TREAT-AD
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IUSM-Purdue TREAT-AD Center Target Enablement Resource

Phospholipase C gamma 2 (PLCG2) Protein Expression, Purification and Cryo-EM Sample Preparation

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Introduction: Phospholipase C gamma 2 (PLCG2) is a member of the phospholipase C (PLC) family that catalyzes the cleavage of phosphatidylinositol 4,5 bisphosphate (PI(4,5)P2) into diacylglycerol (DAG) and inositol triphosphate (IP3). PLCG2 is primarily expressed in immune cells such as B-cells and microglia.[1] Recent genome-wide association studies (GWAS) identified a rare PLCG2 mutant, P522R, linked to a neuroprotective phenotype against Alzheimer's Disease.[2] Structural characterization of wild-type PLCG2 will be pursued to understand its domain organization, which helps to inform its function in the cell.

Proteins Purified: A protein construct containing residues 1-1195 of wild-type human Phospholipase C gamma 2 (PLCG2) was codon optimized for insect cell expression by GenScript (Piscataway, NJ) (**Table 1**). This construct also contains an N-terminal, hexa-histidine tag and short amino acid linker (GVDLGT) followed by a Tobacco Etch Virus (TEV) protease cleavage site (ENLYFQ↓S). The molecular weight of the PLCG2 protein construct is 142 kDa. Initial P1 and P2 viral stocks were generated by GenScript and a single, immobilized metal-ion chromatography (IMAC) step using nickel(II)-charged resin was performed by GenScript to partially purify the PLCG2 protein. This workflow results in a yield of approximately 31 mg from 1L of cells. The purity of this protein stock is shown in **Figure 1**. Subsequent purification methods prior to preparation of cryo-EM grids, as well as grid preparation parameters, are described in more detail below.

Future Plans: We will continue to optimize cryo-EM data processing workflows in order to deposit a fully-refined cryo-EM structure of wild-type PLCG2 into the appropriate online

repositories (Protein Data Bank (PDB), and EM Data Bank (EMDB)). These data processing parameters will be described in future Target Enablement Packages.

Conclusion: In this Target Enablement Package, we show that wild-type PLCG2 can be reliably expressed and purified using an insect cell-based baculovirus expression system that results in a fairly high protein yield. We also show that these proteins can be purified using fast-paced liquid chromatography (FPLC) to a purity suitable for cryo-EM grid preparation.

Methods:

Purification of Wild-Type PLCG2 for cryo-EM. Frozen protein stocks of wild-type (WT) PLCG2 protein corresponding to the sequence shown in **Table 1** were obtained from GenScript (Piscataway, NJ). The protein was sent in a storage buffer consisting of 50 mM Tris-HCl pH 8.0, 500 mM NaCl, and 5% glycerol. Protein stocks were thawed on ice and loaded onto a Nickel HisTrap column (Cytiva Life Sciences) equilibrated in Buffer A (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol). His-tagged PLCG2 was eluted off the HisTrap column using a linear gradient from 0%-100% Buffer B (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, 500 mM imidazole, 2 mM dithiothreitol (DTT)) over 20 column volumes (**Figure 1A**). Fractions containing PLCG2 as determined by denaturing SDS-PAGE gel analysis (**Figure 1B**) were kept as two separate pools (**pool 1**, **pool 2**), with each pool being dialyzed against two liters of Buffer C (50 mM HEPES, pH 7.5, 25 mM NaCl, 2 mM DTT) overnight at 4°C. Dialysis was accomplished by placing the pooled protein in a 10 kilodalton (KDa) molecular weight cutoff (MWCO) Slide-A-Lyzer dialysis cassette (Thermo Scientific). During dialysis, a significant amount of protein precipitate formed in both cassettes, and care was taken to only remove the remaining soluble protein from the cassette for further analysis. A denaturing SDS-PAGE gel showing the relative purities of the pre- and post-dialysis protein samples is shown in **Figure 2A**. A gel showing the relative purity of the soluble fraction of **pool 1** at 5 μg, 10 μg and 20 μg of total protein is shown in **Figure 2B**. Because of the high purity of soluble **pool 1** protein, 200 μL aliquots were made and supplemented with 5% glycerol, flash-frozen in liquid nitrogen, and stored at -80°C for future use in cryo-EM studies. The remainder of the protein pools were subjected to additional purifications steps that are beyond the scope of this TEP.

Preparation of cryo-EM grids containing wild-type PLCG2. WT PLCG2 protein corresponding to **pool 1** in **Figure 2** was thawed on ice. This protein sample was kept in the following buffer for freezing: 50 mM HEPES, pH 7.5, 25 mM NaCl, 2 mM DTT, 5% glycerol. Absorbance at 280 nm was measured using a ClarioStar (BMG Labtech) plate reader and, in conjunction with a calculated extinction coefficient (**Table 1**), was used to determine the protein concentration of the sample. The calculated concentration of the protein stock was 0.80 mg mL^{-1}, and no further dilutions were made to this sample prior to its application on the cryo-EM grids. 3.5 μL of 0.8 mg mL^{-1} WT PLCG2 protein was applied to Quantifoil® 1.2/1.3 400 mesh copper grids (Electron Microscopy Sciences) that had been glow discharged for one minute at 25 mA. Grids were prepared using a Mark IV Vitrobot (Thermo Fisher Scientific) using blotting parameters listed in **Table 2** and were subsequently plunged into liquid ethane for sample vitrification.

These grids were stored in liquid nitrogen until data collection on the Titan Krios G4 electron microscope could be performed. An example of 2D classes that were picked from these grids using the "blob picker" function in cryoSPARC[3] is shown in **Figure 3**. As was previously stated, a subsequent TEP will elaborate on the data processing of these cryo-EM data.

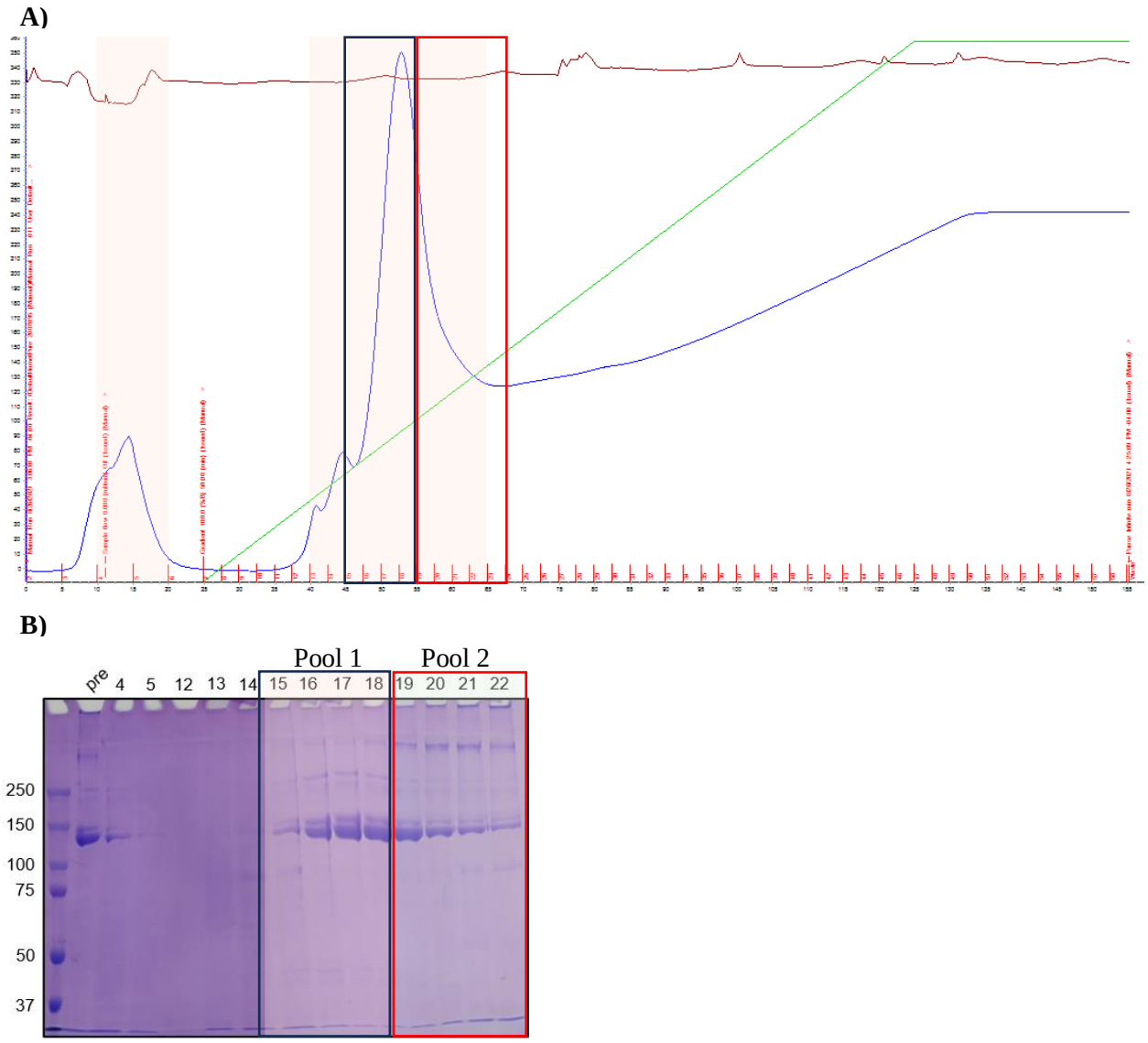


Figure 1. Immobilized Metal-Ion Affinity Chromatography (IMAC) purification using nickel-complexed resin. A) Chromatogram showing the absorbance at 280 nM (A280) in blue, conductivity (brown) and percent Buffer B (green) of the protein sample loaded onto this column. The X axis is in mL and the Y axis shows A280. **B)** Denaturing 7.5% SDS-PAGE gel analysis of the protein peaks that eluted off the IMAC resin. Each lane on the gel was loaded with approximately 3 µg of total protein as determined by a Bradford assay. Fractions outlined in a black box correspond to **pool 1**, and fractions outlined in a red box correspond to **pool 2**. Molecular weight markers show the representative band sizes in kilodaltons (kDa).

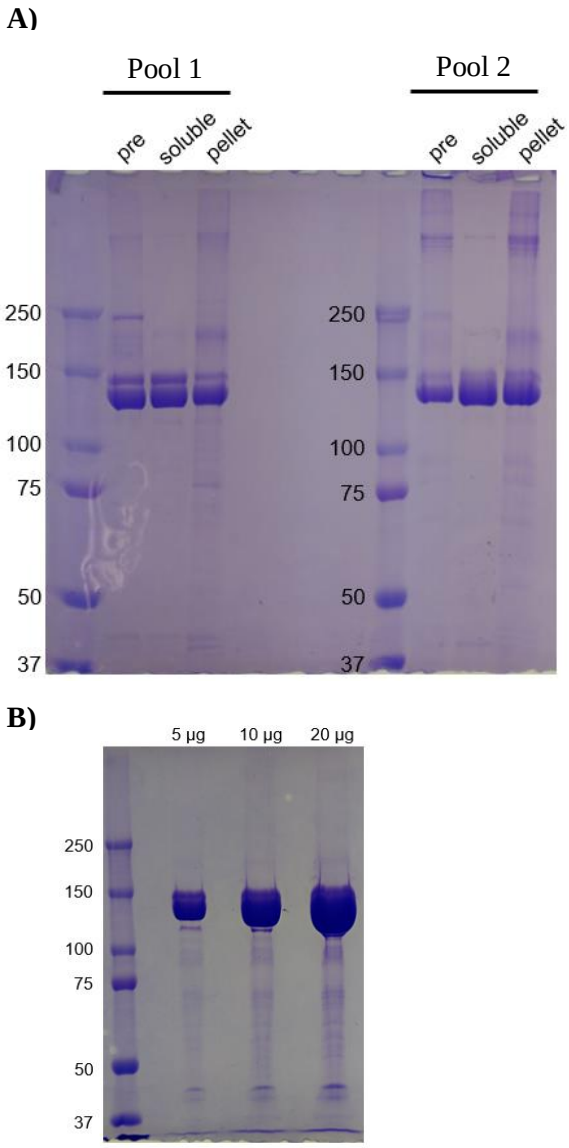


Figure 2. **SDS-PAGE analysis of pre- and post-dialysis WT PLCG2 protein samples. A)** Protein samples from both **pool 1** and **pool 2** were run on a denaturing 7.5% SDS-PAGE. The pre-dialysis ("pre") sample represents the sample that eluted off the HisTrap column in a high-salt buffer, whereas the post-dialysis sample refers to the soluble portion of the sample after dialysis into a low-salt buffer ("soluble"). The precipitate was also recovered from the dialysis cassette, resuspended in dialysis buffer, and run on the gel ("pellet"). All samples shown on the gel contain approximately 3 µg protein as determined by Bradford assay. Molecular weight markers show the representative band sizes in kilodaltons (KDa). B) Either 5 µg, 10 µg, or 20 µg of total protein from the soluble **pool 1** WT PLCG2 protein is run on a denaturing 7.5% SDS-PAGE to demonstrate the high purity of the sample. Molecular weight markers show the representative band sizes in KDa.

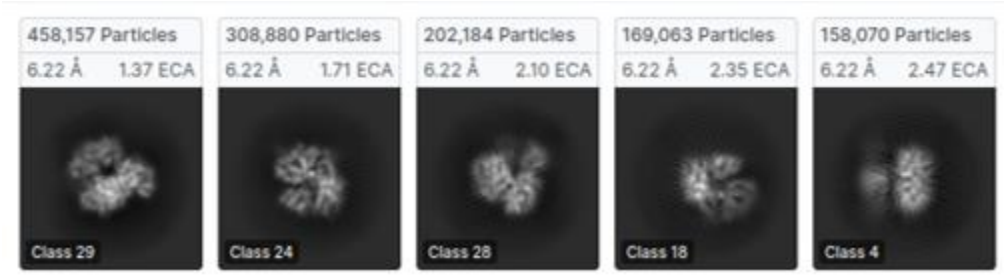


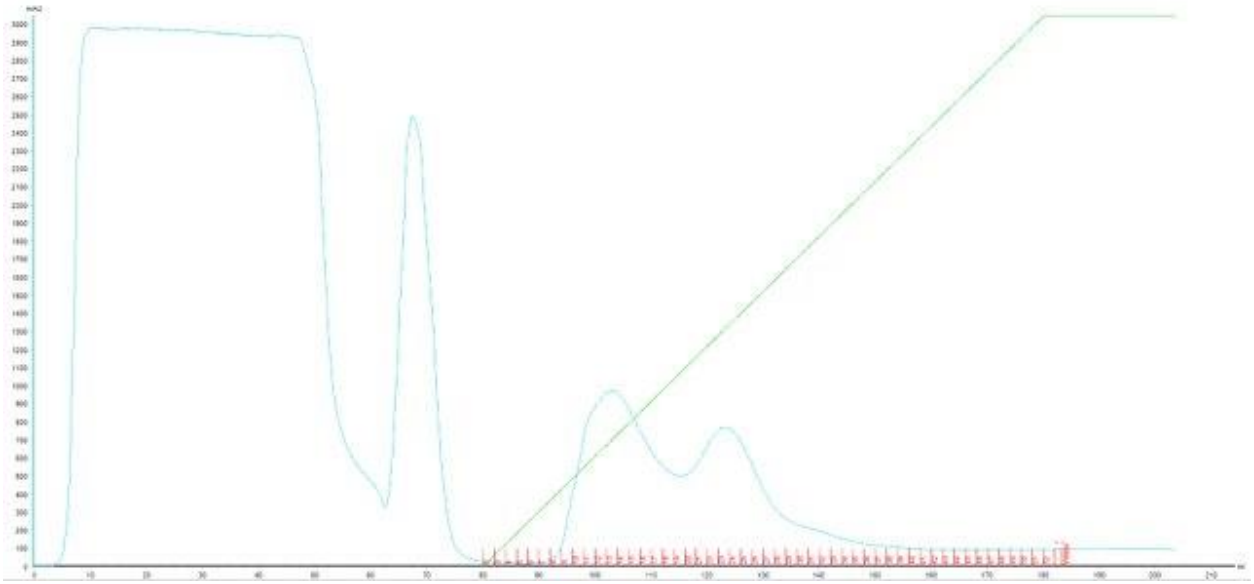
Figure 3. Representative 2D classes for wild-type PLCG2 protein from prepared grids. 5 representative 2D classes extracted from micrographs taken of grids frozen with wild-type human PLCG2 protein. 2D classes are generated using the "blob picker" tool in cryoSPARC.

PLCG2 Genscript P2 Viral Stocks and Expression Genscript (Piscataway, NJ) generated the P2 baculovirus viral stocks for the PLCG2 construct. Expression of PLCG2 was performed in 2L Erlenmeyer flasks containing 500 mL of 2 x 10^6 SF9 cells/mL (viability >95%). The volume of P2 viral stock was adjusted so that all cultures were infected with enough virus to equal 0.1 multiplicity of infection (M.O.I.). Viral infection occurred over 72 hours in a floor shaker set at 27C and shaking at 120 rpm. Cell count, morphology, and viability were checked to verify infection succeeded. Cultures were transferred to 1L centrifuge bottles and centrifuged at 700 x g for 30 minutes. Cell pellets were resuspended in 100 mL Buffer A (table 1) and stored in the -80C freezer for future use. A typical yield of 14.5 mg of PLCG2 protein can be obtained from 1L of 2 x 10^6 SF9 cells/mL infected culture.

PLCG2 Clarified Lysate Cell solutions were thawed at room temperature and shaken until a slushy consistency was obtained. A clarified cell lysate was obtained by centrifuging the cell solution for 30 minutes at 25,000 rpm and 4C. Samples were passed through a 0.45 μm filter before proceeding with immobilized metal affinity chromatography (IMAC). One protease inhibitor cocktail tablet (cOmplete Mini from Sigma, dissolved in buffer A) was added to the clarified lysate to prevent proteolytic degradation.

IMAC FPLC Purification Clarified cell lysate was run on an AKTA pure FPLC attached to a 5 mL nickel HisTrap HP column (Cytiva). After the lysate was fully loaded, the column was rinsed with 20 mL of buffer A (20 mM Hepes pH 7.4, 300 mM NaCl, 10% glycerol) . PLCG2 protein was eluted using 0-100% linear gradient of buffer B (20 mM Hepes pH 7.4, 300 mM NaCl, 10% glycerol, 250 mM imidazole) over 100 mL with PLCG2 eluting around 35% buffer B (figure 4). Fractions 19-27 have the highest ratio of PLCG2 to contaminating proteins, so these fractions were pooled for further purification. Protein concentration was determined by Bradford and purity was assessed by SDS-PAGE.

4A.



4B.

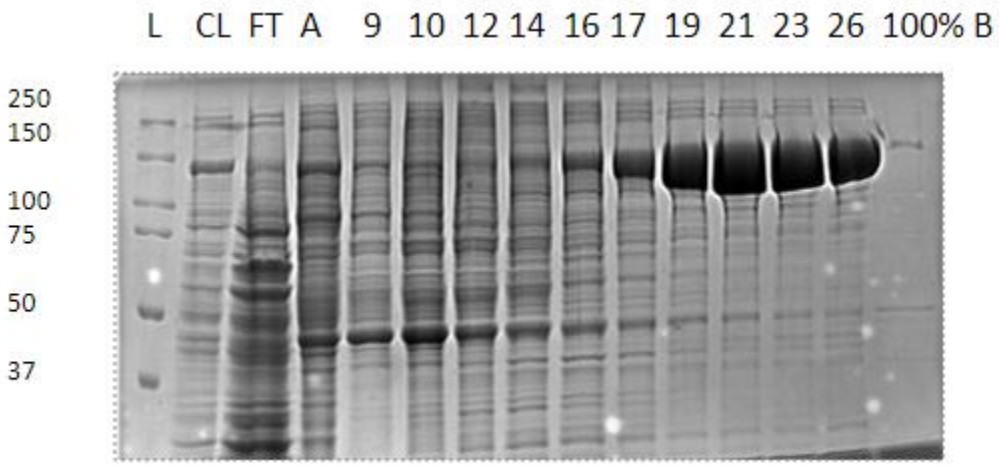
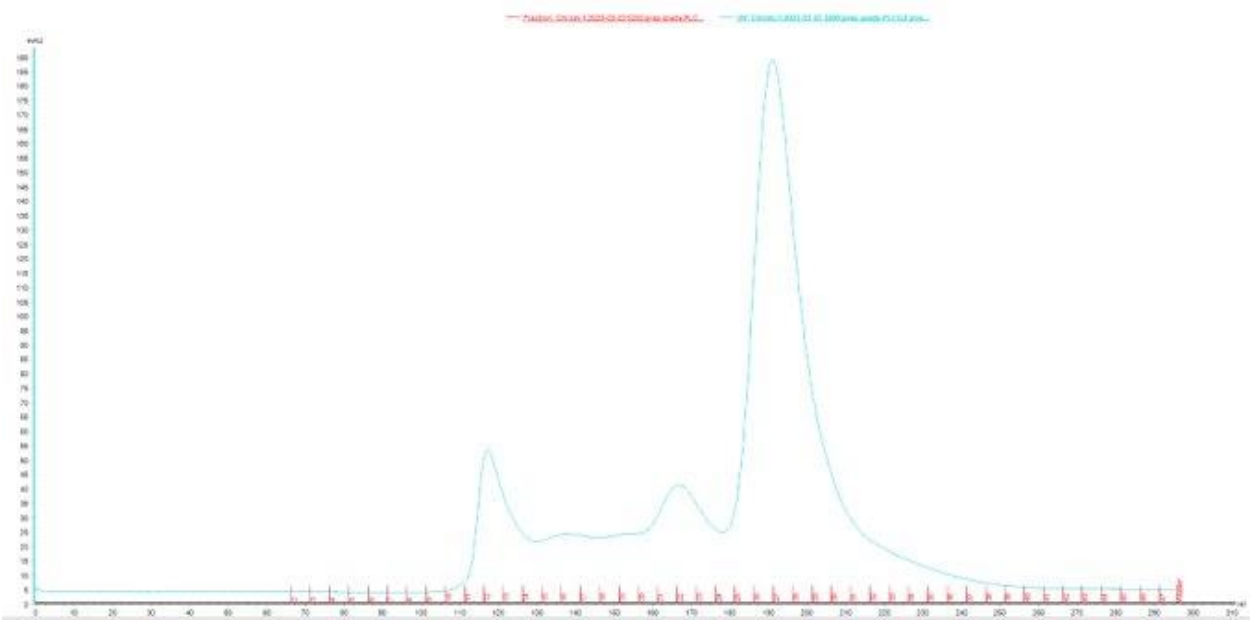


Figure 4. IMAC purification of PLCG2 clarified lysate
A. Chromatogram of IMAC with clarified lysate containing overexpressed PLCG2. During the elution step, there are two major peaks. **B.** SDS-PAGE of protein molecular weight standard (L, weights labeled in kDa), clarified lysate (CL), flowthrough (FT), buffer A wash (A), and fractions 9, 10, 12, 14, 16, 17, 19, 21, 23, 26 and 100% buffer B column wash. A band corresponding to PLCG2 (142 kDa) is seen in several lanes between the 100 and 150 kDa molecular ladder standard.

Gel Filtration FPLC Purification Fraction 19-27 from IMAC was pooled and concentrated to 2 mL volume then loaded on a HiLoad 26/60 Superdex 200 prep grade column (Cytiva). 300 mL of buffer A was used to separate proteins, and PLCG2 fractions came off the column between 180-210 mL of buffer (figure 5). Fractions 21-29 were pooled, concentrated, frozen in liquid nitrogen, and stored at -80C for future use.

5A.



5B.

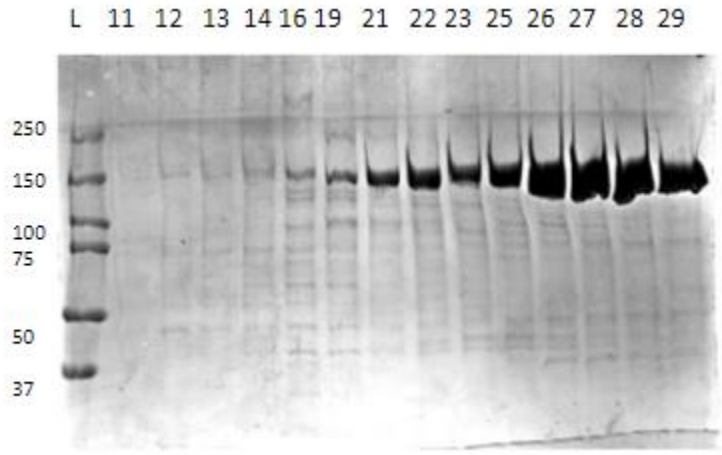
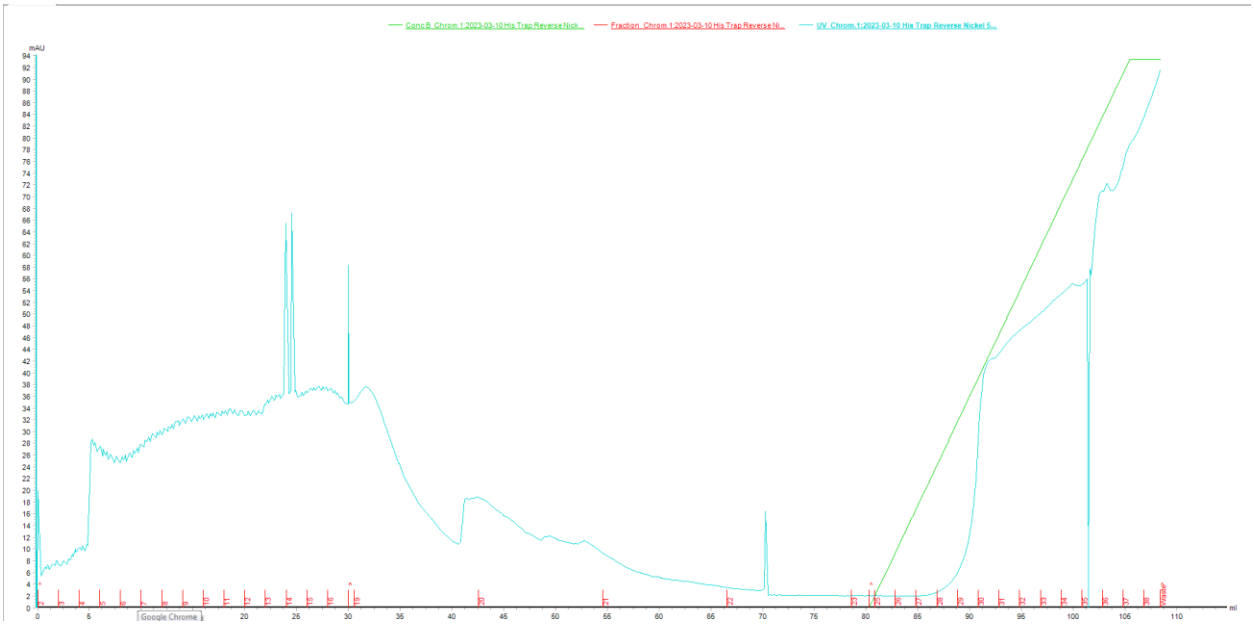


Figure 5. Purification of PLCG2 post IMAC using Gel Filtration Chromatography
A. Chromatogram of PLCG2 pooled IMAC fractions on a 300 mL prep grade sizing column. Three major peaks are present. **B.** SDS-PAGE of gel filtration purification samples (left to right)

protein molecular weight standard (L), fractions 11, 12, 14, 16, 19, 21, 22, 23, 25, 26, 27, 28, 29. 95% pure PLCG2 was found in fractions 21-29, and these were pooled, concentrated, and stored at -80C for further use.

Reverse Nickel Chromatography (optional) Purified PLCG2 up to this point still has a 6x-his tag, and if this tag is to be removed, add 0.5-1.0 mg of TEV protease to the PLCG2 solution and allow the reaction to occur overnight at 4C. The solution was loaded on an AKTA pure FPLC with a 5 mL nickel HisTrap HP column. Protein that is not his-tagged elutes in the flowthrough, but to assess cleavage efficiency, a gradient of buffer B from 0-100% was run over the nickel column (Figure 6). Based on SDS-PAGE, high efficiency of his tag cleavage occurred with 95%+ pure PLCG2. The flowthrough fractions were pooled, concentrated, frozen in liquid nitrogen, and stored at -80C for future use.

6A.



6B.

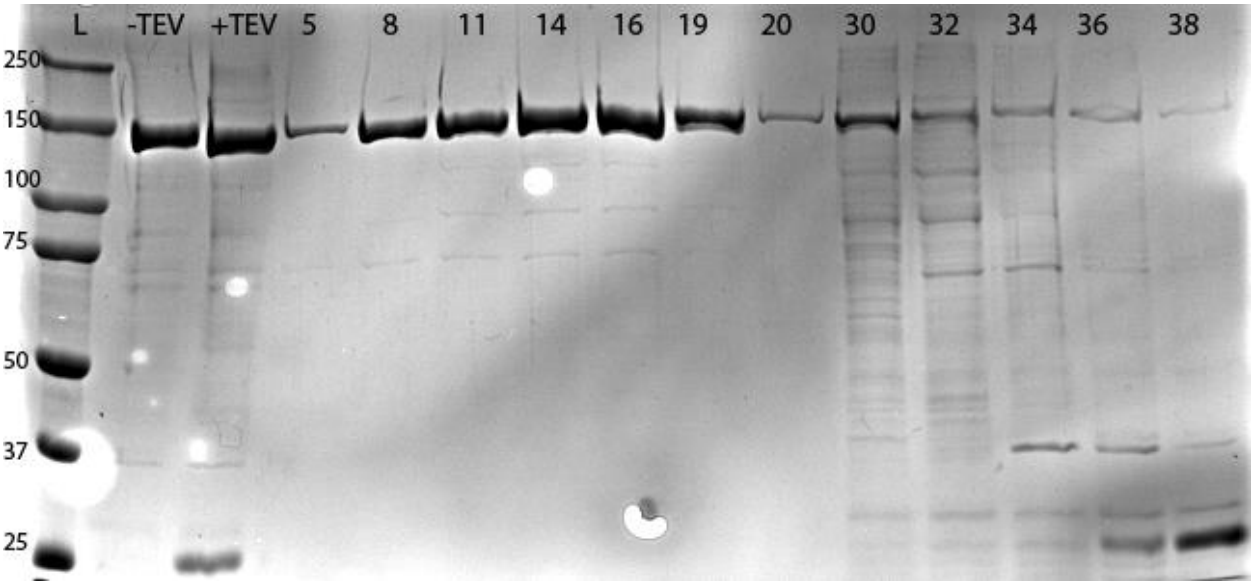


Figure 6. Reverse IMAC purification of PLCG2 with no 6x-his tag
A. Chromatogram of typical reverse nickel purification of PLCG2 without the 6x-his tag. **B.** SDS-PAGE of reverse nickel purification with samples left to right: protein standards ladder (L), PLCG2 before the addition of TEV (-TEV), PLCG2 after overnight TEV treatment before IMAC (+TEV), fraction 5, 8, 11, 14, 16, 19, 20, 30, 32, 34, 36, and 38. Fractions 5-16 are part of the column flowthrough and 19 is part of a column wash with buffer A.

Table 1. Amino acid sequence of human PLCG2 protein construct and calculated extinction coefficients.

Protein Construct	Amino Acid Sequence	Calculated Extinction Coefficients[1]
Wild-type human PLCG2	MHHHHHHGVDLGTENLYFQSNQAMSTTVNVDS LAEYEKSQIKRALELGTVMTVFSFRKSTPERRTV QVIMETRQVAWSKTADKIEGFLDIMEIKEIRPGK NSKDFERAKAVRQKEDCCFTILYGTQFVLSTLSL AADSKEDAVNWLSGLKILHQEAMNASTPTIIESW LRKQIYSVDQTRRNSISLRELKTILPLINFKVSSAK FLKDKFVEIGAHKDELSFEQFHLFYKKLMFEQQK SILDEFKKDSSVFILGNTDRPDASAVYLHDFQRFL IHEQQEHWAQDLNKVRERMTKFIDDTMRETAEP FLFVDEFLTYLFSRENSIWDEKYDAVDMQDMNN PLSHYWISSSHNTYLTGDQLRSESSPEAYIRCLRM GCRCIELDCWDGPDGKPVIYHGWTRTTKIKFDD VVQAIKDHAFVTSSFPVILSIEEHCSVEQQRHMA KAFKEVFGDLLLTKPTEASADQLPSPSQLREKIIIK HKKLGPRGDVDVNMEDKKDEHKQQGELYMWD SIDQKWTRHYCAIADAKLSFSDDIEQTMEEEVPQ DIPPTELHFGEKWFHKKVEKRTSAEKLLQEYCME TGGKDGTFLVRESETFPNDYTLSFWRSGRVQHC RIRSTMEGGTLKYYLTDNLTFSSIYALIQHYRETH LRCAEFELRLTDPVPNPNPHESKPWYYDSLSRGE AEDMLMRIPRDGAFLIRKREGSDSYAITFRARGK VKHCRINRDGRHFVLGTSAYFESLVELVSYYEKH SLYRKMRLRYPVTPELLERYNMERDINSLYDVSR MYVDPSEINPSMPQRTVKALYDYKAKRSDELSF CRGALIHNVSKEPGGWWKGDYGTRIQQYFPSNY VEDISTADFEELEKQIIEDNPLGSLCRGILDLNTYN VVKAPQGKNQKSFVFILEPKQQGDPPVEFATDRV EELFEWFQSIREITWKIDTKENNMKYWEKNQSIA IELSDLVVYCKPTSKTKDNLENPDFREIRSFVETK ADSIIRQKPVDLLKYNQKGLTRVYPKGQRVDSSN YDPFRLWLCGSQMVALNFQTADKYMQMNHALF SLNGRTGYVLQPESMRTEKYDPMPPESQRKILMT LTVKVLGARHLPKLGRSIACPFVEVEICGAEYDN NKFKTTVVNDNGLSPIWAPTQEKVTFEIYDPNLA FLRFVVYEEDMFSDPNFLAHATYPIKAVKSGFRS VPLKNGYSEDIELASLLVFCEMRPVLESEEE	Molar extinction coefficient = 184500 M^{-1} cm^{-1} g/L extinction coefficient = 1.300

[1]The extinction coefficients were calculated using the ProtParam tool available through the online server EXPASY (https://web.expasy.org/protparam/) and assumes all cysteines are reduced.

Table 2. Blotting parameters for cryo-EM grid preparation.

Temperature	4°C
Humidity	100%
Blot time	4 seconds
Wait time	5 seconds
Drain time	0 seconds
Blot force	2
Blot total	1

References:

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2. Sims, R., van der Lee, S., Naj, A. et al. Rare coding variants in PLCG2, ABI3, and TREM2 implicate microglial-mediated innate immunity in Alzheimer's disease. Nat Genet 49, 1373–1384 (2017). https://doi.org/10.1038/ng.3916
3. Punjani, A., Rubinstein, J.L., Fleet, D.J & Brubaker, M.A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. Nature Methods 14, 290-296 (2017).