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A simple protocol for high efficiency protein isolation after RNA isolation from mouse thyroid and other very small tissue samples

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Abstract
As a dedicated hormone-secreting organ, the thyroid gland possesses a complement of proteostatic systems, including antioxidant, unfolded protein, and autophagic responses. The vast majority of animal investigations of thyroid physiology and, more recently, proteostasis, have utilized as model the rat, rather than the mouse. This is due to the very small size of the thyroid gland in the latter, with a total weight of ~2 mg (~1 mg per thyroid lobe). However, this strategy has limited the utilization of genetic approaches, such as taking advantage of the various transgenic and knockout mouse models. Here, we describe a simple and highly effi cient protocol for the simultaneous isolation of mRNA, micro-RNA and 150–200 µg of protein from as little as 1 mg of mouse thyroid tissue, the average weight of one of the two thyroid lobes, thus preserving the other lobe for immunohistochemical or other analyses. While our workflow is similar to other protocols published in the literature and/or proposed by commercial reagent providers, we have introduced a key modification that addresses efficiently the most challenging step of the protein isolation process: the solubilization of the protein pellet after RNA extraction and protein precipitation. We demonstrate the feasibility of our approach and its utility for downstream analyses (including Western blotting) that facilitate the comparative study of proteostatic pathways in the mouse thyroid. We have also successfully applied this protocol on samples from mouse liver, brown and white adipose tissue, as well as from rodent cell lines.

Key words
Simultaneous, Isolation, RNA and protein, Micro-RNA, Guanidinium, TRIzol, QIAzol, TRI reagent, Thyroid, Proteostasis

1. Introduction

The guanidinium thiocyanate–phenol–chloroform (GTPC) extraction method, also called “TRIzol” extraction, introduced by Chomczynski and Sacchi in 1987 [1], is widely used in molecular biology to isolate both nucleic acids and proteins. This method is based on the different solubilities of RNA, DNA, and protein molecules in water and organic solvents such as phenol and chloroform. It also exploits the ability of the chaotropic agent guanidinium thiocyanate to denature all proteins, including those that degrade nucleic acids (DNAses and RNAses). When a cell or tissue lysate that has been incubated with these reagents is subjected to centrifugation, the solution separates into a lower organic phase that contains DNA and proteins, and an upper aqueous phase that contains RNA. The RNA is recovered from the aqueous phase with isopropanol precipitation (after transfer to a different tube); the DNA and proteins can also be recovered from the organic phase following a different procedure (discussed in detail below).

Homemade and commercially available GTPC reagents (such as TRIzol, QIAzol, TRI Reagent, etc.) are widely used in the literature for the extraction of RNA. The simultaneous isolation of protein from the same sample is advantageous for several reasons: (1) it saves time; (2) it permits the reliable assessment of and correlation between coordinated changes in gene and protein expression levels; and (3) it is especially critical when the quantity of the starting biological sample is limited (such as for small, precious, or rare samples). Nevertheless, compared to the huge use of GTPC to isolate RNA in the literature, there are relatively very few reports with simultaneous protein isolation. This is due to the fact that when the standard recommended protocol for protein isolation is used, it is very difficult to dissolve the protein pellet in the final step, leading to experimental failure. Indeed, there are some reports in the literature that have tried to overcome this problem by using different approaches. For example, one approach replaced protein

precipitation with dialysis of phenol–ethanol supernatants against a 100x volume of 1 % SDS; this was repeated three times, and the extracted protein was subsequently concentrated using commercially available columns [2]; this is a laborious and complicated technique. Another approach is to attempt the solubilization of TRIzol-extracted proteins not with the standard 1 % SDS solution but with alternative solutions. Different solutions have been reported to improve solubilization efficiency, such as 9.5 M Urea and 2 % CHAPS ([3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate]) (UREA-CHAPS) [3]; diethylamine [4]; or Urea-SDS solubilization and sonication [5]. Another proposed method is the precipitation of proteins from the phenol–ethanol phase by an ethanol-bromochloropropane-water solution, followed by solubilization of the protein pellet with 4 % SDS and heating at 50 °C [6].

Here, we demonstrate that a simple modification of the standard protocol for protein isolation from the phenol–ethanol supernatant after DNA precipitation facilitates the easy and complete solubilization of the protein pellet in the final step without the need for additional treatments or special reagents. We have found that modification of the steps where the protein pellet is washed with 0.3 M Guanidine hydrochloride in 95 % ethanol in order to remove the remaining phenol from the protein has a dramatic impact on the solubility of the protein in the fi nal step. Specifically, we first completely dissolve the protein pellet in 7 M guanidine hydrochloride solution. Proteins can be efficiently precipitated from a guanidine hydrochloride solution by addition of ethanol [7]; we thus add at least 9 volumes of 100 % ethanol. We believe that this modifi cation improves signifi cantly the removal of phenol from the protein, which in turn increases dramatically protein solubilization in the final step. We demonstrate the feasibility and simplicity of our approach and its utility for downstream analyses that facilitate the comparative study of proteostatic pathways in the mouse thyroid (~1 mg of starting tissue material). Western blotting analyses show that the protocol can isolate detectable amounts of proteins that span a broad range of molecular weights and are present in varying abundance in the starting material. We have also successfully applied this protocol on samples from mouse liver, brown and white adipose tissue, as well as from rodent cell lines.

2. Materials

2.1 Required chemicals, commercial reagents, kits, and equipment

1. Guanidine hydrochloride (Sigma).
2. 1-Bromo-3-chloropropane (1-BCP, Sigma).
3. Isopropyl alcohol.
4. 100 % ethanol (absolute ethanol).
5. Urea.
6. 20 % SDS sodium dodecyl sulfate solution (Applichem).
7. 1 M Tris-Buffer pH 8.0 (Applichem).
8. TRIzol reagent (Life Technologies).
9. RNeasy Mini Kit (QIAGEN).
10. Tissue Grinder homogenizer.
11. Benchtop centrifuge.
12. Swing rotor centrifuge.
13. Vortex device.
14. Water bath or hot plate and magnetic stirrer.
15. 0.22 µm syringe filters.
16. Ultrapure water.
17. 100× Protease inhibitor cocktail (any commercial source).

2.2 Solutions to be prepared before starting the procedure

1. 7 M Guanidine hydrochloride solution (GndCl) solutions: Weigh 66.87 g GndCl in a plastic or glass beaker, add ultrapure water to a volume of 90 ml, and dissolve by stirring (see Note 1). Once dissolved, bring the volume to 100 ml with water and fi lter the solution through a 0.22 µm syringe filter.
2. Protein solubilization solution: 8 M Urea, 40 mM Tris, pH 8, 1 % SDS. Because urea solutions are unstable, always make fresh small quantities. To make 2 ml of this solution, place 961 mg Urea

in a 2 ml tube, add water to about 1.8 ml and dissolve by vortexing, then add 80 µl of 1 M Tris pH 8, and 100 µl of 20 % SDS solution. Finally, bring the volume to 2 ml with water. Just before solubilizing the protein pellet, add to the solution 20 µl of the 100x Protease inhibitor cocktail.

3 Methods

3.1 RNA isolation

1. In fume hood, add 1100 µl TRIzol reagent to a 15 ml Falcon tube (see Notes 2-4). Place the thyroid tissue inside the tube and homogenize immediately until the sample is homogeneous (requires about 30 s using the QIAGEN TissueRuptor with disposable probes at maximum speed; see Note 5).
2. Incubate for 5 min at room temperature, and then centrifuge briefly the samples for 30 s on a bench top centrifuge at maximum speed.
3. Transfer the homogenized samples to 1.5 ml tubes and store them at −70 °C for least 30 min.
4. Move the samples directly from −70 °C to a water bath of 60 °C for 5 min, and then place them on ice for another 5 min (see Note 6).
5. Add 100 µl of 1-BCP (see Note 7) to the tubes containing the samples and shake vigorously by hand or vortexing for 30 s.
6. Leave the samples on the bench top at room temperature for 5 min.
7. Centrifuge for 15 min at 12,000 × *g* at 4 °C. After centrifugation the mixture separates into a lower phenol–chloroform phase of red color, an interphase, and a colorless upper aqueous phase. The upper aqueous phase that contains RNA comprises ~40–50 % of the total volume.
8. Transfer the upper aqueous phase (400–450 µl) to a new collection tube (see Note 8). Add 1.5 volume of 100 % ethanol and mix thoroughly by pipetting. Save the tube containing the interphase and the organic phenol/1-BCP interphase for the protein isolation procedure (see Note 9).
9. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 s at ≥8000 × *g* (≥10,000 rpm). Discard the flow-through. Repeat using the same column and the remainder of the sample. Discard the flow-through.
10. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at ≥8000 × *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
11. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at ≥8000 × *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
12. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently and centrifuge for 2 min at ≥8000 × *g* (≥10,000 rpm) to wash the spin column membrane.
13. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Place the RNeasy Mini spin column into a new 2 ml collection tube. Centrifuge at full speed for 2 min to dry the RNeasy Mini spin column membrane.
14. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at 10,000 × *g* to elute the RNA (see Note 10).
15. The RNA can be stored and used in downstream applications for mRNA and microRNA analyses as usual.

3.2 Protein isolation

1. Proteins are isolated from the interphase and organic phenol/1-BCP interphase that was saved in step 9 of the RNA isolation procedure.
2. Centrifuge the samples briefly (1 min at 10,000 × *g*) and remove any remaining aqueous phase overlying the interphase.
3. Add 0.3 ml of 100 % ethanol per 1 ml TRIzol Reagent used for the initial homogenization to precipitate the DNA. Cap the tube and invert the sample several times to mix. Incubate samples for 5 min at room temperature.
4. Centrifuge the samples for 5 min at 2000 × g at 4 °C to precipitate the DNA into a pellet (see Note 11).
5. Taking care not to dislodge the DNA pellet, transfer the phenol-ethanol supernatant to a 15 ml tube. Add 1.5 volume of isopropanol to the phenol-ethanol supernatant. Incubate the samples for

10–20 min at room temperature until a visible precipitate is formed (see Note 12). This is the precipitated protein.
6. Centrifuge the samples for 10 min at 4000 × *g* at 4 °C in a swing rotor centrifuge to pellet the protein. Remove and discard the supernatant (see Note 13).
7. Resuspend the protein pellet in 100 µl 7 M GndCl solution (see Note 14).
8. Transfer the protein solution to 2 ml tubes. Add 1900 µl 100 % ethanol and vortex for 10 s (see Note 15). Incubate samples for at least 30 min at −70 °C; a visible protein precipitate will be formed (see Note 16).
9. Centrifuge the samples for 5 min at full speed at 4 °C to pellet the protein. Remove and discard the supernatant.
10. Resuspend the protein pellet in 100 µl 7 M GndCl solution (optional, see Note 17). Add 1900 µl 100 % ethanol and vortex for 10 s. Incubate the samples for at least 30 min at −70 °C; a visible protein precipitate is formed.
11. Centrifuge the samples at full speed for 5 min at 4 °C to pellet the protein. Remove and discard the supernatant.
12. Add 2 ml 100 % ethanol and vortex three times for 10 s each time over a period of 10 min at room temperature.
13. Centrifuge the samples for 10 min at full speed at 4 °C to pellet the protein. Discard the ethanol supernatant and air dry the protein pellet for about 10 min (see Note 18).
14. Dissolve the protein pellet in 100–200 µl of 8 M Urea, 40 mM Tris pH 8, 1 % SDS, 1x protease inhibitors solution (see Note 19).
15. The protein can be stored and used in downstream applications as usual. We routinely test visually our extracts on a protein gel (Fig. 1), and use them for Western immunoblotting (Fig. 2) (see Notes 20-23).

4. Notes

1. Warming up the GndCl solution to 40 °C will help to dissolve faster the guanidine hydrochloride. Use a water bath or a hot plate and a magnetic stirrer.
2. TRIzol Reagent contains phenol (toxic and corrosive) and guanidine isothiocyanate (an irritant), and may be a health hazard if not handled properly. The manual and protocol of TRIzol Reagent recommend to always work with TRIzol Reagent in a fume hood, and to always wear a lab coat, gloves, and safety glasses.
3. During the disruption and homogenization of the starting material, we always lose about 5–10 % of the initial TRIzol volume. That is why we use 10 % more than the recommended volume (i.e., 1100 µl instead of 1000 µl).
4. The volume of TRIzol reagent depends on the quantity and the type of starting material. For thyroid tissue, which in mice weighs about 0.5–1 mg per lobe, 1 ml is more than enough. For liver, we use 50 mg of tissue when the tissue is fresh or stored at −70 °C, and no more than 10 mg when it is preserved in RNAlater RNA Stabilization Reagent. For adipose tissue, we use 100–200 mg of fresh tissue, or about 50 mg of tissue preserved in RNAlater. Tissues stored in RNAlater lose most of their water content, and therefore shrink and weigh less than the corresponding amount of fresh tissue.
5. After storage in RNAlater, tissues become harder than fresh or thawed tissues. This must be taken into account during the disruption and homogenization of these tissues, optimizing the duration and magnitude of the disruption and homogenization method.
6. We have found that performing a freeze-thaw treatment of the samples increases RNA yields, possibly due to the complete dissociation of the nucleoprotein complex formed after lysis with TRIzol. In particular, for cells in culture dishes whereas a viscous material is formed after addition of TRIzol, this freeze-thaw cycle eliminates the need to homogenize the samples with the TissueRuptor.
7. Chloroform is commonly used instead of 1-BCP. Since chloroform is a neurotoxicant, an endocrine disruptor, and possibly also a carcinogen, substitution with the less hazardous and less volatile 1-BCP is advantageous, resulting in safer working conditions; see: http://www.subsport.eu/case-stories/071-en?lang=en. 1-BCP can be used as a fully functional substitute for chloroform without any changes to the experimental protocol [9]. Moreover, Ambion supports that use of 1-BCP results in better phase separation, and thus better purification

of the RNA, DNA, and protein fractions; see: https://www.lifetechnologies.com/ch/en/home/references/ambion-techsupport/rna-isolation/tech-notes/rna--dna--and-proteinfrom-a-single-sample.html.
8. At this step, in order to ensure the quality of isolated RNA, it is critical to carefully take the upper aqueous phase without disrupting the interphase. For this reason, we always leave behind 50–100 µl of aqueous phase. For thyroid tissue, where the starting material is very limited, and the expected RNA quantity is accordingly small, we do a second extraction: we add 200 µl of RNAse free water to the remaining sample; vortex; centrifuge; take 200 µl from the aqueous phase and combine with the first one.
9. Store the samples dedicated for protein isolation at 4 °C, if you plan to make the extraction on the same day. Otherwise, place them at −20 °C or at −70 °C for long-term storage.
10. We routinely quantify the RNA on a NanoDrop spectrophotometer. From each mouse thyroid tissue we obtain about 1 µg of total RNA.
11. When the starting material is limited, as is the case with thyroid tissue, most of the time the DNA pellet will not be visible. For this reason, we always leave behind a small portion of phenol-ethanol supernatant (about 50 µl) to ensure that we do not carry over DNA into the protein samples. Moreover, if isolation of DNA is not needed, then it is better in step 4 to centrifuge the samples at full speed for 2 min rather than at 2000 × *g* for 5 min. Carryover of DNA into the protein solution will lead to a solution with some very viscous and sticky parts in the fi nal step. If this is observed, then sonicate the samples briefly (5–10 s) to shear the DNA.
12. If the protein concentration is high, the precipitate will form within 1–2 min. But if the starting tissue material is too little (<1 mg of tissue), or if protein is isolated from small numbers of cells (e.g., cells grown in 24-well culture plates), then place the samples at −20 °C or −70 °C for 1 h to facilitate protein precipitation.
13. After removal of the supernatant, let the tubes drain in an upside down position on a clean piece of absorbent paper for 5 min to completely remove the phenol-isopropanol supernatant.
14. In this step, protein solubilization usually takes 10–20 min. Especially if the protein pellet is big, the volume of GndCl should be increased such that the protein pellet is resuspended in 150–
200 µl. Leave the samples with the GndCl solution at room temperature for 10 min, and then solubilize the pellet by pipetting. Protein degradation under these conditions is not a concern, because GndCl is a potent denaturant, and therefore proteins in the GndCl solution are fully protected from degradation.
15. The minimal volume of ethanol that needs to be added is nine times the volume of the GndCl solution. Thus, if one wants to keep working with 2 ml tubes for practical reasons, then the maximum volume of GndCl solution in which the pellet can be resuspended is 200 µl.
16. Precipitation of proteins will take place also at room temperature or at −4 °C, but we prefer to place the samples at −70 °C in order to ensure the quantitative (i.e., maximal) precipitation of the proteins from GndCl solution, especially for samples with low protein concentration.
17. Perform these extra steps (10, 11) only when the protein pellet has a red tint (indicative of residual phenol), or when it is big (suggesting it may not have been thoroughly washed). Because the protein pellet is completely dissolved in the GndCl solution (step 7), the first precipitation (steps 5 and 6) and the ethanol washes (steps 8 and 12) result in complete removal of the phenol.
18. After the final ethanol wash and centrifugation, the protein pellet detaches from the tube quite easily. Therefore, in order to not lose the pellet, drain off carefully the ethanol supernatant immediately after centrifugation, and then place the tube upside down on absorbent paper for 10 min to drain out the remaining ethanol. Then place the tubes in normal position with the lids open to evaporate the traces of ethanol remaining inside the protein. Depending upon the size of the pellet, this usually needs about 5–10 min. It is very important not to allow the protein pellet to dry completely. Stop the drying when the tube no longer smells of ethanol, and the protein pellet still has a white milky appearance. Waiting too long will result in a protein pellet that has dried completely, has become transparent, and is very difficult to dissolve.
19. The protein pellet is easily solubilized in this buffer. The pellet can also be dissolved in any other buffer that contains at least 1 % SDS.
20. We have applied this protocol to isolate proteins from thyroid, liver, brown and white fat adipose tissue, as well as from cell lines. Simultaneous isolation of both RNA and protein from a single biological sample permits the reliable assessment of coordinated changes in gene and protein expression levels.

21. We measure the protein concentration using the Thermo Scientific Pierce BCA Protein Assay Kit that is compatible with the components of this buffer. For each thyroid tissue we recover about 150–200 µg of protein. We run no more than 5–10 µg of protein per lane for SDS-PAGE and Western immunoblotting, and we use high sensitive ECL reagents like the Amersham ECL Prime Western Blotting Detection Reagent or the Advansta WesternBright Quantum kit.
22. Thyroglobulin, the main protein of the thyroid tissue, accounts for approximately half of the protein content of the thyroid gland. For this reason, when Western blots are performed with thyroid samples coming from different experimental conditions, equal protein loading is not always consistent with the results obtained from the loading controls such as beta-actin, tubulin, etc. This discrepancy is observed when the experimental conditions lead to a significant change in the protein abundance of thyroglobulin.
23. When the total number of samples to be processed does not exceed 10-20, both the RNA and the protein protocols can be completed in a single day. We usually store the phenol/1-BCP supernatant at −20 °C, and we perform the protein extraction the following day. The protein isolation procedure is very flexible regarding time lines and is highly amenable to interruption of the protocol; it can be stopped at any step, storing the samples at −20 °C or −70 °C to continue on the same day or a subsequent day.

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Figure Legends

Fig. 1: SDS-PAGE gel analysis of proteins extracted by our protocol from thyroid, liver, and brown adipose tissue, as well as from the rat thyroid cell line PCCL3 grown in 12-well culture dishes. Various genotypes and/or treatment conditions are used (not indicated). Proteins from liver, brown adipose tissue, and PCCL3 cells were separated on a 10 % Bis–Tris gel (MOPS buffer) that was then stained with Blue Silver G-250 [8]. Proteins from thyroid tissue were separated on a 4–10 % gradient Tris-Glycine gel that was then stained with Coomassie Brilliant Blue R-250.

Fig. 2: Western blot analysis of thyroid-specific or proteostasis-related proteins extracted from mouse thyroid tissues by our protocol. Various genotypes and/or treatment conditions are used (not indicated). Proteins of a wide range of molecular weights are successfully detected, including site-specific phosphorylations. Five to 10 µg of extracted proteins from mouse thyroid tissues were separated under reducing conditions on 7.5 %, 10 % or 12 % Bis–Tris gels and immunoblotted with the indicated antibodies: anti-thyroglobulin anti-TG (A0251, DAKO); anti-sodium-iodide symporter antibody anti-NIS (a kind gift by Prof. Nancy Carrasco, [10]). The following antibodies were all from Cell Signaling Technology: anti-autophagy-related 12 (anti-ATG12, #2011); anti-C/EBP-homologous protein (anti-CHOP, #2895); anti-Phospho-S6 Ribosomal Protein (anti-pS6RP, #8207); anti-LC3I/II (#12741); anti-Phospho-PDK1(Ser241) (#3438); anti-Phospho-Akt (Ser473) (#4060). The anti-beta-actin (ab6276) and anti-Proteasome Subunit Beta type-5 (anti-PSMB5, ab3330) antibodies were from Abcam.

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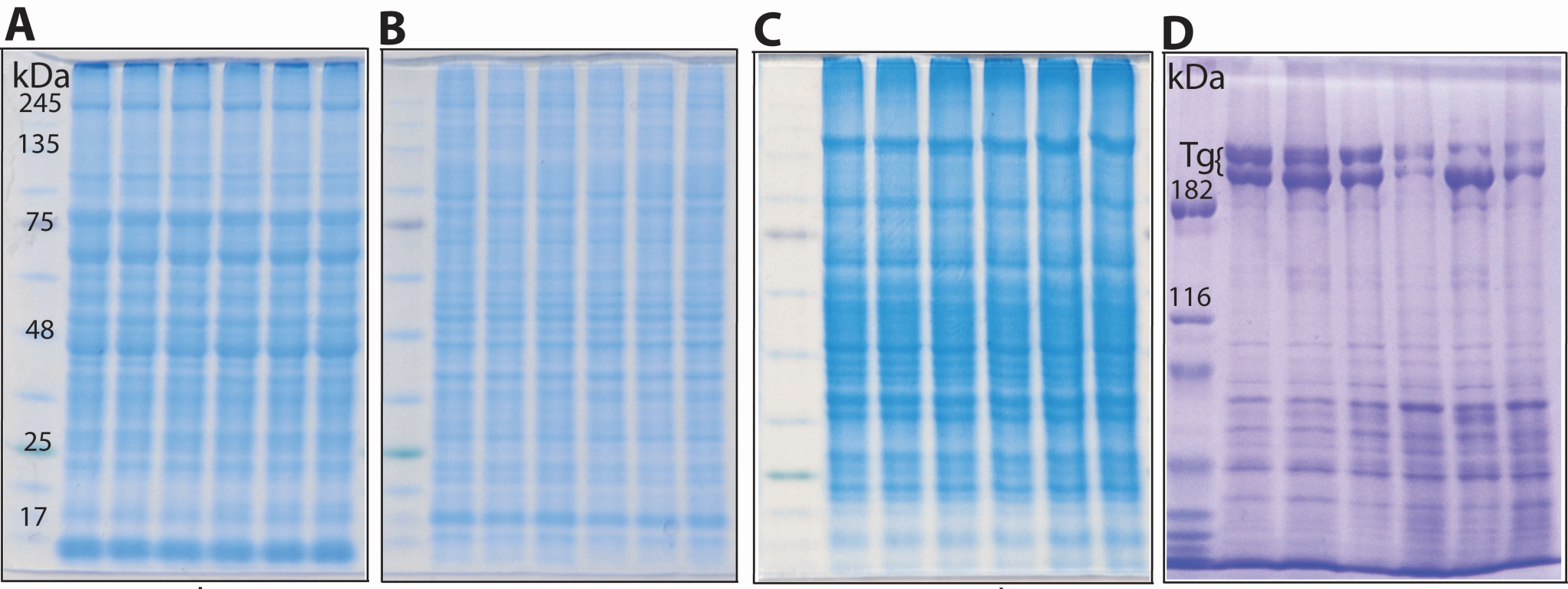
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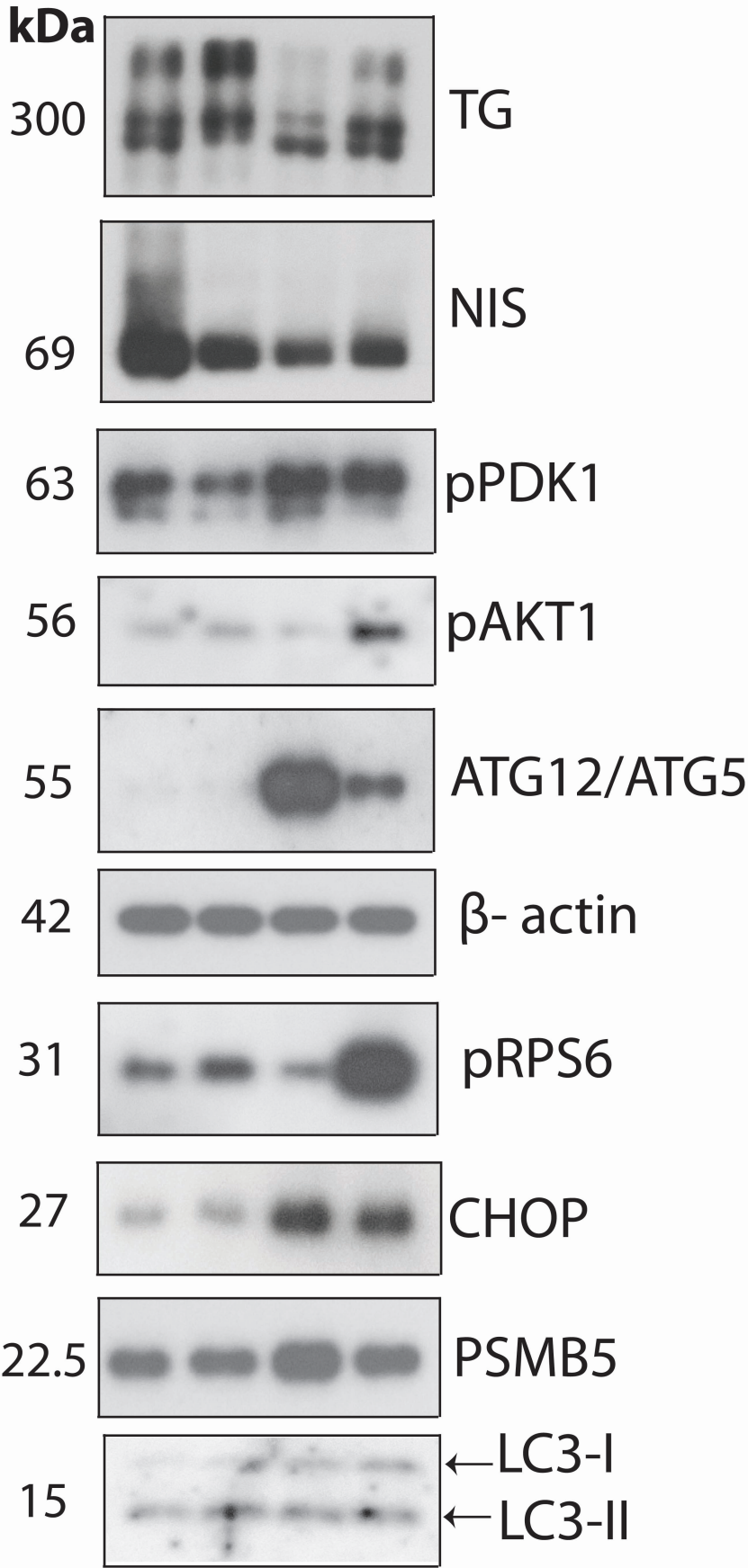
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