Journal of Visualized Experiments

Removal and Replacement of Endogenous Ligands from Lipid-Bound Proteins and Allergens

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61780R1
Full Title:	Removal and Replacement of Endogenous Ligands from Lipid-Bound Proteins and Allergens
Corresponding Author:	Alexander Foo National Institute of Environmental Health Sciences RTP, NC UNITED STATES
Corresponding Author's Institution:	National Institute of Environmental Health Sciences
Corresponding Author E-Mail:	alexander.foo@nih.gov
Order of Authors:	Alexander C.Y. Foo
	Peter M. Thompson
	Geoffrey A. Mueller
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Research Triangle Park, North Carolina, United States of America

TITLE:

Removal and Replacement of Endogenous Ligands from Lipid-Bound Proteins and Allergens

AUTHORS AND AFFILIATIONS:

Alexander C.Y. Foo[1], Peter M. Thompson[1], Geoffrey A. Mueller[1]

[1]Nuclear Magnetic Resonance Group, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

Corresponding Author:
Geoffrey A. Mueller (Geoffrey.Mueller@nih.gov)

E-mail addresses of co-authors:
Alexander C.Y. Foo (Alexander.foo@nih.gov)
Peter M. Thompson (PMThomps@ncsu.edu)

KEYWORDS:

allergens, biochemistry, biophysics, fatty acid-binding proteins, fatty acids, lipids, protein binding, protein stability

SUMMARY:

This protocol describes the removal of endogenous lipids from allergens, and their replacement with user-specified ligands through reverse-phase HPLC coupled with thermal annealing. ^{31}P-NMR and circular dichroism allow for the rapid confirmation of ligand removal/loading, and the recovery of native allergen structure.

ABSTRACT:

Many major allergens bind to hydrophobic lipid-like molecules, including Mus m 1, Bet v 1, Der p 2, and Fel d 1. These ligands are strongly retained and have the potential to influence the sensitization process either through directly stimulating the immune system or altering the biophysical properties of the allergenic protein. In order to control for these variables, techniques are required for the removal of endogenously bound ligands and, if necessary, replacement with lipids of known composition. The cockroach allergen Bla g 1 encloses a large hydrophobic cavity which binds a heterogeneous mixture of endogenous lipids when purified using traditional techniques. Here, we describe a method through which these lipids are removed using reverse-phase HPLC followed by thermal annealing to yield Bla g 1 in either its Apo-form or re-loaded with a user-defined mixture of fatty acid or phospholipid cargos. Coupling this protocol with biochemical assays reveal that fatty acid cargoes significantly alter the thermostability and proteolytic resistance of Bla g 1, with downstream implications for the rate of T-cell epitope generation and allergenicity. These results highlight the importance of lipid removal/re-loading protocols such as the one described herein when studying allergens from both recombinant and natural sources. The protocol is generalizable to other allergen families including lipocalins (Mus m 1), PR-10 (Bet v 1), MD-2 (Der p 2) and Uteroglobin (Fel d 1), providing a valuable tool to study the role of lipids in the allergic response.

INTRODUCTION:

A survey of the allergen database reveals that allergens are found in only 2% of all known protein families, suggesting common functional and biophysical properties contribute to allergenicity[1]. Of these properties, the ability to bind lipid cargoes appears to be strongly over-represented among allergens, suggesting that these cargoes may influence the sensitization process[1]. Indeed, it has been shown that the Brazil Nut allergen Ber e 1 requires co-administration with its endogenous lipid to realize its full sensitizing potential[2]. These lipids could potentially stimulate the immune system directly as illustrated by the mite allergens Der p 2 and Der p 7, both of which share a strong structural homology with LPS-binding proteins[3–5]. Based on this observation it was proposed that Derp 2 and Der p 7 could bind bacterial lipids and directly stimulate the host immune system through TLR4-mediated signaling, facilitating the sensitization process[5,6]. It is also possible that endogenously bound lipids could alter the biophysical properties of allergic proteins themselves. For example, the ability of Sin a 2 (mustard) and Ara h 1 (peanuts) to interact with phospholipid vesicles significantly enhanced their resistance to gastric and endosomal degradation[7], while ligand binding to the major birch pollen allergen Bet v 1 altered both the rate of endosomal processing and the diversity of the resulting peptides[8]. This is particularly relevant to allergenicity given the correlation that has been observed between stability, T-cell epitope generation and allergenicity for proteins such as Bet v 1 and Bla g 1; the latter of which will be the subject of this work[9,10].

Bla g 1 represents the prototypical member of the insect Major Allergen (MA) protein family, and possesses a unique structure composed of 12 amphipathic alpha helices which enclose an abnormally large hydrophobic cavity[9,11]. The available X-ray crystal structure of Bla g 1 shows electron density within this cavity consistent with bound phospholipid or fatty acid ligands; a conjecture confirmed by ^{31}P-NMR and mass spectrometry. These cargoes were heterogeneous in nature and their composition was heavily dependent on the allergen source, with different lipid profiles observed for recombinant Bla g 1 expressed in *E. coli* and *P. pastoris*. Curiously, Bla g 1 purified from its natural allergen source (cockroach frass) contained predominantly fatty acids within its binding site, with a mixture of palmitate, oleate, and stearate being identified as its "natural" ligands[9,11]. The ability of Bla g 1 to retain lipids and fatty acids following multiple purification steps hinders efforts to study the protein in isolation. Conversely, it has been suggested that the natural palmitate, stearate, and oleate ligands of Bla g 1 (henceforth referred to as nMix) play a key role in both its allergenicity and native biological function[9]. However, these ligands are not present in Bla g 1 obtained from recombinant sources, making it difficult to assess this hypothesis. Similar issues have been observed for other lipid binding allergens such as Bet v 1[12,13]. To facilitate the systematic study of lipid-allergen interactions we have developed a protocol through which allergens can be quantitatively stripped of their endogenously bound lipids and reconstituted in either Apo-form or loaded with specific ligands.

Allergens are most commonly purified from their natural or recombinant sources using affinity chromatography and/or size-exclusion chromatography. Here, we introduce an additional purification step in the form of high-performance liquid chromatography (HPLC) employing a reverse-phase C18 column from which the allergen is eluted into an organic solvent similar to

protocols developed for fatty acid binding proteins[14]. The resulting protein is then subjected to a thermal annealing step in the absence or presence of fatty acids and/or phospholipids. In addition to recovering the native Bla g 1 fold, the elevated temperatures increase the solubility and accessibility of the lipid cargoes, yielding Bla g 1 in either the Apo-form or uniformly loaded with the desired hydrophobic ligand. ^{31}P-NMR spectra of Bla g 1 purified in this manner confirms the complete removal of endogenously bound ligands and uniform replacement with the desired compounds, while circular dichroism confirm the successful recovery of the Bla g 1 fold. The utility of this method is highlighted in a recent work in which cargo binding was found to enhance Bla g 1 thermostability and proteolytic resistance, altering the kinetics of T-cell epitope generation with potential implications for sensitization and allergenicity[9].

PROTOCOL:

1. Bla g 1 cloning

1.1. Obtain gene for cockroach allergen Bla g 1.0101 (residues 34-216), representing a single repeat of the MA domain. For the sake of simplicity, Bla g 1 will be used throughout the work to represent this single repeat, rather than the entire Bla g 1.0101 transcript.

1.2. Subclone the Bla g 1 gene into the desired vector. In this study, the gene containing an N-terminal glutathione S-transferase (GST) tag coupled to a tobacco etch virus (TEV) protease cleavage site was inserted into a pGEX vector for expression as described previously[11].

1.3. Transform the Bla g 1 pGEX vector into BL21 DE3 *E. coli* cells.

1.3.1. Prepare a 10 ng/µL stock of the desired vector.

1.3.2. Combine 1 µL of 10 ng/µL DNA stock with 50 µL of BL21 DE3 cells as provided by the manufacturer.

1.3.3. Incubate BL21 DE3-DNA mixture for 30 min on ice. Transfer to a 42 ˚C water bath for 1 min, then immediately transfer back on ice for an additional 1 min incubation.

1.3.4. Add 200 µL of LB media to the cells and incubate for an additional 1 h at 37 ˚C.

1.3.5. Plate the transformed cells on LB-Agar plates containing 100 mg/L ampicillin and grow at 37 ˚C overnight.

2. Initial expression and purification

2.1. Inoculate 1 L of LB media containing 100 mg/L ampicillin with a single colony of BL21 DE3 cells transformed with the Bla g 1 vector as described in 1.3. Grow at 37 ˚C overnight.

2.2. On the next day, harvest cells (OD_{600} ~1.5) via centrifugation at 6,000 *x g* for 10 min and resuspend in 2 L of 2x YT media containing 100 mg/L ampicillin. Allow cells to grow for an additional 1 h at 37 ˚C to an OD_{600} > 0.6.

2.3. Induce protein expression through the addition of 0.5 mM IPTG. Transfer cells to 18 ˚C and incubate overnight.

2.4. On the next day, harvest cells as described in 2.2. The resulting cell pellet can be frozen and stored at -20 ˚C.

2.5. Resuspend pellet obtained from 1 L of culture in 50 mL of lysis buffer (50 mM Tris-HCl pH 8.5, 100 mM NaCl) containing 1 protease inhibitor tablet (or equivalent) and 1 µL of benzonase nuclease.

2.6. Lyse cells using a probe sonicator (500 W, 20 kHz) set to 30–50% power for 4 min with a 50% duty cycle. Keep the lysate in an ice bath during sonication

2.7. Centrifuge lysate at 45,000 *x g* for 20 min. Discard insoluble fraction (pellet).

2.7.1. Remove 28 µL of soluble protein. Combine with 7 µL of 5x SDS-PAGE buffer and store for SDS-PAGE analysis. Repeat this step for the GST column flow-through, wash, and elution fractions before and after incubation with TEV.

2.8. Apply soluble proteins (supernatant) to a glutathione resin column (~10 mL total bed volume) equilibrated in PBS pH 7.4.

2.9. Wash out any unbound proteins using 50 mL of PBS.

2.10. Elute GST-Bla g 1 using 50 mL of PBS containing 10 mM reduced glutathione.

2.11. Incubate eluted protein with 0.2 kU TEV protease overnight at 4 ˚C, or room temperature for 6 h to remove GST tag.

3. Endogenous lipid removal via reverse-phase HPLC

3.1. Collect the cleaved Bla g 1 and concentrate it to ~2 mL using a centrifugal filter unit with a <10 kDa molecular weight cut-off.

3.1.1 Add <12 mL sample to the top of concentrator and spin at 5,000 *x g* for 10–15 min in a swing-bucket rotor.

NOTE: Sample volume and spin speed will vary based on the specific filter and the type of rotor employed. Consult manufacturer documentation prior to use.

3.2. Load the concentrate onto a 250 x 10 mm HPLC system equipped with a C18 reverse-phase chromatography column equilibrated with 97% buffer A (water, 0.1% trifluoroacetic acid) and 3% buffer B (acetonitrile, 0.1% trifluoroacetic acid).

NOTE: Smaller columns may be used, but protein may have to be loaded and eluted using multiple cycles to accommodate the reduced binding capacity. When selecting a column ensure that the resin beads have a particle size of < 5 µm and pore size of >200 Å to permit effective separation of protein-sized molecules

CAUTION: Trifluoroacetic acid is highly corrosive and should be dispensed within a fume hood using appropriate PPE (i.e., nitrile gloves, lab coat and goggles). Acetonitrile is both moderately toxic, volatile, and highly flammable, should be used and dispensed within a fume hood using appropriate PPE (i.e., nitrile gloves, lab coat and goggles).

3.3. Elute Bla g 1 using the protocol shown in **Table 1** at a flow rate of 1.5–4.0 mL/min. Monitor the elution process using the fluorescence absorbance at 280 nm.

3.3.1. Collect and pool Bla g 1 fractions. Bla g 1 normally elutes at >74% buffer B, or ~34–40 min.

NOTE: Elution time will vary slightly depending on the flow rate or column size. Collect fractions based on A_{280} for best results.

[Place **Table 1** Here]

3.4. Aliquot the sample into glass test tubes, filling no test tube more than halfway (~4 mL). Cover tubes with paraffin film and perforate the covering with two holes to allow venting.

3.4.1. Prepare a separate 1 mL aliquot (test aliquot). This will be used to determine the expected yield.

3.5. Freeze the samples and test aliquot by placing them in a -80 ˚C freezer for 1 h, or immersion in liquid nitrogen. In the case of the later, the tube must be rotated continuously to avoid test tube breakage due to expansion of the liquid phase upon freezing.

3.6. Dry the resulting delipidated protein samples using a lyophilizer. Dried protein may be stored at 4 ˚C for several months in a sealed container.

4. Reconstitution of Apo- and cargo-loaded Bla g 1

4.1. Determine the anticipated Bla g 1 yield.

4.1.1. Resuspend lyophilized, delipidated (post-HPLC) test aliquot in 5 mL of refolding buffer, (50 mM HEPES pH 7.4, 100 mM NaCl, 2% DMSO).

4.1.2. Heat the mixture in a water bath (500 mL beaker with 250 mL water and stir bar over a hot plate) to 95 ˚C. Vortex solutions intermittently and incubate at 95 ˚C for 0.5–1 h.

4.1.3. Remove heat and slowly let the water bath equilibrate to room temperature (~1 h). Annealed protein can be stored in this form overnight at 4 ˚C if needed.

4.1.4. Pass annealed Bla g 1-lipid mixture through a 0.22 µM syringe filter to remove particulate matter.

4.1.5. Buffer exchange the filtered protein 3x into PBS pH 7.4 using a centrifugal filter with 10 kDa cutoff as discussed in 3.1 to remove residual free fatty acids and organic solvent.

4.1.6. Assess protein concentration using BCA assay or other preferred method such as OD_{280}. Use this to determine the anticipated yield for the remaining Bla g 1 aliquots.

4.2. Reconstitute Apo- or cargo-loaded Bla g 1

4.2.1. Resuspend Bla g 1 aliquots in refolding buffer as described in 4.1.1.

4.2.2. To produce Apo-Bla g 1, repeat steps 4.1.2–4.1.6 to obtain desired yield.

4.2.3. To load Bla g 1 with fatty acids, prepare 20 mM stock solutions of the desired fatty acid cargo in methanol or DMSO.

4.2.4. To load Bla g 1 with phospholipids, prepare a 10 mg/mL stock of the desired cargo in chloroform inside a glass test tube.

4.2.4.1. Evaporate the chloroform to produce a lipid film. Add PBS to the test tube to produce a final phospholipid concentration of 20 mM.

CAUTION: Chloroform is harmful if inhaled or swallowed. Use in a chemical fume hood or employ respirator if inadequate ventilation is available. Employ nitrile gloves, lab coat and goggles when handling. Consult MSDS prior to use.

4.2.4.2. Rehydrate the lipid film by heating it above the phase transition temperature of the lipid cargo and vortexing until the solution turns cloudy. Note that sonication may be required to fully resuspend and rehydrate some cargoes.

4.2.4.3. If sonication is required, place the test tube in bath sonicator (100 W, 42 kHz) and sonicate at maximum power until cargo is resuspended. Alternatively, a probe sonicator (described in 2.6) may be used an 10–20% power with a 50% duty cycle.

CAUTION: Sonication employs high frequency sound waves which may damage hearing. Employ noise-suppressing PPE (earplugs or mufflers). If possible, place sonicator inside sound-dampening cabinet or chamber.

4.2.5. Add the desired fatty acid or phospholipid cargo to produce a 20x molar excess of ligands relative to Bla g 1 based on the anticipated yield determined in 4.1. The total volume of organic solvent added in this step should not exceed 2%. Vortex to mix.

NOTE: 1 L of Bl 21 DE3 cells typically yields ~0.25–0.4 nmol protein, corresponding to ~400 µM ligand per tube.

4.2.6. Anneal the protein as described in 4.1.

5. Confirming phospholipid cargo removal/loading via ^{31}P-NMR

5.1. Concentrate samples of Apo- or cargo-loaded Bla g 1 to >100 µM using a centrifugal filter unit as described in 3.1.

5.2. Rehydrate reference phospholipid in PBS buffer to final concentrations of 2, 1.5, 1, 0.5, and 0.25 mM.

5.3. Dilute samples 1:1 with cholate buffer (100 mM Tris pH 8.0, 100 mM NaCl, 10% w/v cholate) to a total volume of ~600 µL.

NOTE: Cholate is employed in this step to fully extract and solubilize lipids from the Bla g 1 hydrophobic cavity. This ensures that the chemical environment surrounding the phospholipid headgroups is consistent between different samples, allowing for its quantitative assessment using ^{31}P-NMR. The use of cholate can be substituted for chloroform/methanol as described previously[15].

5.4. Acquire 1D ^{31}P-NMR spectra of the cholate-solubilized Bla g 1 samples and reference phospholipid standards using a broadband probe.

NOTE: The ^{31}P-NMR spectra presented in this work were obtained using a 600 MHz spectrometer. However, previous studies employing similar techniques suggests that acceptable sensitivity can be achieved at fields strengths as low as 150-200 MHz[15].

5.5. Process the resulting data using appropriate software[16].

5.6. Obtain peak intensities using preferred NMR viewing software[17].

5.7. Compare the Bla g 1 ^{31}P-NMR spectra to those obtained for the phospholipid reference samples to confirm removal of endogenously bound ligands and/or binding of desired ligands based on the chemical shifts of the visible peaks (or lack thereof).

5.7.1. Confirm full binding stoichiometry by comparing the peak intensity of the Bla g 1 spectrum to that of the phospholipid reference standards.

6. Confirming Bla g 1 folding

6.1. Prepare 0.5 µM samples of Bla g 1 in CD buffer (100 mM KH_2PO_4, buffer pH 7.5). Load 1 mL of the sample into a 10 mm CD cuvette with magnetic stir bar.

6.2. Measure CD spectrum of Bla g 1 to confirm reconstitution of secondary structure. Ensure that HT voltage does not exceed manufacturer recommendations (generally 1 kV).

6.2.1. Measure CD signal from 260–200 nm at 25 ˚C with a data pitch of 0.2 nm and a scan rate of 20 nm/s with a data integration time of 1 s.

6.3. Increase the temperature in the CD cell from 25 ˚C–95 ˚C at a rate of 0.5 ˚C/min. Activate magnetic stir bar to ensure temperature is uniform across the sample.

6.4. Monitor CD at 222 nm, taking readings every 2 ˚C.

6.5. Fit resulting data to a 2-state Boltzman curve to determine the melting temperature. Due to the high stability of Bla g 1, the melting temperature (MT_{25}) was defined as the temperature at which the protein has lost 25% of its initial CD at 222 nm.

REPRESENTATIVE RESULTS:

Using affinity chromatography, recombinant GST-Bla g 1 was readily isolated to a high level of purity (**Figure 1A**), producing a yield of ~2–4 mg/L of cell culture. Overnight incubation with TEV protease at 4 ˚C is sufficient to remove the GST tag, yielding the final product at ~24 kDa. Note that in this instance there is a significant amount of GST-Bla g 1 in the flow-through and wash fractions, suggesting the Glutathione resin binding capacity was exceeded. The use of more resin or multiple cycles of sample loading and elution could provide remedy for this issue.

Applying the Bla g 1 to a reverse-phase C18 column yields a distinctive elution profile (**Figure 1B**), with two large peaks at ~50% buffer B, and a second large peak at ~75% buffer B. SDS-PAGE analysis of the resulting fractions suggest that the former correspond to the cleaved GST tag, while the latter corresponds to Bla g 1. Occasionally a third, smaller peak will occur in the middle corresponding to residual, un-cleaved GST-Bla g 1. The presence of this un-cleaved product can be eliminated by increasing the amount of TEV employed in the cleavage reaction or extending the incubation time. While incomplete cleavage will reduce the yield, the separation obtained on the C18 column is sufficient to ensure that the purity of the final Bla g 1 product remains uncompromised. A consequence of reverse-phase HPLC is that the final protein product is eluted into an organic solvent environment. While this facilitates removal of any hydrophobic ligands, removal of this solvent via lyophilization is required, yielding a fluffy white powder (**Figure 1C**).

Annealing of the protein is required to reconstitute the native Bla g 1 fold and can be carried out either in the absence or presence of a lipid cargo. Addition of DMSO to the dried Bla g 1 and phospholipid cargoes prior to the refolding buffer facilitates the solubilization process, though some longer chain lipid cargoes will not fully dissolve even at elevated temperatures. However, this was not observed to impact the loading efficacy among the lipids tested in our studies (**Figure 1C**). Similarly, excess lipids will often precipitate out of solution or form large vesicles upon cooling, resulting in a cloudy appearance after annealing (**Figure 1C**). This was also not observed to effect loading efficiency, and any aggregates are readily removed through the filtration and subsequent buffer exchange steps to yield a clear, transparent solution. Despite the harsh conditions, no thermolysis was observed for Bla g 1.

[Place **Figure 1** Here]

^{31}P-NMR spectra of Apo-Bla g 1 purified in this manner show no detectable phospholipids either by NMR (**Figure 2A**) or thin layer chromotography (data not shown). By contrast, similar spectra obtained for Bla g 1 loaded with a distearoylphosphatidylcholine (DSPC) phospholipid show a strong peak corresponding to the phosphatidylcholine headgroup. For comparison, a representative ^{31}P-NMR spectrum of Bla g 1 purified from recombinant *E. coli* without the use of the lipid removal/annealing protocol described herein (ecBla g 1) show a heterogeneous mixture of endogenous lipids extracted from the recombinant expression system (**Figure 2B**). Taking advantage of the quantitative nature of NMR, a standard curve can be produced using reference samples of known DSPC concentrations (**Figure 2C**). Comparing the ^{31}P signal intensity obtained from DSPC-Bla g 1 against this standard curve yields a binding stoichiometry of 4.7 ± 0.5 lipids per protein; a value that compares favorably to the predicted full binding stoichiometry obtained from in silico studies and structural analysis[9]. Note that this technique will only detect ligands which contain a ^{31}P nucleus such as phospholipids, lysophospholipids, lipopolysaccharides etc. However, this protocol can be easily adapted for ^{13}C-NMR analysis. In this case, methyl-^{13}C labeled fatty acids would be recommended due to its favorable NMR relaxation properties. Restricting isotopic labeling to a single site also facilitates spectral interpretation, as only a single peak is expected, while simultaneously reducing the cost relative to uniform ^{13}C-labeled counterparts. An alternative approach would be to employ mass-spec to identify bound ligands, as demonstrated in a previous study which identified a mixture of fatty acids as the natural cargo of Bla g 1 isolated from cockroach frass (nBla g 1) obtained from commercial sources[9]. However, the limited quantitation capabilities of mass spec precluded an accurate measurement of binding stoichiometry without sufficient standards.

[Place **Figure 2** Here]

Crystal structures of Bla g 1 reveal a unique fold consisting of 12 amphipathic alpha-helices. Circular dichroism represents a quick and convenient method to assess whether this fold has been successfully reconstituted after the annealing process. CD spectra for Apo- and lipid (nMix)-loaded Bla g 1 show minima ~220 and 210 nm indicative of a predominantly alpha-helical structure (**Figure 3A**). This spectrum is extremely similar to that obtained for ecBla g 1 and nBla g 1, providing further evidence that the native structure of Bla g 1 is successfully recovered. This

was further confirmed through the use of ^{19}F and ^{1}H-^{15}N solution-NMR, a full discussion of which is available elsewhere[9]. CD-based thermal denaturation assays show a cooperative loss of alpha-helical secondary structure indicative of a folded globular domain (**Figure 3B**). Analysis of the resulting melting temperatures (**Figure 3C**) show a significant increase upon nMix ligand binding. This elevated thermostability is in line with that calculated for nBla g 1, indicating that we are able to fully reproduce the natural state of Bla g 1. Note that ecBla g 1 also shows a similar, if not greater enhancement in thermostability, illustrating the potential for residual endogenously bound lipids to interfere with biophysical characterization of allergens purified using traditional FPLC-based approaches. In contrast, the ability to quantitatively remove and re-load hydrophobic cargoes from allergens such as Bla g 1 provides a unique avenue to examine the role of lipids in the allergic response. Here, we describe a method to examine the influence of lipid cargoes on the structure, stability, and endosomal processing of the allergenic proteins themselves, though other avenues of study could be considered.

FIGURE AND TABLE LEGENDS:

Table 1: Elution protocol for Bla g 1. Table illustrating the elution gradient employed in the isolation of Bla g 1 using a C18 HPLC column.

Figure 1: Initial purification of Bla g 1. (**A**) SDS-PAGE showing the soluble protein fraction following initial lysis (S); flow-through (FT), wash (W), and elution from the glutathione-sepharose column (E); and the final Bla g 1 product following TEV cleavage of the GST tag (TEV). The HPLC elution profile of the resulting Bla g 1 product following TEV cleavage is shown in (**B**). A_{280} is shown in blue, while the elution gradient (% Buffer B) is shown in green. Fractions corresponding to the cleaved GST tag (H1, H2), residual un-cleaved GST-Bla g 1 (H3), and purified Bla g 1 (H4) are indicated with red arrows at ~50%, ~65%, and ~74% Buffer B respectively. SDS-PAGE analysis of fractions H1- H4 are shown in (**A**) and labeled accordingly. (**C**) Representative images showing Bla g 1 at various stages of the annealing process. Note that the precise and extent of precipitate formation as depicted in ii and iii is dependent on the type of lipid cargo employed.

Figure 2: Verifying lipid removal and loading of Bla g 1. (**A**) ^{31}P-NMR spectra of Apo- (black) or DSPC-loaded Bla g 1 (red) prepared using the annealing protocol described in this work demonstrating the complete removal of lipids in the former, and the homogeneous loading of phosphatidylcholine (PC) lipids achieved in the latter. In contrast, Bla g 1 purified from recombinant *E. coli* without lipid stripping and annealing (ecBla g 1) shows a heterogeneous mixture of endogenous phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) lipids when analyzed using this method (**B**). A representative standard curve obtained from DSPC reference samples of known concentrations is shown in (**C**), from which the Bla g 1 binding stoichiometry can be obtained. Figures adapted from Foo et al. (2019) and presented under the Creative Commons CC BY License[9].

Figure 3: Confirming successful recovery of the Bla g 1 fold. (**A**) CD spectra of Apo- (black) or nMix-loaded (red) Bla g 1 purified and annealed using the protocol described herein, with minima at ~220 and 210 nm indicative of a predominantly alpha-helical structure consistent with the

available X-ray crystal structure. Both Apo- and nMix-loaded Bla g 1 spectra are extremely similar with that obtained for Bla g 1 purified from recombinant *E. coli* (ecBla g 1, green) or from its natural allergenic source (nBla g 1, blue) without the lipid removal and annealing protocol, further supporting the successful recovery of the native structure in the former. (**B**) Representative thermal profiles for Apo- (black) and nMix-loaded (red) Bla g 1 showing a sigmoidal curve indicative cooperative unfolding. nBla g 1 (blue) and ecBla g 1 (green) shown as reference. The calculated melting temperatures (MT_{25}) of Bla g 1 are shown in (**C**). Binding of nMix ligands (red) yields a significant increase in thermostability relative to Apo-Bla g 1 (black). This mirrors the trend observed for nBla g 1 (blue), suggesting that we are able to successfully recover the native state. The even greater stability observed for ecBla g 1 highlights the potential of endogenously bound lipids to interfere with biophysical characterization of allergens. MT_{25} values presented in C represent the mean value obtained from at least three independent trials. Error bars represent the corresponding standard deviation values. Figures adapted from Foo et al. (2019) and presented under the Creative Commons CC BY License[9].

DISCUSSION:

The protocol described in this work has been successfully applied to systematically study the lipid binding properties of Bla g 1. This revealed a correlation between cargo binding, thermostability, and endosomal processing, the latter of which was correlated with decrease in the generation of a known T-cell epitope with potential implications for immunogenicity[9,18]. In addition to Bla g 1, other allergens such as Pru p 3 and Bet v 1 have been shown to retain their endogenously-bound cargoes when purified using standard affinity and size-exclusion chromatography methods[13,19-22]. These unwelcome guests could alter the biophysical and immunological properties of these proteins in a similar manner, highlighting the need for techniques to ensure complete delipidation such as the one presented here.

While the use of reverse-phase HPLC in the purification of allergens has been described previously[2], coupling it with a thermal annealing protocol provides the rather unusual opportunity to reconstitute allergens with a range of natural and un-natural ligands, allowing users to probe lipid-allergen interactions. This thermal denaturation step was found to be essential for two main purposes. First, thermal denaturation is required to facilitate ligand access to their binding cavities which, due to their hydrophobic nature, are often buried away from the aqueous solvent[9,22]. Secondly, hydrophobic ligands such as fatty acids and phospholipids often form larger supramolecular structures such as micelles or vesicles when placed in an aqueous environment. The concentration of monomeric, or “free” ligands available for protein binding can be approximated using the critical micelle concentration (CMC). DSPC and other long-chain phospholipids have CMC values in the nM range, indicating that there are virtually no free ligands available for Bla g 1 binding. Even short chain lipids and fatty acids have CMC’s in the low µm to mM range, indicating that a large proportion of these ligands remain in the micellar or bilayer phase[23]. However, the high temperatures employed in our denaturation protocol disperses these larger aggregates, facilitating binding. Previous studies have typically employed prolonged incubation periods to facilitate this process. However, the lack of a thermal denaturation/annealing process raises doubts to the efficacy of loading. For example, incubating the mite allergen Der p 5 with the fluorescent fatty acid analogue 11-(Dansylamino)undecanoic

acid (DAUDA) yielded a binding stoichiometry of 0.66 despite possessing a large hydrophobic cavity on par with Bla g 1[24]. Likewise, the binding specificity and stoichiometry of plant nsLTPs were found to vary greatly depending on whether the lipids and protein are first solubilized in methanol prior to the addition of aqueous buffer, indicating that ligand and/or binding site accessibility was a limiting factor[25].

In addition to Bla g 1, we have successfully applied the same strategy to several other MA domain proteins from cockroaches and mosquito (*A. aegypti*), as well as Der p 2 (data not shown). We noted that both the Bla g 1 homologues and Der p 2 eluted at a different time than Bla g 1 from the C18 column (step 3.3). The elution gradients in this step may need to be optimized for other proteins. Alternatively, HPLC columns with a less hydrophobic stationary phase (e.g., C8) may be employed, though in the case of Bla g 1 the increased hydrophobicity of the C18 column was necessary to completely remove diacyl phospholipid contaminants from ecBlag 1. Despite the differences in biophysical and biochemical properties, we have found this protocol to be extremely robust and could be easily applied to other allergenic proteins. While the harsh conditions employed may present a potential limitation, the increased resilience observed for many allergens reduces its impact[26,27]. Indeed, several food and inhalation allergens such as Der p 2, Ber e 1, Ara a 6 and Lep w 1 have been observed to recover their structure and immunogenicity following thermal denaturation, though optimization of buffer conditions may be required[28–33]; for example reversible denaturation of nsLTP's (Cor a 8) and thaumatins (Mal d 2 and Act d 2) is only observed under acidic (pH <4) conditions[28,30,31]. Additionally, it should be noted that the authors did not attempt to optimize either the timing or temperatures employed in the annealing protocol. It is possible that ligand solubilization and protein folding/unfolding may be achieved using a lower maximum temperature as seen with Ber e 1 for which reversible denaturation is achieved at 82 ˚C[29]. The use of such measures is expected to expand the range of allergens to which this protocol can be applied.

Another important consideration when adapting this protocol to other allergen systems is the concentration of ligands required during the annealing process. In the case of Bla g 1 the expected yield is ~0.25–0.4 µmol of protein per 1 L cell culture. Given the demonstrated binding stoichiometry of 8 fatty acids or 4 diacyl chain lipids per allergen, a 20–40 fold molar excess of cargo (5–10 µmol) was employed. It should be noted that the lipid binding ability of Bla g 1 and its homologues is unique; for example nsLTP's are generally accepted to bind at most two lipid ligands[25] while lipocalins have less than 1 stoichiometry[34]. As such, complete loading of these types of allergens may be accomplished with a smaller excess of ligands. A final consideration when adapting this protocol to other allergen systems is the presence of disulfide bonds, which can be problematic if not properly formed prior to denaturing. One possible approach would be to carry out the annealing process in the presence of a reducing agent such as 2 mM DTT. The native disulfide bonds could be subsequently re-formed through the addition of reduced and oxidized glutathione as described for the peanut allergen fragment studied by Aalberse et al.[35]. In this case, recovery of the correct disulfide bonding should be empirically assessed by mass spectrometry[35].

In this work we describe a technique through which allergens can be delipidated and re-annealed with various phospholipid and fatty acid cargoes. However, there are many other classes of potentially immunogenic or adjuventising ligands present within allergic materials. For example, cat, dog, and mite allergens have been proposed to bind lipopolysaccharides (LPS) and other bacterial lipids from house dust[36], while the Bet v 1 has been shown to extract complex flavonoids from the pollen matrix[13]. The protocol described in this work can be easily adapted to explore the role of these lipids in a more detailed manner. As a proof of concept we have been able to demonstrate that the hydrophobic cavity of Bla g 1 is capable of binding lipoteichoic acid (LTA) from the cell walls of gram positive bacteria, but excludes LPS from gram negative species, potentially reflecting the greater number of acyl chains in the latter[9]. Taking this one step further, one could utilize the thermal denaturation/annealing protocol to incorporate fluorescent probes and other non-natural fatty acid analogues into allergen proteins. Indeed, we were able to load the hydrophobic cavity of the mosquito homologue of Bla g 1 with DAUDA, opening additional avenues to examine the effects of lipid ligands on allergenic disease.

ACKNOWLEDGMENTS:

We would like to thank Dr. Tom Kirby, Scott Gabel, and Dr. Robert London for their help and assistance throughout this work, along with Dr. Bob Petrovich and Lori Edwards for the use of their instrumentation and their assistance in generating the Bla g 1 constructs employed in this study. We thank Andrea Adams for assistance with the mass spectrometry, and Dr. Eugene DeRose for assistance with the NMR instrumentation. This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences, Z01-ES102906 (GAM) and Z01-ES043010 (LP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Radauer, C., Bublin, M., Wagner, S., Mari, A., Breiteneder, H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *Journal of Allergy and Clinical Immunology*. **121** (4), 847–852 (2008).
2. Dearman, R. J., Alcocer, M. J. C., Kimber, I. Influence of plant lipids on immune responses in mice to the major Brazil nut allergen Ber e 1. *Clinical and Experimental Allergy*. **37** (4), 582–591 (2007).
3. Ichikawa, S. et al. Lipopolysaccharide binding of the mite allergen Der f 2. *Genes to Cells*. **14** (9), 1055–1065 (2009).
4. Mueller, G. A. et al. The structure of the dust mite allergen Der p 7 reveals similarities to innate immune proteins. *Journal of Allergy and Clinical Immunology*. **125** (4), 909–917 (2010).
5. Reginald, K., Chew, F. T. The major allergen Der p 2 is a cholesterol binding protein. *Scientific Reports*. **9** (1), 1556 (2019).
6. Trompette, A. et al. Allergenicity resulting from functional mimicry of a Toll-like receptor

complex protein. *Nature*. **457** (7229), 585–589 (2009).
7. Angelina, A. et al. The lipid interaction capacity of Sin a 2 and Ara h 1, major mustard and peanut allergens of the cupin superfamily, endorses allergenicity. *Allergy: European Journal of Allergy and Clinical Immunology*. **71** (9), 1284–1294 (2016).
8. Soh, W. T. et al. Multiple roles of Bet v 1 ligands in allergen stabilization and modulation of endosomal protease activity. *Allergy: European Journal of Allergy and Clinical Immunology*. **74** (12), 2382–2393 (2019).
9. Foo, A. C. Y. et al. Hydrophobic ligands influence the structure, stability, and processing of the major cockroach allergen Bla g 1. *Scientific Reports*. **9** (1), 18294 (2019).
10. Machado, Y. et al. Fold Stability is a key factor for immunogenicity and allergenicity of the major birch pollen allergen Bet v1.0101. *Allergy: European Journal of Allergy and Clinical Immunology*. **137** (5), 1525–1534 (2016).
11. Mueller, G. A. et al. The novel structure of the cockroach allergen Bla g 1 has implications for allergenicity and exposure assessment. *Journal of Allergy and Clinical Immunology*. **132** (6) (2013).
12. Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D., Otzen, D. E. The major birch allergen , Bet v 1 , shows affinity for a broad spectrum of physiological ligands. *The Journal of Biological Chemistry*. **277** (26), 23684–23692 (2002).
13. Seutter von Loetzen, C. et al. Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochemical Journal*. **457** (3), 379–390 (2014).
14. Ibáñez-Shimabukuro, M. et al. Structure and ligand binding of As-p18, an extracellular fatty acid binding protein from the eggs of a parasitic nematode. *Bioscience Reports*. **39** (7), 1–16 (2019).
15. Beyer, K., Klingenberg, M. ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by 31P nuclear magnetic resonance. *Biochemistry*. **24** (15), 3821–3826 (1985).
16. Delaglio, F. et al. Nmrpipe - a multidimensional spectral processing system based on unix pipes. *Journal of Biomolecular NMR*. **6** (3), 277–293 (1995).
17. Johnson, B. A., Blevins, R. A. NMR View: A computer program for the visualization and analysis of NMR data. *Journal of Biomolecular NMR*. **4** (5), 603–614 (1994).
18. Dillon, M. B. C. *et al.* Different Bla-g T cell antigens dominate responses in asthma versus rhinitis subjects. *Clinical and Experimental Allergy*. **45**, 1856–1867 (2015).
19. Pasquato, N. et al. Crystal structure of peach Pru p 3, the prototypic member of the family of plant non-specific lipid transfer protein pan-allergens. *Journal of Molecular Biology*. **356** (3), 684–694 (2006).
20. Dubiela, P. et al. Impact of lipid binding on the tertiary structure and allergenic potential of Jug r 3, the non-specific lipid transfer protein from walnut. *Scientific Reports*. **9** (2007), 1–11 (2019).
21. Abdullah, S. U. et al. Ligand binding to an allergenic lipid transfer protein enhances conformational flexibility resulting in an increase in susceptibility to gastroduodenal proteolysis. *Scientific Reports*. **6**, 30279 (2016).
22. Derewenda, U. et al. The crystal structure of a major dust mite allergen Der p 2 , and its biological implications. *Journal of Molecular Biology.* **318** (1), 189–197 (2002).
23. Lipfert, J., Columbus, L., Chu, V. B., Lesley, S. A., Doniach, S. Size and shape of detergent

613 micelles determined by small-angle X-ray scattering. *The Journal of Physical Chemistry. B*. **111**
614 (43), 12427–38 (2007).
615 24. Pulsawat, P. et al. The house dust mite allergen Der p 5 binds lipid ligands and stimulates
616 airway epithelial cells through a TLR2-dependent pathway. *Clinical and Experimental Allergy*. **49**
617 (3), 378–390 (2019).
618 25. Douliez, J.P., Michon, T., Marion, D. Steady-state tyrosine fluorescence to study the lipid-
619 binding properties of a wheat non-specific lipid-transfer protein (nsLTP1). *Biochimica et*
620 *Biophysica Acta - Biomembranes*. **1467** (1), 65–72 (2000).
621 26. Ogburn, R. N. et al. Are dust mite allergens more abundant and/or more stable than other
622 Dermatophagoides pteronyssinus proteins? *Journal of Allergy and Clinical Immunology*. **139** (3),
623 1030-1032 (2017).
624 27. Cabrera, A. et al. Are allergens more abundant and/or more stable than other proteins in
625 pollens and dust? *Allergy: European Journal of Allergy and Clinical Immunology*. 1267–1269
626 (2019).
627 28. Offermann, L. R. et al. Structural and functional characterization of the hazelnut allergen
628 Cor a 8. *Journal of Agricultural and Food Chemistry*. **63** (41), 9150–9158 (2015).
629 29. Koppelman, S. J. et al. Reversible denaturation of Brazil nut 2S albumin (Ber e1) and
630 implication of structural destabilization on digestion by pepsin. *Journal of Agricultural and Food*
631 *Chemistry*. **53** (1), 123–131 (2005).
632 30. Smole, U., Bublin, M., Radauer, C., Ebner, C., Breiteneder, H. Mal d 2, the thaumatin-like
633 allergen from apple, is highly resistant to gastrointestinal digestion and thermal processing.
634 *International Archives of Allergy and Immunology*. **147** (4), 289–298 (2008).
635 31. Bublin, M. et al. Effects of gastrointestinal digestion and heating on the allergenicity of
636 the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin-like protein. *Molecular Nutrition*
637 *and Food Research*. **52** (10), 1130–1139 (2008).
638 32. Griesmeier, U. *et al.* Physicochemical properties and thermal stability of Lep w 1, the
639 major allergen of whiff. *Molecular Nutrition and Food Research*. **54** (6), 861–869 (2010).
640 33. de Jongh, H. H. J. et al. Effect of heat treatment on the conformational stability of intact
641 and cleaved forms of the peanut allergen Ara h 6 in relation to its IgE-binding potency. *Food*
642 *Chemistry*. **326** (April), 127027 (2020).
643 34. Glasgow, B.J., Abduragimov, A.R. Ligand binding complexes in lipocalins: Underestimation
644 of the stoichiometry parameter (n). *Biochimica et Biophysica Acta - Proteins and Proteomics*.
645 **1866** (10), 1001–1007 (2018).
646 35. Aalberse, R. C. et al. Identification of the amino-terminal fragment of Ara h 1 as a major
647 target of the IgE-binding activity in the basic peanut protein fraction. *Clinical and Experimental*
648 *Allergy*. **50** (3), 401–405 (2020).
649 36. Bublin, M., Eiwegger, T., Breiteneder, H. Do lipids influence the allergic sensitization
650 process? *Journal of Allergy and Clinical Immunology*. **134** (3), 521–529 (2014).
651

Figure 1

Click here to access/download;Figure;Figure1_JoVE61780R1.eps

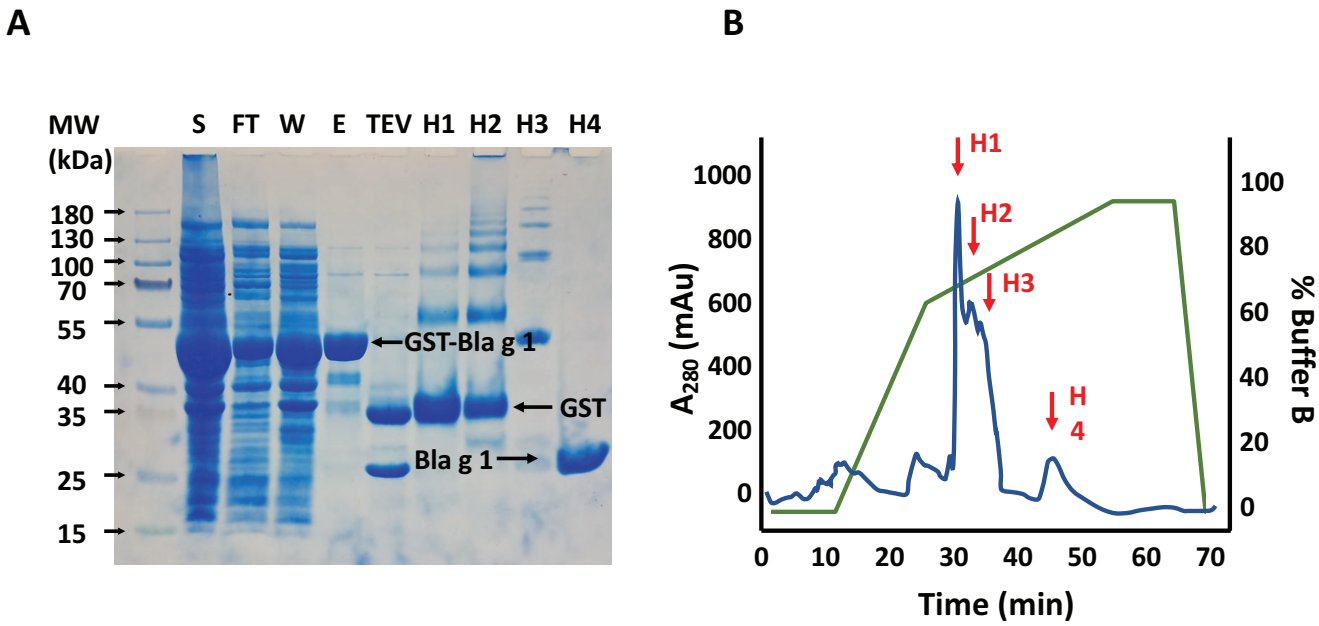

Figure 2

Click here to access/download;Figure;Figure2_JoVE61780R1.eps

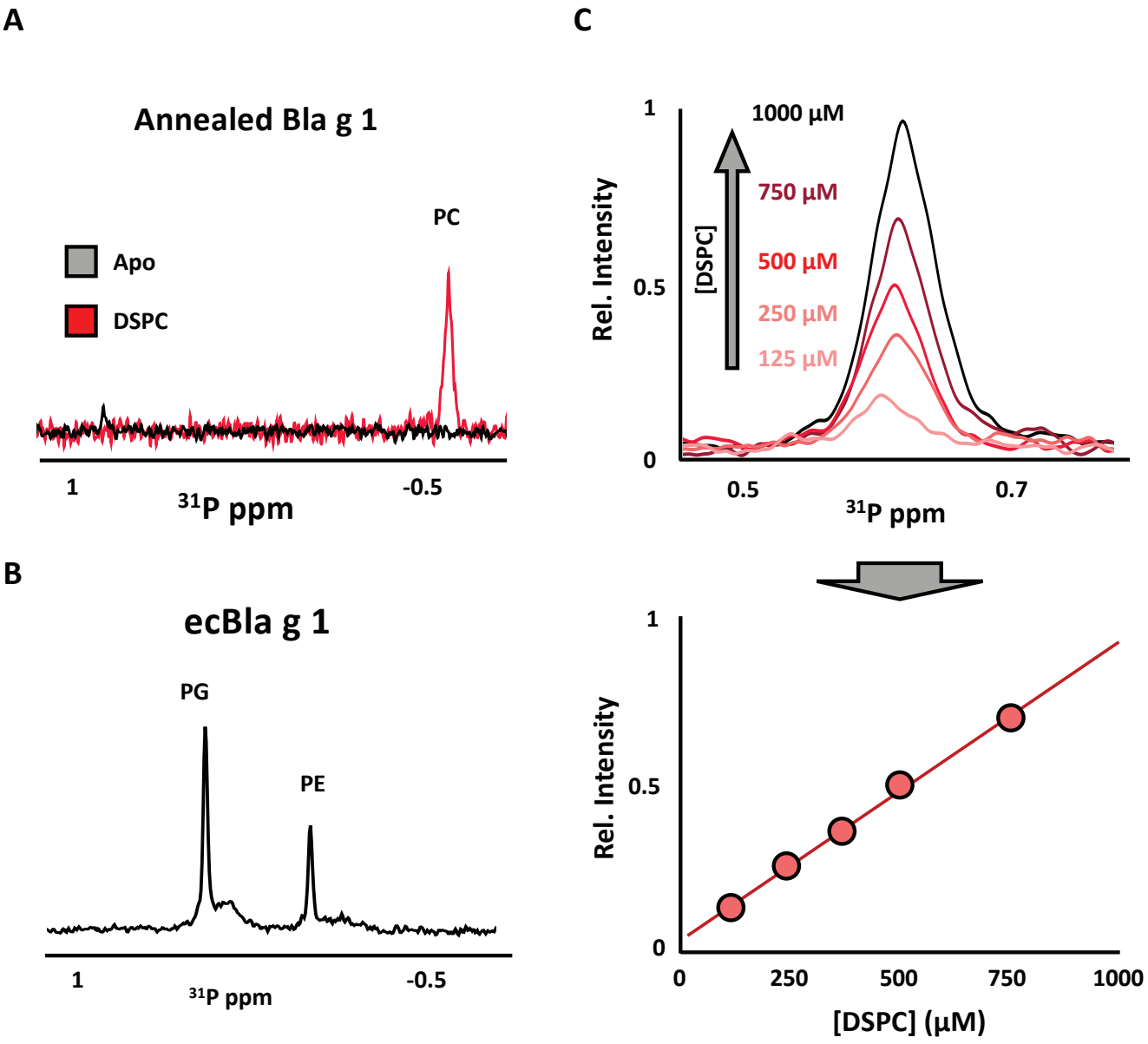

Figure 3

Click here to access/download;Figure;Figure3_JoVE61780R1.eps

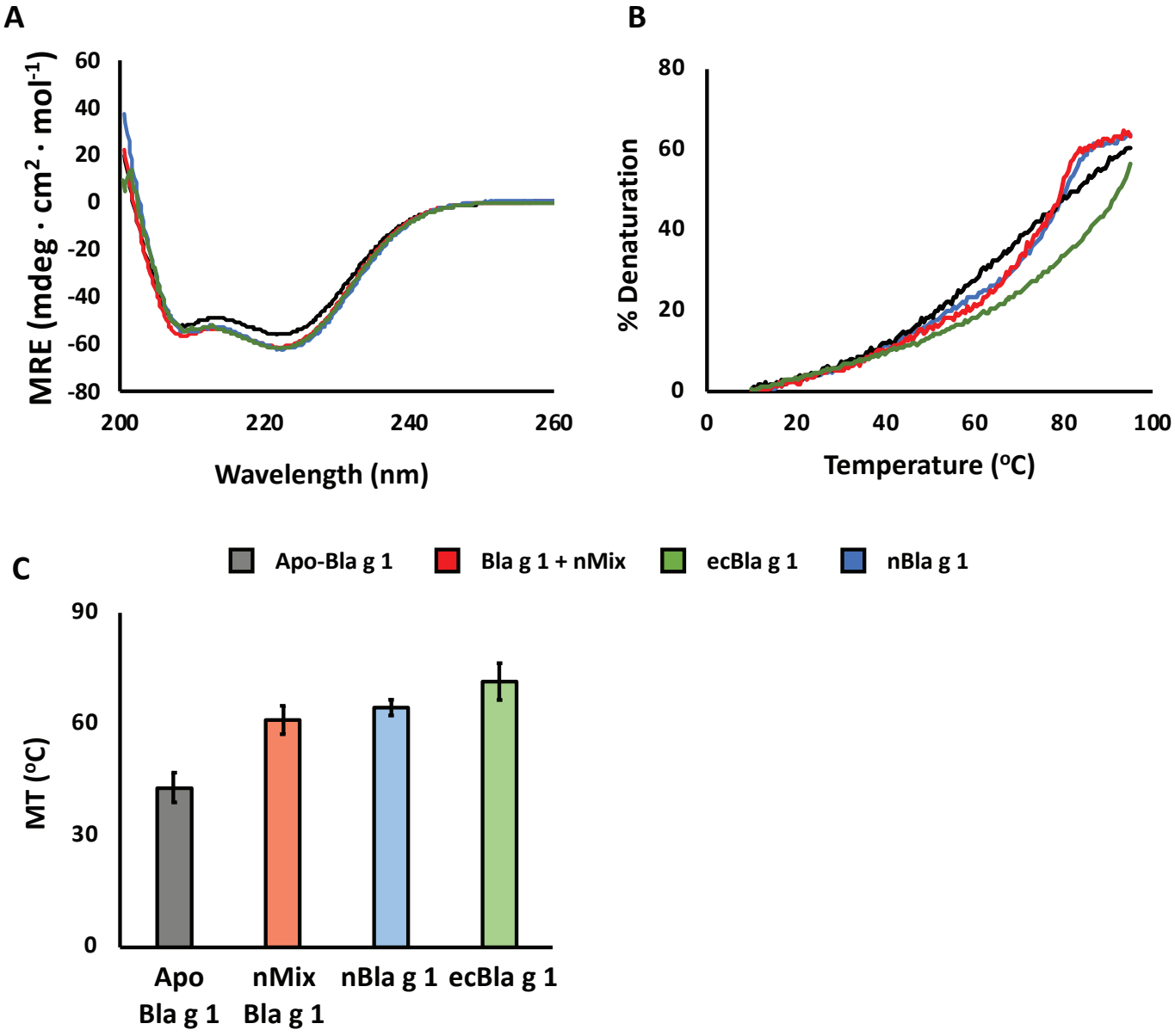

Table 1

Time (Min)	Buffer A (%)	Buffer B (%)
0	97	3
10	97	3
25	35	65
55	5	95
65	5	95
70	97	3

Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials_JoVE61780R1.xls

Name of Material/ Equipment	Company	Catalog Number
Bla g 1 Gene	Genescript	N/a
Affinity purified natural Bla g 1 (nBla g 1)	Indoor biotechnologies	N/a
Agilent 1100 Series HPLC System	Agilent	G1315B, G1311A, G1322A
Agilent DD2 600 MHz spectrometer	Agilent	N/a
Amicon Ultra-15 Centrifugal Filter Unit	Amicon	UFC-1008
Ampicillin	Fisher Scientific	BP1760-5
Benzonase	Sigma-Aldrich	E1014-5KU
Broad- band 5 mm Z-gradient probe	Varian	N/a
ChemStation for LC (Software)	Agilent	N/a
cOmplete Mini Protease Inhibitor Cocktail	Roche	11836153001
Distearoylphosphatidylcholine (18:0 PC)	Avanti Polar Lipids	850365C
E. Coli BL21 DE3 Cells	New England Biolabs	C2530H
Freezone 4.5 Freeze Dry System	Labconco	7750000
Glutathione Resin	Genescript	L00206
Glutathione, Reduced	Fisher Scientific	BP25211
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Fisher Scientific	34060
Jasco CD spectropolarimeter	Jasco	J-815
Millex Syringe Filter Unit	EMD Millipore	SLGS033SS
NMRPipe (Software)	Delaglio et al.	N/a
NMRViewJ (Software)	Johnson et al.	N/a
Oleic acid	Sigma-Aldrich	O1008
Pierce BCA Protein Assay	Sigma-Aldrich	BCA1-1KT
Polaris 5 C18-A 250x10.0 mm HPLC Column	Agilent	SKU: A2000250X100
SD-200 Vacuum Pump	Varian	VP-195
Sodium Cholate Hydrate	Sigma-Aldrich	C6445
Sodium Palmitate	Sigma-Aldrich	P9767
Sodium Stearate	Sigma-Aldrich	S3381
VnmrJ (Software)	Varian	N/a

Comments/Description

Custom gene synthesis service. GenBank Accession no AF072219 Residues 34-216
Custom order
UV Detector, Pump, and Degasser

Delaglio, F. *et al.* Nmrpipe - a Multidimensional Spectral Processing System Based On Unix Pipes. *J. Biomol. NMR* **6**, 277–293 (1995).
Johnson, B. A. & Blevins, R. A. NMR View: A computer program for the visualization and analysis of NMR data. *J. Biomol. NMR* **4**, 603–

614 (1994).

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health
National Institute of
Environmental Health Sciences
MD-MR01
P. O. Box 12233
Research Triangle Park, NC 27709
Website: www.niehs.nih.gov

Dr. Vineeta Bajaj,
Review Editor
Journal of Visualized Experiments

Dear Dr. Bajaj,

Thank you for the opportunity to revise our manuscript (JoVE61780) entitled “Removal and Replacement of Endogenous Ligands from Lipid-Bound Proteins and Allergens”. We found both the editorial and reviewer comments helpful in revising the manuscript. We have carefully considered and responded to each suggestion, and implemented the appropriate changes to the manuscript.

Please find attached our revised manuscript, along with a point-by-point response to both the editorial and reviewer comments. Author comments and responses are coloured in blue, with the corresponding changes highlighted in the manuscript file.

Thank you once again for your consideration

Sincerely,

Geoffrey A. Mueller, Ph.D.

Author comments and response in Blue

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 1.1: List the source in the table of materials.
2) 1.2: How is subcloning done? Please describe or cite appropriate references.
3) 1.3: Describe the transformation step or cite a reference.
4) 2.1: How many cells?
5) 2.2: What is the cell density?
6) 2.4: Mention step number where harvest is described.
7) 2.6, 4.2.3.3: mention sonicator amplitude (Watt) and frequency (Hz).
8) 3.1: Mention centrifugation speed and duration.
9) 4.2.3.1: Mention all safety precautions while handling chloroform.

Changes have been implemented accordingly.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Highlighting has been adjusted accordingly to remove some steps which cannot be easily filmed.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

• **Figures:**
1) Remove the text “Figure #”.
2) Fig 2C: Remove the space between µ and M.
Appropriate changes have been implemented

• **Tables:**
1) Remove the embedded table from Line 186. Please number it and attach it as an Excel file. Ensure that a legend is provided in the manuscript. Appropriate changes have been implemented

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Note that some of the figures in this manuscript are adapted and modified from a previous publication. These figures are available for reuse and adaptation for any purpose (even commercially) as per the Creative Commons CC BY license so long as the appropriate attribution is provided. Note that a link to the license (http://creativecommons.org/licenses/by/4.0/) should be included in the final published version.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors provide a straight forward and apparently generalized protocol for the preparation of lipid binding proteins free of endogenously bound ligands and additional forms where the ligands have been replaced with those of known composition. Removal or replacement of undesired ligands has been demonstrated to impact structure and alter immune responses. As a result, this manuscript will be of interest to a wide number of researchers conducting immunological, biochemical, and structural studies of allergens and the side range of other proteins that strongly bind hydrophobic ligands including lipid and fatty acid moieties. The manuscript is clearly written and well organized with results strongly supporting their findings. I expect that the manuscript will be of general interest and provide a helpful resource to the community.

Major Concerns:
None

Minor Concerns:
How was the timing of the reconstitution step optimized? For example, was a time course for ligand occupancy initially conducted? It would be helpful for the authors to indicate which steps and parameters may warrant further system specific optimization.

The reviewer brings up a good point in that the reconstitution step was not optimized beyond ensuring complete lipid removal. Given the incredible resilience of Bla g 1 there was no need for further optimization beyond ensuring complete lipid removal and re-loading as demonstrated in the representative results. However, this may be an important consideration for other allergen systems as alluded to by the reviewer and we have included a statement in the discussion that optimization of these parameters may be required for other allergen systems. (Lines 568-573)

Lipid binding proteins frequently contain disulfide bonds. While presumably speculative, do the authors have insight as to whether the protocol is amenable to accommodate these proteins and for driving disulfide formation during reconstitution?

We have altered the discussion to include potential modifications one may employ when applying our protocol to disulfide containing proteins (Lines 594-599). The Bla g 1 allergen for which this protocol was developed does not contain any disulfides. As such we have a limited ability to comment on this aspect directly and advise that readers optimize conditions based on their specific allergen system.

Reviewer #2:
Manuscript Summary:
This written manuscript, which would be accompanied by a video tutorial, describes a protocol to delipidate allergen proteins and exchange on a desired phospholipid in a robust and quantitative manner. Often such proteins are recombinantly expressed using a bacterial host, and during purification steps allergens can copurify with bound bacterial lipids. Therefore, methods for removing bacterial lipids and methods to validate binding of a desired ligand should be of interest to the allergen field — and likely to other fields that study lipid binding proteins. A detailed protocol is provided to produce a specific alergen (Bla g 1), remove bacterial lipids and exchange-on a "cargo" ligand such as a phospholipid, and validate the binding of the phosholipid using 31P NMR. The proposed method is a unique use of 31P NMR and should be generally accessible to most research groups that have an on-site NMR spectroscopy facility.

Major Concerns:
None

Minor Concerns:
Where as portions of the protocol could be relevant to lipids in general, the 31P NMR method is very specific to phospholipids. Perhaps this should be stated upfront in the title, abstract, and introduction. The authors do mention it may be possible to generalize the NMR protocol to other types of lipids that do not contain phospholipids by using 13C labeled fatty acids + 13C NMR methods. It would be useful to include product numbers to 13C-labeled lipids as examples, any potential complications (i.e., are the fatty acids isotopically labeled at a single methyl group or throughout? how would that affect the NMR analysis?) and comment on the cost associated with such ligands relative to native lipids that are not isotopically enriched.

The reviewer is correct in that the ^{31}P NMR portion of the protocol is specific for phospholipids. We have altered the title of Step 5 to reflect this. It should be noted that ^{31}P NMR is presented as a confirmational tool only; the rest of the protocol remains broadly applicable to other hydrophobic ligands. Additionally, other biophysical techniques presented in this manuscript and elsewhere (Mueller et. al. 2013; Foo et. al., 2019); Foo et al. Manuscript in Preparation) have been used to further demonstrate the removal and loading of non-phospholipid ligands into Bla g 1 and other MA domain

proteins. As such we believe that the protocol described in this work is generally applicable to a range of hydrophobic ligands, and that the current title, abstract, and introduction accurately reflect this versatility.

With regards to the ^{13}C-NMR experiments, the representative results (Line 419) indicates that methyl-labeled fatty acids are recommended. The relevant section has been modified (Lines 419-423) to highlight this, and to provide further insight into the isotopic labeling scheme suggested. Unfortunately, the author guidelines indicate that the use of company and brand names should be avoided. . With regards to the reviewer's concerns, ^{13}C-fatty acids are readily available from Cambridge Isotope Laboratories, Sigma-Aldrich, and other such suppliers at a cost of ~$200/gm vs ~40/gm for the unlabeled equivalent). Access to such reagents should not represent a major barrier to their use by other researchers.

Reviewer #3:
Manuscript Summary:
The manuscript "Removal and replacement of endogenous ligands from lipid-binding proteins and allergens" by Foo CY et al, describes a method to remove natural lipid ligands from proteins and replace them with user-defined mixture of fatty acid or phospholipids. This very descriptive manuscript contains an interesting work although there are some details that the authors must clarify in order to be ready for publication. The title should be changed because Bla g 1 does not belong to the lipid-binding protein family although is a protein that can bind lipids as many allergens. I suggest to change lipid bind proteins and allergens by lipid biding allergens or something similar to simplify. Although the protocol refers any protein, allergen or not, in this case authors are speaking about Bla g 1, which is an allergen.

The title has been changed accordingly

Major Concerns:
1. Bla g 1 cloning, the number of repeats of the MA domain of the allergen has to be included in the text of the manuscript. Bla g 1 shows a primary structure containing ~100 amino acid repeats, but Bla g 1 is the whole protein?

The reviewer is correct in that the Bla g 1 gene includes many MA domain repeats. For the sake of simplicity, we have isolated a single repeat, and employed that throughout the course of our studies. We have altered 1.1 to make this distinction clearer and provided the appropriate details and references.

2. The reverse-phase HPLC coupling with thermal annealing protocol is adequate in this case, but cannot be generalized to most allergens or proteins, even considering that lipid presence helps to this renaturation, because most of them after the thermal treatment become partially denatured in an irreversible way. In fact, there are few allergen families that recover their 3D-structures after these treatments. Clarify these aspects in the manuscript.

The authors acknowledge that the harsh conditions employed in this protocol represent a potential limitation. In recognition of this, the following statement was included in the discussion of the initial submission.

"While the harsh conditions employed may present a potential limitation, the increased resilience observed for many allergens reduces its impact."

While the authors have not extensively tested the applicability of their protocol across all allergen families, the literature describes several food and inhalation allergen families which display reversible thermal denaturation. Examples of such proteins have been provided in the discussion (Lines 564-573), along with a statement describing the system-specific optimization required to yield these results. Together, we feel that these alterations allow the manuscript to better reflect both the versatility and limitations of this protocol while simultaneously the need to assess the suitability of this approach for a given biological system on a case-by-case basis, and to optimize the refolding conditions accordingly.

The authors would also like to note that the thermal denaturation and annealing process described in this work is conceptually similar to purification of allergens from inclusion bodies. Here, the proteins are completely denatured through the addition of urea or guanidinium hydrochloride, and subsequent refolded through the gradual removal of the denaturing agent. This approach has been successfully applied to a range of proteins including the allergens Bet v 1, Der f 2, Phl p 5, and Gly m 3 [1–4] suggesting that the native fold of many allergenic systems can be successfully recovered following complete denaturation under harsh conditions.

3. Point 2.6. It is important to describe in this type of articles well the characteristics of the columns used for the purification of these allergens by means of HPLC. A protein so large for this type of chromatography needs a very determined particle size to be able to isolate the protein in its native structure. A statement on pore and particle size has been added to 3.2

4. Point 3.3.1. 74% is a very high percentage of acetonitrile at which is difficult to separate proteins. Can authors include the acetonitrile % of H1, H2, H3? In this sense, authors should include the acetonitrile gradient in Figure 1B.

The high acetonitrile concentration reflects the extremely hydrophobic nature of Bla g 1 in its unfolded state. Many allergens will elute at lower concentrations and the protocol indicates that the reader may need to adjust the elution gradient and column material for optimal results based on their specific allergen system. In recognition of the difficulty of separating proteins at this concentration, the elution gradient employed in the purification of Bla g 1 is extremely shallow between 65% and 95% acetonitrile to enhance separation. The reviewer's suggestion of including the elution gradient in figure 1B would be very useful in illustrating this, and has been implemented accordingly. The legend of Figure 1B has been appended to include approximate %Buffer B values.

5. Why do not the authors measure the protein concentration with the extinction coefficient of this protein, theoretical or experimental, and an absorption spectrum? It is clearly a more accurate method, especially in these assays where is critical the lipid-protein stoichiometry.

The Bla g 1 construct employed in this work contains very few aromatic amino acids and no tryptophan residues, giving it a very low extinction coefficient and making it difficult to accurately quantify using UV-VIS spectroscopy, particularly at low concentrations. The use of OD_{280} might be preferable for other allergen systems, and step 4.1.6 has been modified to suggest the use of more accurate methods when possible

6. The incubation of Bla g 1 protein and fatty acids, prepared in ethanol, contains ethanol in the buffer?

Due to the limited solubility of hydrophobic ligands in aqueous solvents, organic solvents such as methanol are required to carry them into solution during the refolding process. As such, a small amount of organic solvent will be present during the refolding process. These solvents are removed during the subsequent buffer exchange steps. We have amended 4.2.5 to clarify this point and provide a warning concerning the use of excessive concentrations of solvents during the refolding process.

The authors note that there was a typo in the initial submission, and the correct solvent should be methanol (corrected in 4.23)

7. Is it correct the optical pathway of the CD cuvette? Is it 10mm? That is correct. A larger cuvette is required to accommodate the magnetic stir bar described in 6.1.

8. The authors should use the ellipticity unit (deg · cm 2 / dmol). Graph has been changed to units of mean residue ellipticity (MRE)

9. Line 309. Is 24 kDa the molecular mass of the allergen? SDS-PAGE indicated a 15 kDa for the protein.

The incorrect SDS-PAGE protein ladder was used to generate the labels in Figure 1A. The authors thank the reviewer for bringing the issue to their attention, and have rectified it accordingly

Minor Concerns:
Introduction section, line 60. Authors make reference to lipid fraction present in the Bertholetia extract that is added together with the recombinant protein Ber e 1. However, Der p 7 is a lipopolysaccharide binding protein (LBP) which specifically binds a lipid as natural ligands. Authors should clarify these differences in the introduction.
Ber e 1 and Der p 2/Der p 7 are presented as examples in which endogenous lipids could enhance allergenicity. However, the statement concerning TLR4-mediated signaling was intended to refer specifically to Der p 2 and Der p 7, and not Ber e 1 as the reviewer has noted. Lines 64-66 have been altered to clarify this distinction.

Introduction, Line 65, Change physical by biophysical properties.
Point 3.5. Specify that tubes in the liquid air must be continuously rotated to distribute the sample well on the surface of the tube walls and to avoid breakage.
Introduction section, Line 74, Major Allergen domain family. Change implemented
Point 4.1.5. Buffer exchange to remove the DMSO…Include in the text.
Line 80, correct P. pistoris by P. pastoris Change implemented
Appropriate changes have been made to address the above points

Line 82, The order that authors follow to describe the different fatty acids considered as natural ligands for this protein, describes the different affinities of each one.

The three fatty acids (oleate, palmitate, and stearate) were identified in Bla g 1 obtained from its natural allergen source. However, neither the relative ratios of these fatty acids within the Bla g 1

binding side, nor the specific affinity of Bla g 1 for these ligands have been quantified. As such we are unable to offer any statement on binding affinity. As a reflection of this reality, Line 85 indicates that the three ligands are present, but does not attempt to offer any further statement on the relative proportions or affinity.

In line 82 you include laurate among the fatty acids specifically bound to Bla g 1. However, in line 85 authors talk about oleate instead laurate. Are both fatty acids natural ligands of the allergen? The correct ligand should be oleate. Laurate was added as a typo, and has since been corrected
Line 173, include the completed name of PPE Change implemented
Line 175, moderately toxic and easily breathable and highly …….. Change implemented to indicate volatility of acetonitrile
Line 178, Point 3.3, Elute Bla g 1. There is a typo in the name of this allergen Change implemented
Line 199, sealed container. It is a typo. Change implemented

Reviewer #4:
Manuscript Summary:
In their manuscript "Removal and Replacement of Endogenous Ligands from Lipid-Binding Proteins and Allergens", Foo et al. describe a protocol to remove lipids and other unwanted cargo from protein allergens that are produced by recombinant expression. In addtion, a protocol for reconstitution of protein loaded with a defined ligand is presented.

Being able to recombinantly produce defined samples of allergens is critical for understanding how these proteins cause allergic sensitization, in particular regarding the fact that ligands have the potential to modulate this process.
The protocols described in the manuscript are generally applicable and should be straightforward to implement in any laboratory. The manuscript is well organized and written, provides all information that is required to perform the experiments. Possible modifications that may be needed for different proteins are discussed (with few exceptions, see below). This manuscript definitely of interest to readers of "The Journal of Visualized Experiments".

Minor Concerns:
Variations of the protocol may be required for different allergens, for example regarding the choice of solvent for the re-loading process. Chloroform and ethanol are described as suitable solvent for fatty acids and phospholipids in Section 4.2 of the protocol. Other solvents (e.g., DMSO) might be better suited for ligands of the PR-10 family of allergens, which are likely flavonoids. The authors may want to comment on this.

4.2.3 has been appended to include the use of DMSO if desired. The initial experiments that were carried out using this protocol employed DMSO to carry out this function, with no apparent influence on Bla g 1 loading and re-folding.

On another note, it might be an option to use 1-dimensional 1H NMR to probe and/or confirm the correct folding of the allergen, complementing circular dichroism (Section 6 of the protocol).

That is correct. The authors have previously used both ^{19}F and ^{1}H-^{15}N NMR to demonstrate recovery of the a-helical Bla g 1 fold. The authors feel that a full discussion of these techniques is beyond the

scope of the current work. Nonetheless the representative results section has been modified to make a note of these possibilities, and direct readers towards the appropriate reference (Lines 445-447)

Figure 2B: 31P NMR spectrum of ecBla g 1: It is not clear what "PG" and "PE" are standing for. Please define in figure caption. Figure legend has been modified accordingly

Some PR-10 allergens are fairly heat sensitive (e.g., Mal d 1) and may percipitate and/or not refold properly after thermal annealing at high temperatures (95 C for Bla g 1, Section 4 of the protocol). If so, this will of course be noted in Section 6, where the folding of the protein is probed, or even before. Nevertheless, do the authors have any experimental hints or evidence that lower temperatures might work as well? This might be an option for more temperature sensitive allergens. Any comment on this would be useful for the reader.

The reviewer introduces a valid point. As mentioned in response to Reviewer 1, the use of lower temperatures could potentially increase the applicability of this protocol to other allergen/ligand systems. While we have not explored this possibility within the context of this work, the manuscript has been modified (see response to reviewer 1) to discuss this possibility. It should be noted that the primary goal of the elevated temperatures is to ensure both the complete unfolding of the target protein, as well as the solubilization of the desired ligand. The resilient nature of Bla g 1 necessitates incubation at 95^{o}C to achieve the former. However lower temperatures may be sufficient to achieve both objectives when applied to other protein systems, an example of which is provided (Line 572) for further guidance.

Reviewer #5:
Manuscript Summary:
The manuscript describes the experimental protocol necessary to remove ligands from a protein and eventually to reload it with selected ligands. In particular the authors concentrate on the allergen Bla g 1 reporting the protocol they devised in the original article Scientific Reports (2019) 9:18294.

Major Concerns:
1) Annealing: a) it should be indicated that this protocol is applicable only to proteins that exhibit reversible thermal unfolding, I believe a check for that ought to be included, b) I am puzzled by the fact that the same annealing procedure can work out both to remove or load ligands. In fact in both cases the unfolded protein is left to return to room temperature and therefore to refold in the presence of the ligand for which the protein has affinity. Why in one case should remain empty and in the other should bind the ligand?

a) The authors acknowledge that this represents a potential limitation to the protocol presented herein. In response to comments from Reviewer 3, a section has been added in the discussion further highlighting this limitation and stressing the need for readers to optimize denaturation and annealing conditions for their specific applications (Lines 564-573). This addition also addresses the limitations raised here by Reviewer 5.

b) Lipid removal and loading are two separate processes. In our protocol, reverse-phase HPLC is employed separate the protein from any endogenously bound lipids. The resulting protein is then subjected to the annealing protocol in order to recover its native protein fold in *either* the absence *or* presence of a defined ligand, yielding Apo- or ligand-loaded Bla g 1 respectively. When endogenous lipids are not present during the annealing phase there is no possibility of these

ligands re-binding Bla g 1. Generally, we test the yield of protein first without the lipids so that we can adjust the stoichiometry for the loading. To clarify this further, step 3 has been re-named and 4.1.1 has been appended to better reflect this separation of functions.

2) Protein refolding: in line 103-104 the authors write that correct refolding will be verified by CD and solution NMR, however subsequently nothing is indicated with respect of solution NMR experiments. If only CD is used, then it is advisable to record also the near-UV spectrum.

The recovery of the Bla g 1 fold using this protocol has been confirmed using ^{1}H-^{15}N NMR and published elsewhere [5]. However, it was decided not to include these results in the current work. Line 109 of the introduction has been altered to reflect this. A note of this has been included in the representative results section directing readers to the appropriate publication.

3) P31 NMR: a) the way it is described the simple detection of a P31 signal does not give absolute certainty that the phospholipid is bound inside the protein pocket. B) I consider necessary to indicate the magnetic field that assures an acceptable sensitivity.

a) The reviewer is correct in that the ^{31}P-NMR experiments described herein are unable to differentiate between Bla g 1-bound and unbound lipids due to the use of cholate to extract and solubilize these ligands as discussed in the note following 5.3. This is performed to accurately quantify the lipid content and not to directly assess binding. Binding is inferred from the experimental design that pellets excess lipids and exchanges the buffer extensively to remove residual excess soluble lipid. Control experiments with no protein have suggested this is more than adequate.[5]

b) A note has been included following 5.4 indicating the magnetic field strength employed in the current study, and to comment on the possibility of carrying out similar experiments on lower-field instruments

Minor Concerns:
1) I do not understand why the title of 4.2 includes also reconstitution of Apo Bla g 1.
Step 4.2.1 has been added to address this issue
2) line 196: "emersion" should be changed for "immersion" Correction implemented.
3) line 436 "to" should be added between "required facilitate" Correction implemented.
4) line 458 "the" between "that the both" should be removed Correction implemented.
5) line 487 "of" should be added between "cavity a" Correction implemented.
6) line 516 pages 909-917 must be added to reference 4 Correction implemented.

Reviewer #6:
Manuscript Summary:
* The authors described a protocol to remove endogenous lipids from allergens and replacement using a mixture of lipids. They presented results obtained for the cockroach allergen Bla g 1. Figure 2 has already been published in a previous article of the group, Scientific reports, 2019 (Ref 7).

Major Concerns:
The confirmation of Bla g 1 and lipids interaction was based in the 31P NMR experiments, but the

protocol is not clear:
* Was it performed in the presence of cholate?

The use of cholate in step 5.3 is to fully extract bound phospholipids from the Bla g 1 cavity into the aqueous phase. As such, cholate is present in the NMR sample, and the wording of step 5.4 has been altered accordingly.

To address the reviewer's subsequent comments, the use of cholate is intended to extract and solubilize the phospholipids from the Bla g 1 hydrophobic cavity. This ensures a consistent local chemical environment for the phospholipid headgroups, allowing for accurate quantitation and comparison between different samples. As such, we do not expect to see a second peak corresponding a Bla g 1-bound species. The note following step 5.4 has been adjusted to clarify this point.

Fig 2C does not represent a titration curve, but rather a standard curve that is obtained by measuring the ^{31}P peak intensity of pure DSPC samples of fixed concentrations. As such, no saturation is expected. The binding stoichiometry of Bla g 1 was calculated by quantifying the ^{31}P peak intensity of DSPC-Bla g 1 and comparing this value to that of the standard curve in order to determine the DSPC concentration. This is then compared with the concentration of Bla g 1 in the sample to obtain a binding stoichiometry (ie: the ratio of DSPC: Bla g 1). Lines 413-416 have been adjusted to better represent this distinction.

* Section 4 mention that 2 % of DMSO was used in the reconstitution step. Was it also present in the NMR sample?

Reconstitution of Ba g 1 is followed by a buffer exchange step in which the DMSO is removed. Step 4.1.5 has been altered to reflect this detail.

* What is the effect of cholate and DMSO on Bla g 1?

As mentioned above, cholate is predicted to denature Bla g 1, allowing for the extraction of any bound ligands into the aqueous phase. DMSO is removed during the refolding and annealing step, and thus should not have any effect on Bla g 1 structure or stability as described in this work.

* The 31P spectrum in the presence of lipids present only one peak, don't you expect two peaks? One for the bound to the protein and another for the lipid in the micelle? See above
* Furthermore, Fig2 shows no saturation. The peak intensity increases with the lipid added. How did the authors calculate the stichometry? See above
* Using this result the authors found 4.7 lipids bound per protein. Is it expected? Usually 2 molecules are able to bind.

Bla g 1 is a unique protein fold expected to have a binding stoichiometry of ~4 phospholipids per protein based on previous structural and molecular dynamics studies. [5] It is true that other lipid-binding proteins such as non-specific lipid transfer proteins (nsLTP's) have a binding stoichiometry of two.[6] We refer to nsLTP's in this manuscript as an example of a lipid binding protein to which our protocol may be applied. However, Bla g 1 is both structurally and evolutionarily distinct from nsLTP's, and as such are not expected to share their binding stoichiometry. This difference is further highlighted in the discussion, in which the binding stoichiometry of Bla g 1 is contrasted with that of

nsLTP's in lines 579-586.

Minor Concerns:
* The legend of Figure 1 is confusing. Legend has been altered accordingly.
* The protocol of nBla purification was not included

nBla g 1 was purchased from a third party. Line 425 has been altered to reflect this, and the relevant details have been added to the of materials and reagents.

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Removal and Replacement of Endogenous Ligands from Lipid-Binding Proteins and Allergens
Author(s):	Alexander C.Y. Foo, Peter M. Thompson, Geoffrey A. Mueller

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☑ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.