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Title:

Production of IgG fusion proteins transiently expressed in *Nicotiana benthamiana*

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Summary:
We describe here a simple method for expression, extraction, and purification of recombinant human IgG fused to GFP in *Nicotiana benthamiana*. This protocol can be extended to purification and visualization of numerous proteins that utilize column chromatography. Moreover, the protocol is adaptable to the in-person and virtual college teaching laboratory, providing project-based exploration.

Abstract
High demand for antibodies as therapeutic interventions for various infectious, metabolic, autoimmune, neoplastic, and other diseases creates a growing need in developing efficient methods for recombinant antibody production. As of 2019, there were more than 70 FDA-approved monoclonal antibodies, and there is exponential growth potential. Despite their promise, limiting factors for widespread use are manufacturing costs and complexity. Potentially, plants offer low-cost, safe, and easily scalable protein manufacturing strategies. Plants like *Nicotiana benthamiana* not only can correctly fold and assemble complex mammalian proteins but also can add critical post-translational modifications similar to those offered by mammalian cell cultures. In this work, by using native GFP and an acid-stable variant of green fluorescent protein (GFP) fused to human monoclonal antibodies, we were able to visualize the entire transient antibody expression and purification process from *N. benthamiana* plants. Depending on the experiment's purpose, native GFP fusion can ensure easier visualization during the expression phase in the plants, while acid-stable GFP fusion allows for visualization during downstream processing. This scalable and straightforward procedure can be performed by a single researcher to produce milligram quantities of highly pure antibody or antibody fusion proteins in a matter of days using only a few small plants. Such a technique can be extended to the visualization of any type of antibody purification process and potentially many other proteins, both in plant and other expression systems. Moreover, these techniques can benefit virtual instructions and be executed in a teaching laboratory by undergraduate students possessing minimal prior experience with molecular biology techniques, providing a foundation for project-based exploration with real-world applications.

Introduction:
Industry reports indicate that thirteen out of the twenty most-highly grossing drugs in the United States were biologics (protein-based pharmaceuticals), of which nine were antibodies. As of 2019, there were over 570 antibody (Ab) therapeutics at various clinical development phases[1–3]. Current global Ab sales exceed 100 billion USD, and the monoclonal Ab (mAb) therapeutic market is expected to generate up to 300 billion USD by 2025[1,4]. With such high demand and projected increases in revenue, researchers have been working to develop ways to produce Ab therapeutics on an ever-larger scale, with higher quality and lower-costs. Plant-based expression systems have several advantages over traditional mammalian cell lines for the affordable and large-scale manufacture of Ab therapeutics[5,6]. Production of protein therapeutics in plants ("molecular pharming") does not require expensive bioreactors or cell culture facilities as do traditional

mammalian cell culture techniques[7,8]. Plants cannot contract human pathogens, minimizing potential contamination[9]. Both transient and transgenic plant-based protein expression can be utilized as lower-cost alternatives to mammalian or bacterial production systems[10]. Though transgenic plants are preferred for crop production, recombinant protein production using this method can require weeks to months. Advances in transient expression using viral vectors through either syringe or vacuum agroinfiltration allow for small- and large-scale production, respectively, of the desired protein in days[11–14]. Production of mAbs against Ebola, Dengue and, Zika, and numerous other recombinant proteins, have been produced and purified quickly and efficiently using transient expression in *N. benthamiana* plants[15–19]. These circumstances make transient plant-based expression an attractive option for developing multiple Ab therapeutics and the methods demonstrated in this protocol[20].

First-generation mAbs were of murine derivation, which resulted in non-specific immunogenicity when used in human trials[21]. Over time, chimeric, humanized, and eventually, fully human Abs were produced to lessen immunogenicity induced by Ab therapeutics. Unfortunately, some of these Abs still cause host immunogenicity due to differences in glycosylation[21]. Developments in plant engineering have allowed for the modification of Ab glycans, which is essential since an Ab's stability and function can significantly be affected by its glycosylation state[22]. Advances have allowed production in plant systems of high-level expression of humanized mAbs, containing human glycans and resultantly the desired biological traits of a mass-produced human pharmaceutical[19, 21].

In addition to recombinant Abs, Ab fusion molecules (Ab fusions) have been explored for various purposes in recent decades. Ab fusions often consist of an Ab or Ab fragment fused to a molecule or protein and are designed to elicit responses from immune effector cells[23]. These molecules have been created as potential therapeutic interventions to treat various pathologies such as cancer and autoimmune diseases[24–27]. Recombinant immune complexes (RICs) are another class of Ab fusions that have been employed as vaccine candidates[28]. RICs take advantage of the immune system's ability to recognize Fc regions of Ab fusions and have been found to improve immunogenicity when combined with other vaccine platforms[29–31].

Green Fluorescent Protein (GFP) is a bioluminescent protein derived from the jellyfish *Aequorea Victoria,* which emits green light when excited by ultraviolet light[32, 33]. Over the years, GFP's use as a visual marker of gene expression has expanded from expression in *Escherichia coli* to numerous protein expression systems, including *N. benthamiana* plants[34–38]. Visible markers, such as GFP, have abundant implications in the teaching and learning of scientific concepts. Numerous entry-level students describe difficulties grasping scientific concepts when the idea being taught is not visible to the naked eye, such as the concepts of molecular biology and related fields[39]. Visual markers, like GFP, can thus contribute to the processing of information related to the scientific processes and could help lessen the difficulties students report in learning numerous scientific concepts.

Although GFP is often used as a marker to indicate gene and expression *in vivo*, it is difficult to visualize it in the downstream processes if using acidic conditions. This circumstance is primarily

because GFP does not maintain its structure and resultant fluorescence at a low pH[40]. Temporary acidic environments are often required in various purification processes, such as protein G, protein A, and protein L chromatography, often utilized for Ab purification[41–44]. GFP mutants have been used to retain fluorescence under acidic conditions[45, 46].

Herein we describe a simple method for expression, extraction, and purification of recombinant IgG fusion proteins in *N. benthamiana* plants. We produced traditional GFP fused to the N-terminus of a humanized IgG heavy chain, creating a GFP-IgG fusion. Simultaneously, we developed the fusion of a plant codon-optimized sequence for an acid-stable GFP (asGFP) to the N-terminus of a humanized IgG heavy chain, creating an asGFP-IgG fusion. The advantages of producing GFP-IgG include the ability to visualize the presence of a target protein during expression, while asGFP-IgG allows seeing the presence of recombinant protein in not only the expression and extraction steps but also in the purification steps of the protein. This protocol can be adapted for the production, purification, and visualization of a range of GFP fusion proteins produced in *N. benthamiana* and purified using chromatography techniques that require low pH. The process can also be tailored to various amounts of leaf material. While Abs and fusion proteins tagged with GFP or asGFP are not intended to be used for therapies, these methods can be useful as controls during experiments and can also be further utilized as a teaching tool for molecular and cellular biology and biotechnology, both in-person and virtually.

Protocol:

1. Cultivate *N. benthamiana* plants

1.1 Place soil peat pellets on a tray and pour previously boiled, still hot (~40-45 °C), water over the peat pellets for full expansion. After pellets are fully expanded, place 2-3 *N. benthamiana* seeds on each peat pellet using tweezers.

1.2. Pour about 0.5 in of water to cover the bottom of the tray. Label the tray with the seeding date. Continue to water the seedlings daily with appropriate amounts of fertilizer. Fertilizer (water-soluble all-purpose plant food) concentration is generally 2.5-2.8 g/L.

1.3. Cover the tray with a humidome top when placed in the growth chamber. Keep the seeded peat pellets in the growth chamber at 23-25 °C, with a 16 h photoperiod and 60% relative humidity.

1.4. After one week, remove extra plants leaving each pellet with only one seedling.

1.5. When the plants are 2-3 weeks old, transfer each peat pellet to an individual pot containing moisture control soil. This demonstration used Miracle-Gro moisture control potting soil.

1.6. Water daily with 1 g/L fertilizer. Never leave the soil completely dry. Plants are ready for infiltration when they are 5-6 weeks old.

2. Preparation of *Agrobacterium tumefaciens* for infiltration

NOTE: GFP-IgG fusion constructs can be obtained as described in this paper[31]. The asGFP gene was obtained and plant-optimized from this study[45]. The following steps must be done next to a Bunsen burner, and basic aseptic techniques should be applied to avoid contamination.

2.1. Streak *A. tumefaciens* EHA105 harboring bean yellow dwarf virus (BeYDV)[19] plant expression vector for each construct (asGFP-IgG, GFP-IgG, light chain) from a glycerol stock on LB agar (10 g/L Tryptone, 10 g/L NaCl, 5 g yeast extract, 15 g/L agar, 50 µg/mL kanamycin) plate.

2.2. Grow for one day in a 30 °C standing incubator. Isolate a single colony for verification by standard colony screen PCR protocol.

2.3. Use verified colony for each construct. Fill conical tube with 10 mL of LB media (10 g/L tryptone, 10 g/L NaCl, 5 g of yeast extract, 50 µg/mL). Next, add 10 µL of 100 µg/mL kanamycin. Add 10 µL of 2.5 µg/mL rifampicin to prevent *E. coli* contamination. Incubate at 30°C and 120-150 rpm overnight.

2.4. The next day, if the *Agrobacterium* culture is grown to OD_{600} = 0.6-0.9, it can be used for infiltration. If it is overgrown (OD_{600}> 1), 1-2 mL should be transferred to fresh LB with antibiotics and grown to the required OD_{600}. Depending on the initial culture's concentration, it may potentially take two days to grow to OD_{600} = 0.6-0.9.

2.5. Once at appropriate OD_{600}, place the cultures in a centrifuge, and pellet the bacteria by centrifugation at 4,500 x g for 20 min, room temperature (RT).

2.6. Decant supernatant from both samples, and then resuspend each pellet in 1x infiltration buffer (10 mM 2-(N-morpholino) ethanesulfonic acid, 10 mM magnesium sulfate, adjusted to pH 5.5 with KOH) to get final OD_{600} = 0.4. This should take approximately 15-45 mL of infiltration buffer, depending on the initial culture density. Combine equal volumes of each IgG fusion construct with the light chain construct to get final OD_{600} = 0.2 per construct in each tube.

3. Needle-less syringe agroinfiltration

3.1. Take a straightened paper clip and 5-6-week-old *N. benthamiana* plants from step 1. Using the paper clip's sharp edge, make a small puncture in the first epidermal layer of the leaf on the adaxial surface. Avoid puncturing it all the way through.

NOTE: The lower leaves are easier for infiltration, whereas the leaves on the top of the plant are harder. Generally, the expression of recombinant proteins is highest in the leaves located in the middle of a plant, and these leaves also get less necrotic.

3.2. Fill a 1 mL syringe, without a needle attached, with the prepared *Agrobacterium* solution from step 2. Cover the hole made in the previous step with the end of the syringe and slowly

push to inject the bacteria into the leaf while applying gentle counterpressure from behind the leaf. Watch the leaf darken as the solution is injected without applying too much pressure on the syringe.

3.3. Try to infiltrate most of the leaf area at a maximum of 3-4 times – excessive leaf damage may hinder protein yield. The infiltrated plant leaf will appear mostly dark from the bottom view.

NOTE: This bacterial solution should be enough for at least 3-4 plants per construct. Autoclave any remaining bacterial solution before discarding.

4. Grow and observe the infiltrated *N. benthamiana*

4.1. Place infiltrated plants back in the growth chamber and continue to water daily.

4.2. Observe the leaves for chlorosis and necrosis in infiltrated areas. Observe plants for GFP fluorescence (if GFP is present) under a long and short-wave UV lamp.

4.3. Day 4-5 shows the highest fluorescence of both GFP constructs in the leaves. Harvest all the leaves at 4-5 dpi (days post-infiltration) and weigh the total leaf material.

4.4. Use it immediately for downstream processing or store at -80 °C until ready to use.

5. Protein extraction

5.1. Keep buffers and blender cups on ice or at 4 °C before use.

5.2. Prepare 2-3 mL of ice-cold extraction buffer (100 mM Tris-HCl, 50 mM NaCl, 2 mM EDTA, pH 8 with HCl) per 1 g of plant material. Add 2 mM phenylmethylsulfonyl fluoride (PMSF) from stock (100 mM) and 50 mM sodium ascorbate to the extraction buffer just before extraction.

5.3. Place plant tissue from step 4 into the prechilled blender cup. Add a measured amount of chilled extraction buffer to the blender cup (as indicated in step 5.2). Place the blender cup on the blender. Take a pre-cut sheet of parafilm and stretch it over the top of the blender cup. Blend to homogeneity with 20-sec intervals, stirring well between blend cycles as needed.

5.4. Transfer blended material to a beaker. Add a stir bar and stir at 4 °C for 30 min to enhance protein solubility and to allow precipitation of solids.

5.5. Place 2 layers of Miracloth over a clean beaker on ice and pour the extract through it to remove large leaf debris. After all the extract is poured, fold the Miracloth to squeeze the residual leaf extract. The extract should appear dark green without visible particulates.

5.6 Transfer 50 µL of this sample to a new 1.5 mL tube and label "total extract" for later analysis. Transfer the extract to centrifuge tubes. Centrifuge the remainder of plant extract at 16,000 x g

for 20 min, 4 °C and transfer the supernatant to a conical tube.

5.7. Filter the soluble extract using a 50 mL syringe and syringe glass fiber filter (0.75 µm).

5.8. Collect 50 µL of a sample after centrifugation, label "soluble extract" for later analysis.

6. Protein G column chromatography procedure

NOTE: The protocol described here is for gravity-flow chromatography using Pierce Protein G agarose resin. If using a different resin, refer to the manufacturer's instructions for adjustments. Never let the resin run dry and prevent all liquid from draining out. Recap the outlet as needed.

6.1. Set up a polypropylene column that holds 20 mL of sample.

6.2. Estimate the amount of slurry needed depending on the target immunoglobulin type and its affinity to the resin. Generally, 3 mL of total slurry with 1.5 mL bed volume is sufficient for the purification of several milligrams of Ab.

6.3. Carefully pour the required amount of resuspended slurry into the capped column. Open the column outlet from the bottom of the column and allow it to drain until most of the buffer is gone.

6.4. Immediately pour 10 mL of wash buffer 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 with HCl) on top. Let it drain and repeat this wash step 2x.

6.5. Apply the filtered sample from step 5 to the column and collect the flowthrough—aliquot 50 µL of flowthrough for later analysis. Save the rest of the flowthrough in case the Ab did not bind to the resin.

NOTE: Re-applying flowthrough to a new column does not usually result in a good yield; hence it is advised to start with new leaf material.

6.6. Wash the resin twice with 10 mL of 1x PBS to reduce non-specific binding. If desired, aliquot 50 µL of wash as the buffer drains through the column to verify that the target Ab is not eluted with a wash buffer.

6.7. Set up and label five tubes with 125 µL of sterile 1 M Tris-HCl at pH 8. This is to neutralize the Abs in the acidic elution buffer to avoid potential structural changes. Alternatively, add 30 µL of 2 M Tris base to get a less diluted sample.

CAUTION: During elution, UV light may be used for visualization. This does not need to be done for the duration of the elution. If UV is being used, be sure to wear appropriate PPE to avoid damage to eyes and skin. A UV light does not need to be used during the elution step.

6.8. Elute the Abs by applying 5 mL of elution buffer (100 mM glycine, pH 2.5 with HCl) to the column and collect 1 mL fractions to each designated tube from the previous step.

6.9. Immediately regenerate the column by applying 20 mL of wash buffer, followed by 10 mL of wash buffer. Ensure that the resin is not left in an acidic environment for an extended time. Elutions should appear fluorescent, often the highest fluorescence is seen in the second elution but can vary from extraction to extraction.

6.10. For storage, wash the resin with 10 mL of 20% ethanol in PBS and let it drain halfway. Recap the top, then the bottom of the column, and keep upright at 4 °C.

NOTE: Generally, protein G resins can be reused up to 10 times without significant loss of efficiency. Refer to the manufacturer's guidelines for specific details.

6.11. Determine Ab concentration using a spectrophotometer by measuring absorbance at 280 nm, using the elution buffer as a blank. Store the eluates in -80 °C and aliquot 50 µL of each fraction to a separate tube for further analysis.

7. SDS-PAGE for GFP-Ig fusion detection

7.1. Prepare all samples before setting up the SDS-PAGE.

7.1.1. Add 4 µL of sample buffer (6x reducing sample buffer: 3.0 mL of glycerol, 0.93 g of DTT, 1 g of SDS, 7 mL of 4x Tris (pH 6.8) 0.5 M, 1.2 mg of bromophenol blue); (6x non-reducing sample buffer: 3.0 mL of glycerol, 1 g of SDS, 7 mL of 4x Tris (pH 6.8) 0.5 M, 1.2 mg of bromophenol blue) to 20 µL of each sample (total extract, soluble extract, flowthrough, wash, all elution fractions) for analysis. Ensure that tube caps are securely fastened.

7.1.2. Treat only reducing samples for 5 min in a boiling water bath, and then put samples for 5 min on ice. Spin samples in a microcentrifuge for ~5 s and load 20 µL of each sample in the order of collection into the gel wells. Load 3 µL of dual-color protein ladder in a separate well.

7.2. Run the SDS-PAGE gel at a constant 100 V to desired protein band separation; it takes about 1.5 hours. Monitor the ladder as an indicator of protein separation.

7.3. Visualize the gel under the UV to observe GFP fluorescence.

7.4. If desired, stain the gel with Coomassie stain to assess total protein in each sample. Alternatively, perform western blot to evaluate target protein using specific Abs.

NOTE: Both Coomassie staining and western blot can be performed by following standard protocols[47,48].

Representative Results:

This study demonstrates an easy and fast method to produce recombinant proteins and visualize them throughout downstream processes. Using *N. benthamiana* and following the provided protocol, recombinant protein production described here can be achieved in less than a week. The overall workflow of plant expression, extraction, and purification is shown in **Figure 1**. The stages of plant growth from 2-week old seedlings, 4-week old plants, and 6-week old plants are displayed in **Figure 1A (1-3),** respectively, while **Figure 1B** depicts leaf morphological changes due to necrosis **(Figure 1B-1)** or chlorosis **(Figure 1B-2)**. Necrosis may occur at the injection site between days 3-5 after infiltration. These changes often depend on the protein's properties being expressed and the infiltrated plants' health (further examined in discussion). Simultaneously, chlorosis can also rely on the health of plants being used (further examined in discussion). The process of *Agrobacterium* growth and preparation for infiltration is shown in **Figure 1C**. **Figure 1C-1** displays isolated colonies of *Agrobacterium*. **Figures 1C (2-5)** display the media's expected appearance after it is inoculated with a single isolated colony. Refer to **Figure 3** for more details on these steps. The plant infiltration process is shown in **Figure 1D** and begins with an un-infiltrated plant and is followed by the infiltration process. The expression, extraction, and clarification of plant proteins are displayed in **Figure 1E**. The leaf material is placed in a blender and is homogenized, shown in **Figures 1E (1-3).** A sample representing total homogenate is then taken. It is then filtered through a Miracloth (gauze or even a coffee filter can substitute for reduced expenses), and the clarified suspension is centrifuged. The centrifugation allows for the separation of the supernatant from the remaining materials, as shown in **Figures 1E (4-6)**. The clarified supernatant is then loaded on a protein G affinity chromatography column, **Figure 1F (1-3).** After most of the protein is bound, **Figure 1F-4**, the proteins are eluted from the resin **Figure 1F (5,6)**.

Table 1 displays the plant optimized nucleic acid sequences used to produce asGFP[45] (upper row) in the pBY!KEAM-GFPasH vector used in this study to express asGFP-IgG fusion and GFP[33] (lower row) in the PBYEAM-GFPHgp vector used in this study to express GFP-IgG fusion. Nucleic acid sequences were examined using the Expasy protein translate tool (https://web.expasy.org/translate/) to determine amino acid sequences.

A representative *Agrobacterium* plate prepared using this protocol is shown in **Figure 2**. Desired colonies should appear round and uniform in shape and color. Colonies closer to the center of the plate have a higher likelihood of expressing kanamycin resistance. The liquid cultures will be prepared from a single isolated colony.

The expected appearance of media containing cultures is shown in **Figure 3.** Upon initial inoculation of an isolated colony, LB media will appear light yellow and translucent, as shown in **Figure 3A**. After incubation of an isolated colony overnight at 30 °C, LB media will appear turbid. As shown in **Figure 3B**, objects can no longer be seen through the media when growth is present in the LB. Following centrifugation, a pellet should form at the bottom of the tube. The tube will have a clear separation of LB media above the pellet and will appear light yellow and translucent, as shown in **Figure 3C**. The LB media supernatant is disposed of, and the pellet is resuspended in the infiltration buffer. At an OD_{600} of 0.2, the media will appear turbid, as shown in **Figure 3D**. OD_{600} should be measured as described in the methods.

Figure 4 represents the process of leaf infiltration. A slight prod of the leaf with a paperclip should yield a break in the leaf epidermis that does not pass entirely through the leaf. The break should barely pierce the leaf so the infiltration buffer can be injected into the leaf, shown in **Figure 4A-C.** The suspension of *Agrobacterium* and infiltration buffer is injected directly into the break in the leaf and slightly alters the infiltrated leaf's color; see **Figure 4D-F.**

The appearance of leaves expressing IgG fusions is represented in **Figure 5.** It displays leaves that express asGFP-IgG fusions **(Figure 5A)** and GFP-IgG fusions **(Figure 5C)** under white light. If the constructs in this protocol are used, when infiltrated at a 0.2 OD_{600}, leaves should appear healthy on days 1-5 for both leaves expressing asGFP-IgG fusions and leaves expressing GFP-IgG fusions. There may be a slight necrotic appearance at injection sites on day 5, which is usually apparent by the lightening of the plant tissue in those areas. **Figure 5** also displays leaves expressing asGFP-IgG fusions **(Figure 5B)** and GFP-IgG fusions **(Figure 5D)**, respectively, under long-wave UV light from the leaf's top view. Fluorescence increases in intensity as the days progress for both constructs expressed. Leaves expressing asGFP-IgG fusions tend to have slightly less intense fluorescence than leaves expressing GFP-IgG fusions on all days.

When the supernatant of the asGFP-IgG extract is added to the Protein G column, the resin will become slightly green under white light due to plant chlorophyll pigments **(Figure 6A).** The addition of supernatant under short-wave UV light results in the accumulation of fluorescence in the Protein G resin, as shown in **Figure 6B**. Note that the supernatant will also be fluorescent alone under UV light. Still, fluorescence is expected to be much more concentrated when the asGFP-IgG fusion begins to bind to the resin.

Following the passing of supernatant of asGFP-IgG through the protein G resin, the resin should illuminate under short wave UV light, as shown in **Figure 7A**. At this point, most of the IgG will be bound to the resin. Upon adding the elution buffer, the fluorescence contained in the protein G resin will still be visible under short-wave UV light and will begin to lose intensity as more elution buffer of low pH passes through the resin. Fluorescence will start to accumulate in the eluates **(Figure 7B)**. Eluate fractions will vary in fluorescence. As seen in **Figure 8**, fluorescence is the lowest intensity in the first elution and highest intensity in the second and third eluates. Results may vary, as the fluorescence will depend on the protein's expression, harvested leaf material, and other conditions used in the experiment.

After finishing the purification process, samples are analyzed on the 10% SDS-PAGE gel under reducing conditions (sample buffer contains DTT and samples were boiled for 5 min) and non-reducing conditions (sample buffer does not contain DTT and samples were not boiled). As shown in **Figure 9A,** only non-reducing samples, such as in Elution 2 NR lane, will fluoresce when exposed to short wave UV light. This lane's first band is fluorescing at the full product's expected size ~200 kDa, indicating that the asGFP is still conformationally correct. The fluorescent bands near the bottom of the gel are dye from the reducing buffer. Note that asGFP loses fluorescence when exposed to temperatures at or above 95 °C for 5 minutes; this is different from eGFP (enhanced GFP), which would maintain some fluorescence under the same conditions[49, 50]. Two bands of the

ladder, 75 kDa and 25 kDa, also fluoresce. **Figure 9B** represents a Coomassie stain of the same gel in **Figure 9A.** Elutions in lanes 6-9 have been prepared under reducing conditions. When run on a gel and Coomassie-stained, these samples should display the asGFP-IgG fusion components separately. These components include the heavy chain fused to GFP (~75 kDa), the heavy chain alone (50 kDa), the light chain (25 kDa), and the asGFP itself (27 kDa). The non-reducing sample was included in the last lane of the gel for comparison purposes and should display a single large band (~200 kDa), which should be made up of two heavy chains fused to the asGFP and respective light chains. Additionally, smaller bands are likely caused by native proteases. This cleavage can be prevented with the addition of protease inhibitors and by keeping the protein extract cold at all times, including when performing the column purification. The IgG fusion protein's individual components will not be distinguishable in the non-reducing samples on the Coomassie gel.

Figure 1: Workflow of plant expression, extraction, and purification processes.

Table 1: Sequences used to create asGFP and GFP

Figure 2: *Agrobacterium* colonies grown on LB plate containing kanamycin.

Figure 3: Appearance of media throughout the growth and processing of *Agrobacterium*. (A) The appearance of LB media immediately following inoculation of isolated *Agrobacterium* colony. **(B)** The presence of media after overnight incubation of an isolated colony at 30°C. **(C)** The appearance of media after cultures are spun down for 20 min at 4,500 x g. **(D)** Pellet resuspended in the infiltration buffer.

Figure 4: Process of infiltration of *N. benthamiana* plants. (A-B) Slightly poking the leaf results in a subtle hole at the top of the leaf. **(C-D)** Injection of *Agrobacterium* suspension into leaf. **(E)** Infiltrated Plant leaf from the top view. **(F)** Plant with multiple leaves infiltrated from the top view.

Figure 5: Visualization of leaves containing asGFP-IgG fusion and GFP-IgG fusion begin on day 1 post infiltration (dpi) in the first row, leading up to day 5 dpi in the last row for all conditions. A) asGFP-IgG fusion under white light. **B)** asGFP-IgG fusion under long-wave UV. **C)** GFP-IgG fusion under white light. **D)** GFP-IgG fusion under long-wave UV.

Figure 6: Supernatant of the asGFP-IgG extract being added to the Protein G column. A) Addition under white light. **B)** Addition under short wave UV light.

Figure 7: Protein G resin under short-wave UV light after supernatant of the asGFP-IgG extract was run through the column. A) Protein G resin under short-wave UV light. **B)** Protein G resin upon elution of proteins under low PH conditions.

Figure 8: Purified elutions of asGFP-IgG obtained after exposure to low pH conditions through purification.

Figure 9: SDS-PAGE of Column Samples. Samples labeled with "R" are in reducing conditions, and samples labeled with "NR" are in non-reducing conditions. **A)** Under UV light, only non-reducing samples fluoresce in the 10% polyacrylamide gel, as seen in Elution 2 NR lane. The 75 kDa and 25 kDa ladder bands also fluoresce. **B)** Coomassie staining of the same gel reveals the presence of all proteins in the sample. In the reduced elutions, the IgG fusion without light chain, the heavy chain, light chain, and possibly degraded GFP are present at ~75 kDa, ~50 kDa, ~25 kDa, and ~10 kDa, respectively. In contrast, in the non-reduced elutions, one prominent band is present, consistent with the size of the entire asGFP-IgG fusion (~200 kDa).

Discussion:

This protocol can be utilized for the visual verification of any recombinant Ab or recombinant protein produced in *N. benthamiana* plants, including those that require temporary exposure to acidic environments for column purification purposes[42–44]. Furthermore, the fusion of asGFP to other proteins in different expression systems can be a useful tool for experimental visualization and education. The protocol herein can further be scaled to larger and smaller amounts of leaf material to produce the desired amount of recombinant protein. The described methods take advantage of previous studies that have identified and made versions of GFP that remain stable under acidic conditions[46]. Comprehensive prior studies have created immunoglobulin domains fused to a protein of interest, often termed IgG-fusions, which this protocol can also accommodate[28]. By creating and expressing an IgG-fusion composed of a humanized IgG1 fused to a GFP and asGFP, we were able to visualize the presence of the desired protein during the expression, extraction, and purification processes.

If a skilled researcher follows this protocol, leaves will begin to display necrosis signs at the infiltration sites between days 4-5. However, when using the described vectors, infiltrated areas of leaves should appear healthy up until day 5 with proper care. It is important to note that *Agrobacterium* infection on its own will eventually result in necrosis of the plant leaves due to the accumulation of reactive oxygen species (ROS) as part of the plant cell immune response[51,52]. This immune response and the resultant level of necrosis can vary based on several factors[51], including the cellular targeting, the protein's location, the type of protein being produced, the expression vector, and the strain of *Agrobacterium* used[53,54]. Also, variations in the optical density (OD_{600}) of the *Agrobacterium* used for infiltration can affect the levels of necrosis[55]. Many expression vectors utilize proteins that bind retinoblastoma protein to keep the plant cells in synthesis (S)-phase and increase cell division and transformation frequency[56,57]. Increases in protein production, such as those caused by binding to retinoblastoma protein, can lead to necrosis[56,57]. Advances in vector design, such as those used in modified versions of the geminivirus BeYDV replicons used in this protocol, have minimized necrosis while maintaining high protein expression levels[58]. Also, BeYDV replicons are non-competing, providing expression of multiple proteins on a single cassette without known size limitations[53,59].

Several factors affect plant growth before and after infiltration, which might eventually lead to low protein yield. When seeding plants, too many seeds per plant pellet can result in many small plant sprouts leading to more modest plant growth. Hence, reducing the seed number per peat pellet and removing the extra sprouts after a week will result in better plant growth. Maintaining

proper soil moisture is another factor affecting overall plant health. Overwatering, underwatering, adding too much or too little fertilizer might contribute to chlorosis and affect the plant health[60–62]. Necrosis and chlorosis can additionally be caused by the production of a protein that causes cell stress. This phenomenon has been seen many times with the expression of recombinant immune complexes (RIC)[56]. We have observed that changes in protein structure and fusion of proteins can help minimize necrosis; however, some proteins can remain toxic to plants even after various modifications. If using the expression vectors outlined herein, extraction of protein may be performed early and before the onset of significant necrosis, resulting in high protein yield[56].

Different growth conditions can slow or even inhibit *Agrobacterium* growth. *Agrobacterium* grows optimally at 28 °C-30 °C and experiences a heat shock when incubated above 30 °C, producing cell division errors[62]. Growth can also be impeded by the addition of too much rifampicin, as different *Agrobacterium* strains are more or less naturally resistant to this antibiotic[62]. The bacterial culture prepared for infiltration with significantly higher OD_{600} than recommended will likely cause necrosis[55]. A slightly higher OD_{600} of the culture usually does not affect the yield, but if it is lower than 0.1, the protein yield might be considerably reduced. Accumulation of dead cells can occur under two circumstances; 1) the culture was overgrown, leading to a significant fraction of the OD being dead cells, and 2) damaging/killing the *Agrobacterium* after growth, such as with chemical residue or high centrifuge speeds. Infiltrations using an increased number of dead cells in the culture might reduce protein expression. Moreover, puncturing the leaves by applying too much pressure can damage the leaves and hence might lead to premature necrosis. Considering these possible factors when expressing recombinant proteins in *Nicotiana benthamiana*, can lead to enhanced protein production.

Obtaining low protein yield could be due to some issues in the extraction and purification steps. Firstly, the extraction buffer might need optimization depending on the protein of interest. During blending, plant material should be homogenous without any visible leaf pieces. Next, some proteins require detergents for solubilization in the extraction buffer, such as Tween-20 or Triton. Other proteins might need urea at high concentrations ~7.5 M for solubilization, while some can be extracted with PBS only. Degradation of protein can occur if buffers, plant tissue, centrifuges, etc. are not kept cool during the extraction process. Lack of protease inhibitors and sodium ascorbate or similar chemicals in the extraction buffer can also cause degradation or aggregation. Some protease inhibitors like PMSF degrade quickly, and sodium ascorbate takes some time to become aqueous. Overall, researchers should determine optimal conditions for their protein of interest.

The purification of IgG-fusions includes few steps that might need modifications if low protein yield is obtained. Analyzing the sample aliquots collected during the entire process by SDS-PAGE and Western will help to identify the fault of the methods. For example, if the flowthrough contains a substantial amount of protein, then the binding of the protein can be facilitated by changing the pH of the buffer. Using high concentrations of detergents during the extraction process might affect the binding property of the resin, especially if the resin is reused several times. Proper storage of the resin, as described in the methods, is vital for the resin's lifespan.

Moreover, if the washing step removes the protein of interest from the resin, the buffers might need to be remade to solve this problem. Other issues with protein purification might be due to misfolded or degraded proteins, which might require further analysis of the overall protein design. Referencing the described troubleshooting may increase the efficiency of the purification using this protocol.

The described GFP-IgG fusion purification is helpful in a teaching environment. Visualization is fundamental to science education because it allows learners to comprehend concepts more easily[39]. Students often report misunderstandings in addition to difficulty understanding concepts at the molecular level[39]. In particular, the experiments that require an understanding of the specific protein location at each step can be modified by tagging protein of interest with fluorescent molecules. Therefore, GFP or asGFP, depending on the pH environment used, can be utilized to harness their fluorescence to facilitate elucidation of the protein purification technique for students.

In summary, we describe a simple method for expression and purification of a recombinant Ab fused to a GFP in *N. benthamiana* plants. This protocol can be used for the purification of an Ab fused to any desired target protein. The process can be edited to accommodate various amounts of leaf material and allows for visual determination of protein presence before, during, and after the conclusion of the protein extraction and purification process. These methods can be useful as controls and can be purposed for teaching techniques.

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Disclosures:

The authors have nothing to disclose.

References:

1. Lu, R.M. et al. Development of therapeutic antibodies for the treatment of diseases. *Journal of Biomedical Science*. **27** (1), doi: 10.1186/s12929-019-0592-z (2020).
2. Kaplon, H., Reichert, J.M. Antibodies to watch in 2019. *mAbs*. **11** (2), 219–238, doi: 10.1080/19420862.2018.1556465 (2019).
3. The top 20 drugs by 2018 U.S. sales | FiercePharma. at <https://www.fiercepharma.com/special-report/top-20-drugs-by-2018-u-s-sales>.
4. Grilo, A.L., Mantalaris, A. The Increasingly Human and Profitable Monoclonal Antibody Market. *Trends in Biotechnology*. **37** (1), 9–16, doi: 10.1016/j.tibtech.2018.05.014 (2019).
5. Kim, M.-Y. et al. Novel vaccination approach for dengue infection based on recombinant immune complex universal platform. *Vaccine*. **33** (15), 1830–1838, doi: 10.1016/J.VACCINE.2015.02.036 (2015).
6. Twyman, R.M., Schillberg, S., Fischer, R. Transgenic plants in the biopharmaceutical

market. *Expert Opinion on Emerging Drugs*. **10** (1), 185–218, doi: 10.1517/14728214.10.1.185 (2005).
7. Stoger, E., Fischer, R., Moloney, M., Ma, J.K.-C. Plant Molecular Pharming for the Treatment of Chronic and Infectious Diseases. *Annual Review of Plant Biology*. **65** (1), 743–768, doi: 10.1146/annurev-arplant-050213-035850 (2014).
8. Buyel, J.F., Fischer, R. Predictive models for transient protein expression in tobacco (Nicotiana tabacum L.) can optimize process time, yield, and downstream costs. *Biotechnology and Bioengineering*. **109** (10), 2575–2588, doi: 10.1002/bit.24523 (2012).
9. Yao, J., Weng, Y., Dickey, A., Wang, K.Y. Plants as factories for human pharmaceuticals: Applications and challenges. *International Journal of Molecular Sciences*. **16** (12), 28549–28565, doi: 10.3390/ijms161226122 (2015).
10. Mor, T.S., Moon, Y.-S., Palmer, K.E., Mason, H.S. Geminivirus vectors for high-level expression of foreign proteins in plant cells. *Biotechnology and Bioengineering*. **81** (4), 430–437, doi: 10.1002/bit.10483 (2003).
11. Hefferon, K.L. Plant virus expression vectors set the stage as production platforms for biopharmaceutical proteins. *Virology*. **433** (1), 1–6, doi: 10.1016/j.virol.2012.06.012 (2012).
12. Yamamoto, T. et al. Improvement of the transient expression system for production of recombinant proteins in plants. *Scientific Reports*. **8** (1), 4755, doi: 10.1038/s41598-018-23024-y (2018).
13. Zhong, G.Y. et al. Commercial production of aprotinin in transgenic maize seeds. *Molecular Breeding*. **5** (4), 345–356, doi: 10.1023/A:1009677809492 (1999).
14. Giddings, G., Allison, G., Brooks, D., Carter, A. Transgenic plants as factories for biopharmaceuticals. *Nature Biotechnology*. **18** (11), 1151–1155, doi: 10.1038/81132 (2000).
15. Fulton, A., Lai, H., Chen, Q., Zhang, C. Purification of monoclonal antibody against Ebola GP1 protein expressed in Nicotiana benthamiana. *Journal of Chromatography A*. **1389**, 128–132, doi: 10.1016/j.chroma.2015.02.013 (2015).
16. Diamos, A. et al. A highly expressing, soluble, and stable plant-made IgG fusion carrying Zika virus envelope domain III elicits potent immunogenic responses in mice without adjuvant. *bioRxiv*. 2020.07.13.199703, doi: 10.1101/2020.07.13.199703 (2020).
17. Hunter, J.G.L. et al. Evaluation of a toxoid fusion protein vaccine produced in plants to protect poultry against necrotic enteritis. *PeerJ*. **2019** (3), e6600, doi: 10.7717/peerj.6600 (2019).
18. Dent, M. et al. Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity. *Journal of General Virology*. **97** (12), 3280–3290, doi: 10.1099/jgv.0.000635 (2016).
19. Diamos, A.G. et al. High Level Production of Monoclonal Antibodies Using an Optimized Plant Expression System. *Frontiers in Bioengineering and Biotechnology*. **7**, 472, doi: 10.3389/fbioe.2019.00472 (2020).
20. Olinger, G.G. et al. Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. *Proceedings of the National Academy of Sciences of the United States of America*. **109** (44), 18030–18035, doi: 10.1073/pnas.1213709109 (2012).

21. Mastrangeli, R., Palinsky, W., Bierau, H. Glycoengineered antibodies: towards the next-generation of immunotherapeutics. *Glycobiology*. **29** (3), 199–210, doi: 10.1093/glycob/cwy092 (2019).
22. Montero-Morales, L., Steinkellner, H. Advanced Plant-Based Glycan Engineering. *Frontiers in Bioengineering and Biotechnology*. **6** (JUN), 81, doi: 10.3389/fbioe.2018.00081 (2018).
23. Joosten, V., Lokman, C., van den Hondel, C.A.M.J.J., Punt, P.J. The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. *Microbial Cell Factories*. **2** (1), 1–15, doi: 10.1186/1475-2859-2-1 (2003).
24. Müller, D. Antibody fusions with immunomodulatory proteins for cancer therapy. *Pharmacology and Therapeutics*. **154**, 57–66, doi: 10.1016/j.pharmthera.2015.07.001 (2015).
25. Bootz, F., Neri, D. Immunocytokines: A novel class of products for the treatment of chronic inflammation and autoimmune conditions. *Drug Discovery Today*. **21** (1), 180–189, doi: 10.1016/j.drudis.2015.10.012 (2016).
26. Jafari, R., Zolbanin, N.M., Rafatpanah, H., Majidi, J., Kazemi, T. Fc-fusion Proteins in Therapy: An Updated View. *Current Medicinal Chemistry*. **24** (12), doi: 10.2174/0929867324666170113112759 (2017).
27. Brinkmann, U., Kontermann, R.E. The making of bispecific antibodies. *mAbs*. **9** (2), 182–212, doi: 10.1080/19420862.2016.1268307 (2017).
28. Mason, H.S. Recombinant immune complexes as versatile and potent vaccines. *Human Vaccines & Immunotherapeutics*. **12** (4), 988–989, doi: 10.1080/21645515.2015.1116655 (2016).
29. Mason, H.S. Recombinant immune complexes as versatile and potent vaccines. *Human Vaccines and Immunotherapeutics*. **12** (4), 988–989, doi: 10.1080/21645515.2015.1116655 (2016).
30. Diamos, A.G. et al. Vaccine synergy with virus-like particle and immune complex platforms for delivery of human papillomavirus L2 antigen. *Vaccine*. **37** (1), 137–144, doi: 10.1016/j.vaccine.2018.11.021 (2019).
31. Diamos, A.G. et al. Codelivery of improved immune complex and virus-like particle vaccines containing Zika virus envelope domain III synergistically enhances immunogenicity. *Vaccine*. **38** (18), 3455–3463, doi: 10.1016/j.vaccine.2020.02.089 (2020).
32. Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J. Primary structure of the Aequorea victoria green-fluorescent protein. *Gene*. **111** (2), 229–233, doi: 10.1016/0378-1119(92)90691-H (1992).
33. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., Prasher, D.C. Green fluorescent protein as a marker for gene expression. *Science*. **263** (5148), 802–805, doi: 10.1126/science.8303295 (1994).
34. Palm, M., Baumstark-Khan, C., Horneck, G. Green Fluorescent Protein (GFP) Expression in Mammalian Cells After UV-Irradiation. *Fundamentals for the Assessment of Risks from Environmental Radiation*. 311–316, doi: 10.1007/978-94-011-4585-5_40 (1999).
35. Paemeleire, K. et al. Intercellular calcium waves in HeLa cells expressing GFP-labeled connexin 43, 32, or 26. *Molecular Biology of the Cell*. **11** (5), 1815–1827, doi: 10.1091/mbc.11.5.1815 (2000).
36. Harper, B.K., Stewart, C.N. Patterns of Green Fluorescent Protein Expression in Transgenic

Plants. *Plant Molecular Biology Reporter*. **18** (2), 141–141, doi: 10.1007/BF02824023 (2000).
37. Kaishima, M., Ishii, J., Matsuno, T., Fukuda, N., Kondo, A. Expression of varied GFPs in Saccharomyces cerevisiae: Codon optimization yields stronger than expected expression and fluorescence intensity. *Scientific Reports*. **6**, doi: 10.1038/srep35932 (2016).
38. Glow in the Dark: Fluorescent Proteins as Cell and Tissue-Specific Markers in Plants - PubMed. at <https://pubmed.ncbi.nlm.nih.gov/21772029/>.
39. Mnguni, L.E. The theoretical cognitive process of visualization for science education. *SpringerPlus*. **3** (1), 1–9, doi: 10.1186/2193-1801-3-184 (2014).
40. Shaner, N.C., Patterson, G.H., Davidson, M.W. Advances in fluorescent protein technology. *Journal of Cell Science*. **120** (24), 4247–4260, doi: 10.1242/jcs.005801 (2007).
41. Bjorck2, L., Kronvall, G. Purification and some properties of streptococcal protein G, a novel IgG-Binding reagent. *The Journal of Immunology.* **133** (2), at <http://www.jimmunol.org/>. (1984).
42. Andrew, S.M., Titus, J.A. Purification of Immunoglobulin G. *Current Protocols in Immunology*. **21** (1), 2.7.1-2.7.12, doi: 10.1002/0471142735.im0207s21 (2001).
43. Hober, S., Nord, K., Linhult, M. Protein A chromatography for antibody purification. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. **848** (1), 40–47, doi: 10.1016/j.jchromb.2006.09.030 (2007).
44. Vola, R., Lombardi, A., Tarditi, L., Björck, L., Mariani, M. Recombinant proteins L and LG: efficient tools for purification of murine immunoglobulin G fragments. *Journal of Chromatography B: Biomedical Sciences and Applications*. **668** (2), 209–218, doi: 10.1016/0378-4347(95)00091-V (1995).
45. Shinoda, H., Ma, Y., Nakashima, R., Sakurai, K., Matsuda, T., Nagai, T. Acid-Tolerant Monomeric GFP from Olindias formosa. *Cell Chemical Biology*. **25** (3), 330-338.e7, doi: 10.1016/j.chembiol.2017.12.005 (2018).
46. Roberts, T.M. et al. Identification and Characterisation of a pH-stable GFP. *Scientific Reports*. **6** (1), 1–9, doi: 10.1038/srep28166 (2016).
47. Brunelle, J.L., Green, R. Coomassie blue staining. *Methods in Enzymology*. **541**, 161–167, doi: 10.1016/B978-0-12-420119-4.00013-6 (2014).
48. Mahmood, T., Yang, P.C. Western blot: Technique, theory, and trouble shooting. *North American Journal of Medical Sciences*. **4** (9), 429–434, doi: 10.4103/1947-2714.100998 (2012).
49. Vessoni Penna, T.C., Ishii, M., Cholewa, O., de Souza, L.C. Thermal characteristics of recombinant green fluorescent protein (GFPuv) extracted from Escherichia coli. *Letters in Applied Microbiology*. **38** (2), 135–139, doi: 10.1111/j.1472-765X.2003.01460.x (2004).
50. Kang, S.J., Park, E.A., Lee, D.H., Hong, K.W. Comparison of the stability of eGFP displayed on the Bacillus subtilis spore surface using CotB and C-terminally truncated CotB proteins as an anchoring motif under extreme conditions. *Applied Biological Chemistry*. **62** (1), 41, doi: 10.1186/s13765-019-0448-y (2019).
51. Kuta, D.D., Tripathi, L. *Agrobacterium*-induced hypersensitive necrotic reaction in plant cells: a resistance response against *Agrobacterium*-mediated DNA transfer. *African Journal of Biotechnology*. **4** (8), 752–757, doi: 10.4314/AJB.V4I8.15199 (2005).
52. Qiusheng, Z., Bao, J., Likun, L., Xianhua, X. Effects of antioxidants on the plant regeneration

and GUS expressive frequency of peanut (Arachis hypogaea) explants by Agrobacterium tumefaciens. *Plant Cell, Tissue and Organ Culture*. **81** (1), 83–90, doi: 10.1007/s11240-004-3176-9 (2005).

53. Diamos, A.G., Rosenthal, S.H., Mason, H.S. 5′ and 3′ Untranslated Regions Strongly Enhance Performance of Geminiviral Replicons in Nicotiana benthamiana Leaves. *Frontiers in Plant Science*. **7** (FEB2016), 200, doi: 10.3389/fpls.2016.00200 (2016).
54. Huang, Z., Chen, Q., Hjelm, B., Arntzen, C., Mason, H. A DNA replicon system for rapid high-level production of virus-like particles in plants. *Biotechnology and Bioengineering*. **103** (4), 706–714, doi: 10.1002/bit.22299 (2009).
55. Diamos, A.G., Mason, H.S. Modifying the Replication of Geminiviral Vectors Reduces Cell Death and Enhances Expression of Biopharmaceutical Proteins in Nicotiana benthamiana Leaves. *Frontiers in Plant Science*. **9**, 1974, doi: 10.3389/fpls.2018.01974 (2019).
56. Gordon-Kamm, W. et al. Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. *Proceedings of the National Academy of Sciences of the United States of America*. **99** (18), 11975–11980, doi: 10.1073/pnas.142409899 (2002).
57. Villemont, E., Dubois, F., Sangwan, R.S., Vasseur, G., Bourgeois, Y., Sangwan-Norreel, B.S. Role of the host cell cycle in the Agrobacterium-mediated genetic transformation of Petunia: Evidence of an S-phase control mechanism for T-DNA transfer. *Planta*. **201** (2), 160–172, doi: 10.1007/BF01007700 (1997).
58. Regnard, G.L., Halley-Stott, R.P., Tanzer, F.L., Hitzeroth, I.I., Rybicki, E.P. High level protein expression in plants through the use of a novel autonomously replicating geminivirus shuttle vector. *Plant Biotechnology Journal*. **8** (1), 38–46, doi: 10.1111/j.1467-7652.2009.00462.x (2010).
59. Chen, Q., He, J., Phoolcharoen, W., Mason, H.S. Geminiviral vectors based on bean yellow dwarf virus for production of vaccine antigens and monoclonal antibodies in plants. *Human Vaccines*. **7** (3), 331–338, doi: 10.4161/hv.7.3.14262 (2011).
60. Morton, T.G., Gold, A.J., Sullivan, W.M. Influence of Overwatering and Fertilization on Nitrogen Losses from Home Lawns. *Journal of Environmental Quality*. **17** (1), 124–130, doi: 10.2134/jeq1988.00472425001700010019x (1988).
61. Brown, J.C. Iron Chlorosis in Plants. *Advances in Agronomy*. **13** (C), 329–369, doi: 10.1016/S0065-2113(08)60963-3 (1961).
62. Morton, E.R., Fuqua, C. Laboratory Maintenance of Agrobacterium . *Current Protocols in Microbiology*. **CHAPTER**, Unit3D.1, doi: 10.1002/9780471729259.mc03d01s24 (2012).

Figure 1

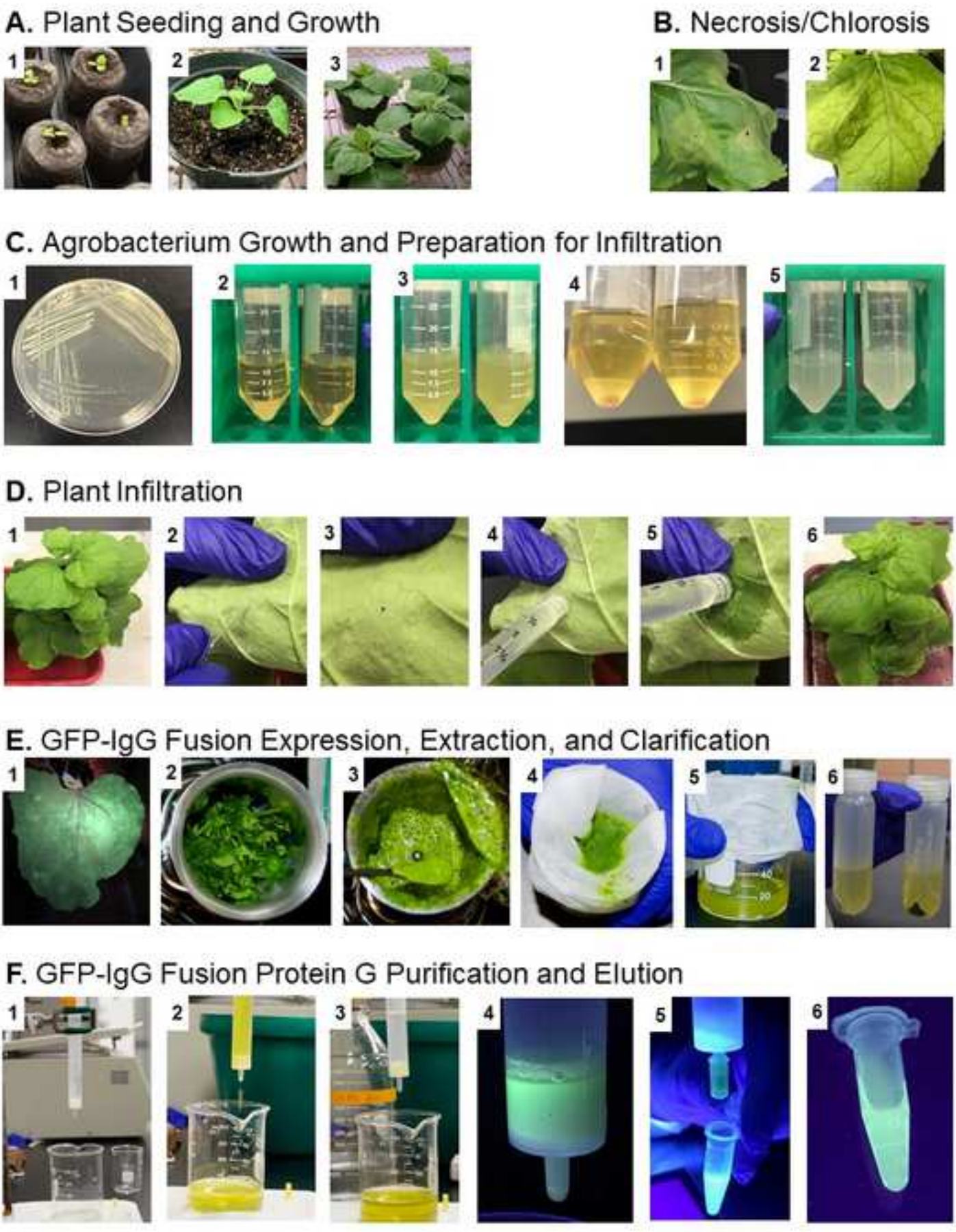

Figure 2

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Figure 3

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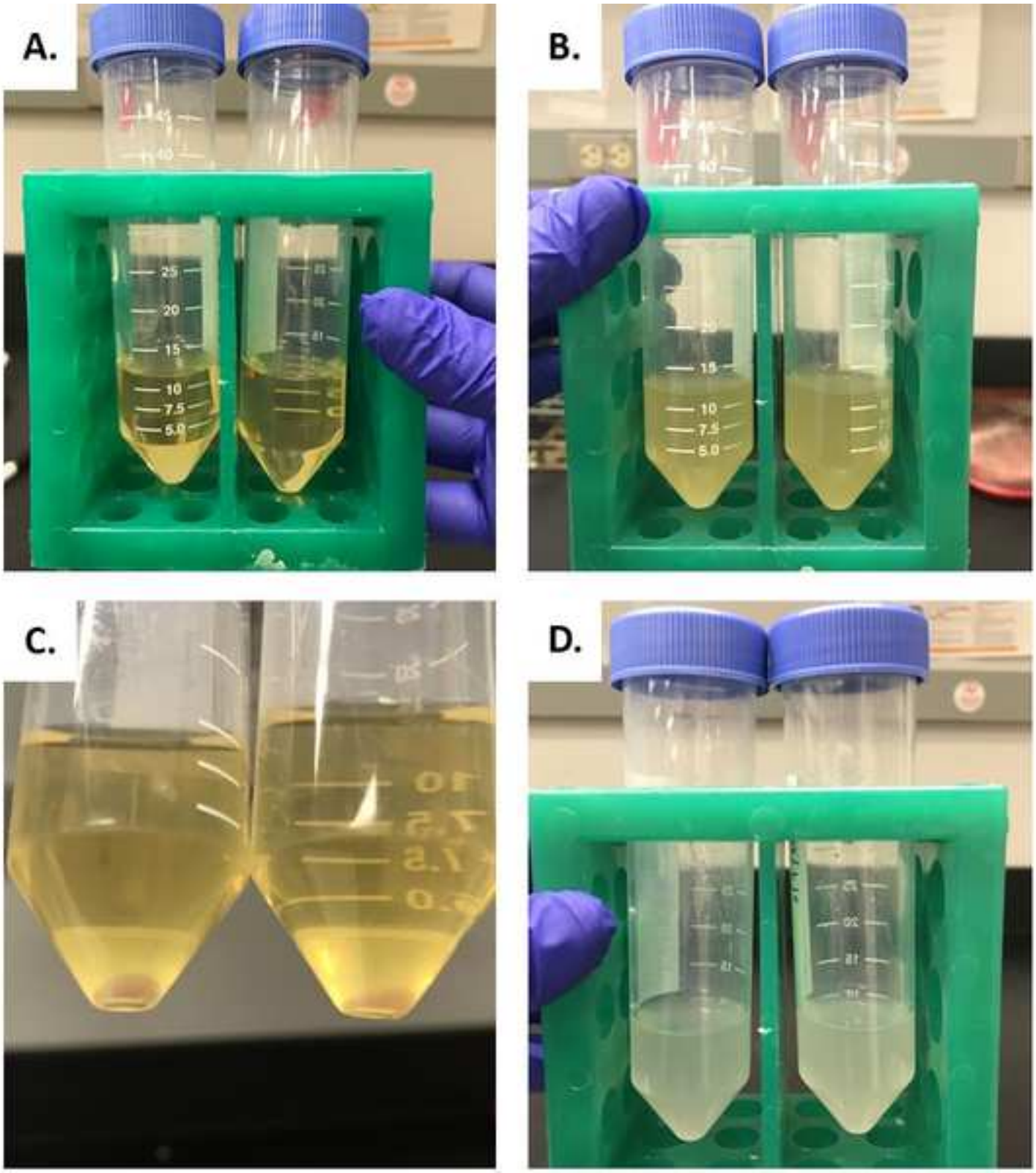

Figure 4

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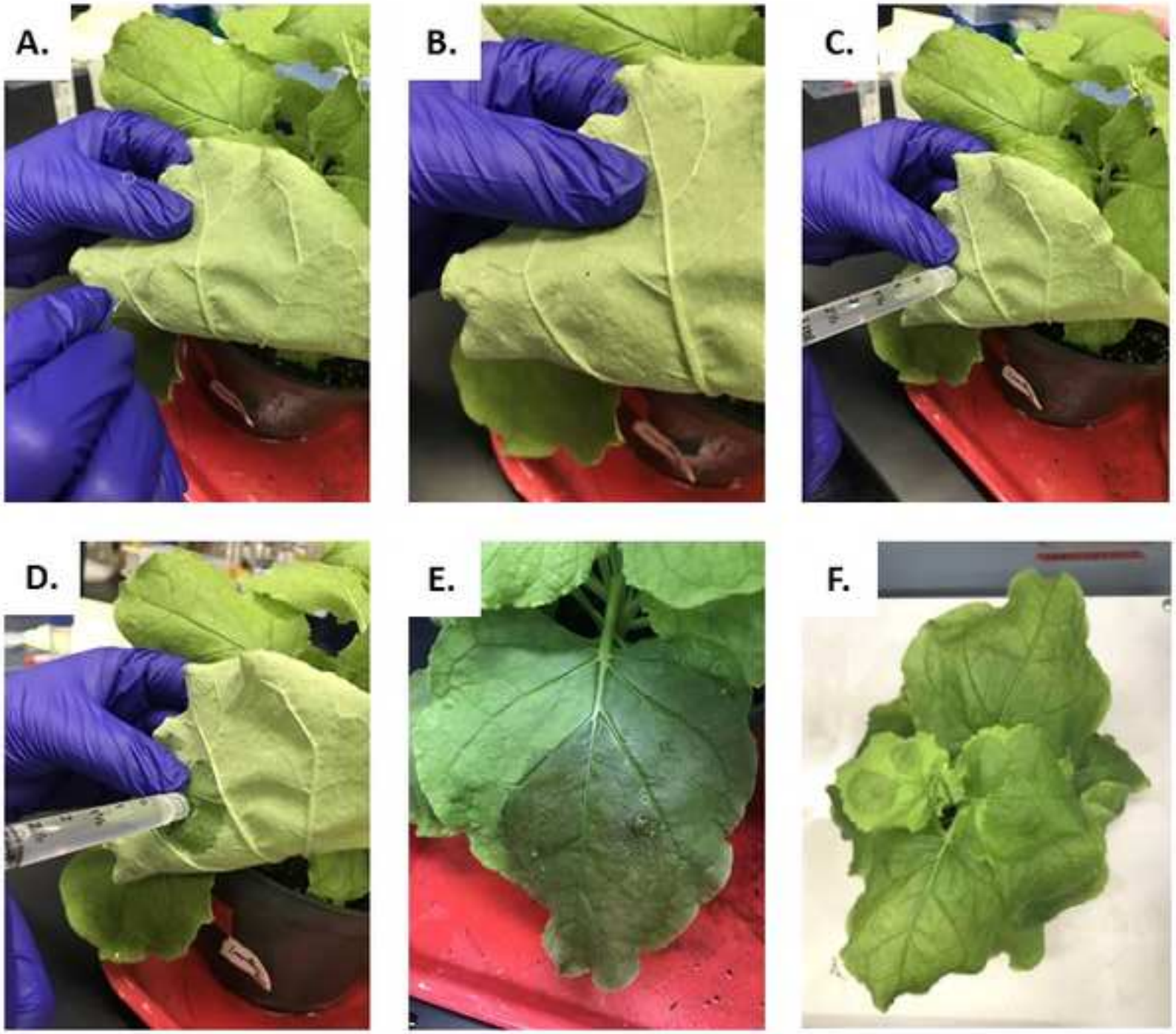

Figure 5

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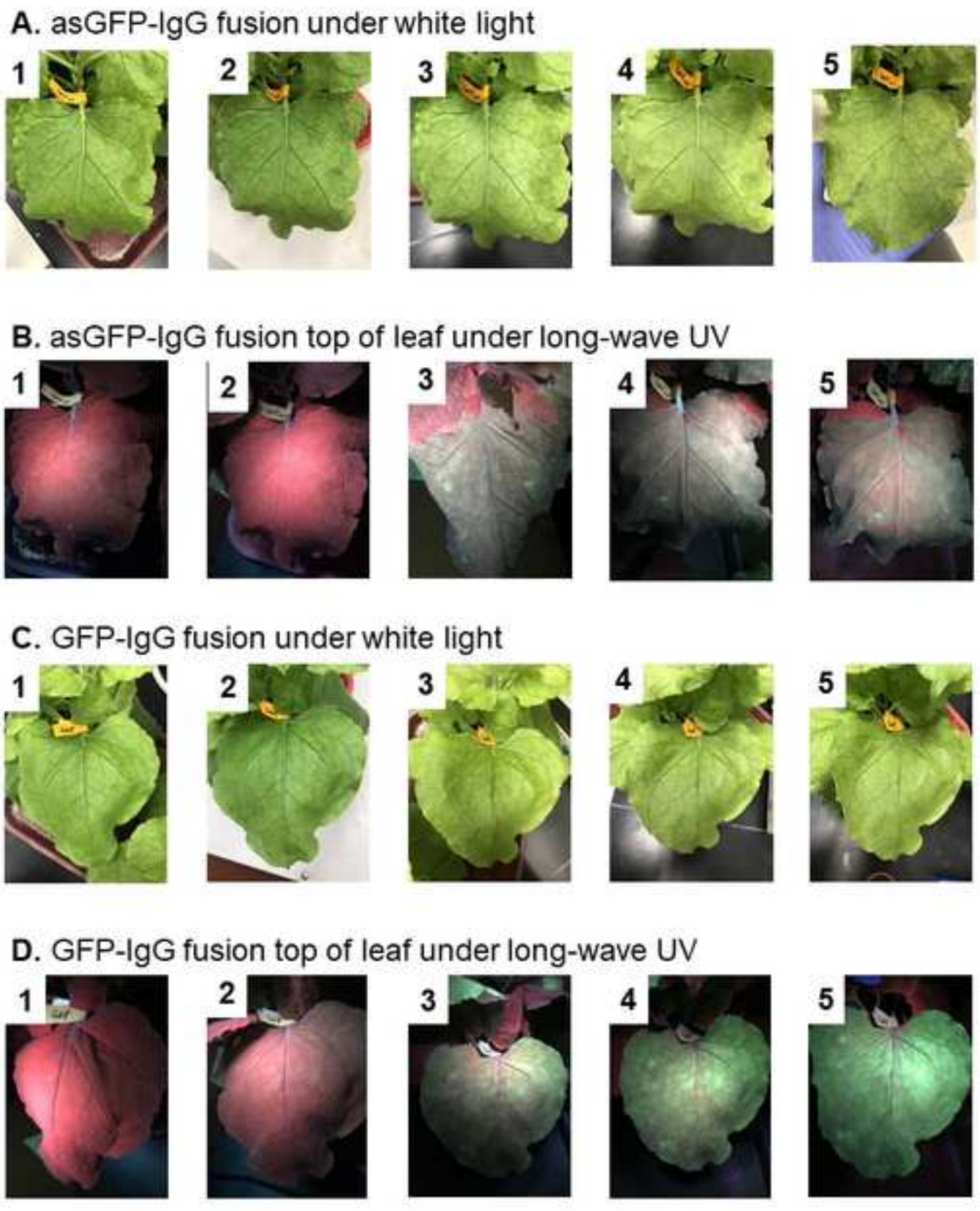

Figure 6

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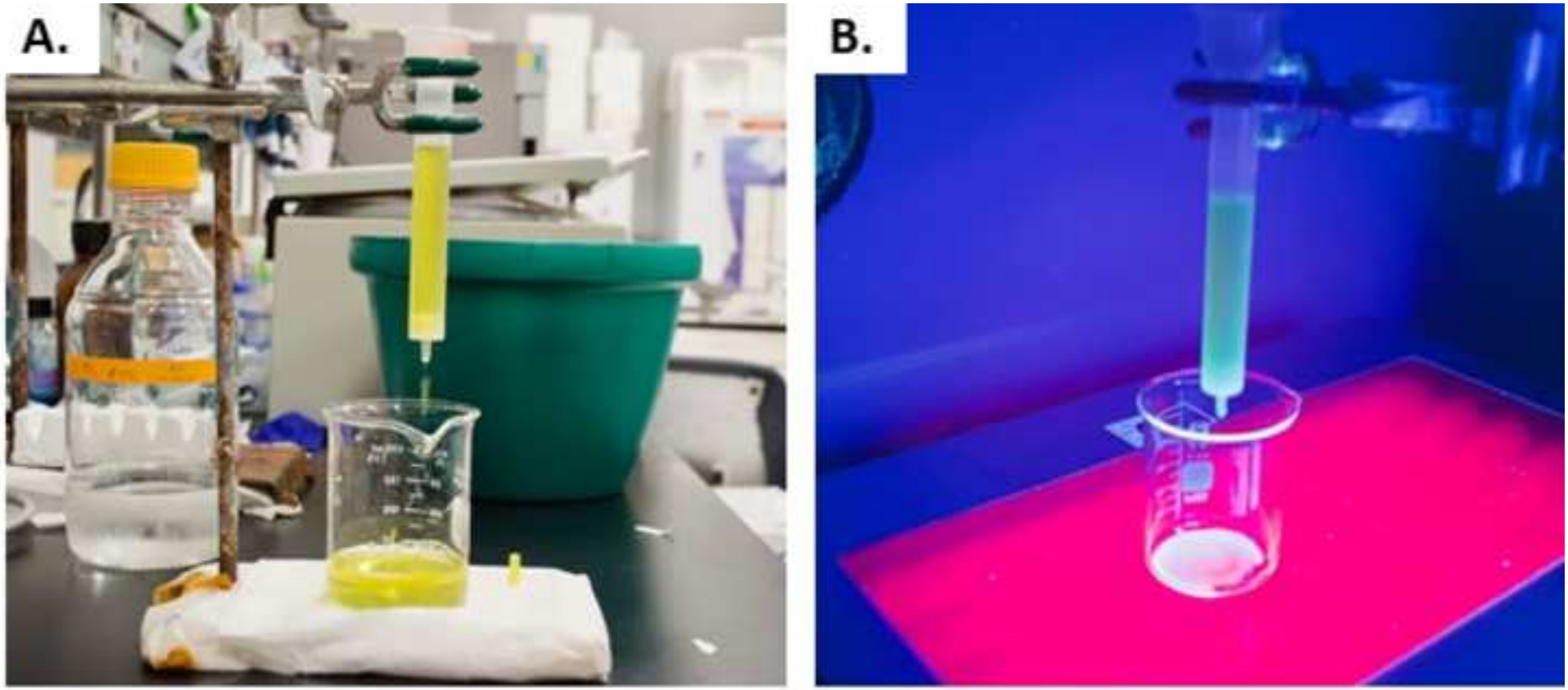

Figure 7

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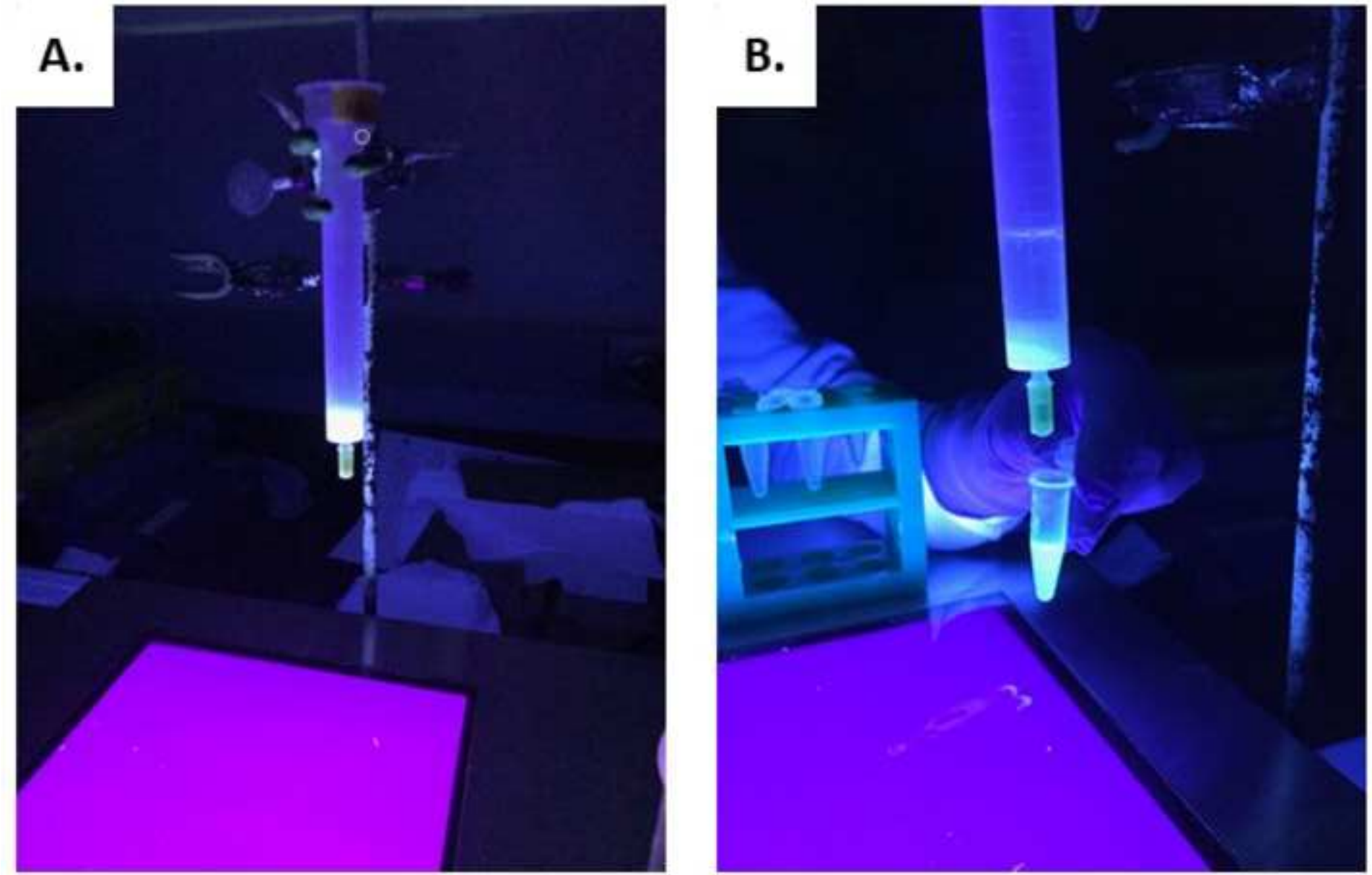

Figure 8

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Figure 9

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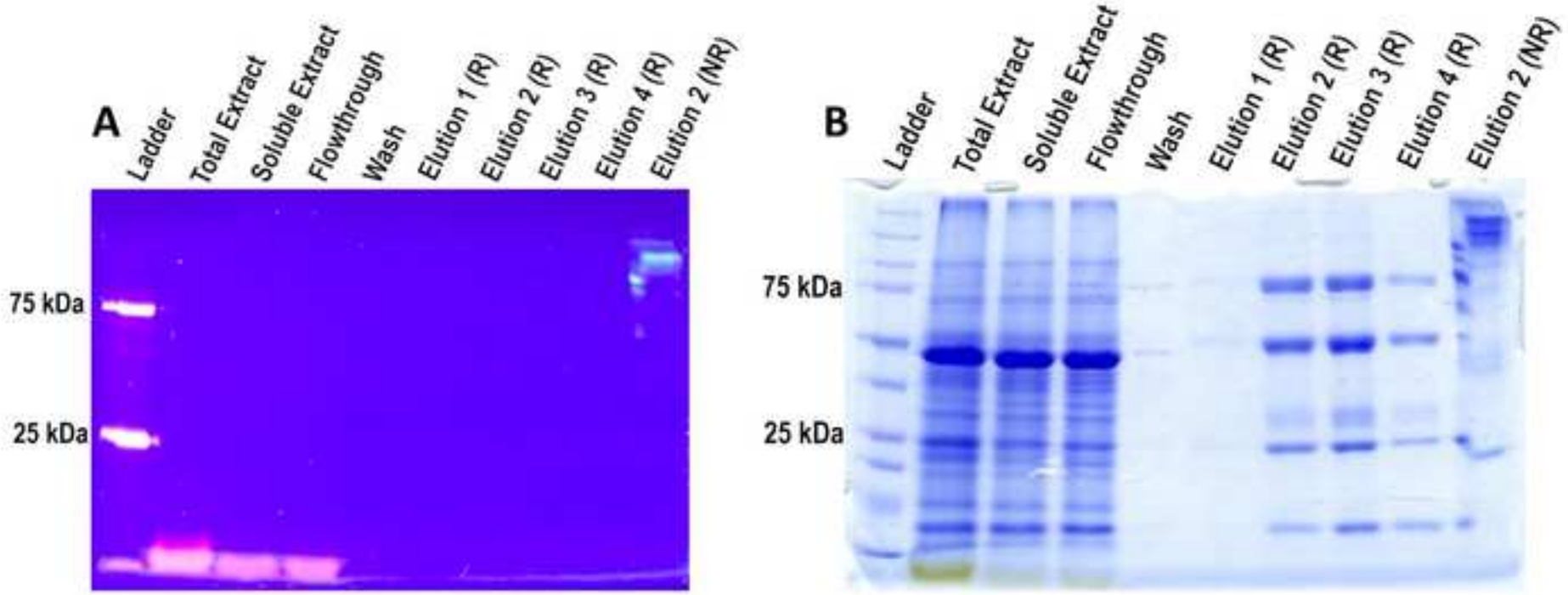

Table 1 Click here to access/download;Table;Table 1.xlsx

	Nucleotide Sequence Used	Amino Acid Sequence
Sequences used for asGFP in pBY!KEAM -GFPasH vector used in the expression of the asGFP-IgG fusion	ATGGTGTCCAAGGGAGAGGAAGCTTCTGGAAGAGCCTTGTTC CAGTACCCTATGACTTCTAAAATCGAGTTGAATGGCGAGATCA ACGGAAAGAAGTTTAAGGTTGCTGGAGAGGGTTTCACCCCTTC ATCTGGAAGATTCAATATGCACGCTTACTGTACTACCGGAGAC TTGCCTATGTCCTGGGTTGTTATAGCTTCCCCGCTTCAGTACGG GTTTCACATGTTTGCCCACTACCCTGAGGATATCACTCACTTCTT CCAAGAATGTTTTCCTGGGTCTTATACTCTCGACAGAACTTTGA GGATGGAGGGAGACGGTACTCTTACTACTCACCACGAGTACTC CCTTGAGGACGGTTGCGTTACTTCCAAGACTACTTTGAACGCTT CTGGATTCGACCCCAAGGGAGCCACTATGACTAAGTCTTTCGT CAAACAGCTCCCAAACGAGGTCAAAATCACCCCACACGGGCCA AATGGTATTAGACTTACTTCCACTGTTCTCTACCTTAAGGAGGA CGGAACTATCCAGATCGGAACTCAAGACTGCATCGTTACCCCA GTTGGCGGCAGAAAAGTTACTCAGCCTAAGGCTCACTTTCTTC ATACTCAGATCATTCAGAAGAAGGACCCAAACGACACCAGAG ATCACATCGTTCAGACTGAGCTTGCCGTTGCTGGAAATCTTTG GCACGGCATGGATGAGCTTTACAAGA	MVSKGEEASGRALF QYPMTSKIELNGEI NGKKFKVAGEGFTP SSGRFNMHAYCTT GDLPMSWVVIASPL QYGFHMFAHYPEDI THFFQECFPGSYTL DRTLRMEGDGTLTT HHEYSLEDGCVTSK TTLNASGFDPKGAT MTKSFVKQLPNEVK ITPHGPNGIRLTSTV LYLKEDGTIQIGTQD CIVTPVGGRKVTQP KAHFLHTQIIQKKDP NDTRDHIVQTELAV AGNLWHGMDELY K
Sequences used for GFP in pBYEAMGFPH gp vector used in the expression of GFP-IgG fusion	ATGGCTAACAAGCACCTCTCATTGTCTCTCTTCCTTGTGCTCCTT GGTCTTTCTGCTTCTCTTGCTTCTGGTATGGTGAGCAAGGGCG AGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGG ACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGG GCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCA TCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGT GACCACCTTCAGCTACGGCGTGCAGTGCTTCAGCCGCTACCCC GACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCG AAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGG CAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACAC CCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAA CAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG CATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGC AGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCG GCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCAC CCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCA CATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCAC GGCATGGACGAGCTGTACAAGA	MANKHLSLSLFLVLL GLSASLASGMVSKG EELFTGVVPILVELD GDVNGHKFSVSGE GEGDATYGKLTLKFI CTTGKLPVPWPTLV TTFSYGVQCFSRYP DHMKQHDFFKSA MPEGYVQERTIFFK DDGNYKTRAEVKFE GDTLVNRIELKGIDF KEDGNILGHKLEYN YNSHNVYIMADKQ KNGIKVNFKIRHNIE DGSVQLADHYQQN TPIGDGPVLLPDNH YLSTQSALSKDPNEK RDHMVLLEFVTAA GITH

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_table_final.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
5 mL syringe	any	N/A	
50 mL syringe	any	N/A	
Agar	SIGMA-ALDRICH	A5306	
Blender with cups	any	N/A	
Bromophenol blue	Bio-Rad	1610404	
DTT (DL-Dithiothreitol)	MP BIOMEDICALS	219482101	
EDTA (Ethylenedinitrilo)tetraacetic acid	SIGMA-ALDRICH	E-6760	
Ethanol	any	N/A	
Glycerol	G-Biosciences	BTNM-0037	
Glycine	SIGMA-ALDRICH	G7126-500G	
HCl (Hydrochloric acid)	EMD MILLIPORE CORPORATION	HX0603-4	
Heating block	any reputable supplier	N/A	
Jiffy-7 727 w/hole peat pellets	Hummert International	14237000	
Kanamycin	Gold Biotechnology Inc	K-120-100	
KCl (Potassium Chloride)	SIGMA-ALDRICH	P9541-500G	
KH2PO4 (Potassium Phosphate)	J.t.baker	3248-05	
KOH (Potassium Hydroxide)	VWR	BDH0262	
Magnesium sulfate heptahydrate	SIGMA-ALDRICH	M2773	
MES (2-(N-Morpholino)ethanesulfonic acid)	SIGMA-ALDRICH	M8250	
Miracloth	Millipore	4 75855-1R	
Moisture control potting mix	Miracle-Gro	755783	
Na2HPO4 (Sodium Phosphate)	J.t.baker	3827-01	
NaCl (Sodium Chloride)	Santa Cruz Biotechnology	sc-203274C	
Nicotiana benthamiana seeds	any reputable supplier	N/A	
PMSF (Phenylmethylsulfonyl Fluoride)	G-Biosciences	786-787	
Polypropylene Column	any	N/A	
Precision Plus Protein Dual Color Standards	Bio-Rad	1610394	
Protein G resin	Thermo Fisher Scientific	20399	
Rifampicin	Gold Biotechnology Inc	R-120-25	
SDS (Sodium Dodecyl Sulfate)	G-Biosciences	DG093	
Sodium Ascorbate	SIGMA-ALDRICH	A7631-500G	
Spectrophotometer	any reputable supplier	N/A	
Titan3 0.75 µm glass fiber filter	ThermoScientific	40725-GM	
Tray for peat pellets with dome	any	N/A	
TRIS Base	J.t.baker	4109-02	
Tris-HCl	Amresco	M108-1KG	
Tryptone	SIGMA-ALDRICH	17221	

UV lamp	any	N/A	
Water Soluble All Purpose Plant Food	Miracle-Gro	2000992	
Yeast extract	SIGMA-ALDRICH	9182	

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REVIEWERS' COMMENTS AND AUTHOR RESPONSES

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for this suggestion. We proofread the manuscript to ensure there are no grammar or spelling issues.

2. Please submit Table 1 as an xls/xlsx file instead of a pdf.

We have attached Table 1 as an excel file.

3. Step 5.6: Presumably the supernatant is saved?

Yes, the supernatant is used in the next step (5.7). We have removed the word “Save” which was left unfinished, and added Step 5.8. “Collect 50 µL of a sample after centrifugation, label "soluble extract" for later analysis.”

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other: Step 2.3 in the written protocol is a bit different from the video narration

Thanks for this suggestion. The homogeneity between the video and the written protocol has been largely increased to make the written manuscript a reflection of the video and vice versa. We have made sure that Step 2.3 written protocol is the same as the video narration. Only portions of the video that would have been awkward to read directly from the protocol vary from the written protocol, but all directions, amounts, and processes now match.

2. Please discuss the aseptic steps in the written protocol: the Bunsen burner usage, etc.

Thank you for this suggestion. To make sure that readers are using aseptic techniques, we have added the following statement: “All the following steps in this section should be done next to the Bunsen burner using basic aseptic techniques to avoid contamination.”

3. Video File Specifications:

• Please export your video at 25.00 frames per second.

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• Please increase the overall audio volume by 3 dB.

The Video specifications listed above have been applied to the video.

4. Video Editing & Pacing:
• There are a couple segments that can be trimmed out since they are just shots of pipettes being assembled or disposed of; common stuff that drags down the pacing a bit. Any narration in these ranges should be moved to coincide with the on-screen action the narration is describing:
○ 02:16-02:28
○ 02:42-03:00

The segments specified have been trimmed out to make the video more fluid. In addition, segments that were only common shots that drag down the pacing have been removed.

Please submit a revised high-resolution video here:
https://www.dropbox.com/request/u27m1My4Q7SDlrIv5hVD?oref=e

Reviewer 1:
Manuscript Summary:
The paper entitled "Production of IgG fusion proteins transiently expressed in Nicotiana benthamiana" authored by Kamzina and colleagues describes the in planta production of IgG fusion proteins and shows a simple method for visualization of expression, extraction, and purification of recombinant proteins. Moreover, authors adapted the protocol to the college teaching laboratory, providing project-based exploration. In this work, by using an acid-stable variant of GFP fused to a human mAb, authors were able to visualize the entire antibody expression and purification process. This protocol can be performed in every lab by a single researcher to produce milligram quantities of highly pure antibody or antibody fusion proteins in a weak using a few plants.
The introduction provides sufficient background and include all relevant references.
The methods are adequately described and clearly presented.
English language and style are fine.

We thank the reviewer for their reading of the manuscript, the positive impression and the constructive comments and corrections provided.

Minor Concerns:

1. *Here some revisions suggested*

Usually, Agrobacterium tumefaciens is cultured at 28◦C and shaking (200 rpm) for 48 h. If the Agro culture is very fresh it can grows faster but usually takes two days.

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Thanks for this comment. We grow our *Agrobacterium* cultures at 30℃ for a day in LB. We made sure to indicate that LB should be used if using this protocol. If we use YENB and incubate in 28℃ it tends to grow slower and we generally allow it to grow for 2 days.

2. Bacterial suspensions (Agro in MMA buffer) are syringe-infiltrated into the N. benthamiana leaves after incubation of 2 - 3 h at room temperature. Do you leave your suspensions for 2-3 h before infiltration?

Yes, some researchers have incubation periods for the agrobacterium, but we do not think it is necessary in our case.

3. Line 346 7.2. Run the gel at constant 100 V to desired protein band separation. Monitor the ladder as an indicator of protein separation. It should explain the time of running.

Thanks for the notice, we have added the time as follows:
“Run the gel at constant 100 V to desired protein band separation, it takes about 1.5 hours.”

Reviewer #2:
Manuscript Summary:
This article describes a method for tracking a recombinantly-produced IgG which is produced transiently in N. benthamiana, through to its extraction from the leaves and subsequent purification by fusing it to gfp and monitoring gfp fluorescence. Apart from being able to track the protein this method also provides a visible demonstration of what is happening at the molecular level.

I think this is a great topic and fulfils a need as many people not familiar with the field of biopharming have great difficulty in visualizing the technology and I think this serves a great purpose for that. In addition, I think it a great method for tracking a specific protein whilst developing a purification protocol, as different optimal protocols are usually required for purification depending on the characteristics of the specific protein being expressed.

We thank the reviewer for their reading of the manuscript, the positive impression and the constructive comments and corrections provided.

Major Concerns:
Manuscript
1. One of the main questions I have about this paper, is why the authors chose to mention the non-mutated gfp in the paper at all. Was it as a control? If so, then it should be used as a comparator all the way through the experiment ie results of both constructs are shown for fluorescence upon infiltration, but no results are shown for the non-mutated gfp beyond this point. The authors would have got their point across much more convincingly if they had shown images of a column with a sample of non-

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mutated gfp-fused IgG to demonstrate that it is not or barely visible under low pH conditions. And it would be interesting to see what the gels would have looked like. On the other hand, a much more convincing control would be the expression of the IgG with no gfp fused to it, hopefully showing the same IgG heavy chain and light chain bands on gels. The authors should decide whether it is indeed necessary to include the use of non-mutated gfp at all.

We appreciate the reviewer for this comment as it will help to make the goals of the experiments clearer. The reason that we use asGFP in chromatography is because it can withstand the low pH buffer that we use to elute, while native GFP cannot as it is known from the literature. Similarly, native GFP fluorescence is much brighter on the leaves than asGFP, hence we used native GFP to show the expression. We revised the abstract and introduction to clarify the use of both constructs for different purposes:

Abstract: … "Depending on the purpose of the experiment, native GFP fusion can be used to ensure easier visualization during the expression phase in the plants, while asGFP fusion allows visualizing during downstream processing."...

Introduction: … "The advantages of producing GFP-IgG include the ability to visualize the presence of a target protein during expression, while asGFP-IgG allows seeing the presence of recombinant protein in the purification steps."...

2. I think the introduction is a little disjointed and it's as if the authors have tried to include too much information.

Thank You for this suggestion. We have deleted information from the introduction that seemed extraneous or unnecessary. Previously these statements were included in the introduction but were cut since they did not add to the overall goal of the protocol:

Original Excerpt: Green Fluorescent Protein (GFP) is a bioluminescent protein derived from the jellyfish *Aequorea Victoria*, which emits green light when excited by ultraviolet light[24]. The protein was initially utilized as a visual marker to indicate the expression of a gene of interest in *Escherichia coli* cells under the control of the T7 promoter, and it was also expressed in the transparent nematode *Caenorhabditis elegans*[29].
Deleted from Introduction: "The protein was initially utilized as a visual marker to indicate the expression of a gene of interest in *Escherichia coli* cells under the T7 promoter's control, and it was also expressed in the transparent nematode *Caenorhabditis elegans* [29]."

Original Excerpt: "Over the years, GFP's use as a visual marker of gene expression has expanded from expression in *Escherichia coli* to numerous protein expression systems, including various mammalian cell lines, *Saccharomyces cerevisiae, Arabidopsis thaliana*, and *N. benthamiana* plants [34–38]."
Deleted From Introduction: "various mammalian cell lines, *Saccharomyces cerevisiae, Arabidopsis thaliana*, and"

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Original Excerpt: Numerous entry-level students describe difficulties grasping scientific concepts when the idea being taught is not visible to the naked eye, such as those concepts present in molecular biology and related fields[31]. Subconscious accumulation of visual information is generally completed with simple eye movement and without significant energy put toward an examination of those tasks in the working memory[32]. High-level visible collection of information often occurs when more effort is put into an interpretation of visual images[31]. Both subconscious and high-level internalization of visual models contribute to the internalization of information. The internalization of material allows for its storage in the working memory where meaning can be generated, and information can be stored as patterns[31, 33]. Visual markers, like GFP, can thus contribute to the processing of information that is related to the scientific processes and could aid in lessening the difficulties students report in learning numerous scientific concepts.
Deleted from Introduction: "Subconscious accumulation of visual information is generally completed with simple eye movement and without significant energy put toward examining those tasks in the working memory[32]. High-level visible collection of information often occurs when more effort is put into interpreting visual images[31]. Both subconscious and high-level internalization of visual models contribute to the internalization of information. The material's internalization allows for its storage in the working memory where meaning can be generated, and information can be stored as patterns[35,37]."

3. From the way it is written, there is the implication that mAbs that are tagged (with gfp) can be used for therapy which is not the case. I think the authors need to clarify this - maybe focus on the advantages of transient plant expression,

Thank you for pointing this out, we made sure to add in the following clarification at the end of the introduction to specify that mAbs tagged with gfp or asGFP cannot be used as therapy:
"While Abs and fusion proteins tagged with GFP or asGFP are not intended to be used for therapies, these methods can be useful as a control during experiments and can also be further utilized as a teaching tool for molecular biology both in-person and virtually."

In addition, we mention the advantages of transient expression earlier in the introduction:
"Advances in transient expression using viral vectors through either syringe or vacuum agroinfiltration allow for small- and large-scale production, respectively, of the desired protein in days[11–14]. Production of mAbs against Ebola, Dengue and Zika, and numerous other recombinant proteins, have been produced and purified quickly and efficiently using transient expression in *N. benthamiana* plants[15–19]. These circumstances make transient plant-based expression an attractive option for developing multiple Ab therapeutics and the methods demonstrated in this protocol[20]."

the fact that there are many examples of mAbs expressed in plants in this way,

This is a great point, we have now included examples of mAbs and recombinant proteins that have been expressed in plants in this way. Examples of some of these are now included in the introduction.

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"Production of mAbs against Ebola, Dengue and Zika, and numerous other recombinant proteins, have been produced and purified quickly and efficiently using transient expression in *N. benthamiana* plants [15–19]."

and how fusion of an acid stable gfp can help track the recombinant protein through the entire expression, extraction and purification process to develop an optimal purification protocol?
Thank you for this suggestion, in the introduction, we first elaborate on the limitations of GFP under acidic conditions.
"Although GFP is often used as a marker to indicate gene and expression *in vivo*, it is difficult to visualize it in the downstream processes if using acidic conditions. This circumstance is primarily because GFP does not maintain its structure and resultant fluorescence at a low pH[38]. Temporary acidic environments are often required in various purification processes, such as protein G, protein A, and protein L chromatography, which is often utilized for antibody purification[39–42]. GFP mutants have been used to retain fluorescence under acidic conditions[43,44]….."

We now have made sure to further elaborate on how the fusion of an acid-stable gfp can help track the recombinant protein through the entire expression, extraction, and purification process:
"We produced traditional GFP fused to the N-terminus of a humanized IgG heavy chain, creating a GFP-IgG fusion. Simultaneously, we developed the fusion of a plant codon-optimized sequence for an acid-stable GFP (asGFP) to the N-terminus of a humanized IgG heavy chain, creating an asGFP-IgG fusion. The advantages of producing GFP-IgG include the ability to visualize the presence of a target protein during expression, while asGFP-IgG allows seeing the presence of recombinant protein in not only the expression and extraction steps but also in the purification steps of the protein. This protocol can be adapted for the production, purification, and visualization of a range of GFP fusion proteins produced in *N. benthamiana* and purified using chromatography techniques that require low pH.

The final words in the conclusion of the movie sum the use of the protocol up well.
We thank the reviewer for this comment and positive feedback.

Lines 439-452 - this description is very confusing. Firstly, no mention is made of preparing non-reducing samples in the methods section;

Thank you for this comment. In the methods section, we have now added how to prepare the non-reducing samples. In the representative results section, we made sure to rephrase the statement indicated in the comments so that it is more clear to the reader. We also now explain that we ran the non-reducing sample onto the gel in order to compare reducing to non-reducing preparations of the same construct.

Original Excerpt in Methods section: "7.1. Prepare all samples before setting up the SDS-PAGE. Add 4 µL of sample buffer (reducing: 3.0 ml glycerol, 0.93 g DTT, 1 g SDS, 7 ml 4x tris (pH 6.8) 0.5 M,

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1.2 mg bromophenol blue; non-reducing conditions does not include DTT in the sample buffer) to 20 µL of each sample (total extract, soluble extract, flowthrough, wash, all elution fractions) for analysis."
Edited Excerpt from methods section: "7.1. Prepare all samples before setting up the SDS-PAGE. Add 4 µL of sample buffer (6X reducing sample buffer: 3.0 ml glycerol, 0.93 g DTT, 1 g SDS, 7 ml 4x tris (pH 6.8) 0.5 M, 1.2 mg bromophenol blue); (6X non-reducing sample buffer: 3.0 ml glycerol, 1 g SDS, 7 ml 4x tris (pH 6.8) 0.5 M, 1.2 mg bromophenol blue) to 20 µL of each sample (total extract, soluble extract, flowthrough, wash, all elution fractions) for analysis. Ensure that tube caps are securely fastened. For reducing samples, treat for 5 min in a boiling water bath (water should be 95°C-100°C), then put samples for 5 min on ice. Non-reducing samples should not be boiled. Spin samples in a microcentrifuge for ~5 seconds and load 20 µL of each sample in the order of collection into the gel wells. Load 3 µL of dual-color protein ladder in a separate well."

Results section:
Original Excerpt in Results section: Elution samples (lanes 6-9) in reducing sample buffer should only show the asGFP-IgG fusion components such as the heavy chain (50 kDa), the light chain (25 kDa), and the asGFP itself (27 kDa). Note that there is also a ~75 kDa band indicating the heavy chain fused to GFP. In contrast, the non-reducing elution samples should have one prominent band that contains all the components of the asGFP-IgG fusion, as seen in lane 10. Additionally, smaller bands are likely caused by native proteases. This cleavage can be prevented with the addition of protease inhibitors."
Rephrased Excerpt to make the description more clear:Elutions in lanes 6-9 have been prepared under reducing conditions described in the methods section 7.1 (reducing sample buffer contained DTT and samples were boiled). When run on a gel and Coomassie-stained, these samples should display the asGFP-IgG fusion components separately. These components include the heavy chain fused to GFP (~75 kDa), the heavy chain alone (50 kDa), the light chain (25 kDa), and the asGFP itself (27 kDa). The non-reducing sample was included in the last lane of the gel for comparison purposes and should display a single large band (~200 kDa), which should be made up of two heavy chains fused to the asGFP and respective light chains. Additionally, smaller bands are likely caused by native proteases. This cleavage can be prevented with the addition of protease inhibitors. The IgG fusion protein's individual components will not be distinguishable in the non-reducing samples on the Coomassie gel.

Figure 9a - arrows indicating the different bands would be helpful; there is no 'R' or 'NR' in the figure - the labels are written out in full - R and NR are labeled in the movie though; no E2 in the figure either;

Thank you for this suggestion; bands have been indicated on the figure to make them more clearly visible to the video reader. We put boxes on the gel because arrows blocked other bands from being clearly visible. We have edited only the video figure because we can make bands appear and disappear as we speak about them in the video. If we put the same boxes on the gel image in the representative results section, it would have caused confusion and overlap the bands, so we instead

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made sure to be more specific as to which bands and rows we are talking about. We also made sure to clearly indicate what “R” and “NR” mean in the representative results section as they are in the video.

Line 497 - reference to lane 10 - lanes aren't numbered - it may be clearer to refer to it as sample in the 'Elution 2 NR lane'; line 451 - what is the expected size in kDa of the full product?

Thank you for this suggestion, we have applied your suggestion to our descriptions and now rather than referring to this lane by a number, it is referred to as “'Elution 2 NR lane” and have now included the expected size in kDa of the full product.

“The first band in this lane is fluorescing at the full product's expected size, which is ~208kDa, indicating that the asGFP is still conformationally correct”

I am also confused as to why the gfp-fused HC protein band is not seen as a fluorescent band in lanes containing the elutions on the gel exposed to UV. In my experience, even in reducing samples containing wildtype egfp, the protein fluoresces. I think this needs explaining.

Thank you for mentioning this, as it could be confusing to some. Past studies have shown that GFP displays a significant drop in fluorescent intensity to no visible fluorescence after approximately 2.5 minutes of exposure to 95℃ [1]. For eGFP, if samples are heated to 80℃ they are still able to maintain around 50% of its fluorescence, however, if you heat the same samples up to above 90℃ or 100℃, the majority of the fluorescence was lost and reduced to below 10% or lower, which would be very difficult to view on a gel though they would still be visible [2]. So native GFP and eGFP should have differences in their ability to fluoresce when exposed to the same high temperatures. We did not use eGFP here, we only used native GFP and the acid stable variant of native GFP which should lose all fluorescence when heated to above 95℃ and subjected to denaturing conditions.

[1] https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1472-765X.2003.01460.x
[2] https://applbiolchem.springeropen.com/articles/10.1186/s13765-019-0448-y

We have also added in this segment to the representative results to make sure this is more clear: “Note that asGFP loses fluorescence when exposed to temperatures at or above 95℃ for 5 minutes, this is different from eGFP (enhanced GFP), which would maintain some fluorescence under the same conditions[49, 50].”

Movie
10:51 - If I understand it correctly, this scene has a UV light turned on to demonstrate fluorescence of the gfp in the column; however, users need to be very careful of exposure to UV and this safety concern needs to be re-iterated here; the narrator should also mention that it is not necessary to run the column with the UV light continuously turned on.

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Thank You for pointing this out; we have added to the video a re-iterated portion. The narrator now mentions that users must be cautious of UV light exposure and have also said that it is not necessary to run the column with the UV light continuously turned on.
These are the specific safety instructions that have been added to both the video and the manuscript: "During elution a UV light may be used for visualization. This does not need to be done for the duration of the elution. If UV is being used be sure to wear appropriate PPE to avoid damage to eyes and skin. A UV light does not need to be used during the elution step."

A few times, one of the narrators says 'negative 80 degrees' and a different narrator says 'minus 80 degrees' - should be consistent with terms.
Thank you for pointing this out for us; all off-screen narration audio should be consistent. Circumstances surrounding COIVD-19 have made it difficult to re-film and insert live film for our lab and our editor who is in a different city. We do believe that the current video with one remaining difference between narrators in how they refer to the temperature conveys the same meaning and hope it can suffice.

Minor Concerns:
Manuscript
Abstract
The title is appropriate and the abstract touches on the salient points, introducing transient plant expression, its advantages and gfp as an appropriate 'tracker' molecule, as well as summarizing the outcomes of the experiment.

We thank the reviewer for the positive feedback.

Lines 93-94 - the authors mention that one is able to 'visualize the entire antibody expression and purification process in the leaves of N. benthamiana plants.' - re-word as purification doesn't occur in the leaves.

We changed this statement to: "visualize the entire antibody expression and purification process from *N. benthamiana plants.*"

Introduction
Lines 121 and 123 - the use of the word 'immunogenicity' is a bit misleading - I thought all mAbs were meant to stimulate immunogenicity? Perhaps you must clarify by indicating 'non-specific immunogenicity?'

We thank the reviewer for indicating this. We added clarification by adding "non-specific" before the term "immunogenicity".

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Lines 164-165 - 'Although GFP is often used as a marker to indicate gene expression in vivo, its use after protein extraction is scarce' - I am not sure what you mean by this - do you mean that you can't visualize it in the downstream process? If so, you need to re-write this sentence.

This sentence was changed to “Although GFP is often used as a marker to indicate gene and expression in vivo, it is difficult to visualize it in the downstream processes if using acidic conditions.”

Protocol
Line 210 - asGFP-IgG, GFP-IgG, light chain - you need to clarify that the GFP is fused to the heavy chain of the IgG.

Thank you for this suggestion. The introduction now clarifies this:
“We produced traditional GFP fused to the N-terminus of a humanized IgG heavy chain, creating a GFP-IgG fusion. Simultaneously, we developed the fusion of a plant codon-optimized sequence for an acid-stable GFP (asGFP) to the N-terminus of a humanized IgG heavy chain, creating an asGFP-IgG fusion.”
We also have cited 2 research papers in the methods which detail all the cloning process:
“**Note:** GFP-IgG fusion constructs can be obtained as described in this paper 31. The asGFP gene was obtained and plant-optimized from this study[45].”

Line 227 - 'then resuspend each pellet in 1X infiltration buffer ' - It would be helpful to give an indication of what volume this is likely to be so that the user can get an idea of how much they are likely to end up with - if you inoculate 10 to 20ml of LB, and you grow to an OD between 0.6 and 0.9 you can probably expect a certain volume range.

Thanks for noticing this. We added the following: “This should take approximately 15-45 mL of infiltration buffer depending on the initial culture density.”

Lines 235-237 - 3.1 - We have found in our lab that leaf age can influence protein yields and also that the larger, lower, older and therefore less-expanding leaves of N benthamiana are much easier to infiltrate than the smaller, newer ones. So, it is important to indicate to the reader/viewer which leaves to infiltrate.

We thank the reviewer for this suggestion. We added the following:
“**Note**: The lower leaves are easier for infiltration, whereas the leaves on the top of the plant are harder. Generally, the expression of recombinant proteins is highest in the leaves located in the middle of a plant and these leaves also get less necrotic.”

Lines 241-242 - I think you need to re-iterate here that you must try and not perforate the leaf with the Luer tip end.

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We added: “If too much pressure applied, it might damage the leaf. “

Line 265 - do you mean 4°C and not -4°C?

We regret this mistake, it was changed to 4°C in both the manuscript and video audio.

Line 268 - 'Add 2 mM phenylmethylsulfonyl fluoride (PMSF) from stock' - what is the stock concentration?

Thank you for pointing this out. The stock concentration is added now: “Add 2 mM phenylmethylsulfonyl fluoride (PMSF) from stock (100 mM)”

Line 271 - 'Add a measured amount of extraction buffer.' - Again here, an indication of what volume to add to a specific mass of plant tissue would be helpful. Usually it is indicated as a ratio of plant mass: buffer volume in publications.

This was already mentioned in previous step, so we added a reference to Step 5.2 as follows:
“5.3. Place plant tissue from step 4 into the prechilled blender cup. Add a measured amount of chilled extraction buffer to the blender cup (as indicated in step 5.2).“

Line 273 - 'if required' - if you use a specific ratio as indicated above, then you wouldn't need this comment.

We have fixed this as recommended above, so deleted this sentence.

Line 288 - 'Collect 50 µL of a sample before' - This should go in section 5.6 ie keep 50µl of total extract for analysis ad centrifuge the remainder…….etc

Thanks for this suggestion, we have changed the sentence:
“5.6. Transfer 50 µL of a sample to a new 1.5 mL tube and label "total extract" for later analysis. Centrifuge the remainder of plant extract at 16,000 x g for 20 min, 4°C and transfer the supernatant to a conical tube.”

Line 322 - you need to indicate why you would want to 'neutralise the antibodies' ie what is the reason.

We added: “This is to neutralize the Abs in the acidic elution buffer to avoid potential structural changes”

Line 323 - 'to get more concentrated eluate' - clarify - surely you mean so as not to dilute the sample fraction volume too much?

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We added this to clarify this sentence: “to get a less diluted sample”

Line 339 - 'using neutralized elution buffer' - I think you should clarify what you mean by this.

We regret this confusion. To resolve this we have deleted the term “neutralized”.

Line 344 - 'the gel' - clarify - ie SDS-polyacrylamide gel (Laemmli).

Thank you for pointing this out, we added:
“7.2 Run the SDS-PAGE gel at a constant 100 V to desired protein band separation”

Line 344 - section 7.1 - I think you should re-iterate/summarise what samples to load on the gel.

We have summarized the samples as suggested:
“... 20 µL of each sample (total extract, soluble extract, flowthrough, wash, all elution fractions) for analysis.”

Representative results
Lines 370-371 - 'The process of Agrobacterium growth and preparation for infiltration is shown in Figure 1C.' - It would be preferable to indicate what the different tubes represent in this image ie what is in which tubes -I see that the same photos are in figure 3 - maybe refer to figure 3 here?

Thank you for pointing this out to us; we have summarized what the different tubes indicate.
Previously the manuscript stated, “The process of Agrobacterium growth and preparation for infiltration is shown in Figure 1C.”
The manuscript now states:
“The process of *Agrobacterium* growth and preparation for infiltration is shown in Figure 1C. Figure 1C-1 displays isolated colonies of Agrobacterium, Figures 1C-2- IC-5 display the expected appearance of media after it is inoculated with a single isolated colony. Refer to Figure 3 for more explanation on these steps.”

This addition also tells readers to refer to Figure 3 for more explanation of the steps in Figure 1C

Line 372 - do you mean 'uninfiltrated' plant?

Thank you for pointing this out to us; the correction has been made.

Line 395 - 'turbid' would be a more appropriate term to use for 'cloudy'

Thank you for this suggestion, we have replaced the following:
“After incubation of an isolated colony overnight at 30°C, LB media will appear cloudy.”

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"At an OD_{600} of 0.2, the media will appear cloudy, as shown in Figure 3d."
With these edits:
"After incubation of an isolated colony overnight at 30°C, LB media will appear turbid."
"At an OD_{600} of 0.2, the media will appear turbid, as shown in Figure 3d."

Line 425 - does the supernatant also fluoresce? It looks like it could do, to me, in which case you should mention this for figure 6b.

Thank you for this suggestion, the previous manuscript contained the following:
"When the supernatant of the asGFP-IgG extract is added to the Protein G column, under white light, the resin will become slightly green due to plant chlorophyll pigments, as seen in Figure 6a. The addition of supernatant under short-wave UV light results in the accumulation of fluorescence in the Protein G resin, as shown in Figure 6b."

Which has been replaced with these edits to indicate that the supernatant is also fluorescing:
"When the supernatant of the asGFP-IgG extract is added to the Protein G column, under white light, the resin will become slightly green due to plant chlorophyll pigments, as seen in Figure 6a. The addition of supernatant under short-wave UV light results in the accumulation of fluorescence in the Protein G resin, as shown in Figure 6b. Note that the supernatant will also be fluorescent alone under UV light. Still, fluorescence is expected to be much more concentrated when the asGFP-IgG fusion begins to bind to the resin."

Line 432 - change 'elutions' to eluate which is what you call them in the movie - same for line 435.

Thank you for this suggestion, the change has been made.

Line 439 - DDT - DTT?

Thank you for noticing this error, the correction has been made.

Discussion
Line 524 - correct the grammar.

Thank you for this suggestion, the grammar in the indicated section of the discussion has been fixed.

Line 554 - in your methods you describe using 30°C - why the difference?

Thank you for noticing this error on our part, the following addition has been made:
"DDifferent growth conditions can slow or even inhibit Agrobacterium growth. Agrobacterium grows optimally at 28°C-30°C and experiences a heat shock when incubated above 30°C, producing cell division errors [62]".

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Materials and methods list
Check that your table fits onto the page - the final column has been put on the next page.

We have fixed this.

Movie
02:21 - EHA205 Agrobacterium strain - this is specific for the expression vector that is used - the narrators should highlight this.

The narrator has made this addition to the movie

02:21 - Concentration of kanamycin in LB plates?

The concentration of the kanamycin in LB plates has been added to the movie

03:11 - 'can also add rifampicin' - sounds like one has a choice to include rifampicin or not - why?

“Rifampicin can be added to prevent *E.coli* contamination since Agrobacterium has innate resistance.” The narrator has added this to the movie.

04:18 - '16000 times g' and at 08:49 - '16000 g's' - be consistent

This segment of the narration has been fixed and is now consistent

10:07 - 1 X PBS - narrator should say '1 times PBS'

This section has now been fixed.

The scenes showing the representative gel results from 12:48 onwards would benefit greatly from some kind of marker/indicator which highlights the individual protein bands that are being spoken about at the times they are mentioned - otherwise the viewer is left looking at a the picture of the gel trying to figure out which lanes the narrator is talking about and by the time they have worked it out, the movie is on to the next scene.

Thank you for this suggestion, we have added marker indicators to the bands in which we are referencing and describing during the results section of the movie.

Reviewer #3:
Manuscript Summary:

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The manuscript entitled "Production of IgG fusion proteins transiently expressed in Nicotiana benthamiana", submitted by Kamzina and co-workers presents a method to produce, purify and visualize an antibody-GFP protein fusion. The manuscript is well written, the procedures are well explained and the protocol, if properly followed by the reader, will lead to the expected outcome. The proposed purposes of the method are (1) use a visual control in purification process experiments and (2) teaching purposes. These purposes are correctly identified by the authors. The submitted video properly presents the protocol described in the manuscript and is self-explanatory. I recommend acceptance of the manuscript for publication in JoVE with minor modifications.

We thank the reviewer for the positive feedback.

Major Concerns:
None

Minor Concerns:
The authors should be clearer on the purpose of using 2 different versions of GFP. It seems from my understanding that the native GFP is easier to visualize during the production phase in the plant whereas the acid-stable version of GFP assures a better visualization during purification. The reader could choose one or another depending on the purpose of the experiment.

We appreciate the reviewer for this comment as it will help to make the goals of the experiments clearer. The reason that we use asGFP in chromatography is because it can withstand the low pH buffer that we use to elute, while native GFP cannot as it is known from the literature. Similarly, native GFP fluorescence is much brighter on the leaves than asGFP, hence we used native GFP to show the expression. We revised the abstract and introduction to clarify the use of both constructs for different purposes:
Abstract: … “Depending on the purpose of the experiment, native GFP fusion can be used to ensure easier visualization during the expression phase in the plants, while asGFP fusion allows for visualization during downstream processing.”...
Introduction: … “The advantages of producing GFP-IgG include the ability to visualize the presence of a target protein during expression, while asGFP-IgG allows seeing the presence of recombinant protein in the purification steps.”...

ON page 4, line 177, the authors state that the protocol "can be extended to allow visualization of any antibody or protein produced in N. benthamiana". This is a stretch, since the purification part of the protocol is specific for antibodies. The author should rather state that the protocol could be adapted for the production, purification and visualization of a range of GFP fusion proteins.

We thank the reviewer for noticing this mistake, we have fixed it as below:

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"This protocol can be adapted for the production, purification, and visualization of a range of GFP fusion proteins produced in *N. benthamiana* and purified using chromatography techniques that require low pH."

Most importantly, the protocol considers that the reader has access to an antibody-GFP expression constructs. Unfortunately, this is rarely the case in the teaching environment. I recommend that the authors refer the reader to specific method publications where assembly of such gene construct is described.

This is a very good point. We have added following citations to the Methods section, step 2 as a note: "**Note**:GFP-IgG fusion constructs can be obtained as described in this paper [31]. The asGFP gene was obtained and plant-optimized from this study [45]."

Reviewer #4:
Manuscript Summary:
The manuscript describes the expression of human IgG fusion to GFP proteins by transient expression in Nicotiana benthamiana. The acid-stable variant of green fluorescent protein (GFP) used is useful to visualize the entire antibody expression and purification process in the leaves of N. benthamiana plants.
A comprehensive study was conducted, and the efficacy of the protocol was demonstrated. All necessary experiments were conducted.

We thank the reviewer for their careful reading of the manuscript, their overall positive impression and their constructive comments and corrections.

Major Concerns:
Is very well known that there are two major approaches to express recombinant proteins in plants: the development of a stable transgenic line and transient expression. Information about the type of expression used is missing in the abstract. Other recommendation is to briefly explain in the introduction the both methods, its advantages and disadvantages and the method chosen for the present study.
Abstract:

Thank you for this suggestion, we added information that specifies some of the advantages and disadvantages of transient and transgenic expression as you have recommended. We also made certain to specify early on, which of these methods is demonstrated in the protocol.

"Both transient and transgenic plant-based protein expression can be utilized as lower-cost alternatives to mammalian or bacterial production systems[10]. Though transgenic plants are preferred for crop production, recombinant protein production using this method can require weeks to months. Advances in transient expression using viral vectors through either syringe or vacuum agroinfiltration allow for

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small- and large-scale production, respectively, of the desired protein in days[11–14]. Production of mAbs against Ebola, Dengue and, Zika, and numerous other recombinant proteins, have been produced and purified quickly and efficiently using transient expression in N. benthamiana plants[15–19]. These circumstances make transient plant-based expression an attractive option for developing multiple Ab therapeutics and the methods demonstrated in this protocol[20].”

Line 170-171 "Herein we describe the fusion of a plant codon-optimized sequence for an acid-stable GFP (asGFP) to the N-terminus of a humanized IgG heavy chain, creating an asGFP-IgG fusion." Based on this statement, it would be appropriate to insert a topic about protein design and cloning in the methodology. Where does the asGFP-IgG and GFP-IgG come from? If it comes from a previous study, it must be cited.

This was a similar suggestion as from reviewer 3. We have added following citations to the Methods section, step 2 as a note:
“**Note**:GFP-IgG fusion constructs can be obtained as described in this paper [31]. The asGFP gene was obtained and plant-optimized from this study [45].”

The "Results" section seems to be detailing the steps approached in the "Methodology" section. I suggest starting with a brief description of the results obtained, addressing each objective in the study, and then proceed detailing its steps. The results should be better explored.

Thank you for this suggestion, we applied your feedback and have inserted a brief description of the results that were obtained rather than parts of this section that could also have been present in the methodology. These sections now contain a brief description of the results obtained, the objective, then the steps that were mentioned. We also have applied your suggestion to better explore the results and have further elaborated where necessary.

Minor Concerns:
Line 562-572: please insert references.

Line 570: "Note that some proteases like PMSF degrade quickly". Correct: protease inhibitors like PMSF.

We regret missing this mistake. We have fixed it as suggested:
“Some protease inhibitors like PMSF degrade quickly..”

Line 507-508: "In addition, emerging vaccine platforms that utilize IgG, such as recombinant immune complex (RIC) vaccines, can be visualized and purified using these methods."
Based on this statement, is it possible to use GFP in the formulation of a vaccine?

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We thought this sentence was a mistake and deleted it.

Reviewer #5:
Manuscript Summary:
This article describes protocols on production of recombinant protein fused with GFP in N. benthamiana. Not only "Protocol" part but also "Discussion" part describe points for protein production using transient expression system in N. benthamiana. Recently, N. benthamiana has been used as an alternative host for production of recombinant protein or biophamaceuticals. So this article would help researchers and facilitate use of benthamiana for protein factory.

We thank the reviewer for their careful reading of the manuscript, their overall positive impression and their constructive comments and corrections.

Major Concerns:
Some stuffs described in Protocol are not clear. Information shown below will help understand Discussion part from lines 528-546.
Page 4; protocol 1.2, what fertilizer should be used.
Page 4; protocol 1.5, what "moisture control soil" is contained in an individual pot.

We have detailed information in the table attached to this manuscript. However, to make it more clearer, we added as below:
“Fertilizer (water-soluble all-purpose plant food) concentration is generally 2.5-2.8 g/L.”
“This demonstration used Miracle Grow moisture control potting soil.”

Page 4; protocol 1.6, Explanation on plant conditions suitable for "needle-less syringe agroinfiltration" should be appreciated. For instance, plant height, how many leaves plant should have, size of leaves, etc

We added the note below to the Methods, step 3.1:
“Note: The lower leaves are easier for infiltration, whereas the leaves on the top of the plant are harder. Generally, the expression of recombinant proteins is highest in the leaves located in the middle of a plant and these leaves also get less necrotic.”

Page 6; protocol 5.7, maybe (there are two "5.6"), what "syringe glass fiber filter" should be used.

We have step 5.7 step in the protocol to describe this, more detailed information is in the table attached to the manuscript:
“5.7. Filter the soluble extract using a 50 mL syringe and syringe glass fiber filter (0.75 µm).”

Page 9, line 410; Authors mentioned "slight necrotic appearance". Indicating the necrotic points by arrows would be helpful.

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Thank you for this suggestion, the image would not display all parts of the leaf if we put arrows to indicate the necrotic parts of the leaf. However, in an effort to still apply this feedback, we explained in the representative results section where the necrotic points are more specifically and included this in the description of the results:

When describing Figure 1B: “Necrosis may occur at the injection site between days 3-5. It often depends on the properties of the protein being expressed and the infiltrated plants' health (further examined in discussion).”

When describing Figure 5: “There may be a slight necrotic appearance at injection sites on days 4-5, which is usually apparent by the lightening of the plant tissue in those areas.”

Joseph G. L. Hunter is a graduate student in Molecular and Cellular Biology at Arizona State University working on a variety of projects covering vaccines, therapies, and material science questions since the summer of 2017. During his journey through higher education, he has managed multiple research projects, taught students in Molecular genetics and genetic engineering, designed courses, and worked on science communication with the general public.

Michelle Di Palma is a Ph. D. student at Arizona State University (ASU) who studies Molecular and Cellular Biology. She received her Master of Science in 2018 in Molecular and Cellular Biology also at Arizona State University under Dr. Tsafrir Mor. Since 2016, she has worked on a variety of vaccines produced in plants for diseases such as HIV, Zika virus, and Schistosomiasis. During her time at ASU, she has taught a variety of subjects, including General Biology, General Genetics, Molecular Genetics, and Genetic Engineering. She hopes to continue research that works to address health disparities.

Aigerim S Kamzina is a Ph.D. student in Molecular and Cellular Biology, Arizona State University. She graduated from University College London, UK, with a Bachelor's degree in Biotechnology in 2010. Currently, she is working on an HIV vaccine utilizing various approaches and recombinantly producing them in plants under Dr. Tsafrir S Mor's supervision. She has been teaching several Molecular Biology classes since 2016 and is involved in the course design.

Tsafrir S Mor is a molecular biologist and biochemist whose research focuses on using plants to produce therapeutic proteins and enzymes. He received his Ph.D. in biochemistry from the Hebrew University of Jerusalem in Israel in 1997. After his post-doctoral studies in plant biotechnology at the Boyce Thompson Institute for Plant Research at Corne University, Ithaca, NY, Dr. Mor joined Arizona State University (ASU) in 2000. He is currently a full professor at Arizona State University in the School of Life Sciences and Biodesign Institute. Since his start at ASU, he has taught and designed the curriculum for many classes, has mentored numerous undergraduate and graduate students, and he has made many contributions to both plant biotechnology and scientific outreach. Tsafrir Supervises the ASU prison biology program, where graduate students and faculties make the knowledge of biology accessible to inmates at Eymen prison's Browning Unit.

Boyd Armer is the Lab Manager and Coordinator for teaching labs in the School of Life Sciences at Arizona State University (ASU) in Tempe, AZ. He received both his Bachelor of Science degree in Biology in 1991 and his Master of Education degree in 1993 from ASU. Boyd has over twenty-seven years of experience in a teaching lab environment including roles as instructor, lab coordinator and manager and involving all aspects of lab operations including designing curriculum and lab activities, developing lab safety protocols and procedures and overseeing lab operations for a variety of courses including General Biology, Plant Biology, Biochemistry, and Molecular Biology.

Andrew Diamos received his Ph. D. from Arizona State University in 2017 under the supervision of Dr. Hugh Mason. During his doctoral studies, Andrew designed, produced, and purified protein therapeutics and vaccines produced in plants. Primary targets of these vaccines and therapies included Ebola virus, Dengue virus, Human papillomavirus, Norovirus, and *Clostridium perfringens*. During his post-doctoral studies, he continues plant biotechnology work but expanded his work on vaccines to target diseases such as Zika virus, Dengue virus, Influenza virus, herpes simplex virus. Hepatitis C. He has made many contributions to the improvement of the Bean Yellow Dwarf Virus (BeYDV) expression system, often used as a vector to produce recombinant plant proteins. He is currently a post-doctoral researcher at the University of Michigan Medical School in Ann Arbor, MI, where he is applying his previous knowledge of molecular genetics to the production of a vaccine targeting Norovirus.

The first to publish peer-reviewed work on the use of plants for production of vaccine antigens in 1992, Hugh Mason has continued to pioneer plants as an expression system for different proteins and therapeutics. He and coworkers are credited with the development of the geminiviral based expression system several labs currently use for plant expression. The development of the original and novel Recombinant Immune Complex (RIC) platform are also achievements of Hugh Mason and coworkers.