Journal of Visualized Experiments

Cellular membrane affinity chromatography columns to identify specialized plant metabolites interacting with immobilized tropomyosin kinase receptor B

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63118R1
Full Title:	Cellular membrane affinity chromatography columns to identify specialized plant metabolites interacting with immobilized tropomyosin kinase receptor B
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
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TITLE:

Cellular Membrane Affinity Chromatography Columns to Identify Specialized Plant Metabolites Interacting with Immobilized Tropomyosin Kinase Receptor B

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SUMMARY:

The protocol describes the preparation of cell membrane affinity chromatography (CMAC) columns with immobilized cell membrane fragments containing functional transmembrane tropomyosin kinase receptor B proteins. The use of CMAC columns in the identification of specialized plant metabolites interacting with these receptors and present in complex natural mixtures is also explained.

ABSTRACT:

Chemicals synthesized by plants, fungi, bacteria, and marine invertebrates have been a rich source of new drug hits and leads. Medicines such as statins, penicillin, paclitaxel, rapamycin, or artemisinin, commonly used in medical practice, have been first identified and isolated from natural products. However, the identification and isolation of biologically active specialized metabolites from natural sources is a challenging and time-consuming process. Traditionally, individual metabolites are isolated and purified from complex mixtures, following the extraction of biomass. Subsequently, the isolated molecules are tested in functional assays to verify their biological activity. Here we present the use of cellular membrane affinity chromatography (CMAC) columns to identify biologically active compounds directly from complex mixtures. CMAC columns allow for the identification of compounds interacting with immobilized functional transmembrane proteins (TMPs) embedded in their native phospholipid bilayer environment. This is a targeted approach, which requires knowing the TMP whose activity one intends to modulate with the newly identified small molecule drug candidate. In this protocol, we present an approach to prepare CMAC columns with immobilized tropomyosin kinase receptor B (TrkB), which has emerged as a viable target for drug discovery for numerous nervous system disorders.

In this article, we provide a detailed protocol to assemble the CMAC column with immobilized TrkB receptors using neuroblastoma cell lines overexpressing TrkB receptors. We further present the approach to investigate the functionality of the column and its use in the identification of specialized plant metabolites interacting with TrkB receptors.

INTRODUCTION:

Botanical mixtures are rich in pharmacologically active compounds[1], serving as a good source for the identification of new drug hits and leads[2–5]. The discovery of new medicines from natural products has been a fruitful approach and many currently approved drugs originated from compounds first identified in nature. The chemical diversity of natural compounds is hard to be matched by man-made libraries of chemically synthesized molecules. Many natural compounds interact with and modulate human protein targets and can be considered evolutionarily optimized drug-like molecules[6]. These natural compounds are particularly well suited for drug lead identification to use in neurological disorders[6]. Two of the currently FDA-approved drugs for the management of Alzheimer's disease (AD) are derived from natural alkaloids, namely: galantamine and rivastigmine (a derivative of physostigmine)[6]. L-DOPA, presently the most commonly prescribed drug for Parkinson's disease, was first identified from the broad bean (*Vicia faba* L.)[7]. Pergolide and lisuride, dopaminergic receptor agonists are the derivatives of natural ergot alkaloids from the parasitic fungus *Claviceps purpurea*[8]. Reserpine, an alkaloid isolated from Indian snakeroot (*Rauvolfia serpentina* (L.) Benth. ex Kurz) was one of the first antipsychotic drugs[9]. Recently, dysregulated immune response and systemic inflammation have been linked to the development of numerous neurological ailments, such as major depressive disorder or neurodegenerative diseases[10]. A plant-based diet together with other lifestyle interventions has been found to improve cognitive and functional abilities in the elderly[11–21]. Certain electrophilic molecules belonging to triterpenes and polyphenols have been found to modulate inflammatory responses in both *in vitro* and *in vivo* models[12]. For instance, natural compounds containing α,β-unsaturated carbonyl (e.g., curcumin, cinnamaldehyde), or isothiocyanate group (e.g., sulforaphane) interfere with Toll-like receptor-4 (TLR4) dimerization inhibiting the downstream synthesis of pro-inflammatory cytokines in a murine interleukin-3 dependent pro-B cell line[12,22]. Epidemiological evidence points strongly that dietary phytochemicals, present in complex food matrices, may also constitute a viable source of new drug leads[6].

One of the major obstacles in the identification of biologically active molecules present in plant extracts, including plant-based food, is the complexity of the investigated samples. Traditionally, the individual compounds are isolated, purified, and subsequently tested for biological activity. This approach usually leads to the identification of the most abundant and well-characterized compounds. Phenotypic drug discovery approaches without a defined molecular target rely on the bio-guided-fractionation of complex mixtures[23]. In this approach, an extract is fractionated into less complex sub-fractions that are subsequently tested in phenotypic assays. The isolation and purification of active compounds are guided by biological activity verified in the assay. The knowledge of the identity of a specified drug target may significantly speed up the identification of pharmacologically active compounds present in complex mixtures. Those approaches are usually based on the immobilization of the molecular target, for example, an enzyme, on a solid surface, like magnetic beads[23]. The immobilized targets are subsequently used in the screening

experiments resulting in the isolation of compounds interacting with the target. While this approach has been extensively used in the identification of compounds targeting cytosolic proteins, it has been less commonly applied in the identification of chemicals interacting with TMPs[23]. An additional challenge in the immobilization of TMPs stems from the fact that the activity of the protein depends on its interaction with cell membrane phospholipids and other molecules in the bilayer such as cholesterol[23,24]. It is important to preserve these subtle interactions between proteins and their native phospholipid bilayer environment when attempting to immobilize the transmembrane target.

In cellular membrane affinity chromatography (CMAC) cell membrane fragments, and not purified proteins, are immobilized on the artificial membrane (IAM) stationary phase particles[23]. IAM stationary phases are prepared by covalently bonding phosphatidylcholine analogs onto silica. Recently novel classes of IAM stationary phases have been developed in which free amine and silanol groups are end-capped (IAM.PC.DD2 particles). During CMAC columns preparation cell membrane fragments are immobilized onto the surface of IAM particles through adsorption. CMAC columns have been used to date to immobilize different classes of TMPs including ion channels (e.g., nicotinic receptors), GPCRs (e.g., opioid receptors), protein transporters (e.g., p-glycoprotein), etc.[24]. The immobilized protein targets have been used in the characterization of pharmacodynamics (e.g., dissociation constant, Kd) or determining binding kinetics (k_{on} and k_{off}) of small molecule ligands interacting with the target as well as in the process of identification of potential new drug leads present in complex matrices[24]. Here we present the preparation of CMAC columns with the immobilized tropomyosin kinase receptor B (TrkB), which has emerged as a viable target for drug discovery for numerous nervous system disorders.

Previous studies showed that the activation of the brain-derived neurotrophic factor (BDNF)/TrkB pathway is associated with the improvement of certain neurological ailments, such as AD or major depressive disorder[25–28]. It was reported that BDNF levels and its receptor TrkB expression decrease in AD, and similar reductions impair hippocampal function in animal models of AD[29]. Decreased levels of BDNF were reported in serum and brain of AD patients[30–32]. Tau overexpression or hyperphosphorylation were found to down-regulate BDNF expression in primary neurons and AD animal models[33–35]. Additionally, BDNF was reported to have protective effects on β-amyloid induced neurotoxicity *in vitro* and *in vivo*[36]. Direct BDNF administration into the rat brain was shown to increase learning and memory in cognitively impaired animals[37]. BDNF/TrkB emerged as a valid target for ameliorating neurological and psychiatric disorders including AD[28,38]. Targeting the BDNF/TrkB signaling pathway for the development of therapies in AD will potentially enhance our understanding of the disease[39]. Unfortunately, BDNF itself cannot be used as a treatment because of its poor pharmacokinetic properties and adverse side effects[40]. Small molecule activators of TrkB/BDNF pathways have been explored as potential TrkB ligands[41–43]. Among tested small molecule agonists, 7,8-dihydroxyflavone (7,8-DHF), has been shown to activate the BDNF/TrkB pathway[41,44–46]. A derivative of 7,8-DHF (R13; 4-Oxo-2-phenyl-4H-chromene-7,8-diyl bis(methylcarbamate)) is currently under consideration as a possible drug for AD[47]. Recently, it was shown that several antidepressants work through directly binding to TrkB and promoting BDNF signaling, further stressing the importance of pursuing TrkB as a valid target to treat various neurological disorders[48].

The protocol describes the process of assembling functional TrkB column and TrkB-NULL negative control column. The columns are characterized using a known natural product small-molecular ligand: 7,8-DHF. Additionally, we describe the process of screening complex matrices, using plant extract as an example, for the identification of compounds interacting with TrkB.

PROTOCOL:

1. Cell culture of SH-SY5Y neuroblastoma cells (TrkB and TrkB-NULL (parental) cell lines)

NOTE: Cell lines (SH-SY5Y Cell Line (TrkB, BR6) and SH-SY5Y Parental Cell Line (Trk NULL))[49,50] were purchased from Kerafast. Cultured cells are used as a source of the transmembrane receptors to be immobilized for the preparation of CMAC columns. The following steps describe how to obtain cell membrane fragments and assemble functional CMAC columns.

1.1. Grow the cells in a cell culture medium prepared by mixing 450 mL of RPMI media, 50 mL of FBS, 5 mL of penicillin/streptomycin solution, and 0.3 mg/mL of geneticin (G418) in a 150 mm cell culture dish at 37 °C in a humid atmosphere with 5% CO_2.

2. Cell harvesting

2.1. Confirm cell confluency (80%-90%) using a microscope, reached after 3–4 days after cell passaging.

2.2. Aspirate cell culture medium from above the cells and wash the cells twice with phosphate-buffered saline (PBS, 1x, pH 7.4). Remove PBS and add 2 mL of low concentrated trypsin (0.25%) to detach cells.

2.3. Gently swirl the plate to evenly cover all the cells with trypsin solution and incubate the cells for approximately 2 min at 37 °C in a humid atmosphere with 5% CO_2. Add 8 mL of cell culture medium to detaching cells and transfer detached cells to a 50 mL conical tube and place it on ice.

2.4. Use a hemocytometer to estimate the number of cells (approximately 3 x 10^7 cells). Mix cell suspension by pipetting up and down to obtain even cell density in the mixture. Place 10 µL of cell suspension under the coverslip and count the cells under a microscope using a 40x objective.

3. Cell homogenization

3.1. Prepare stock solutions of the following protease inhibitors: benzamidine (200 mM), phenylmethylsulfonyl fluoride (PMSF, 10 mM), and commercially available protease inhibitors cocktail (100x concentration) containing 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, and leupeptin.

CAUTION: PMSF should only be handled inside the fume hood.

3.1.1. Prepare benzamidine stock solution (200 mM) by dissolving 120 mg of benzamidine in 5 mL of ultrapure deionized water. Store at 4 °C and use within a day. Freshly prepare solution before each use (recommended).

3.1.2. Prepare PMSF stock solution (10 mM) by dissolving 0.017 g of PMSF in 10 mL of ethanol. Store at - 20 °C.

3.1.3. Prepare protease inhibitor cocktail (100x) by dissolving commercially available protease inhibitor mixture in 1 mL of ultrapure deionized water. Mix thoroughly and aliquot 200–300 µL of the cocktail and store at - 20 °C before use.

3.2. Prepare ATP stock solution (100 mM) by dissolving 55.114 mg of ATP disodium salt hydrate in 1 mL of deionized ultrapure water. Mix thoroughly and aliquot 100 µL of the mixture and store at - 20 °C before use.

3.3. Prepare buffer by dissolving 3.03 g of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 50 mM), 2.9 g of sodium chloride (NaCl, 100 mM), 0.22 g of calcium chloride anhydrous ($CaCl_2$, 3 mM), 0.2 g of magnesium chloride hexahydrate ($MgCl_2$, 2 mM) and 0.19 of potassium chloride (KCl, 5 mM) in 500 mL of ultrapure deionized water.

3.4. Prepare homogenization buffer by mixing 17.3 mL of the buffer prepared in step 3.3 with 0.3 mL (3 mM) of benzamidine stock solution, 0.2 mL (0.1 mM) of PMSF stock solution, 0.2 mL of protease inhibitor cocktail mixture, 20 µL (100 µM) of ATP stock solution, 2 mL (10%) of glycerol, and 0.029 g (5mM) of EDTA (**Table 1**). Adjust pH to 7.4 using hydrochloric acid solution.

3.5. Spin down the cells harvested in step 2.3 at 4 °C for 5 min at 400 x *g*. Remove the supernatant and wash the remaining cell pellet with 10 mL of ice-cold PBS (1x, pH 7.4). Spin down the cells again at 4 °C for 5 min at 400 x *g*.

3.6. Discard the supernatant and replace it with 20 mL of homogenization buffer prepared in step 3.4. Place the conical tube on ice.

3.7. Transfer the cell suspension into a 40 mL Dounce homogenizer tissue grinder and place it on ice. Homogenize the suspension manually, on ice, using 40 up-and-down pestle strokes. Transfer homogenized cell suspension into a 50 mL conical tube.

3.8. Centrifuge the homogenate at 4 °C for 7 min at 400 x *g*. Discard the pellet and transfer the supernatant to a new conical tube and centrifuge it at 4 °C for 30 min at 47900 x *g*. Save the cell membrane pellet and discard the supernatant.

4. Cell membrane solubilization

4.1. Prepare solubilization buffer by mixing 8.7 mL of the buffer solution made in step 3.3 with 0.1 mL (0.1 mM) of PMSF stock solution, 0.15 mL (3 mM) of benzamidine stock solution, 0.1 mL

of protease inhibitor cocktail, 10 µL (100 µM) of ATP stock solution, 1 mL (10%) of glycerol and 0.2 g (2%) of sodium cholate (**Table 2**).

4.2. Transfer the solubilization buffer into the conical tube with cell membrane fragments obtained in step 3.8 and resuspend the pellet. Rotate the resulting mixture at 150 rpm at 4 °C for 18 h.

NOTE: At this point, the experiment can be paused for overnight cell membrane pellet solubilization.

5. Cell membrane immobilization on IAM.PC.DD2 particles

5.1. After 18 h, centrifuge the solubilization mixture at 4 °C for 30 min at 47900 x *g*.

5.2. Keep the supernatant and discard the pellet. Add 100 mg of IAM.PC.DD2 particles, to the supernatant, vortex and rotate the resulting suspension mixture at 150 rpm at 4 °C for 1 h.

5.3. To facilitate the immobilization of cell membrane fragments on the IAM.PC.DD2 particles proceed to the dialysis step as outlined below.

5.3.1. Prepare dialysis buffer in 4 L of ultrapure deionized water by adding 24 g of tris-HCl (50 mM), 23.4 g of NaCl (100 mM), 0.06 g of $CaCl_2$ (0.1 mM), 5.85 g of EDTA (5 mM), and 0.07 g of PMSF (0.1 mM; **Table 3**).

NOTE: PMSF is not soluble in water, therefore dissolve it first in small volume of ethanol and then add slowly into the beaker with the buffer.

5.3.2. Adjust pH to 7.4 with hydrochloric acid solution. Place the buffer at 4 °C for a minimum of 1 h before proceeding to the dialysis step.

5.3.3. Prepare dialysis tube by cutting 10 cm of cellulose membrane dialysis tubing (10 K MWCO, 35 mm) and transfer the suspension containing IAM.PC.DD2 particles and cell membrane fragments into the dialysis tube. Use dialysis tubing clips to close both ends of the dialysis tube.

5.3.4. Place the dialysis tube containing the IAM.PC.DD2 stationary phase in the dialysis buffer and dialyze at 4 °C for 24 h while gently stirring.

5.3.5. After 24 h, place the dialysis tubing in the freshly prepared dialysis buffer and continue dialysis for another 24 h.

6. CMAC column packing

6.1. Prepare ammonium acetate buffer (10 mM, pH 7.4) that will be used to wash the IAM.PC.DD2 particles and in column characterization experiments by dissolving 0.7708 g of ammonium

acetate in 1 L of ultrapure deionized water. Adjust pH to 7.4 with hydrochloric acid solution.

6.2. After 48 h of dialysis, remove the dialysis tube from the dialysis buffer and transfer the content of the dialysis tube into a 15 mL conical tube.

6.3. Centrifuge the mixture at 4 °C for 5 min at 400 x *g*. Discard the supernatant and wash the remaining pellet three times with 10 mL of ammonium acetate buffer, centrifuging the mixture at 4 °C for 5 min at 400 x *g*, after each wash.

6.4. After the third wash, resuspend the remaining pellet in 1 mL of ammonium acetate buffer. Mix it thoroughly and use the resulting slurry to pack the 5/20 glass column to yield CMAC chromatography column.

6.5. To pack the column, first place a bottom filter previously soaked with ammonium acetate buffer, into the filter holder. Fit the filter holder in the glass column and screw the column cap to secure the position of the holder. Place the column vertically in a finger clamp and secure it in the lab stand. Place a beaker below the column.

6.6. Using a single channel pipettor transfer a small volume of the slurry obtained in step 6.4 into the glass column. Pour the material slowly, holding the pipette tip against the glass column wall. Allow the packing material to settle before pouring another volume of slurry.

6.7. To speed up the packing process remove the buffer from above the stationary bed using a micropipette between each step. Repeat these steps until 1 mL of the slurry is packed.

6.8. Place a top filter and screw the adapter unit so that there is no remaining buffer above the stationary phase, as presented in **Figure 1**. Secure the position of the adapter unit with the adapter lock.

6.9. Connect the column to a high-performance liquid chromatography (HPLC) pump, set the flow rate to 0.2 mL/min, and wash the column overnight with ammonium acetate buffer. The column is now ready for the characterization step. Store the column at 4 °C until use.

NOTE: For longer storage (column not used for longer than a week) run the column with 0.05% sodium azide solution in ammonium acetate buffer and store at 4 °C.

CAUTION: Sodium azide is highly toxic when ingested orally or absorbed through the skin; it should only be handled under the fume hood. Make sure to wear a lab coat, safety glasses, and gloves (nitrile preferred) when working with sodium azide.

7. CMAC column characterization

7.1. Confocal microscopy and BDNF binding

7.1.1. Prepare IAM.PCC.DD2 stationary phase particles with immobilized cell membrane fragments obtained separately from SH-SY5Y neuroblastoma cells overexpressing TrkB and SH-SY5Y TrkB-NULL cells by following the instructions from step 1 to step 6.4.

7.1.2. For samples that will be incubated with BDNF prior to antibody staining, prepare BDNF stock solution by dissolving 10 μg of BDNF in 100 μL of ultrapure deionized water. Store the solution at -20 °C.

7.1.2.1. Transfer in separate 1.5 mL microcentrifuge tubes, 100 μL aliquot of IAM.PC.DD2 column packing material with immobilized SH-SY5Y Neuroblastoma cells overexpressing TrkB and 100 μL aliquot of IAM.PC.DD2 column packing material with immobilized SH-SY5Y TrkB-NULL cells suspended in ammonium acetate buffer.

7.1.2.2. Add 390 μL of ammonium acetate buffer, 10 μL of BDNF stock solution, and 0.5 μL of ATP stock solution (prepared in step 3.2) into each tube and incubate for 1 h at room temperature with rocking.

7.1.3. For samples that will not be incubated with BDNF prior to antibody staining, transfer 100 μL aliquot of IAM.PC.DD2 column packing material with immobilized SH-SY5Y Neuroblastoma cells overexpressing TrkB suspended in ammonium acetate into 1.5 mL microcentrifuge tubes.

7.1.4. Add 400 μL of ammonium acetate buffer and 0.5 μL of ATP stock solution (prepared in step 3.2) into each tube and incubate for 1 h at room temperature with rocking.

7.1.5. Spin the samples prepared in steps 7.1.2 and 7.1.4 at 4 °C for 1 min at 10,000 x *g* and discard the supernatant.

7.1.6. Wash the resulting pellets with 500 μL of ammonium acetate buffer for 10 min and spin again at 4 °C for 1 min at 10,000 x *g* and discard the supernatant.

7.1.7. To each of the pellets, add 220 μL of ammonium acetate buffer, 25 μL of 10% normal goat serum, and 5 μL of primary anti-BDNF antibody. Incubate in a cold room (4 °C) overnight with rocking.

7.1.8. Upon completion of the incubation step spin the mixture for 1 min at 10,000 x *g* at 4 °C and discard the supernatant.

7.1.9. Wash the resulting pellet 3 times with ammonium acetate buffer containing 1% mild detergent (e.g., sodium cholate) for 10 min and spin the mixtures for 1 min at 10,000 x *g* at 4 °C and discard the supernatant.

7.1.10. Prepare secondary antibody solution by mixing 900 μL of ammonium acetate buffer, 100 μL of 10% normal goat serum, and 1 μL of fluorophore-conjugated secondary antibody (1:1,000

dilution). Add 300 μL of secondary antibody solution to each of the pellets obtained in step 7.1.9. and incubate overnight at 4 °C with rocking.

7.1.11.Spin the mixture at 10,000 x *g* for 1 min at 4 °C and discard the supernatant.

7.1.12. Wash the resulting pellet 3 times with ammonium acetate buffer containing 1% mild detergent (e.g., sodium cholate) for 10 min and spin the mixture for 1 min at 10,000 x *g* at 4 °C and discard the supernatant.

7.1.13. Resuspend the resulting pellets in 50 μL of ammonium acetate buffer. Place 20 μL of each of the mixtures on a slide and cover with a coverslip.

7.1.14. Image the IAM.PC.DD2 particles with the immobilized cell membrane fragments using a confocal laser scanning microscope with excitation at 488 nm and emission at 520 nm generated using a solid state laser system. Use a 20x objective with a NA of 0.75 and WD of 0.35mm.

7.2. Frontal affinity chromatography using 7,8-DHF as a marker ligand

7.2.1. Connect a CMAC column to an HPLC system with a diode-array detector and/or mass spectrometer.

7.2.2. Prepare 500 mL of 1 mM solution of 7,8-DHF in ammonium acetate buffer containing 5% methanol.

7.2.3. Pump constant concentration of the marker ligand (1 mM) through the CMAC column at 0.4 mL/min flow rate at room temperature. Monitor the elution profile using either a diode array detection (DAD) (wavelength 254 nm) detector or mass spectrometer (use single ion monitoring mode; m/z 253 in negative ionization mode).

7.2.4. After completion of the run perform overnight wash by running ammonium acetate buffer through the column.

7.2.5. Repeat steps 7.2.2. – 7.2.4. using different concentrations of the marker ligand (750 nM, 500 nM, 300 nM), washing the columns overnight after each run. Use frontal affinity chromatography to calculate ligand K_d, as reviewed in detail, elsewhere.[24,51]

7.3. Displacement studies with *Centella asiatica* (gotu kola) extract

7.3.1 Prepare 500 mL of 500 nM solution of 7,8-DHF in ammonium acetate buffer containing 5% methanol and 0.2% aqueous gotu kola extract (10 mg/mL).

7.3.2. Pump constant concentration of the marker ligand (500 nM) with the extract through the column at 0.4 mL/min flow rate at room temperature. Monitor the elution profile using either a DAD detector (wavelength 254 nm) or a mass spectrometer (use single ion monitoring mode;

m/z 253 in negative ionization mode).

7.3.3. After completion of the run perform overnight wash by running ammonium acetate buffer through the column.

7.3.4. Plot the elution profiles for 500 nM 7,8-DHF and the one obtained after running the solution with gotu kola extract to inspect for marker ligand displacement.

8. Missing peak chromatography approach to identify potential TrkB binders from gotu kola extract

8.1. Fractionation of gotu kola extract on CMAC columns

8.1.1. Connect separately the CMAC TrkB and TrkB-NULL columns to an HPLC system and inject 50 μL of the aqueous gotu kola extract (10 mg/mL) on each of the columns. Pump ammonium acetate buffer (10 mM, pH 7.4) containing 5% methanol through the column at 0.4 mL/min flow rate at room temperature.

8.1.2. Collect fractions separately from CMAC TrkB and TrkB-NULL columns as follows: 0–5 min, 5–10 min, 10–15 min, 15–20 min, 20–25 min, 25–30 min, 30–35 min, 35–40 min, 40–45 min, 45–50 min, 50–55 min, 55–60 min.

8.1.3. Freeze and lyophilize the obtained fractions. Resuspend lyophilized fractions in 50 μL of methanol before proceeding to ultra-performance liquid chromatography and mass spectrometry analysis.

8.2. Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) analysis of gotu kola CMAC fractions

8.2.1. Analyze all the fractions obtained in point 8.1.3. using a mass spectrometer coupled with a UPLC system (UPLC-MS^E analysis mode). Analyze the fractions using a C18 column (2.1 x 50 mm, 1.7 μm) with a C18 VanGuard pre-column (2.1 mm x 5 mm, 1.7 μm).

8.2.2. Elute the column using the following gradient solutions at a flow rate of 0.3 mL/min with mobile phase A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid): 0.0 min 99% A 1% B, 1.5 min 84% A 16% B, 5.0 min 80% A 20% B, 7.0 min 75% A 25% B, 10.0 min 65% A 35% B, 20.0-24.0 min 1% A 99% B, 25.0-29.0 min 99% A 1% B.

8.2.3. Set the injection volume for each sample to 3 μL. Perform MS^E in resolution positive and negative ion modes, with collision energy at 4V for low energy and 20–35 V for high energy.

8.2.4. Use the following ESI source conditions in positive ionization mode: capillary voltage 1.5 kV, sampling cone 40 V, source offset 80, source temperature 100 °C, desolvation temp. 350 °C, cone gas flow 38.0 L/h, desolvation gas 400 L/h.

8.2.5.Use the following ESI source conditions in negative ionization mode: capillary voltage 1.45 kV, sampling cone 40 V, source offset 80, source temperature 110 °C, desolvation temp. 300 °C, cone gas flow 50.0 L/h, desolvation gas flow 652 L/h.

REPRESENTATIVE RESULTS:

Following the protocol, two CMAC chromatographic columns were assembled: one with the immobilized SH-SY5Y neuroblastoma cell membrane fragments with overexpressed TrkB and one with SH-SY5Y TrkB-NULL cell membrane fragments. The correctly assembled CMAC column is presented in **Figure 1** and the steps involved in cell membrane fragment immobilization are presented in **Figure 2**.

Since the immobilization of TrkB receptors on IAM.PC.DD2 chromatographic stationary phase had not been attempted before, the successful immobilization of the receptors was confirmed by antibody staining and frontal affinity chromatography using a marker ligand: 7,8-DHF. A schematic representation of the antibody staining experiment is presented in **Figure 2**. Cell membrane fragments obtained from neuroblastoma cell line overexpressing TrkB receptors and cell membrane fragments from parental cell line without TrkB receptors (TrkB-NULL)) were immobilized on IAM.PC.DD2 particles using the optimized protocol. Subsequently, the particles with immobilized cell membrane fragments were incubated with BDNF (physiological ligand) and then with primary and fluorescent-labeled secondary antibodies (**Figure 2**). IAM.PC.DD2 particles with immobilized neuroblastoma TrkB cell membrane fragments and in the presence of BDNF resulted in fluorescently labeled particles (**Figure 3C**). No fluorescence (except for weak background fluorescence) was observed when TrkB-NULL cell membrane encapsulated IAM particles were investigated (**Figure 3A**) or when no BDNF was used in the case of TrkB cell membrane encapsulated IAM particles (**Figure 3B**).

To characterize the binding of a small marker ligand (7,8-DHF) to the immobilized TrkB receptors frontal affinity chromatography was performed with different concentrations of 7,8-DHF. A typical chromatogram of increasing 7,8-DHF concentrations on a functional CMAC column is presented in **Figure 4**. Specific binding of 7,8-DHF to the immobilized TrkB was confirmed by the concentration-dependent decreases in the retention time of the marker ligand. Frontal affinity chromatography results obtained on a non-functional CMAC column are presented in **Figure 5**. The lack of concentration-dependent changes in retention of the marker ligand indicates the unsuccessful attempt in CMAC preparation.

Compounds injected onto the CMAC column interact not only specifically but can also non-specifically interact with IAM.PC.DD2 particles, phospholipid bilayer of the immobilized cell membrane fragments, and other proteins present in the bilayer. To rule out compounds non-specifically interacting with the CMAC column during the process of screening of complex extracts for TrkB binders it is important to prepare CMAC negative control column by immobilizing cell membrane fragments of the parental cell line not expressing the targeted protein (in this case TrkB-NULL). Frontal affinity chromatography results obtained on a negative CMAC TrkB-NULL column are presented in **Figure 6**.

Functional CMAC columns can be used for different purposes, such as characterization of the targeted protein, studying interactions of individual compounds with the immobilized targets (e.g., obtaining dissociation constant, Kd) or determining binding kinetics (k_{on} and k_{off}) etc.[24]. The columns can also be used to screen complex samples, such as plant extracts for the presence of compounds binding to TrkB receptors, therefore potentially containing molecules that act as TrkB agonists or antagonists. To verify if an extract potentially contains compounds interacting with the immobilized receptors a simple displacement experiment is performed. The result of the competition experiment performed with gotu kola extract and 500 nM of 7,8-DHF on a functional TrkB column is presented in **Figure 7**. The addition of 0.2% aqueous gotu kola extract (10 mg/mL) resulted in a significant reduction of 7,8-DHF retention indicating the presence of competing ligand(s) for the agonist binding site. The result of the displacement experiment performed on the CMAC negative TrkB-NULL column is presented in **Figure 8**. The lack of reduction of 7,8-DHF retention on that column further confirms the lack of functional TrkB receptors on the CMAC TrkB-NULL column.

To identify specialized metabolites specifically interacting with the immobilized targets, CMAC columns are used in an approach called missing-peak chromatography[52] following the displacement experiment. In this approach, a small volume of the investigated extract is chromatographed in parallel on the column containing the investigated immobilized target and CMAC negative control column. These two columns differ in the expression of the target, therefore any differences in the retention patterns of individual molecules are due to the specific nature of interactions with the investigated targets on the CMAC column containing these TMPs. Timed fractions from both columns are collected, concentrated, and subsequently analyzed on a C18 column. The obtained chromatograms are compared, and compounds represented by peaks similarly retained on both columns are labeled as non-specifically interacting molecules. The presence of a peak in later fractions on the CMAC column with the immobilized receptors and lack of a peak representing the same compound in early fractions of CMAC negative column represents specific interaction of the compound with the immobilized target. The compounds identified in this step can be targeted for isolation and further testing, without the need to perform bio-guided fractionation. The missing-peak chromatography approach was used to identify compounds specifically interacting with TrkB receptors from gotu kola extract. Gotu kola extract was fractionated on both TrkB and TrkB-NULL columns and compounds present in these fractions were analyzed using UPLC-QTOF-MS. Investigation of the chromatograms of each of the fractions led to the identification of a compound strongly retained on TrkB column (50–55 min fraction), while eluting early on TrkB-NULL column (0–5 min fraction), indicating specific interaction with the immobilized TrkB receptors (**Figure 9**). The compound eluting at ~ 17.48 min (**Figure 9**) is now targeted for isolation and testing in functional assays.

FIGURE AND TABLE LEGENDS:

Figure 1. Correctly assembled CMAC column.

Figure 2. CMAC preparation steps and fluorescent antibody labelling of the immobilized receptors. Schematic representation of the preparation of a CMAC column (1–4) and the antibody labeling experiment (5–8). (1) Homogenized cell membrane fragments containing

targeted transmembrane protein, TrkB. (2) Solubilized cell membrane fragments in a micellar structure. (3) Cell membrane fragments immobilized on the surface of the IAM particles. (4) IAM particles with immobilized cell membrane fragments packed into a glass column. (5) Immobilized TrkB receptor in the cell membrane fragments. (6) Binding of the natural ligand BDNF to the functional TrkB receptor. (7) Binding of the primary antibodies to BDNF molecules. (8) Binding of fluorophore-labeled secondary antibodies to the primary antibodies resulting in green fluorescence.

Figure 3. Immobilization of cell membrane fragments with TrkB receptors onto IAM particles. Confocal microscopy images showing the immobilization of cell membrane fragments with functional TrkB receptors on IAM particles. (**A**) Cell membrane fragments from SH-SY5Y TrkB-NULL cell line immobilized on IAM particles after incubation with BDNF, primary antibody, and fluorophore-labeled secondary antibody. (**B**) Cell membrane fragments from SH-SY5Y Neuroblastoma cell lines expressing TrkB immobilized on IAM particles after incubation with primary antibody and GFP-tagged secondary antibody without BDNF. (**C**) Cell membrane fragments from SH-SY5Y neuroblastoma cell lines expressing TrkB immobilized on IAM particles after incubation with BDNF, primary antibody, and GFP-tagged secondary antibody.

Figure 4. Frontal affinity chromatograms of 7,8-DHF on the TrkB CMAC column. Frontal chromatogram of increasing concentrations of 7,8-DHF on the TrkB CMAC column, where A - 1 mM, B - 750 nM, C - 500 nM, and D - 300 nM. Ammonium acetate buffer (10 mM, pH 7.4) with 5% methanol was used as eluent at a flow rate of 0.4 mL/min.

Figure 5. Frontal affinity chromatograms of 7,8-DHF on nonfunctional TrkB CMAC column. Frontal chromatogram of increasing concentrations of 7,8-DHF on the non-functional TrkB CMAC column, where A -1 mM, B - 1 mM, C - 750 nM, and D - 500 nM. Ammonium acetate buffer (10 mM, pH 7.4) with 5% methanol was used as eluent at a flow rate of 0.4 mL/min.

Figure 6. Frontal affinity chromatograms of 7,8-DHF on the TrkB-NULL CMAC column. Frontal chromatogram of increasing concentrations of 7,8-DHF on the TrkB-NULL CMAC column, where A - 1 mM, B - 750 nM, C - 500 nM, and D - 300 nM. Ammonium acetate buffer (10 mM, pH 7.4) with 5 % methanol was used as eluent at a flow rate of 0.4 mL/min.

Figure 7. Frontal affinity chromatograms of 7,8-DHF with 0.2% gotu kola extract on the TrkB CMAC column. Representative frontal elution profile of (**A**) 500 nM 7,8-DHF + 0.2% gotu kola extract (10 mg/ml) and (**B**) 500 nM 7,8-DHF on the CMAC TrkB column. Ammonium acetate buffer (10 mM, pH 7.4) with 5% methanol was used as eluent at a flow rate of 0.4 mL/min.

Figure 8. Frontal affinity chromatograms of 7,8-DHF with 0.2% gotu kola extract on the TrkB-NULL CMAC column. Representative frontal elution profile of (**A**) 500 nM 7,8-DHF and (**B**) 500 nM 7,8-DHF + 0.2% gotu kola extract (10 mg/mL) on the TrkB-NULL CMAC column. Ammonium acetate buffer (10 mM, pH 7.4) with 5% methanol was used as eluent at a flow rate of 0.4 mL/min.

Figure 9. UPLC-MS chromatograms of gotu kola extract fractions. UPLC-MS chromatograms of gotu kola extract fractions (**A**) eluted between 0–5 min from TrkB-NULL CMAC column and (**C**) eluted between 50–55 min from CMAC TrkB column. A compound with a retention time of 17.48 min present in both fractions, as confirmed by matching mass spectra (**B** and **D**) was identified as a potential TrkB binder.

Table 1. Homogenization buffer composition.

Table 2. Solubilization buffer composition.

Table 3. Dialysis buffer composition.

DISCUSSION:

Identification of active compounds present in complex mixtures of specialized metabolites is a very challenging task[23]. Traditionally, individual compounds are isolated, and their activity is tested in different assays. This approach is time-consuming and costly and often leads to isolation and identification of the most abundant and well-characterized compounds[23]. Currently used high-throughput screening assays rely heavily on screening combinatorial chemistry libraries with already known targets and are not designed to identify and isolate biologically active compounds present in complex mixtures[53].

CMAC allows for the identification of compounds targeting TMPs[23,24,54]. In this technique cell membrane fragments with TMPs are immobilized on the surface of IAM stationary phase particles[23,24]. CMAC is a unique approach that allows for the immobilization of cell membrane fragments with targeted proteins on the solid support imitating cell membrane phospholipid bilayer[24]. This allows preserving the functionality of immobilized protein targets and provides close to physiological conditions while using CMAC to identify small molecules interacting with TMPs. CMAC offers the advantage of opportunities for the discovery of novel chemical scaffolds from unique natural products libraries, that cannot be tested using any other of the currently used assays[23]. The proposed approach allows for the identification of compounds interacting with TMPs without unraveling the nature of this interaction. The mode of interaction (allosteric or orthosteric interaction; inhibition or activation) needs to be verified using functional cell-based assays. CMAC with the immobilized protein targets can also be used in the characterization of pharmacodynamics (e.g. dissociation constant, Kd) or determining binding kinetics (k_{on} and k_{off}) of small molecule ligands interacting with the target as well as in the process of characterization of the binding sites on the immobilized protein[24]. Although CMAC only allows identification of compounds binding to TMPs, it is an innovative approach that significantly speeds up the first step in the drug discovery pipeline, as it does not require compound purification, which has been currently the major bottleneck in the drug discovery process from natural mixtures.

Although the presented protocol focuses on the immobilization of one transmembrane receptor (TrkB), CMAC technology can be utilized in the preparation of columns with other targets. One of the crucial aspects of CMAC preparation is the choice of cell line or tissue that is used to isolate cell membrane fragments. If columns are used in the screening of complex mixtures for

compounds interacting with the immobilized receptors, it is advised to use cell lines overexpressing the targeted protein. Using such a cell line will result in a higher number of immobilized targets and increase the chance of identification of compounds with a lower affinity towards the target or less abundant molecules. If one focuses on the characterization of the immobilized protein, the use of a native cell line is recommended, as post-translational modifications the protein undergoes as well as the phospholipid environment may significantly differ in the transfected cell line.

Some aspects of cell membrane isolation and immobilization on IAM particles are critical and may require modifications depending on the receptor type or the source of the protein. To prevent proteolytic cleavage of the immobilized proteins, it is essential to use proper protease inhibitors in the homogenization and solubilization buffers. A literature search is recommended to identify required protease inhibitors. In the process of protocol optimization, we determined that the addition of ATP and glycerol increases the number of binding sites (B_{max}) on CMAC columns when immobilizing TrkB. Other cofactors may be necessary when optimizing the immobilization of other types of transmembrane targets[24]. Currently, we are investigating the addition of cholesterol in the process of TrkB immobilization, as recently cholesterol was found to modify the effects of TrkB ligands[48]. It was previously reported that the addition of cholesterol and/or other lipids may be required for obtaining functional CMAC columns[24].

Different types of detectors may be used to monitor the column eluate including diode array detector for ligands with chromophores, mass spectrometry, or radio flow detector for radioactive ligands.

Monitoring the stability of CMAC columns is of critical importance. CMAC columns may be stable for up to several months, depending on the type of immobilized protein, frequency of use, and storage conditions. It is recommended to store the column in 0.05% sodium azide solution in ammonium acetate buffer at 4 °C if the column remains unused for more than a week. It is important to thoroughly wash the column with ammonium acetate buffer after longer periods before attempting to use it. It is advised to monitor the functionality of the column by injecting a selected concentration of a marker ligand weekly.

Despite numerous advantages, CMAC technology has several limitations that need to be taken into consideration when using the columns in drug discovery endeavors. Firstly, the number of immobilized transmembrane targets decreases with time, and therefore it is recommended that the total number of binding sites is monitored weekly[24]. One of the reasons for this decrease is the consequence of the immobilization process that is based on adsorption and does not involve the introduction of covalent bonds. Lipophilic compounds may be strongly retained on CMAC columns due to significant nonspecific binding to the IAM surface and phospholipid bilayers. This significantly increases the analysis time decreasing throughput. The process of CMAC column preparation is cell line-specific and requires a thorough understanding of the nature of immobilized proteins, making it less suitable for less characterized targets.

ACKNOWLEDGMENTS:

Z.C.A. was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) 2219- International Postdoctoral Research Fellowship Program. Research reported in this publication was supported by the National Center for Complimentary and Integrative Medicine of the National Institutes of Health under award number 1R41AT011716-01. This work was also partially supported by American of Pharmacognosy Research Starter Grant, Regis Technologies grant to L.C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES:

Lukasz Ciesla collaborates with Regis Technologies, the provider of the IAM.PC.DD2 particles.

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Figure 1

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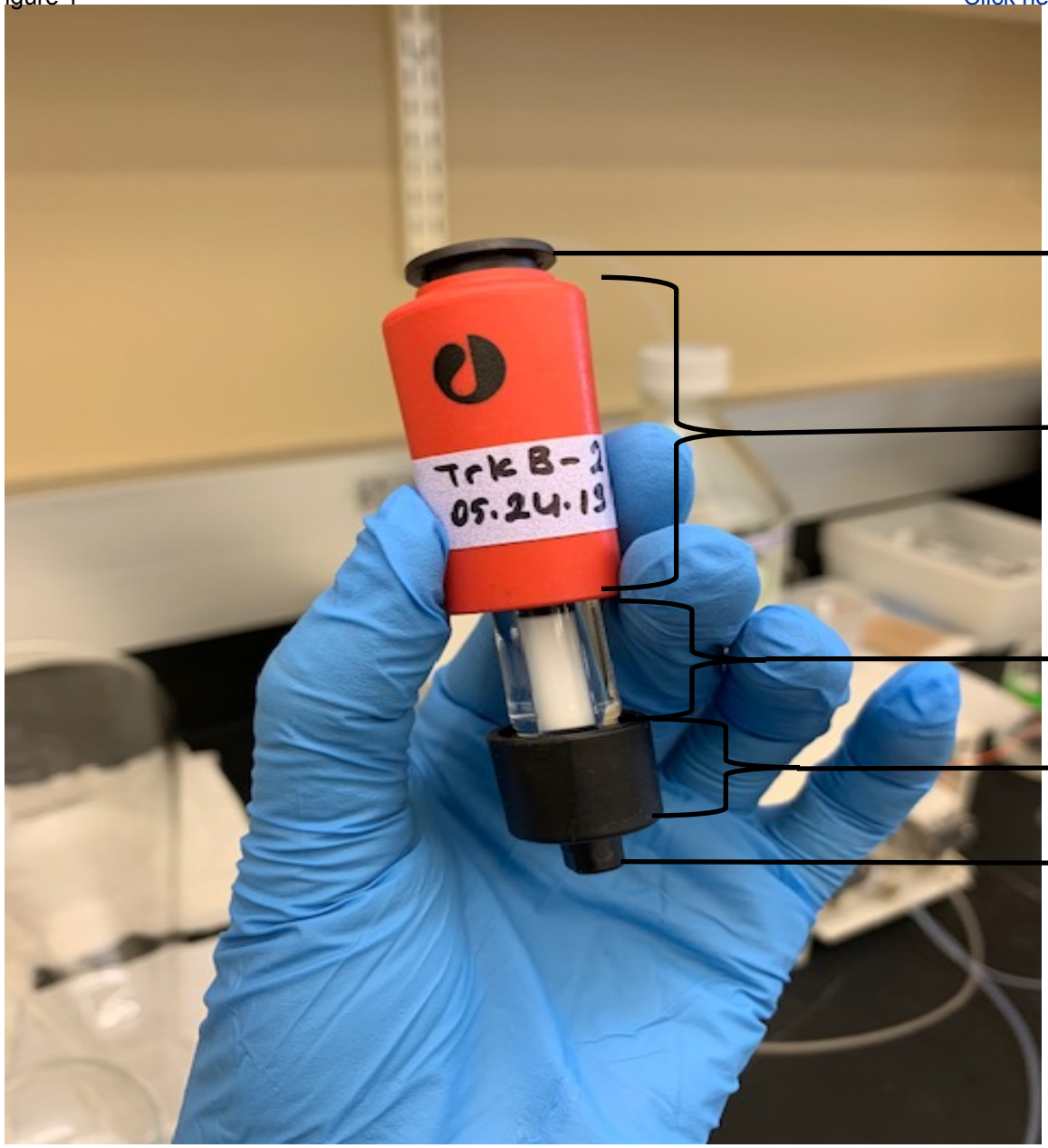

Figure 2

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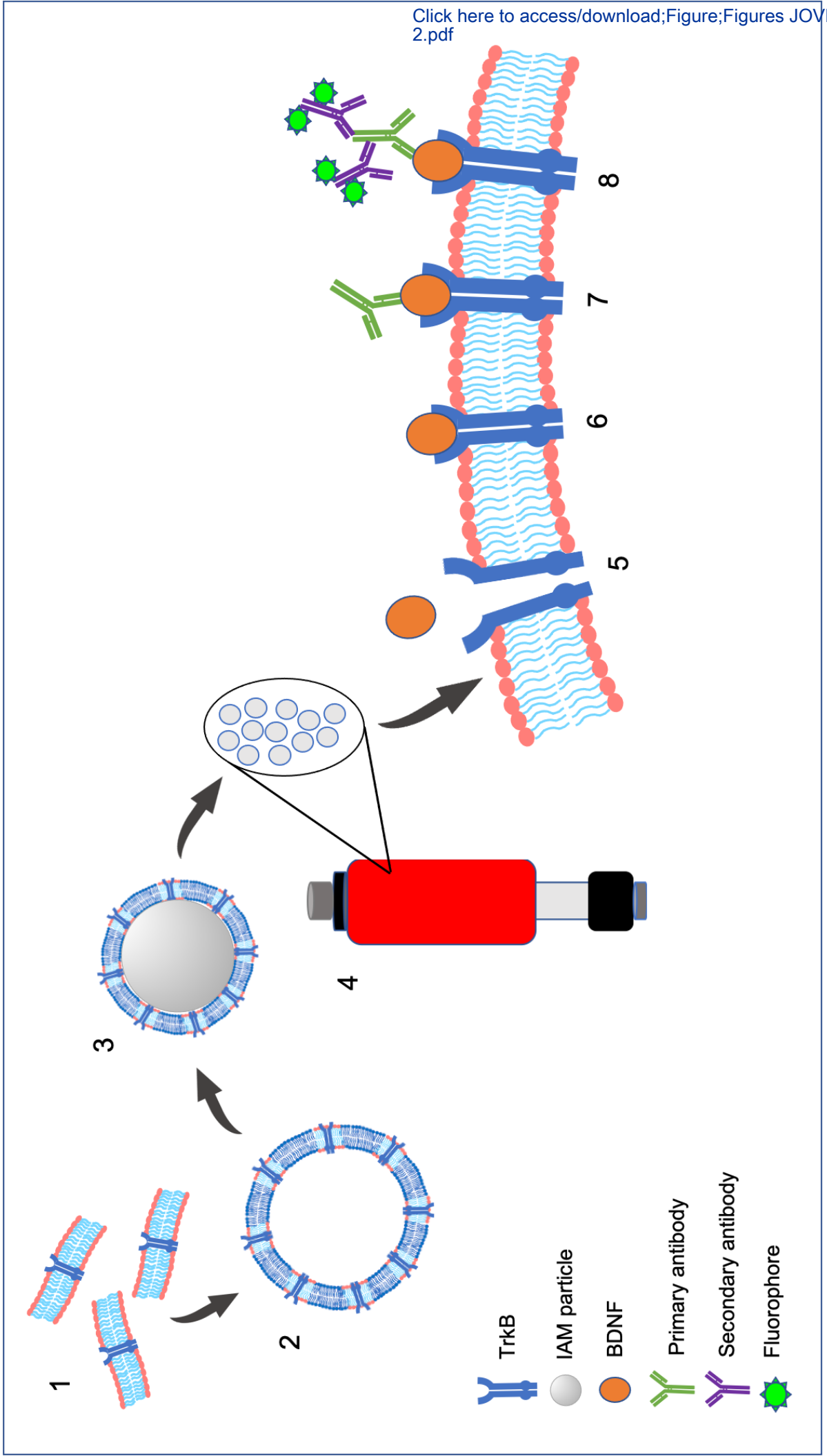

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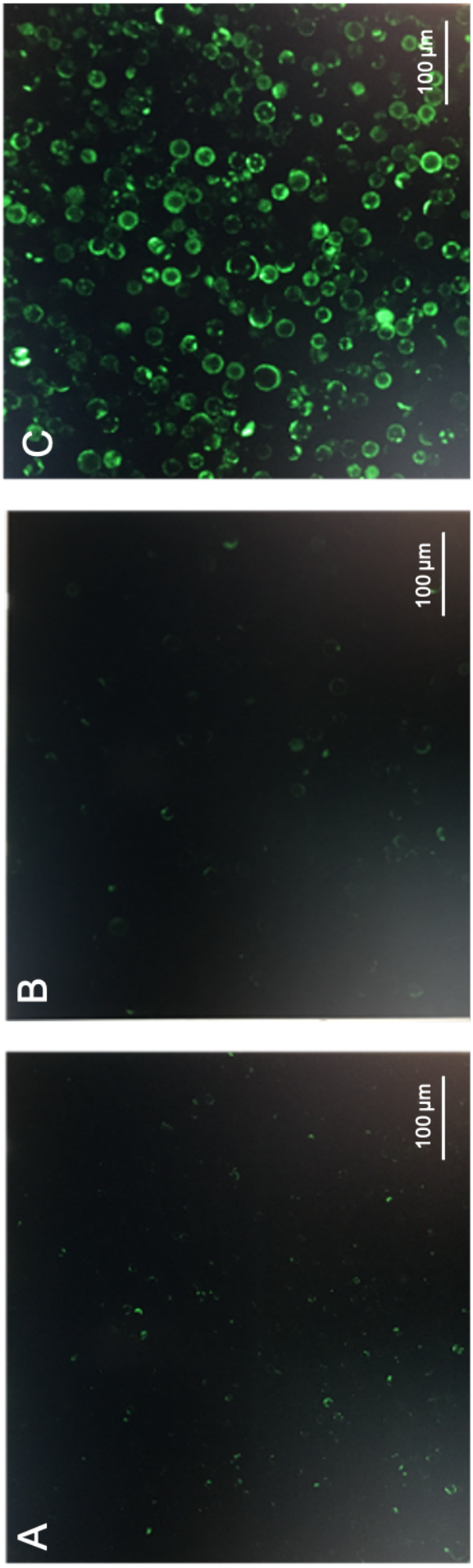

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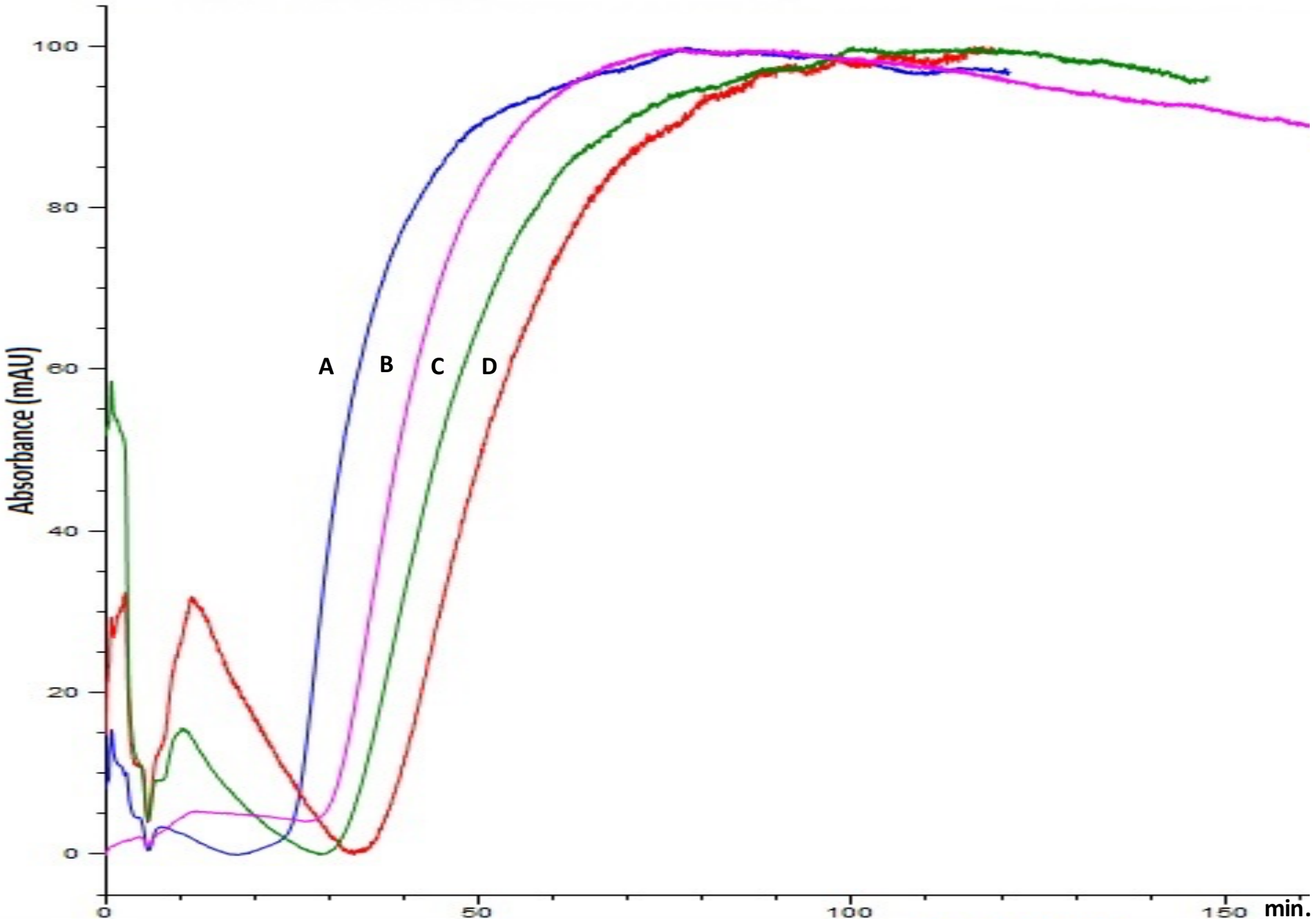

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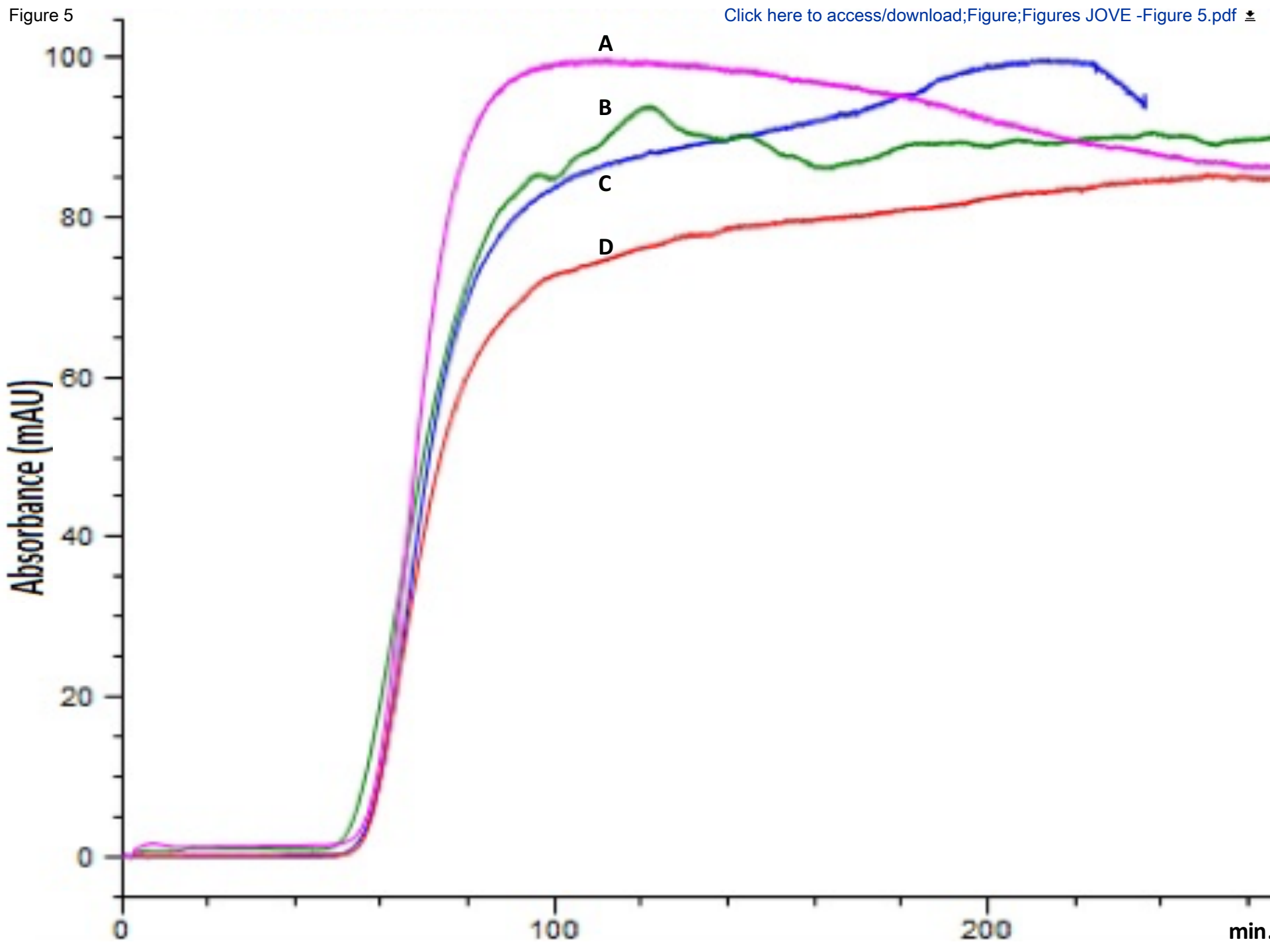

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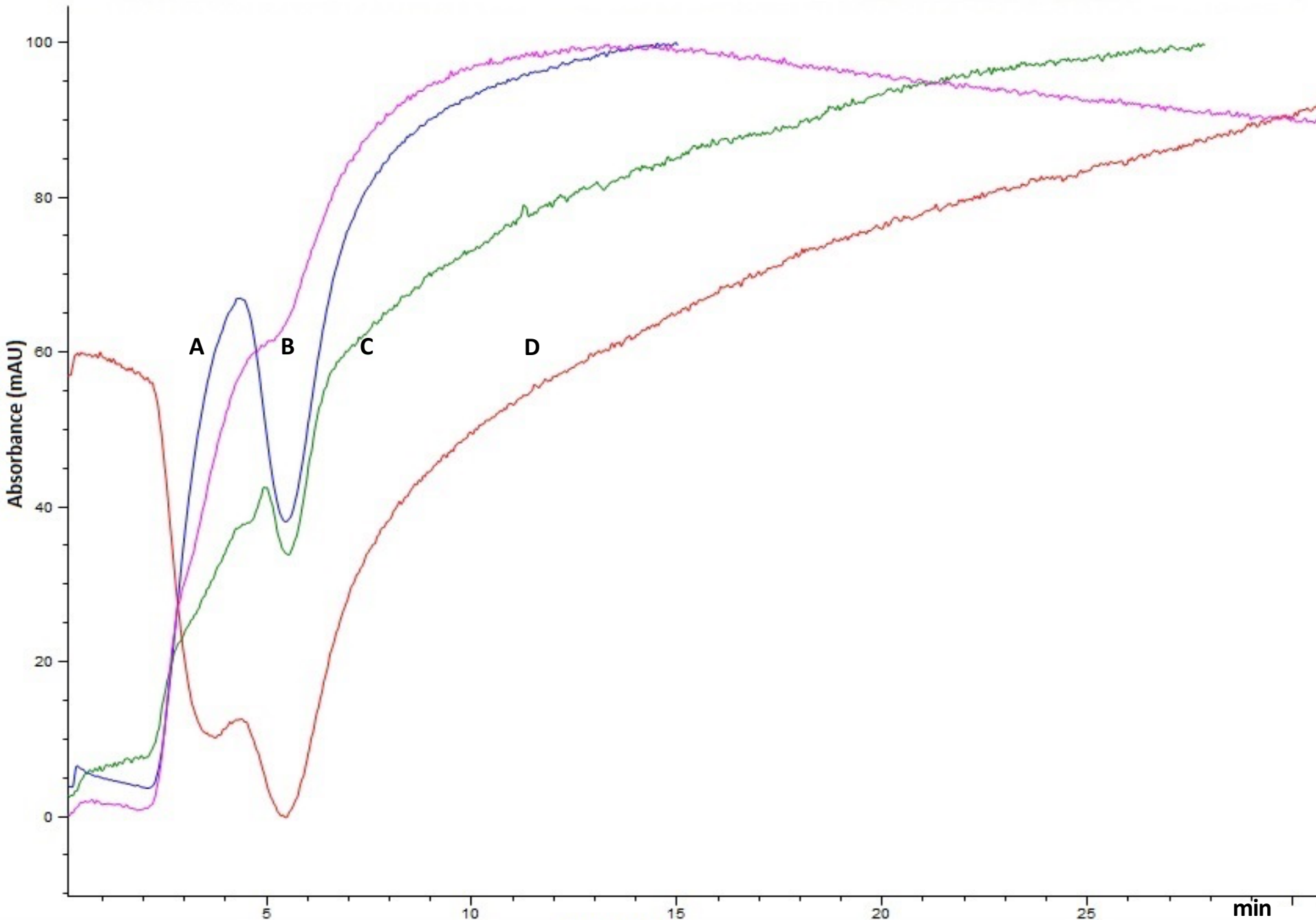

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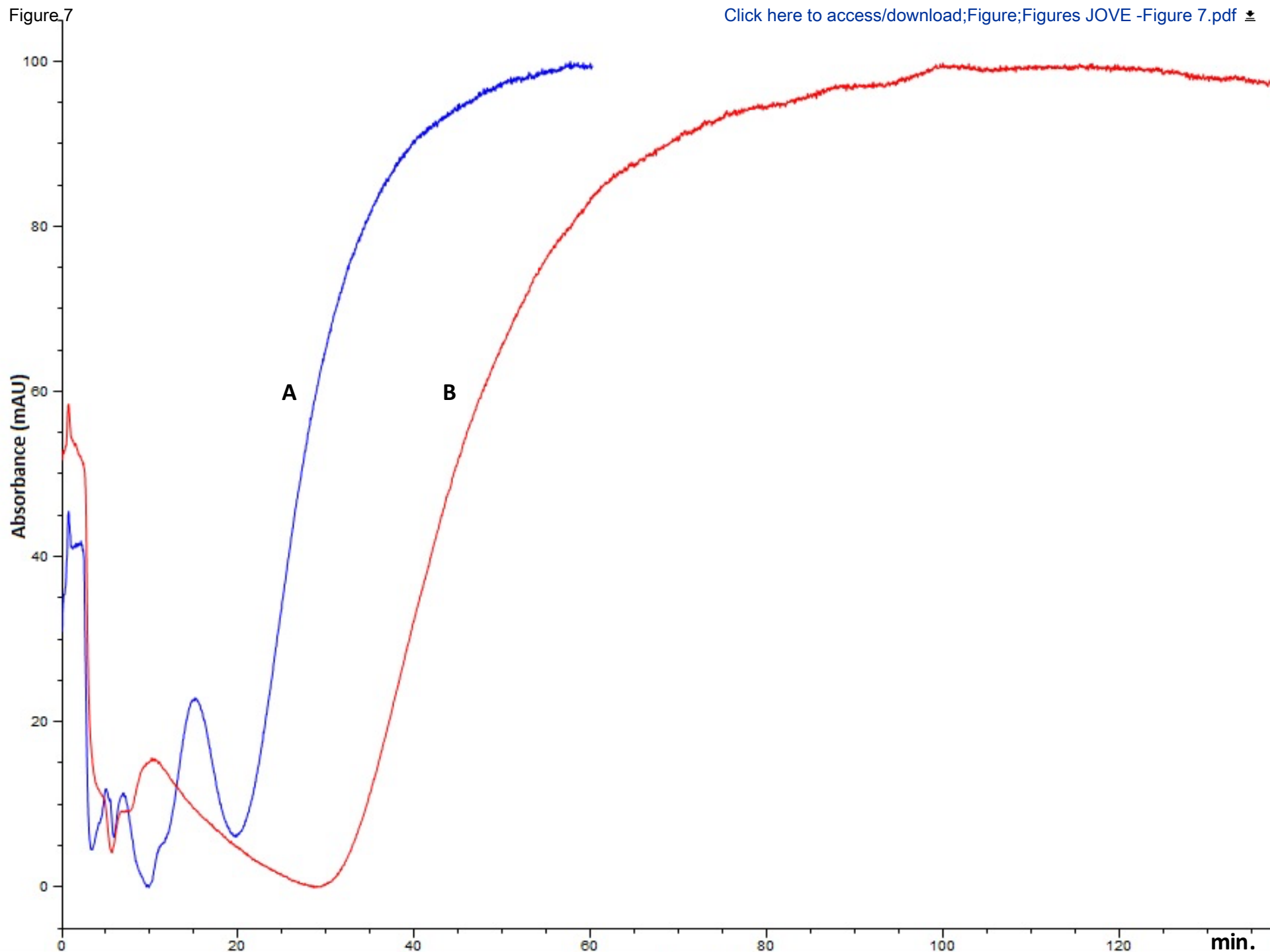

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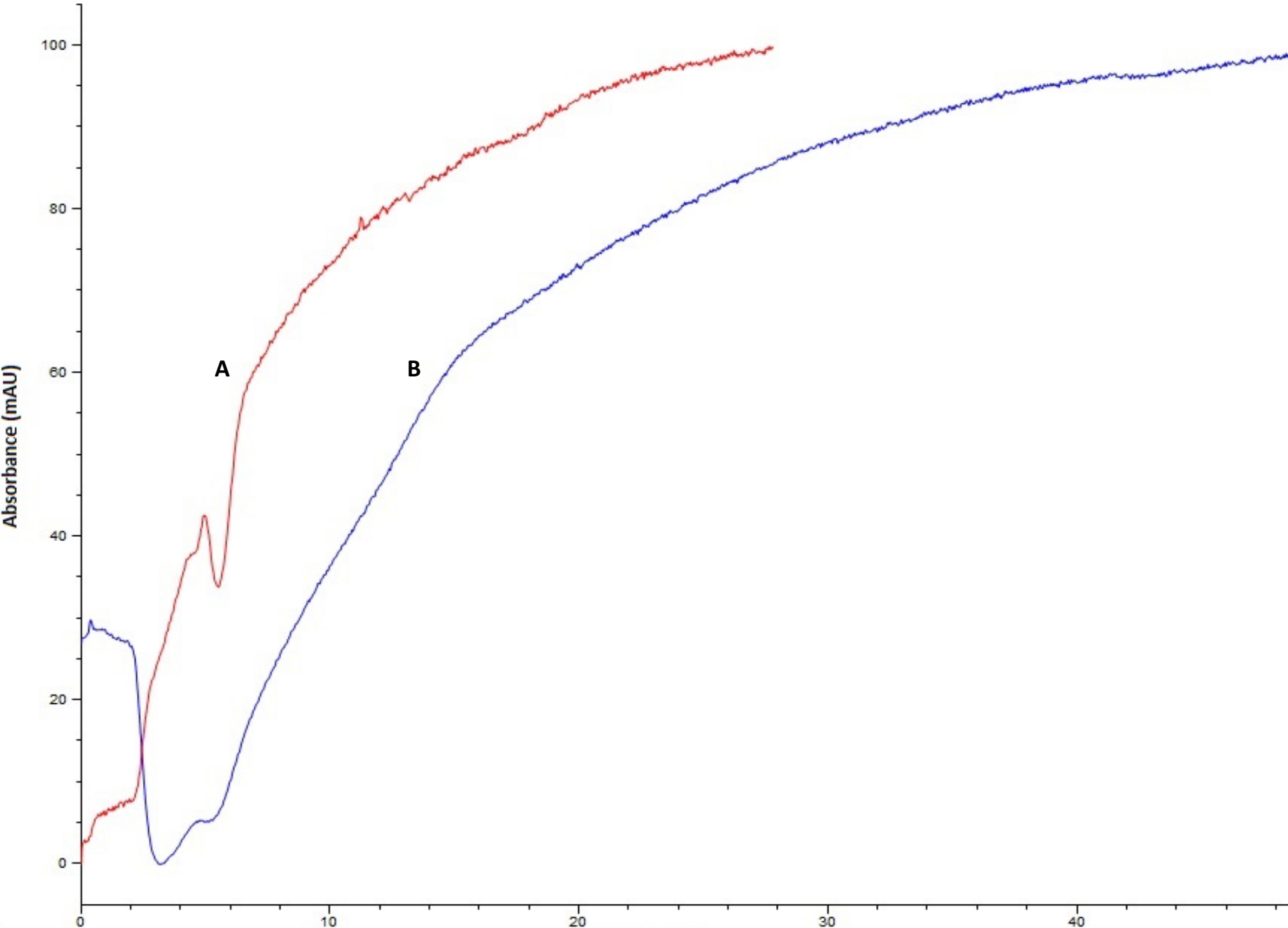

Figure 9

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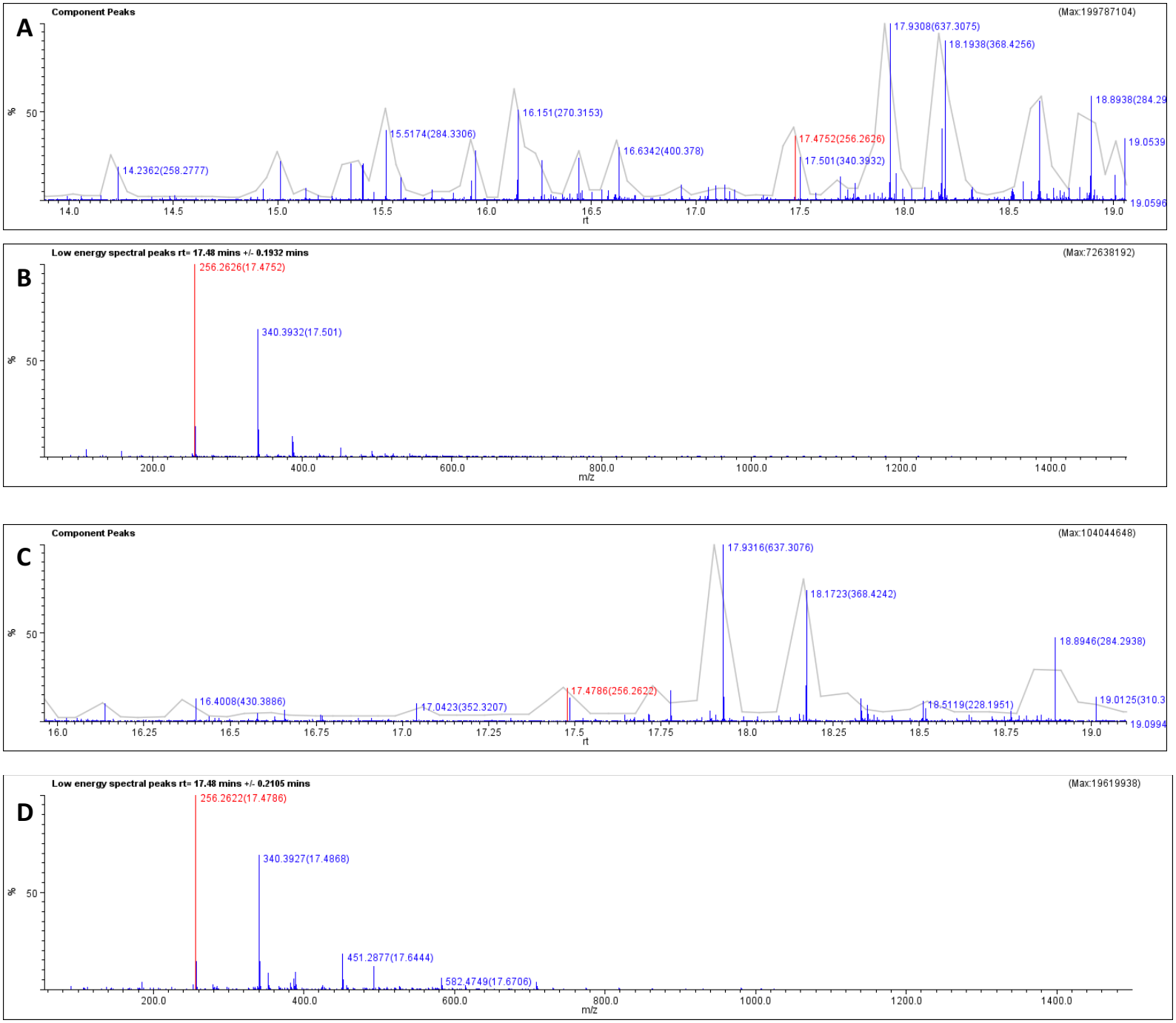

Table 1

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	Homogenization Buffer	
1	Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)	50 mM
2	Sodium chloride (NaCl)	100 mM
3	Calcium chloride anhydrous ($CaCl_2$)	3 mM
4	Magnesium chloride hexahydrate ($MgCl_2$)	2 mM
5	Potassium chloride (KCl)	5 mM
6	Phenylmethanesulfonyl fluoride (PMSF)	0.1 mM
7	Benzamidine	3 mM
8	Protease inhibitor cocktail (100X)	(1/100)
9	Glycerol	10%
10	Adenosine 5'-triphosphate (ATP) disodium salt hydrate	100 µM
11	Ethylenediaminetetraacetic acid (EDTA)	5mM

Table 2

Click here to access/download;Table;Solubilization Buffer.xlsx

	Solubilization Buffer	
1	Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)	50 mM
2	Sodium chloride (NaCl)	100 mM
3	Calcium chloride anhydrous ($CaCl_2$)	3 mM
4	Magnesium chloride hexahydrate ($MgCl_2$)	2 mM
5	Potassium chloride (KCl)	5 mM
6	Phenylmethanesulfonyl fluoride (PMSF)	0.1 mM
7	Benzamidine	3 mM
8	Protease inhibitor cocktail (100X)	(1/100)
9	Glycerol	10%
10	Adenosine 5'-triphosphate (ATP) disodium salt hydrate	100 µM
11	Sodium cholate	2%

Table 3

Click here to access/download;Table;Dialysis Buffer.xlsx

	Dialysis Buffer	
1	Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)	50 mM
2	Sodium chloride (NaCl)	100 mM
3	Calcium chloride anhydrous ($CaCl_2$)	0.1 mM
4	Ethylenediaminetetraacetic acid (EDTA)	5 mM
5	Phenylmethanesulfonyl fluoride (PMSF)	0.1 mM

Click here to access/download
Table of Materials
JoVE_Materials_RE.xlsx

Rebuttal Letter
Click here to access/download;Rebuttal Letter;Rebuttal letter.docx

THE UNIVERSITY OF ALABAMA | College of Arts & Sciences
Biological Sciences

Dr. Vidhya Iyer, Ph.D.
Review Editor
JOVE

Dear Dr. Iyer,

We would like to thank JOVE Editorial team and the Reviewers for their feedback on our manuscript entitled: "**Cellular membrane affinity chromatography columns to identify specialized plant metabolites interacting with immobilized tropomyosin kinase receptor B**". We have thoroughly revised our paper and all the introduced changes have been clearly indicated in the manuscript. Below you will find our detailed responses to the Reviewers' concerns and all the editorial comments. We have also added one more author: Dr. Urmila Maitra who has contributed to the experiments performed for the resubmission. All the co-authors agreed to add Dr. Maitra as a co-author.

With best regards,

Lukasz Ciesla
Assistant professor
Department of Biological Sciences
The University of Alabama

Editorial comments:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have thoroughly proofread the manuscript and revised it to ensure that there are no spelling or grammar issues. All the abbreviations have been defined at first use in the manuscript.

2. Please avoid abbreviations in the title and shorten it to be within the 150 character limit (including spaces).

We have removed the abbreviation in the title and revised it so that it stays within the 150-character limit.

3. Please revise the following lines to avoid previously published work: 94-96, 111-117, 120-121, 196-198, 291-292, 419-420, 516-519.

We have revised the abovementioned lines.

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Proper in-text formatting has been incorporated throughout the manuscript.

5. Please consider providing solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

We have provided Tables with solution compositions and uploaded them as separate files into Editorial Manager.

6. Please use x g for centrifuge speeds.

"x g" has been used for centrifuge speeds.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be

enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We thoroughly went through the protocol, specifically over the highlighted portions and provided more details, where needed to make sure sufficient experimental details are provided.

8. 7.1.13: Please provide all microscope parameters and settings.

Microscope parameters and settings have been provided in the manuscript.

9. 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 and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

The manuscript has been formatted in accordance with this comment.

10. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Scale bar has been provided.

11. Please revise the Discussion to explicitly cover any limitations of the technique.

Discussion has been revised and a paragraph on method limitations has been added.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

All references have been modified to follow the requested formatting.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The work by Arituluk et al provided a detailed protocol to assemble CMAC column with immobilized TrkB receptors using neuroblastoma cell line overexpressing TrkB receptors. Such work is possible to provide a strategy for the immobilization of other receptors. These

are encouraged by Journal of Visualized Experiments. My recommendation for the work is published with the consideration of the comments as below:

Major Concerns:

1. Although the authors organized their work logically and presented the method in a relatively clear way, the applications of CMAC columns need to be supplemented otherwise the section of disscussion would not be valid. Such applications of CMAC columns were crucial for the integrity of this article.

We have now performed additional experiments using CMAC TrkB columns to identify natural compounds interacting with TrkB receptors. Using the "missing peak" chromatography approach we identified a compound strongly retained on TrkB column, while eluting early on TrkB NULL column, indicating specific interaction with the immobilized TrkB receptors. New Figure (Figure 9) has been added and the representative results and discussion sections have been expanded.

Minor Concerns:

2. The comments of Figure 4 and Figure 5 were confused. In general, the specificity of the CMAC column should be investigated under the same conditions as the control column.

We closely inspected both the Figures 4 and 5 and checked the provided captions and comments made in the text. They correctly present the data, clearly showing the difference between functional and nonfunctional CMAC TrkB column.

3. I noticed that the present tense was used in this article, which is inconsistent with the past tense used in other articles. Advise the author to make changes if necessary.

We thoroughly read the manuscript and made proper changes where necessary.

<u>Reviewer #2:</u>

Manuscript Summary:

This paper presents the preparation and application of cellular membrane affinity chromatography (CMAC) columns in the identification of biologically active plant substances that interact with tropomyosin kinase receptor B. Cellular membrane affinity chromatography is a technique that is based on the immobilization of a target trans-membrane protein onto a stationary phase. The columns may have been used in drug discovery. The great advantage of CMAC is not only the possibility of isolation of ligands, but also able to determine the pharmacodynamic parameters that are critical in the development of potential drugs.

In the presented work the process of assembling of CMAC columns: one with immobilized SH-SY5Y neuroblastoma cell membrane fragments with overexpressed TrkB and one with SH-SY5Y TrkB-NULL cell membrane fragments as a negative control, is described in great details.

The authors confirmed immobilization of cell membrane fragments with functional TrkB receptors on IAM particles by using confocal microscopy.

The correct function of the columns was checked with the small marker ligand: 7,8 -

dihydroxyflavone.
The authors of the publication presented detailed protocols, performed all the necessary experiments, drew the correct conclusions and this paper is suitable to be published in JoVE.

Minor Concerns:
What, in my opinion, would facilitate the perception of the protocol, is the change in sections: 3.1.1, 3.1.2, 3.2, 3.3, 4.1, 5.3.1, 6.1. Instead of masses of chemical compounds, molar concentrations would be better. In laboratory work, when specifying the composition of the buffer, molar concentrations of individual components of the buffer are given.

We have provided molar concentrations of chemicals in the sections mentioned above.

Reviewer #3:
Manuscript Summary:
Arituluk et al. report a protocol about the use of cellular membrane affinity chromatography column to characterize
specialized metabolites interacting with tropomyosin kinase receptor B cellular membrane.

Major Concerns:
No major concerns

Minor Concerns:
1-It is not clear to me how the isolated membrane fraction of neuroblastome cells, over expressing tropomyosin kinase receptor B, are combined to Immobilized Artificial Membrane (IAM.PCC.DD2) in a stable structure.

Cell membrane fragments are immobilized through adsorption, facilitated by the dialysis process. We have included specific comments on the process of immobilization in the manuscript to allow the reader to better understand the process. We provided the following sentence in the Introduction:
"During CMAC columns preparation cell membrane fragments are immobilized on to the surface of IAM particles through adsorption." and further in the Discussion we also commented on the immobilization mechanism: "Firstly, the number of immobilized transmembrane targets decreases with time and therefore it is recommended that the total number of binding sites is monitored weekly[24]. One of the reasons for this decrease is the consequence of the immobilization process that is based on adsorption and does not involve the introduction of covalent bonds."

2-Would be better to add a figure with the complete pipeline, including a cartoon showing the expected membrane composition/structure/immobilization after combination with IAM.PCC.DD2 (figure 2 does not represent well how the membrane is immobilized).

We have updated Figure 2 to clearly present the process of cell membrane immobilization.

3-It would be nice to comment that complex mixtures of metabolites interacting with tropomyosin kinase receptor B cellular membrane can be monitored via mass spectrometry, for example, and a lot of information from several metabolites can be extracted from single analysis (I suppose).

We have now added additional data (Figure 9), that presents the use of high resolution mass spectrometry in the identification of compounds interacting with TrkB receptors and present in complex natural mixture.