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Title of resource
GN 22 Egg-white ovalbumin ELISA protocol
Authored by
When using this protocol, the following should be referenced: Christopher Sanders (The Pirbright Institute, UK), Rod Dillon, Fernando Genta, Luigi Sedda. Adapted from Hagler and Jones (2010) and Sanders *et al*. (2017).
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Description
This protocol is for the detection of egg-white ovalbumin on small marked arthropods including Phlebotomine sand flies and *Culicoides* biting midges for dispersal and habitat studies. Adapted from Hagler and Jones (2010) and Sanders *et al*. (2017).
Intended use
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Resource history

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A. Introduction

This protocol is for the detection of egg-white ovalbumin on small marked arthropods including Phlebotomine sand flies and *Culicoides* biting midges for dispersal and habitat studies. Adapted from Hagler and Jones (2010) and Sanders *et al*. (2017).

This assay can detect minute quantities of ovalbumin protein on individual insects. Due to the sensitivity of the assay to, there is a risk of contamination and the generation of false positives if due care and attention is not paid. Ideally, different users should mark the insects in the field or laboratory to those that use this protocol to determine marking of the insects. To reduce the possibility of contamination, it is essential to use and replace gloves between the different stages of the protocol and avoid splashes or drips. Sterile filter tips should be used, with a new tip used per well to prevent cross-contamination. The working area should be cleaned with 70% ethanol before and after use. Negative controls should be used to monitor potential cross-contamination and determine thresholds to identify positive wells.

Containers of insects to be tested for the presence of ovalbumin should be placed at -20°C as soon as possible after capture for a period of 24 hours to kill all insects present.

B. Materials

Equipment

- Single channel pipettes 20-200 µl and 100-1000 µl
- Multichannel pipette 30-300 µl
- Fridge (+4°C)
- Freezer (-20°C)
- Incubator (37°C)
- Pure water

- Vortex
- ELISA plate washer
- ELISA plate reader
- 96 well ELISA plates (e.g. Nunc Maxisorp flat bottom plates Thermofisher 44-2404-21)
- 250 ml amber glass Duran glass bottle with PBT chemical resistant red lid and pouring ring (e.g. Fisher Scientific 13114344, 13184344, 13197154)
- 1 L plastic storage bottles (e.g. Corning® 1 L Easy Grip Polystyrene Storage Bottles with 45 mm Caps #430518))
- 50 ml centrifuge tubes (e.g. Falcon Tubes)
- 1 L spray bottle
- Stereomicroscope (1-40 x magnification)
- Label printer (e.g. Brady BP33)
- Timer
- Fume hood with filters suitable for use with sulphuric acid
- 15 ml centrifuge tube rack
- 300 µl sterile filter pipette tips (e.g. Finntipp Flex 300 94056580)
- 200 µl sterile filter pipette tips (e.g. Axygen MAXYMum Recovery AX-TF-200-L-R-S-CS)
- Disposable reagent reservoirs 100 ml (e.g. Thermo Scientific 95128085) or 25 ml (e.g. Thermo Scientific 95128093)
- 1.5 ml collection microtubes and caps (e.g. Qiagen 19560 and 19566)
- Adhesive PCR sealing foil (e.g. Abgene AB-0626)
- 15 ml centrifuge tubes
- 70% Ethanol
- Blue roll
- Cocktail sticks
- A4 white paper
- Nitrile gloves

Reagents

- Tris buffered saline (TBS) (pH 7.4) (e.g. 10x TBS Alfa Aesar J62938.K2)
- Tween-20 (Sigma Aldrich 1379)
- Phosphate buffered saline with tween-20 (PBST) (e.g. Sigma P3563)
- Phosphate buffered saline with bovine serum albumin (PBS-BSA) (e.g. Sigma P3688)
- Rabbit anti-chicken egg ovalbumin antibody (Sigma C6534)
- Anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase produced in goat (Sigma A6154)
- Sulphuric acid (H_2SO_4) 95-99 % (e.g. Sigma 258105) made to 2N solution.
- 1-Step Ultra TMB-ELISA Substrate Solution (Life Technologies 34029)
- Elix pure water (or equivalent)
- Chicken egg white solution (e.g. Bulk Powders™ [Sports Supplements Ltd, UK])
- 1:10 serial dilution standards from an initial concentration of 30 µl egg white in 270 µl TBS, (1x to -7x)

Solutions

- **Sample wash**: 1 x TBS with 0.05% Tween 20
- **PBST**: 1 x PBS with 0.05% Tween 20
- **Blocker**: PBS-BSA with 0.05% Tween 20
- **Primary antibody**: 1:8000 rabbit anti-chicken egg ovalbumin antibody in PBS-BSA + 0.05 % Tween 20
- **Secondary antibody**: 1:2000 goat-antirabbit IG conjugated to horseradish peroxidase in PBS-BSA+ 0.05 % Tween 20

- **2N H_2SO_4 stop solution**: very slowly add **11.222** mL of 95% w/w (35.644 N) H_2SO_4 to **50** mL pure water. Adjust the final volume of solution to **200** mL with pure water.

C. Method

C.1 Prepare 1.5 ml sample microtubes, adding 300 µl **Sample Wash** per tube. The addition of Tween wets hydrophobic insects allowing them to sink.

C.2 Empty container of potentially marked insects onto clean piece of paper, spreading insects evenly and lightly over the paper. This can be done under a stereomicroscope if necessary. Use new paper for each insect container.

C.3 Use single-use cocktail sticks to pick up individual sandflies and place in sample microtubes wash tubes. Use a fresh stick end for each individual and record the location of each individual, including information such as species, sex or parity.

C.4 Place caps on tubes. Return samples of insects in sample wash.

C.5 Vortex and/or centrifuge plate to ensure insect is within sample wash. Soak overnight (up to 24 hrs) at +4°C.

C.6 Vortex to mix, and centrifuge at 1000 rpm to remove sample wash from tube cap.

C.7 Carefully remove caps, then place 80 µl of each sample into a well on an ELISA plate. Leave space for negative controls and positive dilution standards on each plate.

C.8 Cover plate with plate sealer.

C.9 Incubate for 1 hr at +37°C.

C.10 Remove plate sealer and discard contents and wash wells x 2 with **PBST**.

C.11 Add **blocker** of 360 µl PBS-BSA with Tween 20. Cover with plate sealer.

C.12 Incubate for 1 hr at room temperature.

C.13 Remove plate sealer and discard contents and wash wells x 2 with **PBST** .

C.14 Add 80 µl **Primary antibody** (1:8000 rabbit anti-chicken egg ovalbumin antibody in PBS-BSA + Tween 20) per well. Cover with plate sealer.

C.15 Incubate for 1 hr at 37°C.

C.16 Remove plate sealer and discard contents and wash wells x 5 with **PBST** .

C.17 Add 80 µl **Secondary antibody** (1:2000 goat-antirabbit IG conjugated to horseradish peroxidase in PBS-BSA + tween 20). Cover with plate sealer.

C.18 Incubate for 2 hrs at 37°C.

C.19 Remove plate sealer and discard contents and wash x 5 with PBST (PBS + 0.05% Tween 20).

C.20 Add 80 µl **TMB substrate**.

C.21 Incubate at room temperature for 15 mins.

C.22 Add 80 µl **2N H_2SO_4 as stop solution**.

C.23 Read optical density of wells at 430 nm in plate reader.

D. Results (determination of positives)

Wells are considered to be positive for ovalbumin if the optical density (OD) for that well is greater than a selected threshold. One of several methods for the selection of this threshold may be used:

Threshold 1:

A conventional threshold defined as the mean level of mark in unmarked individuals (negative controls) plus three times the standard deviation of the unmarked distribution (Stimmann, 1974) including a correction for the between plate variation observed in ELISAs (Clark, 1977) may be used such that the threshold is equal to $\mu_j + 3s_j$ where μ_j and s_j are the mean and sample standard deviation respectively of the negative controls on plate *j*.

Threshold 2:

All ODs can be transformed using the standard normal variate (SNV) transformation (*eqn 1*) (Sivakoff *et al.*, 2011). The SNV transformation (*z*) is the OD score (*X*) of a sample in well *i* on plate *j,* where $\hat{\mu}$ is an estimate of the mean of the negative controls on plate *j*.

$$z_{ij} = \frac{(X_{ij} - \hat{\mu}_j)}{c\hat{\mu}_j^k}$$

Equation 1

Where $c\hat{\mu}_j^k$ represents the estimate of the standard deviation (*s*) of the negative controls on plate *j* calculated using the power law function (Sivakoff *et al.*, 2011). Parameters *c* (the *y* intercept) and *k* (the slope parameter) are estimated by taking the natural logarithm (*ln*) of *eqn 2* and regressing the ln of *s* on the ln of $\hat{\mu}_j$ (Sivakoff *et al.*, 2011). For *eqn2* the values used in the regression are the μ and *s* of all negative controls on each plate.

$$\ln(s_j) = \ln c + k\ln(\hat{\mu}_j)$$

Equation 2

However for *eqn 1*, as per (Sivakoff *et al*., 2011) to avoid masking the false positive rate when transforming the negative controls, $\hat{\mu}_j$ is calculated using the all the negative controls for that plate excluding the one being transformed. In order to minimise the false positive rate, the threshold score should be set using the 'maximum negative control' algorithm i.e. the highest negative control *z* observed across all ELISA plates in the study (Hooper, 1991; Sivakoff *et al*. 2011). With specimens classified as positive for marker if their *z* was greater than the selected threshold.

Following this non-destructive protocol, specimens returned to appropriate storage (+4°C short term, -20°C or -80°C longer term, are still suited to identification by molecular means (Sanders *et al*., 2017).

E. References

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4. Sanders, C., Harrup, L.E., Tugwell, L.A. Brugman, V.A. England, M. and Carpenter, S. (2017). Quantification of within- and between-farm dispersal of *Culicoides* using and immunomarking technique. *Journal of Applied Ecology*. 54(5): 1429-1439. doi:10.1111/1365-2664.12875.

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