

Chapter 3

Recombinant Allergen Production in *E. coli*

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Abstract

Recombinant protein allergens have been used in allergy studies, allergy diagnosis, and epitope mapping. Messenger RNAs (mRNAs) are isolated from tissues of interest for complementary DNA (cDNA) library construction. Subsequently, the allergen gene is amplified by polymerase chain reaction (PCR) and sequenced. The amplified gene is then cloned into an expression vector, expressed in *Escherichia coli* cells, and purified from the cell lysate. This chapter describes the protocols for recombinant allergen production.

Key words Allergy, Food allergen, Recombinant allergen

1 Introduction

Type I food allergies are induced by food proteins (allergens) through cross-linking immunoglobulin E (IgE) molecules on the surface of mast cells and basophils [1]. Approximately 400 proteins in >170 foods have been reported to cause type I allergic reactions [2]. In the US, milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybean are the most common offending foods, accounting for over 90% of food allergies [3]. Considerable research has been conducted to identify and characterize the specific allergens in these foods. Although native allergens are the optimum choice for analysis, they are difficult to isolate and their properties may vary between samples due to presence of multiple isoforms, cultivar variations, different developmental stages, and environmental influences. Alternatively, molecular cloning techniques are useful in producing large quantities of molecularly defined homogenous allergens of consistent purity and quality. Therefore, recombinant allergens have been widely used in food allergy diagnosis, assessing cross-reactivity, epitope mapping, and analyzing the effects of food matrix, food processing, and digestion on allergen immunoreactivity [4]. A comparison between the immunoreactivity of native and recombinant almond major allergen amandin is illustrated in Fig. 1. Detailed information on

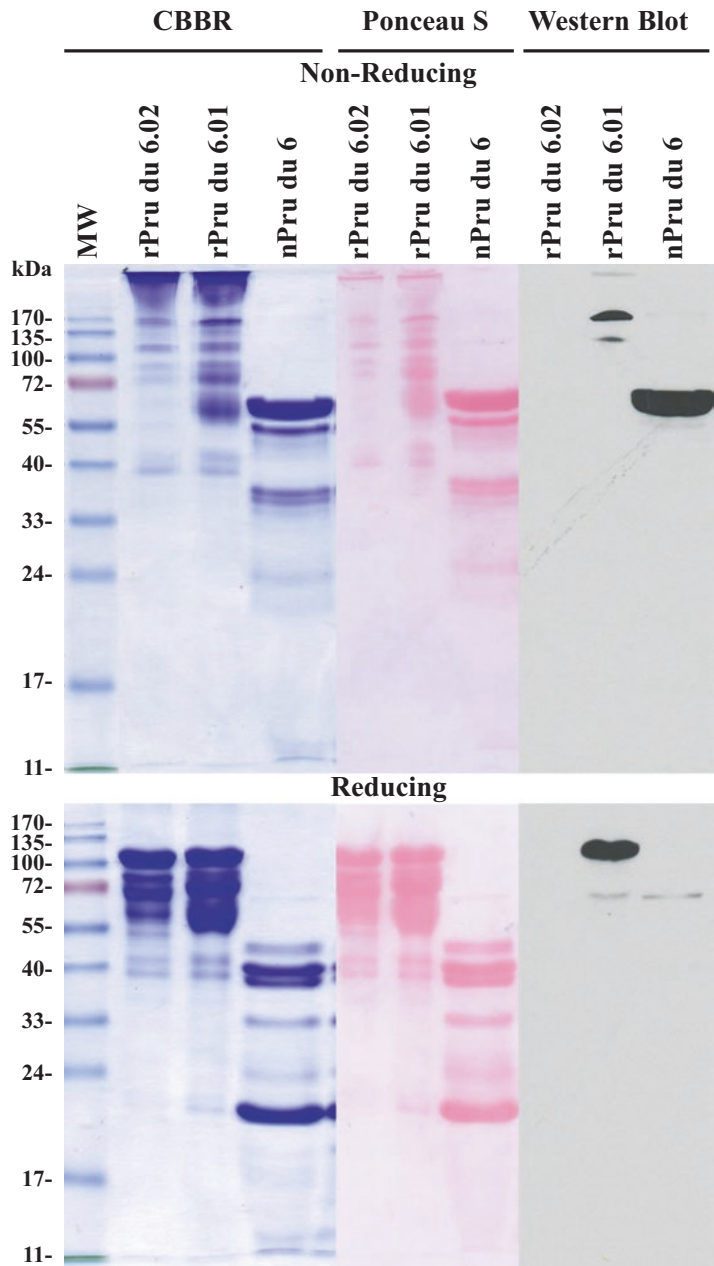


Fig. 1 Comparison of the native and recombinant almond major allergen amandin (Pru du 6). Proteins on polyacrylamide gel and nitrocellulose membrane were stained by Coomassie Brilliant Blue R (CBBR) and Ponceau S, respectively. Immunoreactive polypeptides were recognized by murine anti-amandin monoclonal antibody 4C10 in Western blot. MW: molecular weight standards; rPru du 6.01: recombinant Pru du 6.01-maltose binding protein (MBP) fusion protein; rPru du 6.02: recombinant Pru du 6.02-MBP fusion protein; nPru du 6: native amandin purified by column chromatography

production of recombinant food allergens is available in references [5, 6] and specific kit manuals. Protocols we used to produce recombinant allergens of almond [7, 8], cashew [9–11], pecan [12, 13], and pistachio [14] are described in this chapter.

2 Materials

2.1 RNA Extraction Reagents

1. Liquid nitrogen.
2. Denaturing solution (solution D): 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) *N*-lauroylsarcosine (Sarkosyl), and 0.1 M β -mercaptoethanol. Prepare stock solution by dissolving 250 g guanidinium thiocyanate in 293 mL deionized water at 65 °C, then adding 17.6 mL 0.75 M sodium citrate (pH 7.0) and 26.4 mL 10% (w/v) Sarkosyl. Prepare working solution by adding 0.36 mL 98% β -mercaptoethanol to 50 mL stock solution. The stock and working solutions can be stored at room temperature (RT, 25 °C) for 3 and 1 months, respectively. Solution D is also commercially available as TRIzol (Thermo Fisher Scientific) and TRI reagents (Sigma-Aldrich).
3. Diethylpyrocarbonate (DEPC)-treated water: Add 0.2 mL DEPC to 100 mL deionized water and shake vigorously. Autoclave the solution to inactivate DEPC. DEPC is used to inactivate RNase and prevent RNA degradation. DEPC-treated water should always be used when handling RNA.
4. Sodium acetate buffer (2 M, pH 4.0): Add 16.42 g anhydrous sodium acetate to 40 mL deionized water and 35 mL glacial acetic acid. Adjust to pH 4.0 with glacial acetic acid and bring the final volume to 100 mL with DEPC-treated water. Store up to 1 year at RT.
5. Water-saturated phenol: Dissolve 100 g phenol crystals in deionized water at 65 °C. Aspirate the upper water phase and store at 4 °C for 1 month.
6. Chloroform:isoamyl alcohol (49:1, v/v).
7. Isopropanol.
8. Ethanol (75%, v/v): Add 75 mL absolute ethanol to 25 mL DEPC-treated water.

2.2 mRNA Isolation Reagents

PolyATtract mRNA isolation kit (Promega Corporation). This kit includes biotinylated Oligo(dT) probe solution (50 pmol/ μ L), saline-sodium citrate (SSC) buffer 20 $\times$ concentrate, streptavidin MagneSphere paramagnetic particles (SA-PMPs), nuclease-free water, mRNA user tubes, and a MagneSphere magnetic separation stand.

2.3 cDNA Production Reagents

ZAP Express cDNA Gigapack III Gold cloning kit (Agilent Technologies). This kit includes ZAP Express vector, pBR322 test insert, XL1-Blue MRF⁺ strain, Gigapack III Gold packaging extract, VCS257 host strain, AccuScript reverse transcriptase (AccuScript RT), RNase block ribonuclease inhibitor, first-strand methyl nucleotide mixture, 10× first-strand buffer, linker-primer, test mRNA, DEPC-treated water, 10× second-strand buffer, second-strand dNTP mixture, *E. coli* RNase H, *E. coli* DNA polymerase, 3 M sodium acetate, blunting dNTP mixture, cloned *Pfu* DNA polymerase, *EcoR* I adapters, 10× ligase buffer, 10 mM rATP, T4 DNA ligase, T4 polynucleotide kinase, *Xho* I, *Xho* I buffer, Sepharose CL-2B gel filtration medium, column-loading dye, and STE buffer (10×). Additional reagents and media required include:

1. SM buffer: Add 5.8 g NaCl, 2.0 g MgSO₄•7H₂O, and 5.0 mL 2% (w/v) gelatin to 50 mL 1 M Tris-HCl (pH 7.5), add deionized water to a final volume of 1 L.
 2. 20× SSC buffer: Add 175.3 g NaCl and 88.2 g sodium citrate to 800 mL deionized water, adjust pH to 7.0, add deionized water to a final volume of 1 L.
 3. LB broth: Add 10 g NaCl, 10 g tryptone, and 5 g yeast extract to 800 mL deionized water, adjust pH to 7.0, add deionized water to a final volume of 1 L. Autoclave the broth.
 4. LB agar: Add 10 g NaCl, 10 g tryptone, 5 g yeast extract, and 20 g agar to 800 mL deionized water, adjust pH to 7.0, add deionized water to a final volume of 1 L. Autoclave and pour into petri dishes. Let solidify at RT, then store at 4 °C until use.
 5. LB broth with supplements: Add 10 mL 1 M MgSO₄, 3 mL 2 M maltose solution in 1 L of LB broth. Store at 4 °C until use.
 6. NZY agar: Add 5 g NaCl, 2 g MgSO₄•7H₂O, 5 g yeast extract, 10 g NZ amine, and 15 g agar to 800 mL deionized water, adjust pH to 7.5, add deionized water to a final volume of 1 L. Autoclave and pour into petri dishes. Let solidify at RT, then store at 4 °C until use.
 7. NYZ broth: Add 5 g NaCl, 2 g MgSO₄•7H₂O, 5 g yeast extract, and 10 g NZ amine to 800 mL deionized water, adjust pH to 7.5, add deionized water to a final volume of 1 L. Autoclave, allow to cool, and store at 4 °C until use.
 8. NYZ top agar: Add 0.7% (w/v) agarose to 1 L NYZ broth. Autoclave and store at 4 °C until use.
- See **Note 1** concerning media storage.

2.4 Rapid Amplification of cDNA Ends (RACE) Reagents

SMARTer RACE cDNA Amplification kit (Clontech Laboratories). This kit includes SMARTer II A oligonucleotide (oligo), 3' and 5' RACE CDS Primer A, 5× first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 30 mM MgCl₂, pH 8.3), 20 mM dithiothreitol

(DTT), deionized water, RNase inhibitor, SMARTScribe reverse transcriptase, 10× universal primer A mix, dNTP mix, and Tricine EDTA buffer (10 mM Tricine-KOH, 1.0 mM EDTA, pH 8.5). Prepare enough of the following reagents:

1. Buffer Mix (for each 10 µL cDNA synthesis reaction): Mix 2.0 µL 5× first-strand buffer, 1.0 µL 20 mM DTT, and 1.0 µL 10 mM dNTP mix.
2. Master Mix (for each 10 µL cDNA synthesis reaction): Mix 4.0 µL Buffer Mix, 0.2 µL RNase inhibitor, and 1.0 µL SMARTScribe reverse transcriptase.
3. RACE Mix (for each 50 µL PCR reaction): Mix 34.5 µL PCR-grade water, 5.0 µL 10× Advantage 2 PCR buffer, 1.0 µL dNTP mix, and 1.0 µL 50× Advantage 2 polymerase mix.

2.5 TA Cloning Reagent

TOPO TA Cloning Kit (Invitrogen). This kit includes: pCR 2.1-TOPO vector (10 ng/µL plasmid DNA in 50% glycerol, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 100 µg/mL BSA, phenol red), salt solution (1.2 M NaCl, 0.06 M MgCl₂), and One Shot competent cells.

2.6 pMAL Protein Fusion and Purification Reagent

pMAL Protein Fusion and Purification System (New England BioLabs). This kit includes: pMAL-c5X vector, amylose resin, Factor Xa, anti-maltose binding protein (MBP) monoclonal antibody, MBP5 protein, and NEB Express *E. coli* ER2523. Additional solutions and media are required:

1. Rich medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose in 1 L, autoclave and add sterile ampicillin to 100 µg/mL.
2. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 M) stock: 1.19 g IPTG, add deionized water to 50 mL, filter sterilize, store at −20 °C.
3. Column buffer: 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM β-mercaptoethanol, pH 7.4.
4. Sodium phosphate buffer: 20 mM sodium phosphate, 25 mM NaCl, pH 5.5.
5. Elution buffer (10 mM maltose): dissolve 0.0342 g maltose in 10 mL column buffer. Store at −20 °C until use.

3 Methods

An overall flow of the recombinant allergen production procedures is illustrated in Fig. 2.

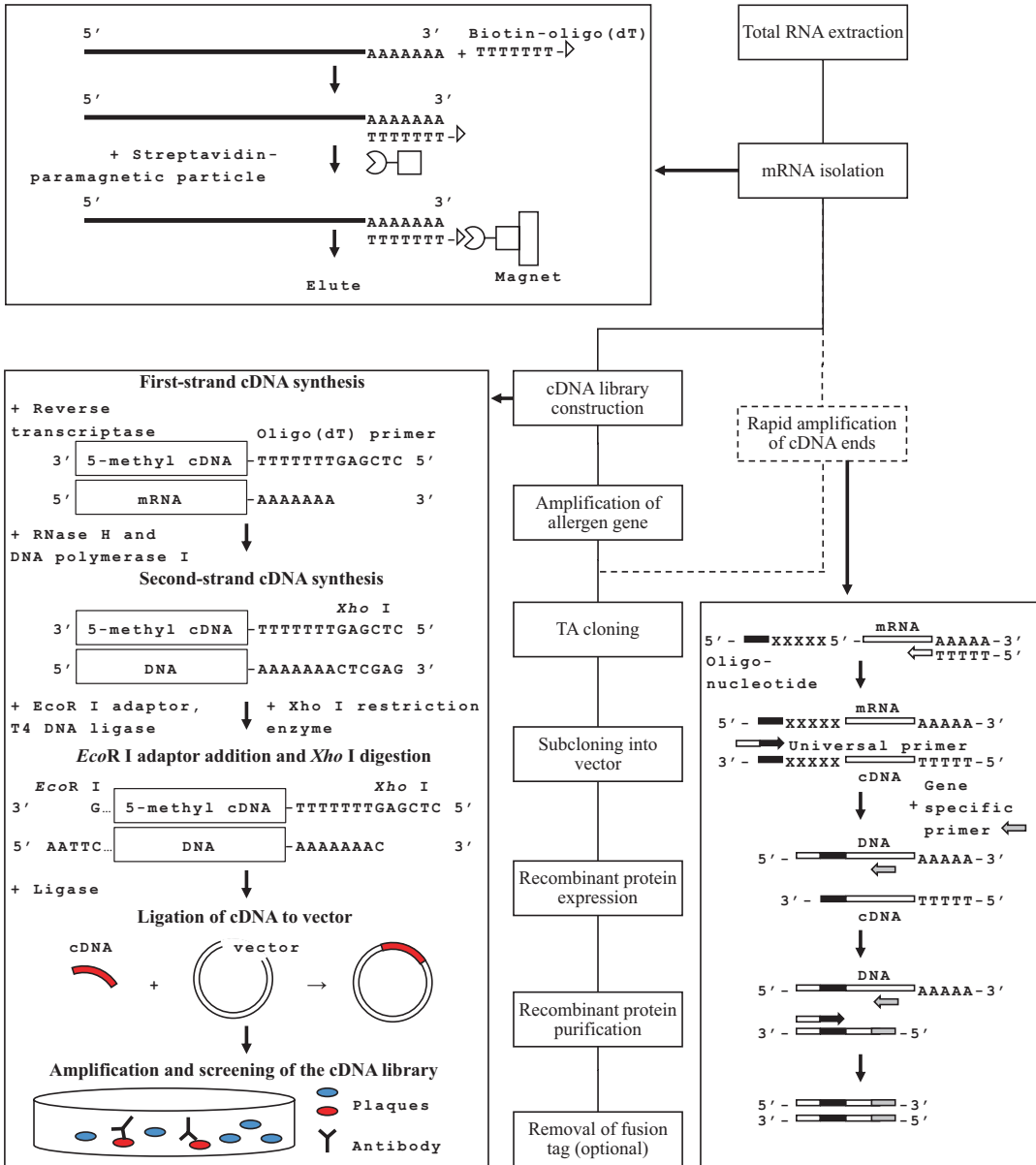


Fig. 2 Schematic diagram of the recombinant allergen production procedures

3.1 Tissue Collection

1. Collect tissues at various development stages to ensure the presence of the desired mRNA.
2. Proceed to Subheading 3.2 or freeze the tissues in liquid nitrogen immediately after harvest and store at -80°C .

3.2 Total RNA Extraction

Extract total RNA as described by Chomczynski and Sacchi [15] or by using commercial total RNA extraction kit (e.g. Qiagen RNeasy kit).

1. Freeze the tissues in liquid nitrogen and grind to a fine powder with a mortar and pestle, add 1 mL solution D per 100 mg frozen tissue and mix well manually (*see* **Note 2**).
2. Transfer the tissue lysate to a polypropylene tube and add the following to 1 mL lysate in sequence: 0.1 mL 2 M sodium acetate, pH 4.0, mix thoroughly by inversion; 1 mL water-saturated phenol, mix thoroughly by inversion; 0.2 mL chloroform/isoamyl alcohol (49:1 v/v), shake vigorously by hand for 10 s.
3. Cool the samples on ice for 15 min followed by centrifugation at $10,000 \times g$, 4 °C for 20 min.
4. Transfer the upper aqueous phase containing RNA to a clean tube, add 1 mL isopropanol to precipitate RNA, and incubate the samples for at least 1 h at −20 °C.
5. Centrifuge at $10,000 \times g$, 4 °C for 20 min, and discard the supernatant.
6. Dissolve the RNA pellet in 0.3 mL solution D, transfer to a 1.5 mL microcentrifuge tube, add 0.3 mL isopropanol, and incubate at −20 °C for at least 30 min.
7. Centrifuge at $10,000 \times g$, 4 °C for 10 min, and discard the supernatant.
8. Resuspend the RNA pellet with 0.5–1 mL 75% ethanol and vortex for a few seconds.
9. Incubate samples at RT for 10–15 min, centrifuge at $10,000 \times g$, 4 °C for 5 min, and discard the supernatant.
10. Air-dry the RNA pellet at RT for 5–10 min (*see* **Note 3**), re-dissolve in 100–200 μ L DEPC-treated water, and incubate at 60 °C for 10–15 min for complete solubilization.
11. The concentration of isolated RNA can be determined by measuring the absorbance at 260 nm (OD_{260}) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 40 μ g of RNA per mL ($OD_{260} = 1 = 40 \mu\text{g/mL}$). It is estimated that 1–5% of total RNA is mRNA [16].
12. Store the samples at −80 °C.

3.3 mRNA Isolation

mRNA is conventionally separated from total RNA using oligo(dT) cellulose gravity column that binds the poly(A) tail of the mRNA [17]. Faster and more convenient methods using oligo(dT) cellulose packed in spin columns or bound to magnetic particles are available (Thermo Fisher Scientific, New England BioLabs, ClonTech Laboratories, or Invitrogen). Detailed protocols for oligo(dT) cellulose packed gravity and spin column can be found in reference [18]. We describe here the recommended protocol for the PolyATtract mRNA isolation kit available from Promega (Fig. 2). This kit uses a biotinylated oligo (dT) primer to

hybridize mRNA. The hybrids are captured and separated using streptavidin-coupled paramagnetic particles and a magnetic separation stand [19].

1. Transfer 1–5 mg total RNA to a sterile, RNase-free tube, add nuclease-free water to a final volume of 2.43 mL.
2. Heat at 65 °C for 10 min, add 10 µL biotinylated-oligo(dT) probe solution and 60 µL 20× SSC. Mix gently and cool to RT.
3. Resuspend the SA-PMPs by gently flicking the tube, then capture the particles by placing the tube in the magnetic stand until the particles are collected at the side of the tube.
4. Carefully remove the supernatant and wash the SA-PMPs three times with equal volume of 0.5× SSC (discard supernatant each time), then resuspend the washed SA-PMPs in 0.5 mL 0.5× SSC (*see Note 4*).
5. Add the entire contents of the **step 2** to the washed SA-PMPs, incubate for 10 min at RT, gently mix by inverting every 1–2 min.
6. Capture the SA-PMPs using the magnetic stand and carefully remove the supernatant.
7. Wash the SA-PMPs four times with 0.1× SSC by gently flicking the tube until all particles are resuspended. Remove as much supernatant as possible without disturbing the SA-PMPs.
8. Resuspend the final SA-PMP pellet in 1.0 mL nuclease-free water and gently flicking the tube to resuspend the particles.
9. Magnetically capture the SA-PMPs and transfer the eluted mRNA to a sterile, RNase-free tube (*see Note 5*).
10. The concentration and purity of eluted mRNA can be determined by spectrophotometry. Pure mRNA has an OD₂₆₀/OD₂₈₀ absorbance ratio greater than or equal to 2.0.

3.4 cDNA Library Construction

Synthesis of cDNA can be accomplished by a number of different methods. For detailed information refer to protocols in [5]. cDNA production protocols for cDNA library construction (Subheading 3.4) and RACE (Subheading 3.5) are described below (Fig. 2). The protocol provided by ZAP Express cDNA Gigapack III Gold cloning kit (Agilent Technologies) is used to prepare cDNA library [20].

3.4.1 First-Strand cDNA Synthesis

1. Gently mix 5 µg mRNA isolated in Subheading 3.3 with 5 µL 10× first-strand buffer, 3 µL first-strand methyl nucleotide mixture, 2 µL linker-primer, and 1 µL RNase block ribonuclease inhibitor in a RNase-free microcentrifuge tube. Add DEPC-treated water to a final volume of 47 µL. Prepare a control mixture by replacing the isolated mRNA with the kit-provided mRNA.

2. Incubate at RT for 10 min then add 3 μL of AccuScript RT to synthesize the first-strand cDNA sample. Mix gently and spin down the contents.
3. Transfer 5 μL of the mixture to another tube containing 0.5 μL [α - ^{32}P]dNTP as the first-strand synthesis control.
4. Incubate the first-strand cDNA sample and the first-strand control at 42 °C for 1 h.
5. Keep the first-strand cDNA sample on ice and freeze the first-strand control at -20 °C.

3.4.2 Second-Strand cDNA Synthesis

1. Add 20 μL 10 $\times$ second-strand buffer, 6 μL second-strand dNTP mixture, 114 μL sterile deionized water, and 2 μL [α - ^{32}P]dNTP to the first-strand cDNA sample.
2. Add 2 μL RNase H and 11 μL DNA polymerase I to start the second-strand cDNA synthesis.
3. Gently vortex and centrifuge (16,100 $\times g$, RT) the mixture and incubate in a 16 °C water bath for 2.5 h (*see Note 6*), then immediately place the tube on ice.

3.4.3 Blunting the cDNA Termini

After synthesis, the frayed termini of the double-strand cDNA are repaired by the *Pfu* DNA polymerase.

1. Add 23 μL blunting dNTP mix and 2 μL *Pfu* DNA polymerase to the double-strand cDNA.
2. Quickly vortex and centrifuge (16,100 $\times g$, RT) the mixture and incubate at 72 °C for exactly 30 min (do not exceed).
3. Add 200 μL phenol-chloroform, vortex and centrifuge at 16,100 $\times g$ for 2 min at RT. Transfer the upper aqueous layer containing the cDNA to a new tube.
4. Add equal volume of chloroform and repeat vortex mixing and centrifugation. Transfer the upper layer to a new tube.
5. Add 2 μL 3 M sodium acetate, 400 μL 100% (v/v) ethanol, vortex and precipitate the cDNA overnight at -20 °C.
6. Centrifuge at 16,100 $\times g$ for 60 min at 4 °C. Carefully remove the supernatant and discard in a radioactive waste container.
7. Gently wash the pellet with 500 μL 70% (v/v) ethanol. Do not mix or vortex.
8. Centrifuge at 16,100 $\times g$ for 2 min at RT, and dry the pellet by vacuum centrifugation.
9. Resuspend the pellet in 9 μL *EcoR* I adapters and incubate for 30 min at 4 °C. Transfer the cDNA to a new tube and ensure the cDNA is in solution.
10. Transfer 1 μL of the solution to a new tube as the second-strand control.

11. Run the first-strand and second-strand samples on an alkaline agarose gel to determine the cDNA size range and the secondary structures.

3.4.4 Addition of the *EcoR* I Adaptors

Addition of adaptors to the termini of the cDNAs is required to attach the generated cDNAs into the vector.

1. Add 1 μL 10 $\times$ ligase buffer, 1 μL 10 mM rATP, and 1 μL T4 DNA ligase to the tube containing the blunted cDNA and the *EcoR* I adaptors.
2. Centrifuge at $16,100 \times g$ and incubate at 8 °C overnight.
3. Heat in a 70 °C water bath for 30 min to inactivate the ligase.
4. Centrifuge at $16,100 \times g$ for 2 s and cool at RT for 5 min.
5. Add 1 μL 10 $\times$ ligase buffer, 2 μL 10 mM rATP, 5 μL sterile water, and 2 μL T4 polynucleotide kinase and incubate at 37 °C for 30 min to phosphorylate the adapter ends.
6. Inactivate the kinase by heating at 70 °C for 30 min.
7. Centrifuge at $16,100 \times g$ for 2 s and cool to RT for 5 min.
8. Add 28 μL *Xho* I buffer and 3 μL *Xho* I to the reaction and incubate at 37 °C for 1.5 h.
9. Add 5 μL 10 $\times$ STE buffer and 125 μL 100% (v/v) ethanol and precipitate the cDNA at -20 °C overnight.
10. Centrifuge at $16,100 \times g$ for 60 min at 4 °C, discard the supernatant, dry the pellet and resuspend the pellet in 14 μL 1 $\times$ STE buffer.
11. Add 3.5 μL column loading dye to each sample.

3.4.5 Fractionation of cDNA

Before insertion into the vector, cDNA is fractionated by gel filtration to remove excess adaptors and low molecular weight DNA.

1. Pack a sterile 1 mL pipette with a cotton plug at the narrow end with Sepharose CL-2B medium. Add the resin till the packed bed is 0.25 inch below the wide end of the pipette. Wash the column with at least 10 mL 1 $\times$ STE buffer.
2. Gently load the cDNA sample using a pipettor and keep the column filled with 1 $\times$ STE buffer.
3. Collect three drops per fraction in microcentrifuge tubes when the leading edge of the dye reaches the 0.4 mL gradation on the pipette. Stop fraction collection when the trailing edge of the dye reaches the 0.3 mL gradation or until all radioactive nucleotides are eluted.
4. Remove 8 μL sample from each fraction. Electrophorese the aliquots on a 5% non-denaturing acrylamide gel to assess the effectiveness of the fractionation and to determine which fractions to be used for ligation.

5. Adding an equal volume of phenol–chloroform (1:1, v/v) to the collected fractions. Vortex and centrifuge at $16,100 \times g$ at RT for 2 min.
6. Collect the upper aqueous layer, add an equal volume of chloroform, vortex and centrifuge at $16,100 \times g$ at RT for 2 min. Transfer the upper aqueous layer to a new microcentrifuge tube.
7. Add twice volume of 100% (v/v) ethanol and precipitate the cDNA at $-20\text{ }^{\circ}\text{C}$ overnight.
8. Centrifuge at $16,100 \times g$ at $4\text{ }^{\circ}\text{C}$ for 60 min. Discard the supernatant if the majority of radioactivity is present in the pellet. Otherwise repeat the centrifugation.
9. Carefully wash the pellet with 200 μL 80% (v/v) ethanol without vortex mixing. Centrifuge at $16,100 \times g$ at RT for 2 min. Remove the ethanol and vacuum evaporate the pellet until dry (*see Note 7*).
10. Measure the counts per second (cps) of each fraction using a handheld Geiger counter. Resuspend each cDNA pellet in 3.5 μL (<30 cps) or 5 μL (>30 cps) sterile water and mix by pipetting up and down.
11. Quantify the cDNA using ethidium bromide plate assay.

3.4.6 Ligation of cDNA to the Vector

1. Prepare a control ligation by mixing 1 μL ZAP Express vector, 1.6 μL test insert, 0.5 μL 10 $\times$ ligase buffer, 0.5 μL 10 mM rATP, and 0.9 μL deionized water.
2. Prepare the sample ligation by mixing 100 ng resuspended cDNA, 0.5 μL 10 $\times$ ligase buffer, 0.5 μL 10 mM rATP, and 1 μL ZAP Express vector, then add deionized water to a final volume of 4.5 μL .
3. Add 0.5 μL T4 DNA ligase to the control and sample ligations, incubate at $12\text{ }^{\circ}\text{C}$ overnight.
4. Remove Gigapack III Gold packaging extracts from $-80\text{ }^{\circ}\text{C}$ freezer and place on dry ice.
5. Add 1–4 μL experimental DNA (0.1–1 μg ligated DNA) to the packaging extract once it begins to thaw.
6. Gently mix with a pipette tip, centrifuge at $16,100 \times g$ at RT for 3–5 s, and incubate at RT for 2 h (*see Note 8*).
7. Add 500 μL SM buffer, 20 μL chloroform and mix gently.
8. Centrifuge at $16,100 \times g$ at RT for 3–5 s, collect the supernatant containing the phage, store at $4\text{ }^{\circ}\text{C}$.

3.4.7 cDNA Library Titration and Amplification

1. Streak the XL1-Blue MRF['] and VCS257 cells onto LB agar plates containing appropriate antibiotic and incubate at $37\text{ }^{\circ}\text{C}$ overnight.

2. Grow separate 50 mL cultures of XL1-Blue MRF' and VCS257 cells in LB broth with supplements at 37 °C for 4–6 h.
3. Centrifuge the cells at $1,000 \times g$ for 10 min. Resuspend the cell pellet in 25 mL sterile 10 mM MgSO_4 . Dilute the cell suspensions to an OD_{600} of 0.5 in 10 mM MgSO_4 .
4. Add 1 μL lambda phage to 200 μL VCS257 cell suspension. Incubate at 37 °C for 15 min.
5. Add 2–3 mL melted NZY top agar cooled to ~ 48 °C, 15 μL 0.5 M IPTG, and 50 μL 250 mg/mL X-gal in dimethylformamide.
6. Plate immediately onto NZY agar plates, set for 10 min, invert, and incubate at 37 °C overnight.
7. Count the number of plaques and determine the titer using the following formula:

$$\frac{\text{Number of plaques} \times \text{dilution factor} \times \text{total packing volume}}{\text{Micrograms packed} \times \text{Microliters plated}}$$

8. Combine aliquots of the packaged mixture containing $\sim 5 \times 10^4$ pfu bacteriophage with 600 μL XL1-Blue MRF' cells. Incubate at 37 °C for 15 min.
9. Mix each aliquot with 6.5 mL melted NZY top agar cooled to ~ 48 °C and spread evenly onto a freshly poured NZY agar plate, set for 10 min, invert the plates, and incubate at 37 °C for 6–8 h.
10. Add 8–10 mL SM buffer, store at 4 °C overnight.
11. Collect bacteriophage suspension from each plate, rinse the plates with 2 mL SM buffer and pool. Add chloroform to a 5% (v/v) final concentration. Mix and incubate at RT for 15 min.
12. Centrifuge at $500 \times g$ for 10 min and collect the supernatant.
13. Repeat **steps 11 and 12** until the supernatant is clear. Add chloroform to a 0.3% (v/v) final concentration and store at 4 °C. Store aliquots of the amplified library in 7% (v/v) dimethyl sulfoxide (DMSO) at -80 °C.
14. Check the titer of the amplified library.

3.4.8 Screening of the cDNA Library

1. Combine 5×10^4 pfu/plate bacteriophage and 600 μL freshly prepared XL1-Blue MRF' cells at an OD_{600} of 0.5, incubate at 37 °C for 15 min.
2. Add 6.5 mL melted NZY top agar cooled to ~ 48 °C, quickly pour onto a NZY agar plate (at least 2 days old). Spread the cells evenly, allow set for 10 min, invert the plates, and incubate at 37 °C for 8 h.
3. Cool the plates at 4 °C for 2 h. Place a nitrocellulose membrane onto each NYZ agar plate for 2 min (*see Note 9*).

4. Submerge the membrane in a 1.5 M NaCl and 0.5 M NaOH solution for 2 min followed by submerging the membrane in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) solution for 5 min.
5. Submerge the membrane in a 0.2 M Tris-HCl (pH 7.5) and 2× SSC buffer for <30 s and blot dry on a Whatman 3MM paper.
6. Oven-bake the membrane at 80 °C for 1.5–2 h. Store the stock agar plates at 4 °C.
7. Screen the membrane with antibodies of interest to identify the immunopositive plaques. Follow a basic Western blotting protocol.
8. Pick and purify the immunopositive clones, and store in SM buffer with 2% (v/v) chloroform at 4 °C.
9. Proceed to Subheading 3.6.

3.5 Generating RACE-Ready cDNA

When antisera and a cDNA library are not available and the specific sequence of the targeted gene is unknown, 5' or 3' RACE using degenerate primers can be performed to derive the genetic sequence of the target of interest. The SMARTer RACE cDNA amplification kit (Clontech Laboratories) protocol is used to synthesis the first-strand cDNA [21]. This first-strand cDNA can be directly used in 5' and 3' RACE without the need for second-strand synthesis and adaptor ligation.

1. Combine 1.0–2.75 µL mRNA and 1.0 µL 5' CDS Primer A for 5' RACE, add sterile water to a final volume of 3.75 µL. In a separate tube, combine 1.0–3.75 µL mRNA and 1.0 µL 3' CDS Primer A for 3' RACE, add sterile water to a final volume of 4.75 µL.
2. Mix and spin briefly in a microcentrifuge.
3. Incubate at 72 °C for 3 min, then cool to 42 °C for 2 min. Centrifuge at $14,000 \times g$ for 10 s.
4. Add 1 µL SMARTer IIA oligo to the 5' RACE.
5. Add 5.25 µL Master Mix to the 5' RACE and 3' RACE.
6. Mix by gentle pipetting and centrifuge briefly.
7. Incubate the tubes at 42 °C for 90 min followed by heating at 70 °C for 10 min.
8. Add 250 µL Tricine-EDTA buffer and store the synthesized first-strand cDNA at –20 °C.
9. Proceed to Subheading 3.7.

3.6 Amplification of the Immunopositive Phage Clones (cDNA Library)

1. Add the immunopositive phage clones containing 1–200 ng DNA, 200 nM each forward and reverse primers (primers designed based on the known target gene sequence or M13 primers), and 45 µL Platinum PCR SuperMix High Fidelity reagent (Invitrogen) to a microcentrifuge tube and mix.

2. Load the tube into a thermal cycler, incubate at 94 °C for 30 s to 2 min.
3. Perform 35 cycles of PCR amplification as follows: denature at 94 °C for 15–30 s, anneal at 55 °C for 15–30 s, extend at 68 °C for 1 min/kb [22].

3.7 Rapid Amplification of cDNA Ends (RACE)

3.7.1 Primer Design

Conserved DNA sequences in homologous proteins of related species are used to design the degenerate primers. For example, conserved homologous sequences in cashew, hazelnut, sesame, soybean, and fava bean 7S vicilins were used to design primers for pistachio vicilin gene amplification [14].

3.7.2 cDNA Amplification

1. Combine 2.5 µL 5' RACE-ready cDNA, 5 µL universal primer A mix, 1 µL 5' degenerate primer (10 µM), and 41.5 µL RACE Mix for 5' RACE. Combine 2.5 µL 3' RACE-ready cDNA, 5 µL universal primer A mix, 1 µL 3' degenerate primer (10 µM), and 41.5 µL RACE Mix for 3' RACE.
2. Start the following thermal cycling program:
Five cycles: 94 °C 30 s, 72 °C 3 min.
Five cycles: 94 °C 30 s, 70 °C 30 s, 72 °C 3 min.
20 cycles: 94 °C 30 s, 68 °C 30 s, 72 °C 3 min.

3.8 Gel Purification of PCR Products

Check the purity of the PCR product by agarose gel electrophoresis. If a single, discrete band is not obtained, gel purify the fragment using the following protocol [23].

1. Electrophorese the PCR products on a low-melt TAE agarose gel (0.8–12%).
2. Visualize the DNA fragment under UV light and excise the band of interest.
3. Place the gel slice in a microcentrifuge tube and heat at 65 °C until the gel melts.
4. Keep the tube at 37 °C and use 4 µL of the melted agarose for TA cloning (Subheading 3.9).

3.9 TA Cloning of the Targeting Allergen cDNA

The PCR amplicon is TA cloned according to [23].

1. Mix 0.5–4 µL fresh PCR product with 1 µL salt solution, add deionized water to a total volume of 5 µL. Add 1 µL TOPO vector, mix gently, and incubate at RT for 5 min.
2. Add 4 µL of the mixture into a vial of One Shot chemically competent *E. coli*, mix gently, and incubate on ice for 5 min.
3. Spread 50 µL of the cells on a LB plate containing 50–100 µg/mL ampicillin and incubate at 37 °C overnight.

3.10 Sequencing of the Plasmid cDNA

The M13 forward and reverse primer sites are used for sequencing the PCR inserts on an ABI 3100 Genetic analyzer by using capillary electrophoresis and Version 2 Big Dye Terminators.

3.11 Subcloning and Expression of Recombinant Allergens

A wide variety of expression systems are available for the production of the recombinant allergen [4]. The selection of a suitable system depends on the desired yield, a need for post-translational modifications, ease of purification, the overall cost, and size of the allergen. The most common systems for recombinant allergen production are the pGEX (GE Healthcare Biosciences) which generates an allergen GST fusion protein, pET (Novagen) which generates a His tagged allergen protein, and pMAL (New England BioLabs) which generates an allergen MBP fusion protein [24, 25]. Schematic representation of common vectors used to generate recombinant allergens is illustrated in Fig. 3. The pMAL expression vector is demonstrated in this chapter [26].

3.11.1 Ligation of the cDNA into the Expression Vector

1. Digest 0.5 µg pMAL vector DNA in 20 µL 1× CutSmart buffer with 10 units each of *Xmn*I and *Sbf*I at 37 °C for 1 h. Inactivate the enzyme at 65 °C for 10 min.
2. Run 4 µL pMAL digest on an agarose gel to check for complete digestion.
3. Digest 0.5 µg cDNA insert in 20 µL 1× CutSmart Buffer with 10 units of *Sbf*I at 37 °C for 1 h.
4. Purify the pMAL vector backbone and the cut cDNA insert by gel extraction. A kit such as the QIAquick Gel Extraction Kit (QIAGEN) can be used.
5. Run the vector backbone and cDNA insert on a gel to check the concentrations.
6. Prepare the ligation reaction. Mix 20–40 ng vector backbone, 20 ng cDNA insert, 10 µL deionized water, and 10 µL 2× Quick Ligation reaction buffer. Add 1 µL Quick T4 DNA ligase, incubate at RT for 10 min.

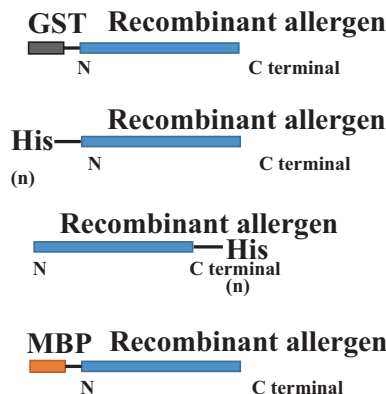


Fig. 3 Schematic representation of common vectors used to generate recombinant allergens. His tags can vary in length and location based upon the particular pET vector selected for expression. The molecular mass of GST and MBP is approximately 26 and 43 kDa, respectively

3.11.2 Transformation and Expression of the Recombinant Allergens

1. Mix 2 μ L of the ligated vectors with 50 μ L BL21 Competent *E. coli* cells (*see Note 10*). Incubate on ice for 5 min then heat to 42 °C for 30 s (*see Note 11*).
2. Add 0.2 mL LB and incubate at 37 °C for 20 min (*see Note 12*). Spread on an LB plate containing 100 μ g/mL ampicillin. Incubate, inverted at 37 °C overnight.
3. Inoculate 10 mL overnight culture in 1 L rich broth containing glucose and ampicillin.
4. Grow to 0.5 OD₆₀₀, add IPTG to a 0.3 mM final concentration, incubate at 37 °C for 2 h (*see Note 13*).

3.12 Purification of Recombinant Allergens

3.12.1 Affinity Chromatography

1. Harvest the cells by centrifugation at $4,000 \times g$ at 4 °C for 20 min and discard the supernatant. Resuspend the cells in 25 mL column buffer.
2. Freeze sample at –20 °C overnight. Thaw in cold water.
3. Keep sample in an ice-water bath and sonicate in 15 s short pulses until the released protein reaches a maximum concentration. Protein concentration can be monitored by mixing 5 μ L sonicate with 1 mL Bradford reagent.
4. Centrifuge at $20,000 \times g$ at 4 °C for 20 min to pellet cell debris and collect the supernatant. Dilute the supernatant 1:6 (v/v) in column buffer.
5. Pack amylose resin in a 2.5×10 cm column. The volume of resin is dependent upon the site of recombinant protein expression. Larger volumes of recombinant protein will be expressed in cytoplasm-directed expression as opposed to periplasm-directed expression (*see Note 14*).
6. Wash the column with five bed volumes of column buffer.
7. Load the diluted supernatant at a flow rate of 5 mL/min.
8. Wash with 12 bed volumes of column buffer at 10 mL/min.
9. Elute the fusion protein with 10 mM maltose in column buffer. Collect 10–20 fractions of 3 mL each to ensure all the recombinant allergen is eluted and collected from the column.
10. Pool the protein-containing fractions. Concentrate to ~1 mg/mL.
11. Amylose resin can be regenerated by washing with three column volumes of deionized water, three column volumes of 0.1% (w/v) SDS, one column volume of deionized water and five column volumes of column buffer (*see Note 15*). Store the regenerated resin at 4 °C.

3.12.2 Cleavage of the Fusion Protein

The fusion tag can be removed using the following protocol. However, removal of the tag may result in nonspecific degradation or protein insolubilization. In such cases, the recombinant allergens can be analyzed without cleavage of the fusion protein [8].

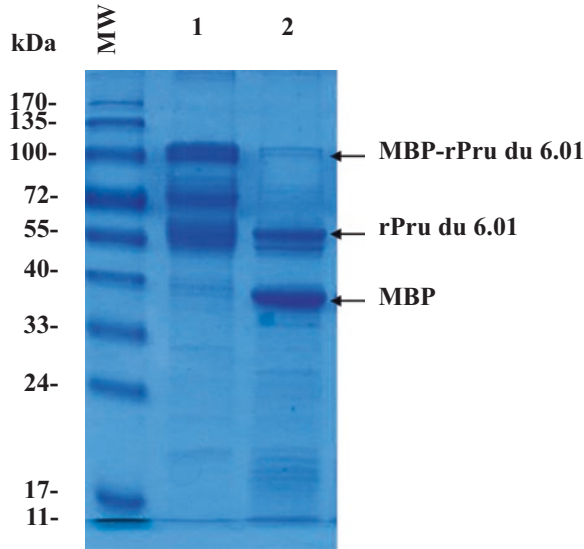


Fig. 4 Digestion of the recombinant almond allergen rPru du 6.01 expressed in the pMAL expression system by Factor Xa. *MW* Molecular weight marker, *1* undigested recombinant allergen, *2* recombinant allergen digested for 2 h at RT with Factor Xa. The liberated MBP must be removed from the sample by chromatography

SDS-PAGE of recombinant almond allergen rPru du 6.01 in presence and absence of fusion tag is illustrated in Fig. 4.

1. Mix 20 μ L 1 mg/mL fusion protein with 1 μ L 200 μ g/mL Factor Xa. In a separate tube, add 5 μ L fusion protein with no protease as control. At 2, 4, 8, and 24 h, remove 5 μ L of the cleavage reaction.
2. Perform SDS-PAGE analysis of the cleavage samples to determine the success of the digestion. Add 5 μ L SDS-PAGE sample buffer to the control and samples (*see Note 16*).
3. Boil for 5 min and run on an SDS-PAGE gel.
4. Scale up for the fusion proteins to be cleaved.
5. Stain gels using Colloidal Coomassie blue to visualize protein bands. MBP should be present at ~43 kDa. Recombinant allergen size will vary depending on the specific allergen expressed (*see Note 17*).

3.12.3 Separating the Recombinant Protein from MBP

Removal of MBP and Factor Xa can be performed using various column chromatography methods. If the recombinant allergen is <20 kDa or >100 kDa it can be separated from MBP using a sizing column. If the recombinant allergen and MBP are similar in size ion exchange chromatography is ideal for separation. Anion exchange chromatography is typically used as MBP and Factor Xa bind to a positively charged resin at pH 5.5, where most other proteins will flow through the column. Therefore, the recombinant

allergen can be separated from the cleavage mixture by passage over an anion exchange column.

1. Dialyze the cleavage mixture in 100 volumes of running buffer (20 mM sodium phosphate buffer, 25 mM NaCl, pH 5.5) with three buffer changes, 2 h each.
2. Wash a HiTrap Q FF column with 15 mL running buffer.
3. Load the cleavage mixture onto the column. Collect 2.5 mL fractions of the column flow-through.
4. Wash the column with 3–5 mL running buffer until the OD₂₈₀ of the flow through reaches baseline.
5. Regenerate the column by washing with five column volumes of regeneration buffer (running buffer with 1 M NaCl) followed by 5–10 columns volumes of running buffer. This will removed bound MBP and Factor Xa.
6. The column can now be stored at 4 °C to purify additional recombinant allergen.
7. Assess the purity of the recombinant allergen by SDS-PAGE (as described above) to ensure all contaminating MBP has been removed.
8. The purified recombinant allergen can be dialyzed into a desired phosphate containing buffer like phosphate buffered saline (PBS)-10 mM PO₄³⁻, 137 mM NaCl, and 2.7 mM KCl, pH 7.4 for subsequent immunoassays.

3.13 Characterization and Applications of Recombinant Allergens

Recombinant allergens provide a source of molecularly defined homogenous protein preparations. This makes them ideal for both in vitro and ex vivo assays. Recombinant allergens are routinely used in a variety of immunoassays including: Western blots, dot blots, skin prick tests, basophil degranulation assays, ELISA, site directed mutagenesis, and epitope mapping studies. These assays aim to assess patient IgE reactivity, investigate cross-reactivity between various foods, assist in structural studies, identify IgE reactive epitopes, and analyze the effects of enzymatic digestion, and denaturation on allergenicity. The information gathered is important in diagnosis of allergies, furthering our understanding of the mechanism by which allergies are induced, and understanding the effect of the food matrices/digestions on the allergenicity of a protein. Commonly used physicochemical and immunological characterization methods for the recombinant allergens are listed in Table 1.

4 Notes

1. All agar plates and antibiotic containing media should be stored at 4 °C. This will prevent contaminating bacteria growth and antibiotic breakdown. Allow media to reach RT before use. Plates should be used within several weeks.

Table 1
Physicochemical and immunological characterization methods of recombinant food allergens

Parameter	Method	Example	Reference
Molecular mass	SDS-PAGE	rAna o 1, 2, 3; rAra h 8; rBer e 1; rCar i 1, 4; rCor a 1, 2, 8, 11, 12, 13, 14; rCyp c 1; rJug r 1, 2, 4; rMal d 4; rPis v 1, 2, 3, 4; rPru av 1, 4; rPru du 4, 5, 6	[7–14, 27–43]
	MALDI-TOF-MS	rBer e 1	[27]
	ESI-QTOF-MS	rMal d 4	[41]
Sequence verification	N-terminal sequence	rBer e 1; rCor a 1, 2, 8; rMal d 4	[27, 30, 41]
	MALDI-TOF-MS	rCor a 1, 2, 8; rCyp c 1	[30, 40]
Protein structure	Circular dichroism	rAra h 8; rBer e 1; rCor a 1, 2, 8, 11; rCyp c 1; rJug r 1; rMal d 4	[27, 28, 30, 31, 40, 41, 43, 44]
	NMR	rBer e 1; rCor a 1, 2, 8	[30, 45]
	Electron microscopy	rAna o 2	[46]
	Small angle X-ray scattering	rMal d 4	[41]
Disulfide mapping	HPLC-MS/MS	rBer e 1	[27]
Glycan analysis	Agglutinin binding	rBer e 1	[27]
	1D-NMR	rBer e 1	[27]
Digestibility	SGF digestion	rBer e 1; rMal d 4	[28, 41]
Epitope mapping	Solid-phase peptide synthesis	rAna o 1, 2, 3; rCar i 1, 4; rJug r 1; rPru du 6	[8–13, 44]
	LC-ESI FT-ICR MS (H/D exchange)	rAna o 1, 2; rPru du 6	[47–50]
	Chimeric molecule	rAna o 2; rPru du 6	[46, 48]
	Alanine mutation	rPru du 6	[48]
Immunoreactivity	Immunoblotting	rAna o 1, 2, 3; rAra h 8; rBer e 1; rCar i 1, 4; rCor a 1, 2, 8, 11, 12, 13, 14; rCyp c 1; rJug r 1, 2, 4; rMal d 4; rPis v 1, 2, 3, 4; rPru av 1, 4; rPru du 4, 5, 6	[7–14, 27–43]
	ELISA	rBer e 1; rCar i 1; rCyp c 1; rJug r 1; rMal d 4; rPru du 4, 6	[7, 8, 12, 28, 40, 41, 44]
	EAST	rAra h 8; rCor a 1; rPru av 1, 4	[29, 42, 43]
	RAST	rAra h 8; rMal d 4	[41, 43]
	Histamine/ β -hexosaminidase release	rAra h 8; rCor a 1, 11; rCyp c 1; rJug r 1; rMal d 4	[29, 31, 40, 41, 43, 44]
	ImmunoCAP	rAra h 8; rCor a 1, 2, 8, 11, 14; rJug r 1	[29–31, 33, 34, 43]
	Skin prick test	rMal d 4	[41]

2. Samples should not be mixed with solution D for more than 30 min.
3. Never dry the RNA pellet completely, which greatly decreases RNA solubility.

4. The washing step is important as SA-PMPs are provided in a solution of phosphate buffered saline, 1 mg/mL bovine serum albumin, and 0.02% (w/v) sodium azide. Use the SA-PMPs within 30 min after washing for optimal performance.
5. If particles are transferred with the mRNA, remove by centrifugation at $12,000 \times g$ for 1 min. The SA-PMPs cannot be washed and reused.
6. Temperatures above 16 °C may cause hairpin structure formation. It is therefore important to keep all reagents under 16 °C before adding the DNA polymerase I.
7. Do not over dry the pellet or the cDNA becomes difficult to solubilize.
8. Do not incubate for longer than 2 h as the packaging efficiency may drop.
9. It is essential to mark both the nitrocellulose membrane and plate with identifying symbols such as pencil annotations or indentions in the agar. This will ensure that the membrane can be correctly re-aligned with the plate to pick immunoreactive plaques identified in screening.
10. If desired, the ligation reaction can initially be transformed into DH5- α competent cells (Thermo Fisher Scientific). These cells increase insert stability and improve the quality of plasmid DNA. Plasmid can be isolated from these cells using a DNA plasmid miniprep kit. The obtained plasmid can be sequenced using an ABI 3100 Genetic analyzer to verify insertion of the allergen PCR product and to check for possible mutations, then transformed into BL21 Competent *E. coli* cells (New England BioLabs).
11. A short heat shock is essential for facilitating the uptake of plasmid DNA.
12. This short outgrowth is necessary for expression of the ampicillin resistance gene in newly transformed cells. This will allow the selective growth of transformed cells on ampicillin containing media.
13. Smaller culture volumes can be inoculated and expression induced with IPTG. If small scale, trial expression is desired 5 mL of culture is sufficient for induction and detection of the expressed allergen MBP fusion protein by SDS-PAGE.
14. Amylose resin can bind 7–10 mg/mL of MBP fusion protein. Typically 3–6 mL of resin is sufficient to bind recombinant proteins expressed in a 1 L culture of *E. coli*.
15. Resin may be regenerated up to five times.

16. For fusions resistant to cleavage, adding 0.005–0.05% (w/v) SDS in the reaction or denaturing the fusion by 6 M guanidine hydrochloride may improve the cleavage.
17. Alternative staining options are available such as silver stain or fluorescent dye stains.

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