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TITLE:

Direct Detection of Isolevuglandins in Tissues using a D11 scFv-Alkaline Phosphatase Fusion Protein and Immunofluorescence

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KEYWORDS:

isolevuglandins, immunofluorescence, alkaline phosphatase conjugated ScFv D11 antibody, and Hypertension

SUMMARY:

This article provides a detailed methodology for the measurement of isolevuglandins in tissues by immunofluorescence using alkaline phosphatase-conjugated ScFv D11 antibody. Hypertension models in both mice and humans are used to explain the step-by-step procedures and fundamental principles associated with isolevuglandin measurement in tissue samples.

ABSTRACT:

Isolevuglandins (IsoLGs) are highly reactive gamma ketoaldehydes formed from H2-isoprostanes through lipid peroxidation and crosslink proteins leading to inflammation and various diseases

including hypertension. Detection of IsoLG accumulation in tissues is crucial in shedding light on their involvement in the disease processes. However, measurement of IsoLGs in tissues is extremely difficult, and currently available tools, including mass spectrometry analysis, are laborious and extremely expensive. Here we describe a novel method for *in situ* detection of IsoLGs in tissues using alkaline phosphatase-conjugated D11 ScFv and a recombinant phage-display antibody produced in *E. coli* by immunofluorescent microscopy. Four controls were used for validating the staining: (1) staining with and without D11, (2) staining with bacterial periplasmic extract with the alkaline phosphatase linker, (3) irrelevant scFV antibody staining, and (4) competitive control with IsoLG prior to the staining. We demonstrate the effectiveness of the alkaline phosphatase-conjugated D11 in both human and mouse tissues with or without hypertension. This method will likely serve as an important tool to study the role of IsoLGs in a wide variety of disease processes.

INTRODUCTION:

Isolevuglandins (IsoLGs), also known as isoketals, are isomers of the 4-ketoaldehyde family, which are products of lipid peroxidation, and react with and adduct to primary amines on proteins[1,2]. IsoLGs have been implicated in several diseases, including cardiovascular, Alzheimer's, lung and liver diseases, and many types of cancer[3]. IsoLGs have been most extensively studied in their contribution to cardiovascular disease (CVD), which is a significant health and economic burden globally, including the United States. It is estimated that 92.1 million US adults have at least one type of CVD, with 2030 estimated projections reaching to 43.9% of the US adult population[4]. Reducing blood pressure, cholesterol, and smoking reduces the overall risk and reduction in CVD events[5].

High blood pressure or hypertension is a major risk factor for cardiovascular disease and affects approximately half of the US population[6]. Previous studies have found that inflammation is an underlying cause of hypertension and that IsoLGs play a role[7]. Hypertensive stimuli, including angiotensin II, catecholamines, aldosterone, and excess dietary salt, induce IsoLG accumulation in antigen presenting cells including dendritic cells (DCs), which in turn activate T cells to proliferate and produce inflammatory cytokines that contribute to hypertension[8,9].

Previously, IsoLGs have been measured by immunohistochemistry, mass spectrometry, enzyme-linked immunosorbent assay, and flow cytometry[10,11]. To facilitate the measurement of IsoLGs, a single-chain fragment variable (scfv) recombinant antibody (D11) was developed against IsoLGs[12]. Initially, this D11 antibody contained an 11 amino acid E-tag and required a secondary antibody for immunohistochemistry detection[11]. However, it was difficult to find a reliable secondary antibody against the E-tag after the discontinuation of its production by the manufacturer. Therefore, we have developed a reliable protocol for immunofluorescent staining of IsoLGs using D11 conjugated with alkaline phosphatase (D11-AP), which we have demonstrated in mouse and human tissues with and without hypertension.

PROTOCOL:

Vanderbilt University's Institutional Animal Care and Use Committee approved all procedures described in this manuscript. Mice are housed and cared for in accordance with the Guide for the

Care and Use of Laboratory Animals. All subjects gave written informed consent before enrolling in the study as approved by the Institutional Review Board of Vanderbilt University. All procedures were performed according to the Declaration of Helsinki.

1. Preparation of plasmids encoding D11-alkaline phosphatase fusion protein and negative control vectors

1.1. Construct a modified version of the pCANTAB5E plasmid[13,14] in which the single chain fragment variable (scFv) segment is linked at its 3' end to a sequence encoding bacterial alkaline phosphatase (AP).

NOTE: Immediately downstream of the scFv sequence's NotI restriction site, the modified plasmid no longer contains the coding sequence of the E-tag and instead encodes the linker sequence GGSGGHMGSGG, followed by the sequence for AP (GenBank accession number AXY87039.1, T16-K464)[15,16]. Downstream of AP, the plasmid will encode 8xHis and DYKDDDDK tags. At the 3' end of the tags, the coding sequence ends with an Amber stop codon. The modified plasmid is called pCANTAB5-AP.

1.2. Clone D11 scFv (GenBank accession number AAW28931.1) into pCANTAB5-AP with the SfiI/NotI restriction sites.

1.3. Clone D20 scFv into pCANTAB5-AP with the SfiI/NotI restriction sites.

NOTE: D20 scFv is a negative control designed to assess the tissue interaction pattern of an irrelevant scFv. This scFv was originally selected by phage display for its ability to interact with a glycan group known as A2.

1.4. Generate an "empty" vector by removing the scFv portion from the plasmid entirely and replacing it with an "AP linker" coding sequence consisting of amino acids GGGGSGRAGSGGGGS.

1.5. Transform all plasmids into competent TG1 *E. coli* and grow bacteria overnight at 30 ˚C, on 0.5% agar plates containing 2xYT supplemented with 100 µg/mL Ampicillin and 2% glucose (2xYTAG) medium.

NOTE: Strains with the supE gene (such as TG1 *E. coli*) do not suppress the amber stop codon 100%. Estimates range from 0.8 – 20% of the time, so there is still a lot of product that terminates just downstream of BAP, as it is intended for these experiments[17]. TG1 *E. coli* are mainly used for practical reasons, such as the reduction of time and costs, their compatibility with the pCANTAB protein expression system, and their use in phage display, which is commonly conducted in the lab. Downstream of the amber stop codon following BAP is a sequence for geneIII. GeneIII fusion proteins need to be expressed rarely for phage display to function as intended because scFv-geneIII fusion proteins interfere with that particular geneIII protein to re-infect naïve bacteria. Therefore, fusion-free geneIII proteins need to exist during virion assembly for the generation of functioning phage i.e., scFv-geneIII protein products are usually relatively rare inside the

bacterium. D11-AP, D20-AP, empty vector, and all constructs used in this article are all affected similarly by the occasional geneIII fusion protein expression. There are still observed differences in how these proteins behave.

1.6. Pick an individual colony and inoculate a fresh 5 mL of 2xYTAG culture. Allow bacteria to grow overnight at 30 ˚C and shaking at 150 rpm.

1.7. Pellet the cells by centrifugation at 3,000 x *g* for 10 min at room temperature. Discard the supernatant and resuspend the pellet in 2 mL of 85% 2xYTAG and 15% glycerol.

1.8. Maintain glycerol stocks in a -80 ˚C freezer.

2. Protein expression and generation of periplasmic extract

NOTE: Generation of the periplasmic extract is a commonly used method for protein expression in phage display, mainly because the formation of disulfide bonds is important in scFv and antibody generation. The method avoids the need to generate lysates (commonly containing inclusion bodies) and ensures that proteins are properly folded. pCANTAB has a gIII signal sequence upstream of the scFv portion of the D11-BAP fusion protein. The signal sequence ensures that the protein is shuttled to the periplasmic space of the bacterium, and then the signal sequence is cleaved. The periplasmic space provides an oxidizing environment, which is crucial to the proper formation of disulfide bridges. Osmotic shock is used to derive periplasmic extracts because it disrupts the outer membrane enough to release the periplasmic proteins into the surrounding medium, while keeping the bacterium intact.

2.1. Inoculate TG1 *E. coli* glycerol stocks containing the relevant plasmids into a 60 mL culture of 2xYTAG and culture overnight at 30 ˚C with shaking at 150 rpm.

2.2. To induce protein expression, pellet bacterial cultures at 3,000 x *g* for 10 min, resuspend in 60 mL of 2xYTA medium, and culture overnight at 30 ˚C with shaking at 150 rpm.

NOTE: The switch from 2xTYAG to 2xYTA medium helps in sufficient induction of protein expression[18]. When glucose is present in the bacterial medium, the lac promoter is suppressed because bacteria preferentially consume glucose and ignore lactose, as glucose takes less energy to process. When glucose is removed following the medium switch, bacteria rely on the carbohydrates provided by the 2xYT medium. Yeast extract (the Y component in 2xYT) contains carbohydrates, among them lactose, and is, therefore, able to drive protein expression through the lac promoter. IPTG is not necessary with the pCANTAB construct and can result in excessive protein expression, along with inclusion bodies that result in non-functional protein.

2.3. Generate periplasmic extracts via osmotic shock.

2.3.1 Pellet bacterial cultures at 3,000 x *g* for 10 min.

2.3.2 Resuspend in 20 mL of 1xTES (0.2 M Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) and incubate for 1 h on ice.

2.3.3. Pellet again at 3,000 x *g* for 10 min, and resuspend in 15 mL of 0.05 M Tris, pH 7.6.

2.4. Incubate this suspension on ice for 1 h, then clarify by centrifugation at 5,000 x *g* for 10 min.

2.5. Transfer supernatants to a fresh tube and store at -20 ˚C until needed for either purification or direct use in experiments.

2.6. Assess the cell lysis by the presence of active D11-BAP in the ELISA. Add 3-5 µL of the periplasmic extract to one mL of pNPP, then observe if a color change happens over the next 10 min (color goes from clear-yellow to intense yellow).

NOTE: The color can be compared side-by-side to a tube of pNPP which has not received any lysate or a tube of pNPP which received lysate of naïve TG1 bacteria. Quantify with absorbance at 405 nm.

4. Characterization of D11-AP titer in ELISA

4.1. Coat a 384-well polystyrene plate overnight at 4 ˚C with 25 µL/well phosphate-buffered saline (PBS) (1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl) containing either 5 µg/mL of mouse serum albumin (MSA) (negative control), or 5 µg/mL of IsoLG/MSA (positive control).

4.2. Empty the plate and tap dry. Wash once with PBS + 0.1% Tween (PBS-T). Empty the plate and tap dry.

4.3. Fill the plate with PBS-T as a blocking buffer (120 µL/well). Incubate for 1 h at room temperature.

4.4. Empty the plate and tap dry. Apply serial 1:2 dilutions of D11-AP periplasmic extracts at 25 µL/well and diluted in PBS-T. Include one well containing only PBS-T as a negative control.

NOTE: Typical dilution ranges for periplasmic extracts are 1:8 – 1:4096. For a 25 µL assay volume, start with 50 µL of the starting “1:8” concentration: 6.25 µL periplasmic extract and 43.75 µL PBS-T. Then, perform a 2-fold dilution by removing 25 µL of this solution and adding it to the next well containing 25 µL of PBS-T and pipette it up and down. This well now contains the “1:16” dilution. Keep repeating the 2-fold dilution to generate the described 1:2 dilution series.

4.5. Incubate for 1.5 h at room temperature.

4.6. Empty the plate and tap dry. Wash 5 times with PBS-T. Empty and tap dry.

4.6. Prepare pNPP solution by dissolving 1 g of pNPP in 1 L of 930 mM diethanolamine (98% stock solution diluted 1:10 in H_2O), with 0.5 mM $MgCl_2$ and adjusted to pH 9.5 with HCl.

4.7. Apply 25 µL/well pNPP solution to develop AP. Incubate for 1 h at room temperature and determine the absorbance at 405 nm within each well using a compatible plate reader.

4.8. Compare the signal generated from the IsoLG/MSA wells to the noise generated from the MSA wells and find the range of dilutions in which the signal is at least 5-fold higher than the noise.

4.9. Plot this D11-AP signal dilution series on a graph, determine the linear range of the curve, and establish the dilution where 50% of the signal can be observed.

NOTE: If this dilution is equivalent to approximately 1:1,000, then the D11-AP solution may be used at a concentration of 1:10 in IHC/IF.

5. Immunofluorescence

5.1. Cut serial sections of mouse and human paraffin-embedded tissues (5 µm thick) using a microtome and place in a warm water bath (37 ˚C). Mount tissue sections on glass slides and allow to dry overnight.

NOTE: For this study, aortae were obtained from mice. Colon sections of normotensive and hypertensive humans were obtained from the Vanderbilt Cooperative Human Tissue Network.

5.2. Immerse slides in xylene three times for 5 min to deparaffinize tissues.

5.3. Rehydrate tissues in 2 washes each of 95%, 70% and 50% ethanol in H_2O.

5.4. Wash slides in Tris-buffered saline (TBS) with 0.1% Tween20 (TBST) three times with quick washes by filling the slide holder with TBST then discarding TBST.

NOTE: Hydrated slides can be stored in TBST at 4 ˚C for no longer than a week before antigen retrieval.

5.5. To perform heat-induced antigen retrieval of slides, place slides in pre-heated (80-95 ˚C) sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and incubate in a pressure cooker set to 4 min on high pressure for a total antigen retrieval time of 20 min.

5.6. Remove slides from the pressure cooker and allow them to cool for 20 min to room temperature.

5.7. Wash slides in TBST three times with quick washes.

NOTE: Slides can be stored in TBST after antigen retrieval for no longer than a week before staining.

5.8. Add 2% BSA dissolved in TBST to block slides. Cover slides with a strip of paraffin film and incubate at room temperature for 15 min.

5.9. Discard the blocking buffer from the slides.

5.10. Add 200 µL of 1:10 D11-AP in TBST to the slides and cover with a strip of paraffin film.

5.11. Incubate in a humidified chamber to minimize the antibody solution evaporation for 3 h at room temperature.

5.12. Wash slides three times in TBST.

5.13. Develop with a colorimetric or fluorescent alkaline phosphatase developer for immunohistochemistry or immunofluorescence, respectively. Wash slides once with TBST to remove excess developer and prevent further color development.

5.12. Counterstain slides with Hoechst nuclear stain at 1 µg/mL in PBS for immunofluorescence. Wash slides once in TBST to remove any excess counterstain.

5.13. Apply coverslips using the mounting medium.

5.14. View slides under an inverted light microscope for immunohistochemistry or a confocal fluorescent microscope for immunofluorescence.

6. Negative controls

NOTE: Four negative control experiments can be performed to confirm the specificity of D11-AP staining for IsoLG. Negative control experiments should be performed in the same staining set under the same conditions.

6.1. In the first negative control experiment, incubate tissues with D11-AP diluted in TBST or TBST alone.

6.2. Incubate tissues with diluted D11-AP in TBST and bacterial periplasmic extract without D11-AP (AP Linker) diluted in TBST.

6.3. Perform a competitive assay with IsoLG/MSA or non-adducted MSA as previously described[12].

6.3.1. Prepare IsoLG and IsoLG adducted to mouse serum albumin (MSA) as previously described[19], at a molar ratio of 8 IsoLG: 1 MSA (8:1 IsoLG/MSA).

6.3.2. Dilute D11-AP 1:10 in TBST.

6.3.3. Incubate diluted D11-AP with 50 µg/mL IsoLG/MSA or non-adducted MSA for 1 h at room temperature.

6.3.4. Add D11-AP with IsoLG/MSA or D11-AP with non-adducted MSA to tissues for staining.

6.4. Use irrelevant scFv antibody, D20, to stain tissues for the final negative control set.

REPRESENTATIVE RESULTS:

In representative experiments, D11 scfv with an alkaline phosphatase conjugation (D11-AP) was used in immunofluorescence to detect IsoLGs present in angiotensin II-treated mice compared to normal sham mice and humans with hypertension compared to normotensive humans. Mice were treated with angiotensin II at a dose of 490 ng/kg/min for two weeks, and hypertension was confirmed with increased systolic blood pressure compared to sham mice[10]. To ensure specificity of D11-AP, tissues were stained with or without the presence of D11-AP. As demonstrated by D11-AP staining, the aorta of mice with angiotensin II-induced hypertension showed elevated concentration of IsoLGs when compared to control mice (**Figure 1**). Background staining or autofluorescence was limited, as shown by negative controls that were not stained with D11-AP.

D11-AP was used to detect IsoLGs present in intestinal tissues of human patients with hypertension (HTN) or normotensive humans (NTN). Hypertension status was established from the hospital records as systolic blood pressure above 140 and diastolic blood pressure above 80 mmHg. Researchers developing immunofluorescence protocol for D11-AP were blinded to the hypertension status of human tissues. Sections were stained in the presence and absence of D11-AP to ensure antibody specificity and show background staining or autofluorescence. As shown in **Figure 2**, we found that tissues from patients with HTN had elevated concentrations of IsoLGs compared to patients with NTN. Staining without D11-AP also showed minimal background staining and autofluorescence. Endogenous alkaline phosphatase is expressed by intestinal epithelium, so the limited fluorescence of tissues stained without D11-AP shows the antigen retrieval used in this protocol was sufficient in inactivating endogenous alkaline phosphatase present in tissues. In combination with the results in mice, these results also show that the immunofluorescence protocol effectively shows elevated IsoLGs in hypertension when compared to normotensive status.

D11-AP was isolated and stored in the bacterial periplasmic extract. Mouse and human tissues were stained with D11-AP and periplasmic extract containing the AP linker without D11 to ensure that other factors that may be present in the periplasmic extract, such as excess or non-conjugated alkaline phosphatase, do not contribute to the staining observed in tissues treated with D11-AP (**Figure 3**). Tissues stained with D11-AP resulted in brighter staining compared to tissues stained with periplasmic extract. These results confirm that D11-AP is staining the tissues, and the staining is not due to non-conjugated bacterial alkaline phosphatase that can potentially

be present in the periplasmic extract and result in false staining of IsoLGs or contribute to background staining.

A competitive control was performed by pre-incubating D11-AP with IsoLG adducted to MSA or non-adducted MSA before staining tissues to show the specificity of D11-AP to IsoLG. If D11-AP is specific for IsoLG, the antibody would bind to IsoLG-MSA, resulting in a depleted availability of D11-AP to stain tissues, and D11-AP incubated with non-adducted MSA would have similar staining to normal D11-AP. In tissues stained with D11-AP pre-incubated with the IsoLG competitor, we found diminished staining compared to tissues that were stained with D11-AP without any preincubation (**Figure 4**). In tissues stained with D11-AP preincubated with non-adducted MSA, we found staining to be similar to staining observed in tissues with D11-AP. These results show the specificity of D11-AP to IsoLGs due to reduced staining of tissues when D11-AP was pre-incubated with IsoLG/MSA, but not non-adducted MSA. In the final negative control, mouse tissue was stained with D11-AP or irrelevant scFv antibody, D20. Staining mouse aorta with D11-AP resulted in strong staining compared to D20 indicating the specificity of D11-AP to IsoLGs in hypertensive aortae (**Figure 5**).

FIGURE LEGENDS:

Figure 1: Immunofluorescence of the aorta in hypertensive and normotensive mice. Arteries from angiotensin II and sham infused mice were stained with and without D11-AP (D11) to show presence of IsoLGs. (**A**) Artery from an angiotensin II treated mouse probed with D11-AP (green) and nuclear counterstain (magenta), (**B**) Artery from a control sham treated mouse probed with D11-AP, (**C**) Artery from an angiotensin II treated mouse without D11-AP, (**D**) Artery from a control sham treated mouse without D11-AP (scale bar =100 μm).

Figure 2: Immunofluorescence of human intestinal tissues from hypertensive and normotensive patients. Tissues from patients with hypertension (HTN) and normotensive humans (NTN) were stained with and without D11-AP (D11) to show presence of IsoLGs in patients with HTN. (**A**) HTN tissues stained with D11-AP (green) and nuclear counterstain (magenta), (**B**) NTN tissues stained with D11-AP, (**C**) HTN tissues stained without D11-AP, (**D**) NTN tissues stained without D11-AP (scale bar =100 μm).

Figure 3: Mouse and human tissues stained with periplasmic extract with and without D11-AP. Mouse and human tissues were stained with periplasmic extracts with and without D11-AP. Images show limited staining of tissues with periplasmic extract without D11-AP, which show staining is mostly due to D11-AP binding rather than another component that may be present in periplasmic extract. (**A**) Ang mouse aorta stained with D11-AP (green) and nuclear counterstain (magenta), (**B**) Ang mouse aorta stained with periplasmic extract, (**C**) Sham mouse aorta stained with D11-AP, (**D**) Sham mouse aorta stained with periplasmic extract, (**E**) hypertensive human intestinal tissue stained with D11-AP, (**F**) hypertensive human intestinal tissue stained with periplasmic extract, (**G**) normotensive human intestinal tissue stained with D11-AP, (**H**) normotensive human intestinal tissue stained with periplasmic extract (scale bar =100 μm).

Figure 4: Competitive control in vessels of Ang and Sham mice. In this competitive control, D11-AP was pre-incubated with IsoLG-adducted MSA or non-adducted MSA. D11-AP without any pre-incubation was used as a control. These results show the specificity of D11-AP to IsoLGs because there is reduced staining of tissues with the IsoLG-MSA competitor compared to D11-AP. This reduction is due to IsoLG and not MSA because the non-adducted MSA pre-incubation resulted in staining similar to D11-AP control. Angiotensin II (**A**) and Sham (**B**) mouse aortae stained with D11-AP (green) and nuclear counterstain (magenta), Angiotensin II (**C**) and Sham (**D**) mouse aortae stained with D11-AP after incubating with IsoLG-MSA, Angiotensin II (**E**) and Sham (**F**) mouse aortae stained with D11-AP after incubating with non-adducted MSA (scale bar =100 µm).

Figure 5: Mouse aortae stained with D11 and irrelevant scFv, D20. Mouse tissue were stained with D11-AP and compared to an irrelevant control antibody, D20, which is specific for glycoprotein A2. Staining of tissue with D11-AP (green) and nuclear counterstain (magenta) (**A**) resulted in intense immunofluorescence compared to D20 (green) and nuclear counterstain (magenta) (**B**) which indicates specificity of D11-AP to IsoLGs (scale bar =100 µm).

DISCUSSION:

D11 has been used extensively to detect IsoLG-adducted proteins in cells or tissues as a marker for inflammation or oxidative stress in disease[8,9,20]. Previously, D11 contained an E tag and IHC development required the use of a secondary anti-E tag antibody conjugated with HRP[10,20,21]. Here, we have developed and optimized protocol for detection of IsoLG-adducted proteins using the D11 antibody conjugated with alkaline phosphatase in place of the E-tag, which eliminates the need for a secondary antibody incubation.

To determine the specificity of D11-AP, four negative control experiments were performed. We performed the protocol without the presence of D11 and had minimal development. These results have a two-fold indication: endogenous alkaline phosphatase is not contributing to development, and the staining observed is due to D11 and not another contributing factor. Next, we stained slides with the AP linker without D11. This experiment resulted in little staining, which indicates free AP or other factors in the periplasmic extract is not causing the stain we observe in the presence of D11. To ensure the specificity of D11 to IsoLG, we preincubated D11-AP with purified IsoLG before staining slides. We saw a decrease in development which indicates that the D11-AP was bound to IsoLG protein, thus exhausting the amount of free D11-AP to bind to IsoLG present in the tissue. Finally, to ensure D11-AP was binding to IsoLG and not the MSA protein IsoLG was bound to, we preincubated D11-AP with MSA only. There were no changes in development, indicating D11-AP was not binding to MSA but the IsoLG protein. Lastly, researchers developing the staining protocol were blind to hypertensive status of human intestinal tissue. The differences in staining observed between patients with hypertension and patients with normotension were not due to bias and have been previously described[22,23].

Although our protocol for detection of IsoLG-adducted proteins using the D11 antibody conjugated with alkaline phosphatase in place of the E-tag is rigorous and robust and eliminates the need for a secondary antibody incubation, it has some limitations. One limitation is that we used D11 conjugated with alkaline phosphatase in the periplasmic extract, and there could be

false staining of endogenous alkaline phosphatase in the periplasmic extract or certain tissues, such as intestine[24]. However, the first step to develop this protocol included disabling endogenous alkaline phosphatase that may be present in tissues[25]. Initially, cold acetic acid, BME, and Levamisole[26] were tested for efficiency. None of these completely diminished the presence of active endogenous alkaline phosphatase. Heat has been used to deactivate alkaline phosphatase[27], so we tested heat deactivation of alkaline phosphatase in different buffers. We found heating mounted and hydrated slides in citrate buffer eliminated most endogenous alkaline phosphatase. Slides were developed initially using a Chemiluminescent/Fluorescent Substrate, but when imaged without this substrate, there was a high amount of autofluorescence. VectorRed is a substrate which develops in the presence of alkaline phosphatase to produce a chromogen which can be visualized in the Texas Red/TRITC channel range. Using this substrate, we were able to more easily observe signal above background autofluorescence. Care should be taken during the staining process to minimize artifactual staining. Drying of tissues on slides after hydration until imaging has resulted in heightened development. D11-AP should be aliquoted and stored at -20C. Multiple freeze-thaw cycles should be avoided when working with D11-AP. Phosphate-buffer saline (PBS) can also affect the enzymatic activity of alkaline phosphatase and should not be used as a wash buffer[28]. As with any antibody-based approach, thorough testing and optimization must be carried out to ensure that staining is specific, and that signal is not over or under amplified.

In conclusion, we have developed a powerful, rigorous, and robust optimized protocol to detect IsoLG-adducted proteins using the D11 antibody conjugated with alkaline phosphatase in place of the E-tag. This protocol presents several advantages: First, using D11 as an alkaline phosphatase fusion protein is cheaper. D11 was originally derived from a phage antibody library that could not be commercialized and was expensive to purify. Although D11 in E. coli periplasmic extract could provide an inexpensive alternative, it was ineffective in most assays. Second, the alkaline phosphatase fusion approach allows D11 scfv to have a useful reporter[15] (alkaline phosphatase) fused to it and would not need to be purified for use in immunoassays as substrates are commercially available. Third, the E. coli alkaline phosphatase forms dimers[29]. So D11, when fused to the alkaline phosphatase would also form dimers and this increase the antibody's avidity and binding activity[30]. Finally, D11 conjugated with alkaline phosphatase in periplasmic extract can easily be cleaned using Cibacron Blue Sepharose. D11 has a high isoelectric point (~9.2 pH). As such, it is positively charged and can bind to Cibacron Blue through pi-cation interactions. Most of the impurities in the E. coli periplasmic extract can be eluted off the resin. The D11 conjugated with alkaline phosphatase can then be eluted using high salt (~1.5M NaCl) in water. The eluted D11 conjugated with alkaline phosphatase is quite stable at 4-8C in the high salt solution. Thus, we have developed a protocol which not only makes the D11 antibody available at a low cost, but also eliminates the extra steps and need for the secondary antibody incubation. This protocol facilitates reproducible measurement of IsoLGs, which accumulate in tissues in multiple diseases where increased oxidative stress plays a role.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1.tif

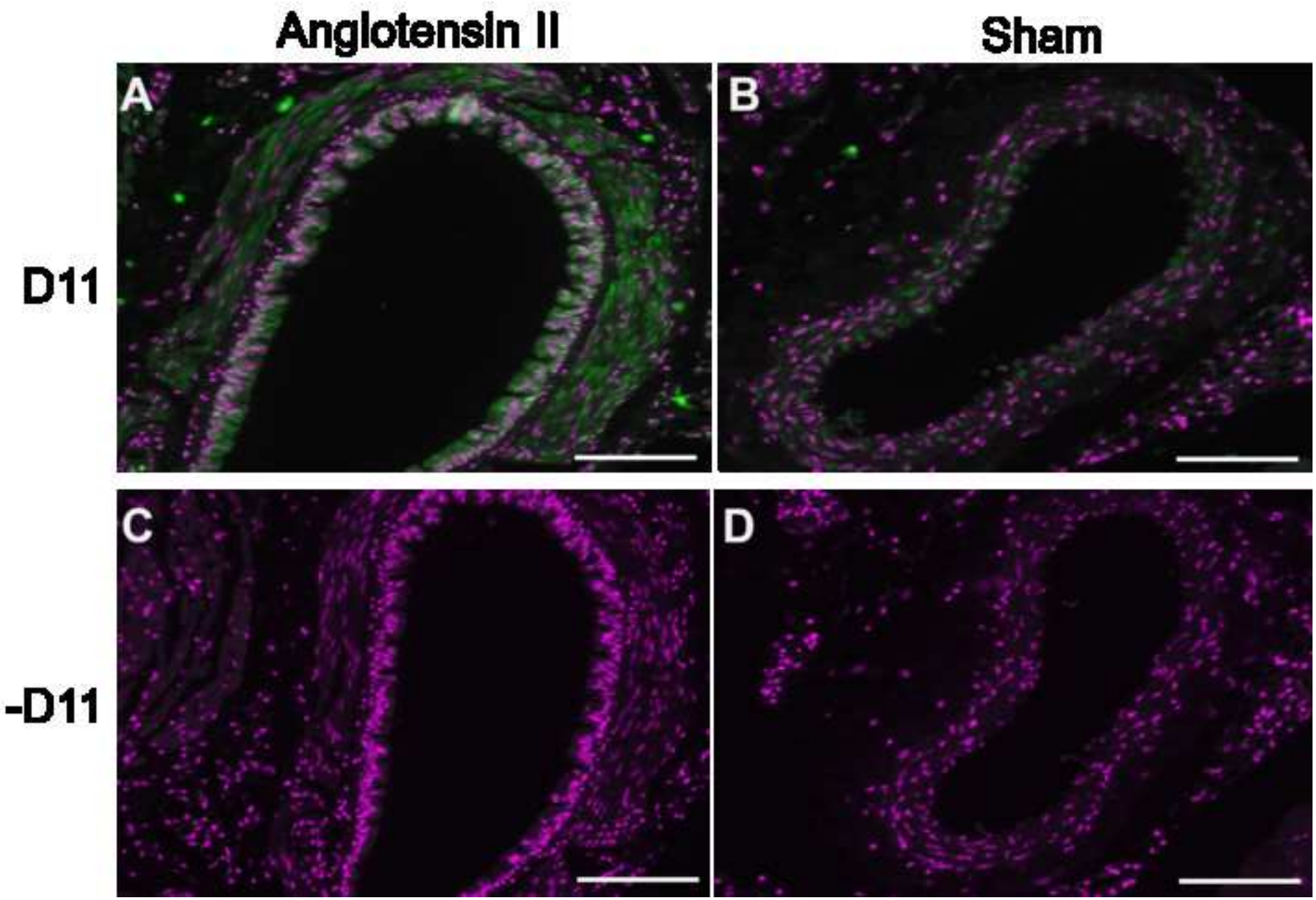


Figure 2

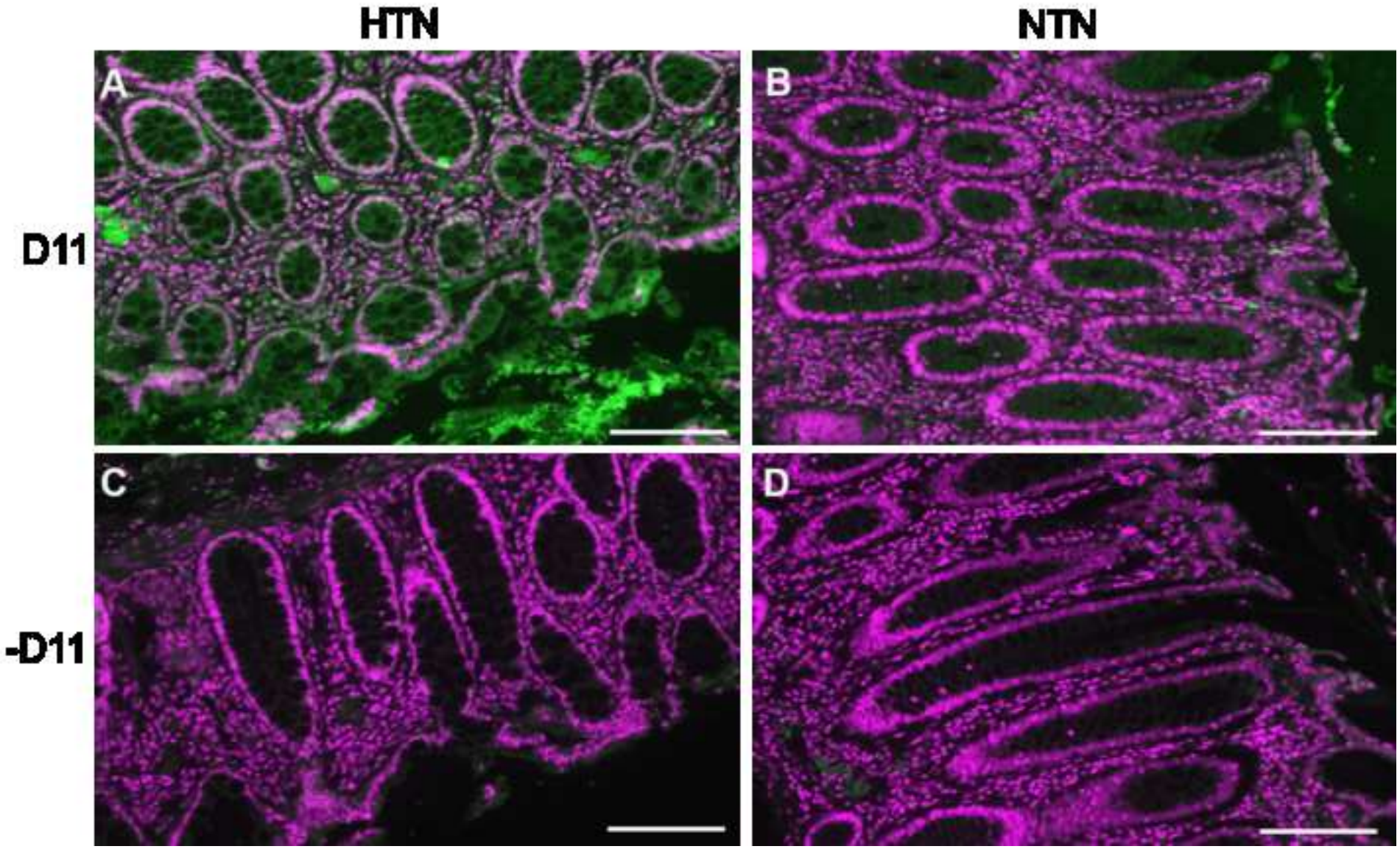


Figure 3

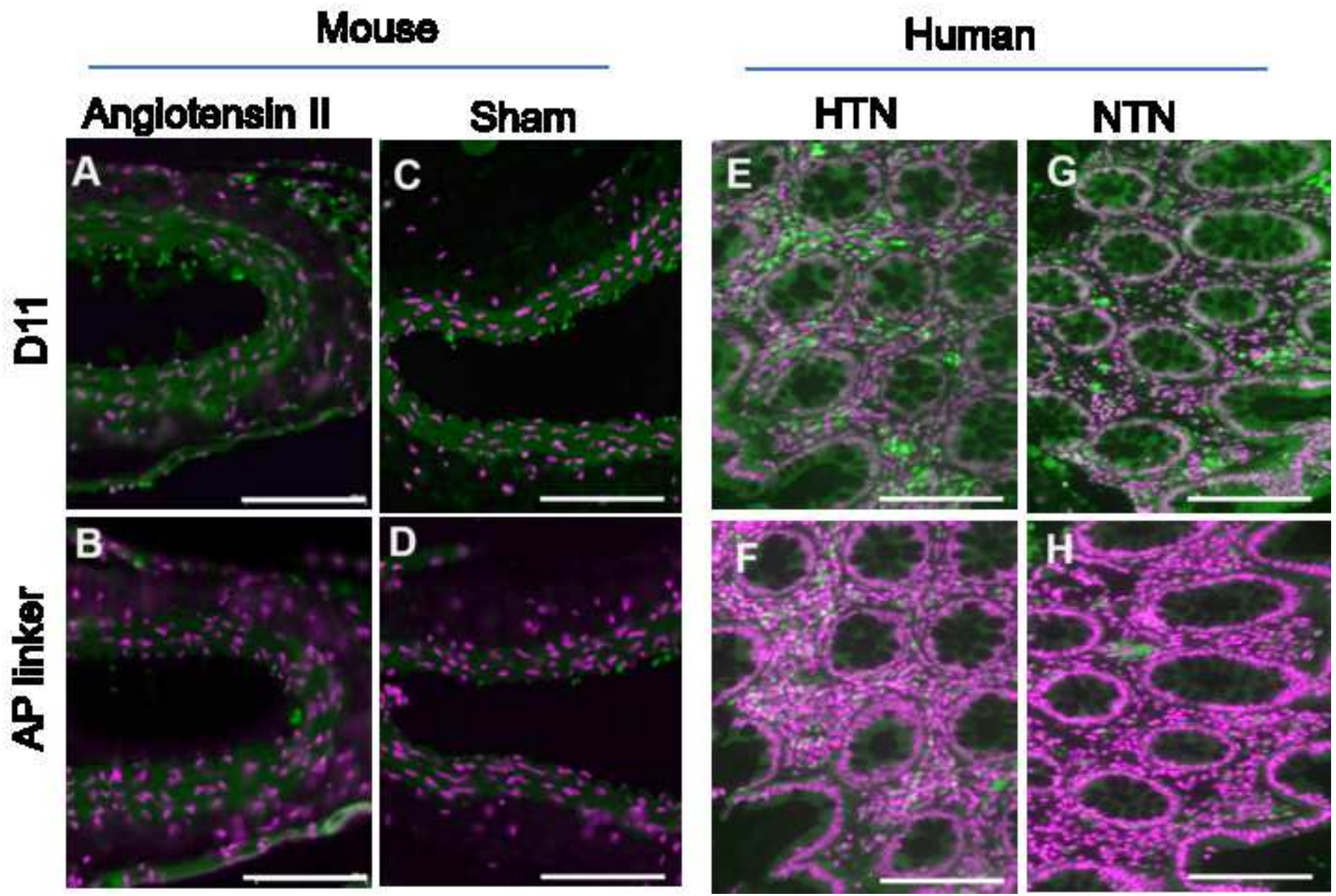


Figure 4

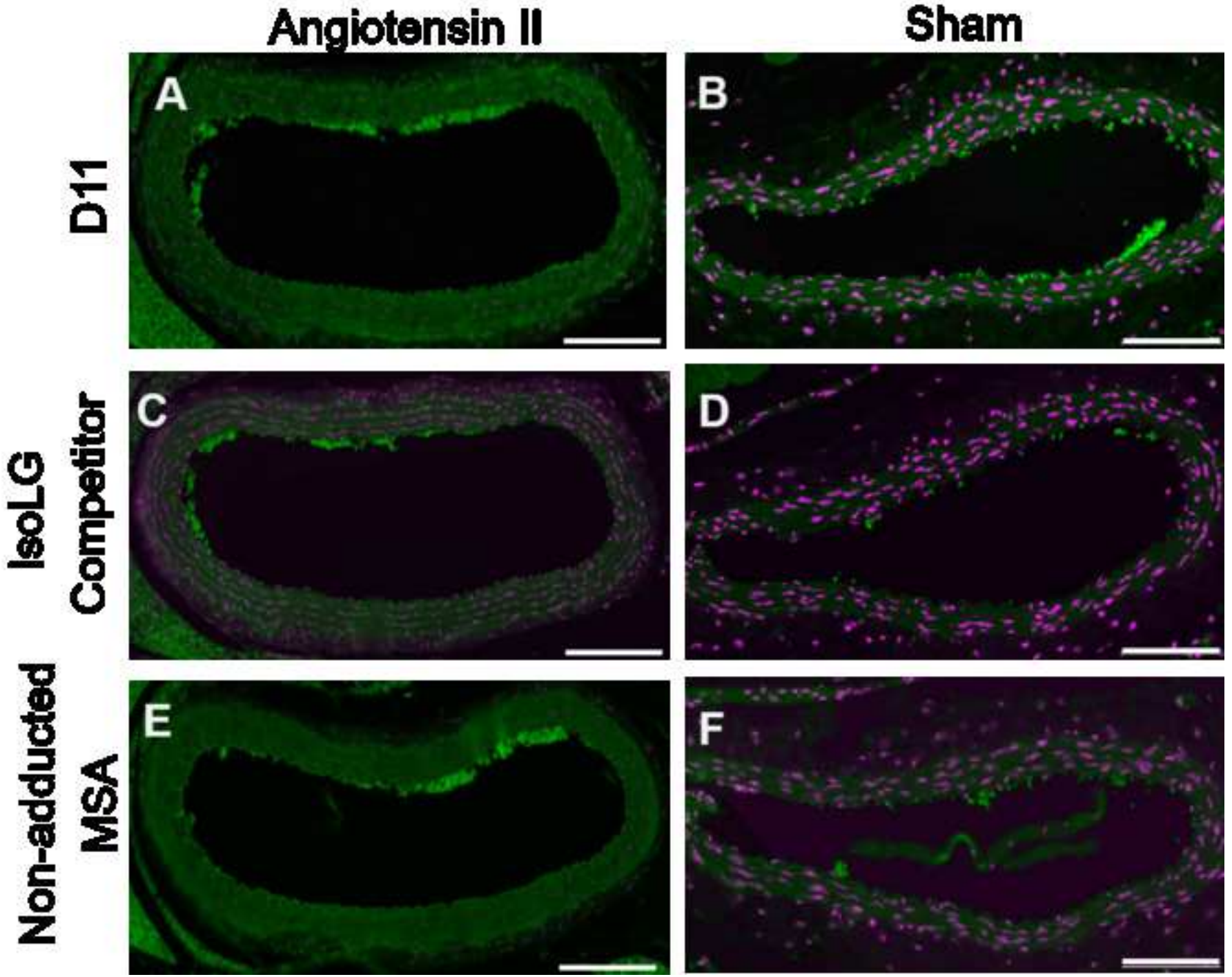
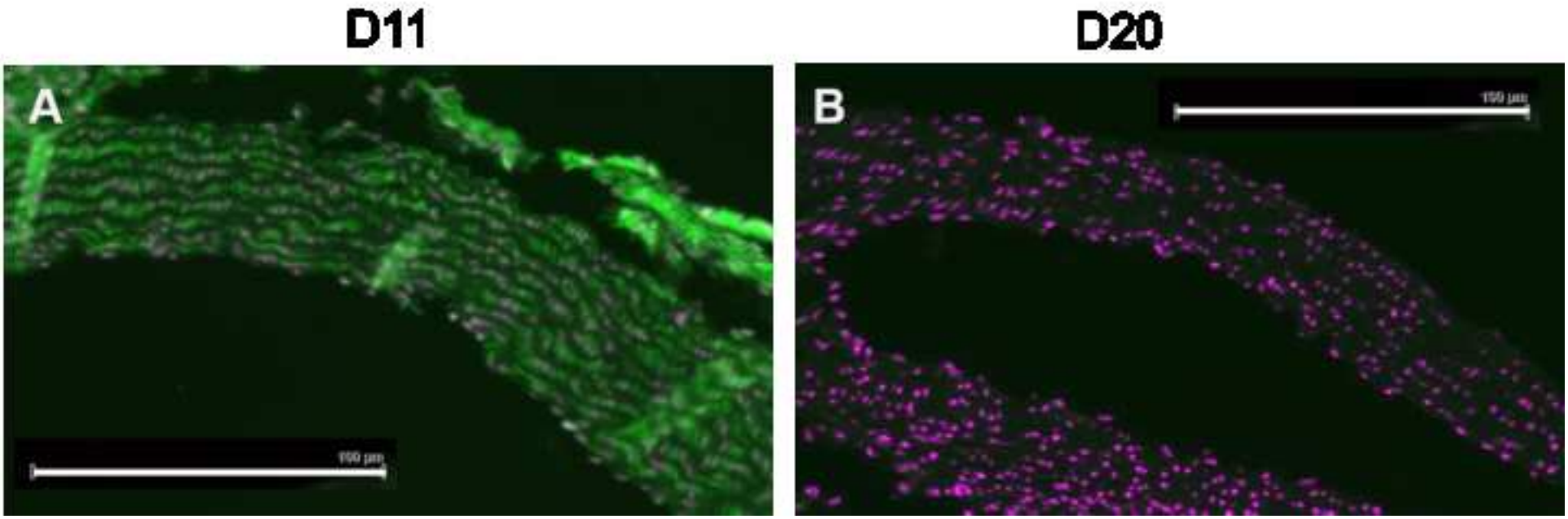


Figure 5



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 ml TALON HiTrap column (Cobalt-CMA)	Cytiva	28953766	
200 Proof Ethanol	Pharmco	111000200	
2xYT powder	MP Biomedicals	3012-032	
384-well, clear, flat-bottom polystyrene microplates	ThermoFisher (NUNC)	242757	
4-Nitrophenyl phosphate disodium salt hexahydrate (pNPP)	Carbosynth	EN08508	
5-Bromo-4-chloro-3indoxyl phosphate, p-toluidine salt (BCIP)	Carbosynth	EB09335	
Ampicillin, sodium salt	Research Products International (RPI)	A40040	
Bovine Serum Albumin	RPI	A30075	
Chemically competent TG1 *E. coli*	Amid Biosciences	TG1-201	
Diethanolamine, >98%	Sigma-Aldrich	D8885	
EDTA	Sigma-Aldrich	ED	
Fluoromount-G	SouthernBiotech	0100-01	Mouting medium
Glucose	Research Products International (RPI)	G32045	
Glycerol	Sigma-Aldrich	G7893	
Histoclear	National Diagnostics	HS-200	Xylene alternative
Hoechst 33342	ThermoFisher	H3570	stock solution = 10 mg/mL
Hydrochloric acid (HCl), 30%, Macron Fine Chemicals	ThermoFisher	MK-2624-212	
Imidazole	Research Products International (RPI)	I52000	
$MgCl_2$ (anhydrous)	Sigma-Aldrich	M8266	
Mouse Serum Albumin (MSA)	Sigma-Aldrich/Calbiochem	126674	
Nitroblue tetrazolium chloride (NBT)	Carbosynth	EN13587	
Potassium chloride (KCl)	Sigma-Aldrich	P4504	

Potassium phosphate, monobasic (KH_2PO_4)	Sigma-Aldrich	P0662	
Pressure Cooker	Cuisinart	CPC-600	
Slide-a-Lyzer Dialysis cassettes, 10K MWCO, 3 ml	ThermoFisher	66380	
Sodium chloride (NaCl)	Research Products International (RPI)	S23020	
Sodium Citrate	Sigma-Aldrich	1064461000	
Sodium phosphate, dibasic (Na_2HPO_4)	Research Products International (RPI)	S23100	
Sucrose	Research Products International (RPI)	S24065	
Tris base	Research Products International (RPI)	T60040	
Tris-buffered Saline	Boston Bio-Products		25mM Tris, 2.7mM KCl, 137 mM NaCl, pH 7.4
Tris-HCl	Research Products International (RPI)	T60050	
Tween20	Sigma-Aldrich	P9416	
Vector Red	Vector Labs	SK-5105	

We very much appreciate the constructive and helpful comments of the reviewers and the editors and have revised our manuscript in accordance to the various concerns raised. We believe that these responses have considerably improved our manuscript and hope that it is now acceptable for publication in JoVE. Below is an outline of these responses:

RESPONSE TO EDITORS:

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations during the first-time use.

Response 1: We have carefully revised the Manuscript to ensure all abbreviations are defined during first-time use.

Comment 2: Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

Response 2: We have reformatted the Manuscript to the suggested format.

Comment 3: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Fluoro-mount, (NUNC 242757), TALON, etc.

Response 3: We have removed all commercial products or trademark names from the Manuscript, which are included in the Table of Materials and Reagents.

Comment 4: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Response 4: We have ensured the language is written in imperative tense throughout. Any information relevant to a step that is not an action has been added as a “Note.”

Comment 5: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Response 5: We have revised and simplified the Protocol section to limit each step to no more than 2-3 actions or 4 sentences.

Comment 6: The Protocol should contain only action items that direct the reader to do something.

Response 6: We have removed any non-action items or added non-action items as a Note.

Comment 7: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response 7: We have added more details to our steps in order to answer the "how" question.

Comment 8: For the materials section: this can either be moved to a table format and table can be referenced as and wherever applicable. Otherwise please rewrite as if you are instructing someone how to make the solutions.

Response 8: We have removed the Materials section from the Protocol and have either added those to the Materials Table or added it as steps throughout the protocol.

Comment 9: How do you perform the cloning? Do you perform PCR amplification of the whole genome? How is this done? Do you perform ligation? How do you generate empty vector? How do you purify the product? Please include all details associated with the step.

Response 9: We are legally constrained from providing anyone with D11 DNA. The legal agreement we signed when we agreed to make the phage antibody library from which D11 was obtained, prevented us from sharing recombinant antibody DNA stemming from that library with anybody outside of Vanderbilt. As such, when others wanted to use D11 in their studies, we could not give them the D11 DNA; but we were obligated to the scientific community to provide D11 antibody for use in their studies. D11 purification and labeling was too time-consuming and expensive for us to perform for others. Thus, the purpose in developing D11-BAP was to bypass the need to purify and label the D11 scfv. Providing unpurified, labeled (i.e. BAP = bacterial alkaline phosphatase) D11-BAP in periplasmic extract inexpensively solved these problems when others asked us to provide them with D11.

The purpose of these manuscript is to tell others how to use unpurified D11-BAP in periplasmic extract when Vanderbilt provides it to them, free of all charges - except shipping. The D11 DNA and the D11 protein cannot be sold, as per our legal agreement. But the D11 protein can be provided free of charge since it is just too costly to purify D11 scfv protein.

For these reasons, we have now entirely removed any sections on protein purification of D11 and only mention cloning for completion, without adding details, in order to comply with the legal agreement.

Comment 10: How do you ensure that this leads to protein expression?

Response 10: The switch from 2xTYAG to 2xYTA medium serves as sufficient induction of protein expression. When glucose is present in the bacterial medium, the lac promoter is suppressed because bacteria preferentially consume glucose and ignore lactose, as glucose takes less energy to process. When glucose is removed following the medium switch, the bacteria have to rely on the carbohydrates provided by the 2xYT medium. Yeast extract (the Y

component in 2xYT) contains carbohydrates, among them lactose, and is therefore able to drive protein expression through the lac promoter. IPTG is not necessary with the pCANTAB construct and can result in excessive protein expression, along with inclusion bodies that result in non-functional protein.

We have added this as a Note to the revised manuscript.

Comment 11: Rationale for generating periplasmic extracts?

Response 11: Please see response to your comment 9. This is a commonly used method for protein expression in phage display, particularly because the formation of disulfide bonds is so important in scFv and antibody generation. The method avoids the need to generate lysates (which commonly contain inclusion bodies) and ensures that proteins are properly folded. pCANTAB has a gIII signal sequence upstream of the scFv portion of the D11-BAP fusion protein. The signal sequence ensures that the protein is shuttled to the periplasmic space of the bacterium, and then the signal sequence is cleaved. The periplasmic space provides an oxidizing environment, which is crucial to the proper formation of disulfide bridges. Osmotic shock is used to derive periplasmic extracts because it disrupts the outer membrane to enough of an extent that periplasmic proteins flow into the surrounding medium, while the rest of the bacterium stays intact.

Comment 12: How do you ensure the cell lysis for the generation of protein extracts?

Response 12: We assess lysis by the presence of active D11-BAP in the ELISA. If we ever need to check for the presence of BAP in the lysate, we add 3-5 µl of the periplasmic extract to one ml of pNPP, then observe if a color change happens over the next 10 minutes (color goes from clear-yellow to intense yellow). The color can be compared side-by-side to a tube of pNPP which has not received any lysate, or a tube of pNPP which received lysate of naïve TG1 bacteria. If this color intensity needs to be quantified, it can be measured with absorbance at 405 nm.

Comment 13: Volume of the extract loaded?

Response 13: For a 25 µl assay volume, start off with 50 µl of the starting "1:8" concentration: 6.25 µl periplasmic extract and 43.75 µl PBS-T. Then, perform a 2-fold dilution by removing 25 µl of this solution and adding it to the next well, which contains 25 µl PBS-T, and pipet it up and down. This well now contains the "1:16" dilution. Keep repeating the 2-fold dilution to generate the described 1:2 dilution series.

Comment 14: How do you check for the presence of purified DP11-AP in this case?

Response 14: Please see response to your comment #9.

Comment 15: How do you ensure that the tissue is from hypertensive animals or humans. Do you perform and marker staining to confirm this? How do you ensure what part of the tissue is to be taken? How is the cutting performed?

Response 15: We have added the following details, in blue, to Step 5.1 for cutting tissues:

Lines 248-250: 5.1. Cut serial sections of mouse and human paraffin-embedded tissues (5µM thick) using a microtome and place in a warm water bath (37˚C). Mount tissue sections on glass slides and allow to dry overnight.

We have added the following details, in blue, to the Representative Results to confirm tissue is from hypertensive animals or humans:

Lines 328-330: Mice were treated with angiotensin II at a dose of 490 ng/kg/min for two weeks, and hypertension was confirmed with increased systolic blood pressure compared to sham mice (Kirabo 2014).

Lines 337-338: Hypertension status was established from the hospital records as systolic blood pressure above 140 and diastolic blood pressure above 80 mmHg.

Comment 16: Please reword for clarity.

Response 16: We have reworded Step 5.5 to clarify how heat-induced antigen retrieval is performed:

Lines 263-266: To perform heat-induced antigen retrieval of slides, place slides in pre-heated (80-95 ˚C) sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0) and incubate in a pressure cooker set to 4 minutes on high pressure for a total antigen retrieval time of 20 minutes.

Comment 17: There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response 17: Done

Comment 18: Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

Response 18: We have ensured all figures are discussed in the Representative Results

Comment 19: Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Response 19: We have ensured that the results show the immunofluorescent protocol designed for D11-AP is robust and the staining is true to the presence of isolevuglandins and not the result of background/nonspecific staining or autofluorescence.

Comment 20: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response 20: We have not used any figures from previous publications.

Comment 21: Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. What does different color represent? Please include the scale bar for all the images taken by microscope and define it in the figure legend.

Response 21: We have added color representation to each figure legend as D11-AP staining represented as green staining and nuclear counterstain with magenta. Scalebars are also added and defined.

Comment 22: As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response 22: Done

Comment 23: Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Response 23: All figures embedded in the Manuscript have been removed and will be uploaded separately. Each figure is accompanied by a title and a description under the "Figure Legend" section after the Representative Results.

Comment 24: Please sort the materials table in alphabetical order.

Response 24: We have sorted the Materials table in alphabetical order.

RESPONSE TO REVIEWER #1:

This manuscript presented by Warden et al. offers a novel protocol for immunofluorescent staining of Isolevuglandin (IsoLGs) by using a single chain fragment variable (scfv) recombinant

antibody (D11) conjugated with alkaline phosphatase, which can be used in mouse and human tissues.

Comment 1: Since the success and sensibility of this protocol can variate depending on the tissues studied, it is recommended to add and specify some information that can help to the readers. For instance, in the protocol used in mice, it is recommended to mention: days and dose of AngII (Is similar the IsoLGs generation in animals with 180mmHg of systolic blood pressure, with respect to mice with 140mmHg?) and some technical information related to the plasmid preparation (for instance, gels images and/or schemes of empty or cloned vectors).

Response 1: We have added the following details to the Results to describe the protocol used in mice:

Mice were treated with angiotensin II at a dose of 490 ng/kg/min for two weeks, and hypertension was confirmed with increased systolic blood pressure compared to sham mice using telemetry (Kirabo 2014).

We believe that technical information related to the plasmid preparation (for instance, gels images and/or schemes of empty or cloned vectors) is beyond the scope and we have just included it briefly for completion purposes. Please see our response to the editor comment #9 and #10.

Comment 2: Abstract: please define 'AP' (alkaline phosphatase?)

Response 2: This has been defined

Comment 3: Introduction: Line 73, says 'ccardiovascular' and, in line 75, is not defined CVD (cardiovascular)

Response 3: This has been defined at first mention

Comment 4: Materials: Since this protocol involves different steps that include different strategies and techniques, it should be convenient that some points can be explained in an extended way. In particular, it is recommended to include an extension (explanation) of point 1.4 of methodology.
In step 2.5, please add the temperature of centrifugation before freezing at -80ºC.
4) Results: Please, add dose and days of AngII protocol. What kind of vasculature was analyzed? Aorta? Please, specify.

Response 4: Thank you. We have added the suggested details.

Comment 5: Discussion: Line 356, says 'PBR' refereed to saline buffer (it should be 'PBS')

Response 5: This has been changed to the appropriate abbreviation.

RESPONSE TO REVIEWER #2:

In the manuscript entitled: "Immunofluorescence Isolevuglandin Detection by Alkaline Phosphatase-Conjugated ScFv D11 Single Chain Antibody" the authors describe a methodology for measurement of isolevuglandins in tissues by immunofluorescence in using a recombinant scFvD11-alkaline phosphatase immunoconjugate. The experimental protocol is clear and well designed. The manuscript is well written, well organized, overall accurate and concise in compliance with international requirements. However, I have only some minor points to address (see below), this will contribute to make the paper even more reliable. Therefore, the paper can be accepted after minor revision.

Comment 1: Line 131: E. coli TG1 is used for the production of the chimeric protein. TG1 is an amber-suppressing E. coli strain in which the amber stop codon is read as a codon for Gln. How do you explain this in your construction? While HB2151 is an amber codon non-suppressor strain!!!

Response 1: Strains with the supE gene (such as TG1 E. coli) do not suppress the amber stop codon 100%. Estimates range from 0.8 – 20% of the time, so there is still a lot of product that terminates just downstream of BAP, as it is intended to for our experiments. TG1 E. coli are mainly used for practical reasons, such as reduction of time and costs, their compatibility with the pCANTAB protein expression system, and their use in phage display, which is commonly conducted in the lab. Downstream of the amber stop codon following BAP is a sequence for geneIII. GeneIII fusion proteins need to be expressed rarely in order for phage display to function as intended. This is because a scFv-geneIII fusion proteins interfere with that particular geneIII protein to re-infect naïve bacteria. Therefore, fusion-free geneIII proteins need to exist during virion assembly in order for functioning phage to be generated, meaning that scFv-geneIII protein products are usually relatively rare inside the bacterium. D11-AP, D20-AP, empty vector, and all constructs used in this article are all affected similarly by the occasional geneIII fusion protein expression. There are still observed differences in how these proteins behave. We have incorporated this in the manuscript as a note under procedure 1.5.

Comment 2: Line 150: 3.2. Protein induction must be defined: if that requires IPTG or the presence of other sugars? Inducer concentration must be indicated? Idem for the recommended antibiotics?
Line 178: 25 µl/well: can you translate this in concentration/well after D11-AP immunoconjugate purification and quantification? Idem for line 203, to estimate 1:10 of the recombinant immunoconjuate in concentration.

Response 2: Please see our responses to the editor's comments #9 and #10.

Comment 3: Line 196: sodium citrate buffer: molarity, pH… ?

Response 3: We have clarified the composition of citrate buffer as follows:

5.5. To perform heat-induced antigen retrieval of slides, place slides in pre-heated (80-95 ˚C) sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and incubate in a pressure cooker set to 4 minutes on high pressure for a total antigen retrieval time of 20 minutes.

Comment 4: Line 234: The authors claim "To ensure specificity of D11-AP, tissues were stained with or without the presence of D11-AP". It's partially correct if there is no-cross reactivity with isolevuglandins isomers. This point should be clarified.

Response 4: Sorry for the confusion. We have clarified this.