard deviation of the unmarked individuals. This method has also been used as the conventional method for scoring insects for the presence of protein marks[9,17]. However, in some cases, researchers have intuitively selected more conservative threshold values to reduce the likelihood of obtaining false positives. The easiest method is to just add four to six standard deviations to the mean of the negative controls instead of three[18,34]. A more sophisticated method to further reduce the chance of obtaining false positives was proposed by Sivakoff *et al.*[12]. This method entails calculating the mean absorbance value of the eight negative control insects on each ELISA plate and then calculating the standard deviation based on the pooled negative controls from all of the ELISA plates of a given study.

In summary, a reliable method to tag arthropods is critical to the success of most dispersal studies. Various methods have been used to mark arthropods with proteins over the past two decades. Protein immunomarking and the detection of the marks are relatively easy and inexpensive and very adaptable to a myriad of studies. Future users of the immunomarking technique should ensure that their methodology is suitable to the specifics of their research. The concentration and volume of the mark needed and how the mark is applied will depend on factors like behavior, body type and size of the target species[40].

Disclosures

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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