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Combining non-reducing SDS-PAGE analysis and chemical crosslinking to detect multimeric protein complexes in mammalian cells in culture

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Cover Letter

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December 3, 2018

Dear Editorial board:

We are most happy to accept your invitation, through your science editor Anna Justis, to submit a methods manuscript. This work is based on a recently published article in Protein Science entitled "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)"

Please accept the enclosed files as a start to the review process.

Sincerely,

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TITLE:

Combining Non-Reducing SDS-PAGE Analysis and Chemical Crosslinking to Detect Multimeric Complexes Stabilized by Disulfide Linkages in Mammalian Cells in Culture

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KEYWORDS:

non-reducing SDS-PAGE, chemical crosslinking, formaldehyde, disulfide linkages, iodoacetamide, multimeric complexes, mammalian cell culture.

SUMMARY:

Disulfide linkages have long been known to stabilize the structure of many proteins. A simple method to analyze multimeric complexes stabilized by these linkages is through non-reducing SDS-PAGE analysis. Here, this method is illustrated by analyzing the nuclear isoform of dUTPase from the human bone osteosarcoma cell line U-2 OS.

ABSTRACT:

The structures of many proteins are stabilized through covalent disulfide linkages. In recent work, this bond has also been classified as a post-translational modification. Thus, it is important to be able to study this modification in living cells. A simple method to analyze these cysteine-stabilized multimeric complexes is through a two-step method of non-reducing SDS-PAGE analysis and formaldehyde cross-linking. This two-step method is advantageous as the first step to uncovering multimeric complexes stabilized by disulfide linkages due to its technical ease and low cost of operation. Here, the human bone osteosarcoma cell line U-2 OS is used to illustrate this method by specifically analyzing the nuclear isoform of dUTPase.

INTRODUCTION:

Disulfide linkages have long been known to stabilize the structure of many proteins. In recent work, this bond has also been classified as a reversible post-translational modification, acting as a cysteine-based “redox switch” allowing for the modulation of protein function, location and interaction[1–4]. Thus, it is important to be able to study this modification. A simple method to analyze these cysteine-stabilized multimeric complexes is through non-reducing SDS-PAGE

analysis[5]. SDS-PAGE analysis is a technique used in many laboratories, where the results can be obtained and interpreted quickly, easily, and with minimal costs, and is advantageous over other techniques used to identify disulfide linkages such as mass spectrometry[6,7] and circular dichroism[8].

One important step in determining if this method is an appropriate technique to aid in a study is to thoroughly examine the primary sequence of the protein of interest to insure there are cysteine residue(s) present. Another helpful step is to research any previous crystal structures published or use a bioinformatics application to explore the three dimensional structure of the protein of interest to visualize where the cysteine residue(s) may be located. If the residue(s) is present on the outside surface it may be a better candidate to form a disulfide linkage rather than a cysteine residue buried on the inside of the structure. However, it is important to note that proteins may undergo structural changes upon substrate interactions or protein-protein interactions allowing these residues to then become exposed to the environment as well.

Identified multimeric complexes can then be verified with chemical cross-linking using formaldehyde. Formaldehyde is an ideal cross-linker for this verification technique due to the high cell permeability and short cross-linking span of ~2-3 Å, ensuring detection of specific protein-protein interactions[9,10]. Here, this method is illustrated by analyzing the nuclear isoform of dUTPase from the human bone osteosarcoma cell line U-2 OS[11]. However, this protocol can be adapted for other cell lines, tissues and organisms.

PROTOCOL:

1. Blocking free cysteine residues using iodoacetamide

1.1. Grow U-2 OS cells in a 6 cm^2 dish to 50%–60% confluency in minimum essential medium with high glucose containing 10% fetal bovine serum and 1% sodium pyruvate at 37 °C in 5% CO_2.

1.2. Make a fresh stock of 10 mM iodoacetamide just prior to use, then discard any unused reagent.

1.3. Add 0.1 mM final concentration iodoacetamide directly to the cell culture media. Gently rock the dish at room temperature for 2 min.

2. Harvesting the cells

2.1. Aspirate the media from the cells.

2.2. Wash the cells with 5 mL of cold phosphate-buffered saline (PBS) three times. Aspirate the final PBS wash solution then add 1 mL of cold PBS.

2.3. Scrape the cells off the bottom of the dish using a cell scraper. Using a 1 mL pipette draw up the PBS and cell suspension and dispense all the liquid into a 1.5 mL microcentrifuge tube.

2.4. Spin the cells at 7,500 x *g* for three min at 4 °C. Aspirate the PBS, leaving the cell pellet behind. The cell pellet can be stored at -80 °C until processing

3. Extraction of protein

3.1. Prepare 100 µL of a 1x lysis buffer diluted in ddH_2O (See **Table of Materials**). Add phenylmethylsulfonyl fluoride (PMSF) immediately before use to a 1 mM final concentration.

3.2. Add 50 µL of the 1x lysis buffer directly to cell pellet and suspend. Incubate this on ice for 5 min.

3.3. Sonicate for 8 s using the constant pulse mode at 40% (see **Table of Materials** for sonicator; adjust as necessary for different sonicators), keeping the extract on ice.

3.4. Spin the extract at 4 °C for 5 min at 16,000 x *g*. The supernatant is the soluble protein fraction. Bradford analysis can be performed to determine protein concentration if desired.

4. Sample preparation

4.1. Take 10 µL of the soluble protein extract and add 10 µL of a 1x Laemmli SDS-sample buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl, pH 6.8). Do not add any reducing reagents.

4.2. Keep samples on ice if SDS PAGE analysis will be performed that day; for long term storage, -20 °C is appropriate. Right before running the gel, heat the sample for 5 min at 85 °C.

5. In vivo formaldehyde cross-linking of endogenous proteins in U-2 OS cells

5.1. Grow U-2 OS cells in a 175 cm^2 flask to 70%–80% confluency (see section 1).

5.2. Perform the formaldehyde crosslinking reaction

5.2.1. In a fume hood, aliquot a 37% formaldehyde solution purchased from commercial sources. Add the formaldehyde fixative directly to the medium to a final concentration of 1% and incubate at room temperature with gentle agitation for 15 min.

5.2.2. To quench the reaction, add 1.25 M glycine to a final concentration of 0.125 M and incubate at room temperature with gentle agitation on a rocker for 5 min.

5.3. Wash the cells with 5 mL of cold PBS three times. Aspirate the final PBS wash solution and add 10 mL of PBS. Scrape the cells off the bottom of the flask using a cell scraper.

5.4. Using a 10 mL pipette, draw up the PBS and cell suspension and dispense all of the liquid into a 15 mL conical centrifuge tube. Spin the cells at 500 x g for 2 min at 4 °C. Aspirate the PBS, leaving the cell pellet behind.

6. Fractionation of nuclei.

6.1. Prepare 10 mL of homogenization buffer: 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, and 0.5% BSA at pH 7.4. Add PMSF immediately before use to a 1 mM final concentration and 3mL of nuclei suspension buffer (0.1% Triton X-100 in PBS).

6.2. Add 5 mL homogenization buffer directly to the cell pellet and suspend completely. Centrifuge the suspension at 500 x *g* for 2 min at 4 °C then discard the supernatant.

6.3. Suspend the pellet in 5 mL of homogenization buffer. Use a tight-fitting glass-Teflon homogenizer, dounce homogenize cells at 10 strokes/500 rpm.

6.4. Centrifuge the suspension at 1,500 x *g* for 10 min at 4 °C, then discard the supernatant.

NOTE: Use the supernatant for isolation of mitochondria by centrifuging at 10,000 x *g* for10 min.

6.5. Suspend the pellet in 1 mL nuclei suspension buffer and incubate on ice for 10 min.

6.6. Centrifuge at 600 x g for 10 min. Discard the supernatant.

6.7. Suspend the pellet in 1 mL nuclei suspension buffer and centrifuge again. Discard the supernatant. The final pellet will be isolated nuclei.

7. Extraction of protein

7.1. Repeat section 4, except adding 25 µL of the 1x lysis buffer directly to the cell pellet then suspending.

8. Sample preparation

8.1. Prepare two samples for SDS-PAGE by taking 10 µL each of the soluble protein extract and add 10 µL of 2x Laemmli SDS-sample buffer and 1 µL of 2-Mercaptoethanol (BME).

8.2. Heat one sample for 5 min at 37 °C and the second sample for 15 min at 98 °C to reverse the formaldehyde cross link.

9. SDS-PAGE analysis.

9.1. Prepare 1 L of 1x Tris-glycine running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS).

9.2. Set up the SDS-PAGE running apparatus.

NOTE: This protocol uses a 16% precast TGX SDS-Page. Of note, any percentage gel can be used.

9.2.1. Per the manufacturer protocol, open the package the gel is stored in and remove the cassette.

9.2.2. Remove the comb that is lining the wells and the tape from the bottom of the cassette.

9.2.3. Place the gel into the running apparatus.

9.2.4. Fill the chamber with the 1x running buffer until the wells are submerged in liquid. Using a plastic pipet, rinse out the wells with the running buffer.

9.3. Load the samples on the gel along with 10 µL prestained standard marker.

9.4. Run the gel at 200 V until the dye front is approximately 1 cm from the bottom of the gel.

10. Western Blot

10.1. Prepare 1 L of 1x transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol) and store at 4 °C until use.

10.2. Carefully remove the gel and open the cassette. Using a razor, carefully cut and discard the stacking gel. Pick up the gel using one corner and place it into a tray with transfer buffer, allowing it to rock gently for 5 min.

10.3. While the gel is rocking, place an ice block (stored in -20 °C) into the transfer tank and add the transfer buffer to ¾ full.

10.4. Wet the polyvinylidene fluoride (PVDF) membrane by soaking it in 100% methanol for 30 s.

10.5. Once the 5 min have passed, prepare to set up the transfer in a tray with transfer buffer. The transfer buffer should be enough to completely submerge the blot. Set up the blot transfer as follows: at bottom, the bottom of the cassette holder (black); then a thick sponge, extra thick blotting paper, the polyacrylamide gel, a PVDF membrane, extra thick blotting paper, and a thick sponge; then finally the top of the cassette holder (white) at top.

10.6. Lock the cassette and place the unit into the transfer tank with the PVDF membrane towards the positive anode and the gel towards the negative. Top off the unit with transfer buffer until your transfer is completely submerged.

10.7. Place the unit on top of a stir plate. Add a stir bar to the unit and begin stirring at 125 rpm.

10.8. Run at 100 V for 60 min.

10.9. While the transfer is running, prepare a solution of 5% powdered milk dissolved in Tris-buffered saline, with Tween-20, pH 7.5 (TBST).

10.10. Dismantle the unit and remove the membrane, noting which side was in contact with the gel; ensure this side stays up in the tray for the remaining steps.

10.11. Soak the membrane for 2 min in Tris-buffered saline (TBS) followed by blocking the membrane by incubating in 5 mL of 5% milk-TBST solution for 30 min at room temperature.

10.12. Replace the milk with a fresh 4 mL of 5% milk-TBST solution and add the primary antibody at the proper dilution (in this case, our dilution is 1:2000 for the dUTPase antibody) and incubate over night with gentle rocking at 4 °C.

10.13. The following day, remove the blot from the 4 °C rocker to a room temperature rocker, discarding the primary antibody solution.

10.14. Do three quick washes with TBST, using enough liquid to completely submerge the blot, followed by 3 washes, 5 min each, rocking slowly.

10.15. Following the last wash, add 5 mL of the 5% milk-TBST solution and rock the blot for 15 min then discard.

10.16. Add secondary antibody diluted in 5% milk-TBST at the desired concentration. (In this case, 1:5000 dilution of goat anti-rabbit diluted in 5 mL of 5% milk-TBST solution)

10.17. Incubate at room temperature, rocking for 1 h, then discard the solution.

10.18. Do three quick washes with TBST followed by 3 washes, 5 min each, rocking slowly, then discard the final wash and add 5 mL of TBS.

10.19. Prepare a 1:1 mixture of an ECL chemiluminescent detection solutions (1 mL of each reagent for a final volume of 2 mL) and add this directly to the blot incubating for 1 min.

10.20. Remove the membrane using tweezers and dab the corner with a laboratory wipe, removing any excess solution. Place the membrane in shrink wrap and place the protein side up in an x-ray cassette.

10.21. Expose the membrane to x-ray film. The time of exposure will vary.

REPRESENTATIVE RESULTS:

Nuclear dUTPase forms an intermolecular disulfide linkage forming a stable dimer configuration

through the interaction of two cysteine residues positioned at the third amino acid of each monomeric protein[11]. This is demonstrated in **Figure 1A,B**. To ensure this disulfide linkage was not a nonspecific interaction due to migration abnormalities in the non-reduced environment,t the inclusion of a proper control was essential. Of note, nuclear dUTPase is one of four isoforms present in humans. Three of the four isoforms have a unique amino-terminal domain while sharing a common catalytic core[11,12]. As seen on the western blot, the monomeric confirmation of dUTPase in human cells at the time of harvesting is a combination of at least three of the isoforms of dUTPase (the mitochondria isoform, the nuclear isoform, and a truncated version notated at M24), all of which are recognized by our polyclonal antibody. The nuclear isoform is the only isoform that contains a cysteine residue in its unique amino terminal domain. Due to the nuclear isoform being only a small percentage of the monomeric state of the proteins seen on the western blot, it was exposed longer to demonstrate the dimeric state of that isoform.

The mitochondrial isoform lacks the cysteine residue present in nuclear isoform and was used as a control for this intermolecular disulfide linkage. As seen in **Figure 1C**, isolation of mitochondria followed by a western blot analysis demonstrated that this isoform under non-reducing conditions did not form a disulfide linkage and migrated to the predicted molecular weight for the monomeric protein.

To confirm this complex can form a multimeric complex, formaldehyde cross-linking was performed. Isolated nuclei were subjected to 1% formaldehyde treatment followed by denaturing SDS-PAGE/western blot analysis under reducing conditions. As demonstrated in **Figure 2**, the dimerization was visualized. When the cross-link was reversed by incubating the sample at 95 °C for 15 min, the complex was destabilized and could be visualized in its monomeric state.

FIGURE LEGENDS:

Figure 1. Demonstration of intermolecular disulfide bond formation in the nuclear dUTPase protein. (**A**) A western blot analysis of total cell extracts (TCE) in the absence of the reducing agent, beta-mercaptoethanol (BME), demonstrates a multimeric complex formation in asynchronous populations of U-2 OS, Saos2, A549, and 18CO as indicated by the black box. (**B**) This complex disappears with the addition of BME in all four cell lines examined, indicating the presence of a disulfide linkage. (**C**) A western blot analysis of TCE and purified mitochondrial extracts (Mito) derived from U-2 OS cells, ±BME, shows no multimeric complex formation in the –BME sample. The lower panels in panels A, B, and C demonstrate exposure to X-ray film for 10 s, showing the monomeric state of the three isoforms of dUTPase. The upper panels in A and B were exposed for 1 min, while the upper panel in C was exposed for 2 min. Equivalent amounts of protein were applied to each lane. Blots were probed with a polyclonal specific antibody against the conserved carboxyl-terminal domain of dUTPase. This figure has been modified from Rotoli et al.[11]

Figure 2. Formaldehyde cross-linking of nuclear dUTPase demonstrate multimeric complex formation. U-2 OS cells were incubated with 1% formaldehyde for 15 min. Nuclei (N) were

isolated then analyzed by western blot using a specific polyclonal antibody against the conserved carboxyl-terminal domain of dUTPase (+ formaldehyde). To reverse the formaldehyde cross-links, extracts derived from the nuclear preparations were mixed with SDS-PAGE buffer then heated to 98 °C for 15 min in the presence of BME (+formaldehyde, 98 °C for 15 min). The observed heterogeneity (i.e. doublet bands of nDut) seen with the preparations remain to be explained, but may be due to anomalous migration due to the formaldehyde treatment. The lower panel is exposed to X-ray film for 10 s, while the upper panel is exposed to X-ray film for 2 min. This figure has been modified from Rotoli et al. [11]

DISCUSSION:

The method outlined here gives a straight-forward protocol for the analysis of multimeric complexes stabilized through disulfide linkages. This protocol can easily be adapted to other cell culture lines, tissues and organisms allowing for a broad range of applications.

An important step in this procedure is to ensure the disulfide linkages are not a consequence of the extraction procedure. Any free cysteine residues can be blocked using iodoacetamide[13]. This alkylating agent will bind covalently to cysteine residues though their thiol group, blocking the formation of new disulfide bonds. However, if the disulfide bond is present at the time of treatment this agent will not disrupt it. Optimization of the iodoacetamide can be done using a variation of concentration and time. However, over exposure to this reagent will cause cell death.

An additional step to this protocol that may be helpful is the optimization of the formaldehyde cross-linking. A variation of the percent of formaldehyde can be used as well as the time of cross-linking[9,14]. It is important to note that as the percent of formaldehyde as well as time increases, so do the chances of forming non-specific interactions. Downstream confirmation of the multimeric complex is also necessary. A useful technique to determine the molecular weight and identity, if the complex is a heteromultimeric, is mass spectrometry. Also, site directed mutagenesis can be a suitable technique to determine the cysteine residues responsible for the disulfide linkage.

Lastly, this two-step method of non-reduced SDS-PAGE analysis and formaldehyde cross-linking verification is beneficial due to its technical ease and low cost. It can be a first step in uncovering multimeric complexes stabilized by disulfide linkages.

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DISCLOSURES:
The authors declare that they have no conflicts of interest with the contents of this manuscript.

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Figure 1

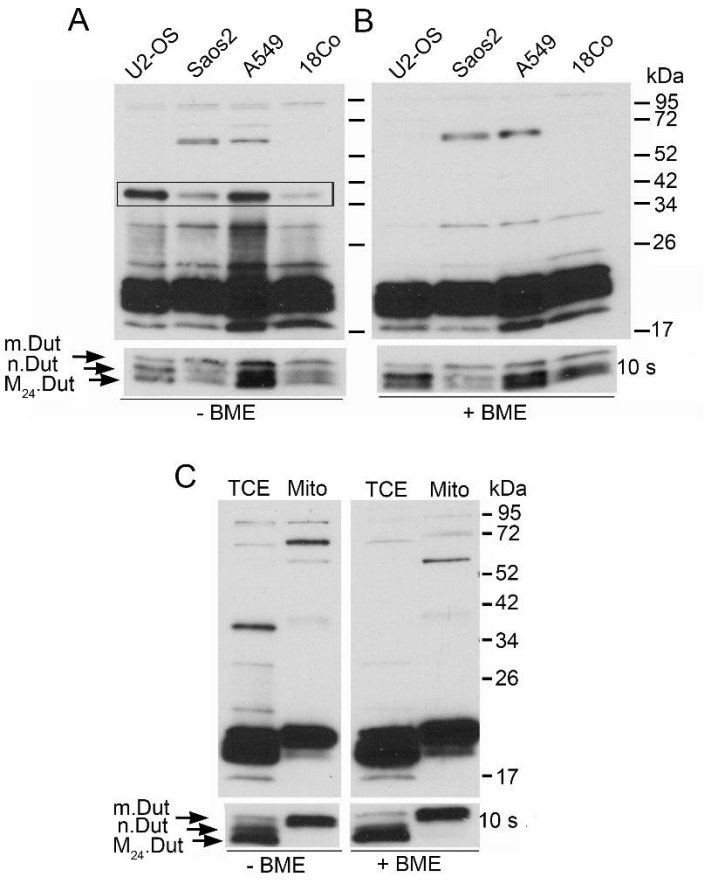

Figure 2

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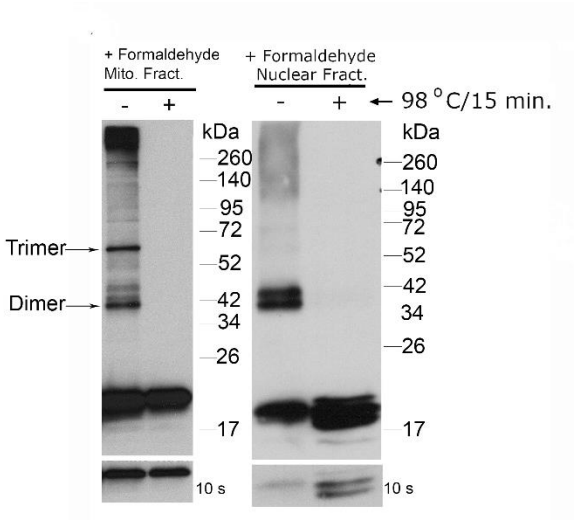

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
16% precast TGX gels	ThermoFisher	Xp00160	
175 cm^2 Flask	Cell star	658175	
18CO	ATCC	CRL-1459	
6 cm^2 dish	VWR	10861-588	
A549	ATCC	CCL-185	
Amersham ECL detection kit	GE	16817200	
Blot transfer apparatus	Biorad	153BR76789	
BME	Sigma Aldrich	M3148	
Bradford protein reagent	Biorad	5000006	
Bromophenol Blue			
BSA	Cell signaling	99985	
Cell lysis buffer	Cell signaling	9803	
Centrifuge	Eppendor	5415D	
DMEM	Gibco	11330-032	
Drill			
EDTA	Sigma Aldrich	M101	
Electrophoresis apparatus	Invitrogen	A25977	
Extra thick western blotting paper	ThermoFisher	88610	
Fetal bovine serum	Gibco	1932693	
Formaldehyde	ThermoFisher	28908	
Glass-teflon homogenizer			
Glycerol	Sigma Aldrich	65516	
Glycine	RPI	636050	
Heat block	Denville	10285-D	
Hepes	Sigma Aldrich	H0527	
Hydrochloric acid	VWR	2018010431	
Iodoacetamide	ThermoFisher	90034	
Kimwipe	Kimtech	34155	
Methanol	Pharmco	339000000	
Non-fat dry milk	Cell signaling	99995	
PBS	Sigma Aldrich	P3813	

PMSF	Sigma Aldrich	329-98-6
Posi-click tube	Denville	C2170
Power supply	Biorad	200120
Prestained marker	ThermoFisher	26619
PVDF membrane	Biorad	162-0177
Rocker	Reliable Scientific	55
Saos2	ATCC	HTB-85
SDS	Biorad	161-0302
Secondary antibody	Cell signaling	70748
Small cell scraper	Tygon	S-50HL class VI
Sodium chloride	RPI	S23020
Sodium pyruvate	Gibco	
Sonicator	Branson	450
Sponge pad for blotting	Invitrogen	E19051
Stir plate	Corning	PC353
Sucrose	Sigma Aldrich	S-1888
Tris Base	RPI	T60040
		SRE0031
Tris Buffered Saline, with Tween 20, pH 7.5	Sigma Aldrich	
Tris-Glycine running buffer	VWR	J61006
Triton X-100	Sigma Aldrich	T8787
Tween 20	Sigma Aldrich	P9416
U-2 OS	ATCC	HTB-96
X-ray film	ThermoFisher	34090

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Date:	December 3, 2018

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

RESPONSE: The manuscript has been thoroughly proofread.

2. Affiliations: Please provide an email address for each author.

RESPONSE: The email address for each author has been added to the text.

3. Abstract: Please do not include references here.

RESPONSE: The abstract has been updated without the use of references.

4. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

RESPONSE: The introduction has been updated as the following: A simple method to analyze these cysteine-stabilized multimeric complexes is through non-reducing SDS-PAGE analysis [5]. SDS-PAGE analysis is a technique used in many laboratories, where the results can be obtained and interpreted quickly, easily and with minimal costs which is advantageous over other techniques used to identify disulfide linkages such as mass spectrometry, [6,7] and circular dichroism[8].

One important step in determining if this method is an appropriate technique to aid in a study is to thoroughly examine the primary sequence of the protein of interest to insure there are cysteine residue(s) present. Another helpful step is to research any previous crystal structures published or use a bioinformatics application to explore the three dimensional structure of the protein of interest to visualize where the cysteine residue(s) may be located. If the residue(s) is present on the outside surface it may be a better candidate to form a disulfide linkage rather than a cysteine residue buried on the inside of the structure. However, it is important to note that proteins may undergo structural changes upon substrate interactions or protein-protein interactions allowing these residues to then become exposed to the environment as well.

5. Please define all abbreviations before use.

RESPONSE: The protocol has been updated to define all abbreviations.

6. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

RESPONSE: The protocol has been updated to the appropriate abbreviations.

7. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

RESPONSE: The protocol has been updated by including a space between all numerical values and their corresponding units.

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RESPONSE: The protocol has been updated by removing all commercial language.

9. Line 51, step 1: Please specify culture medium and conditions.

RESPONSE: The protocol has been updated to include culture medium and conditions.

10. 9.1: Please list an approximate volume of the buffer to prepare.

RESPONSE: The protocol has been updated to include approximate volumes of the buffer to prepare.

11. 9.2, 10.1, 10.2, etc.: The Protocol should contain only action items that direct the reader to do something. Please write the text in imperative tense in complete sentences.

RESPONSE: The protocol has been updated to imperative tense.

12. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

RESPONSE: The shorter protocol steps have been combined having each step contain 2-3 actions and maximum of 4 sentences per step.

13. Please apply single line spacing throughout the manuscript, and include single-line spaces between all paragraphs, headings, steps, etc.

RESPONSE: Single line spacing has been applied throughout the manuscript.

14. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

RESPONSE: The highlighted steps have been updated.

15. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

RESPONSE: The highlighted steps have been updated.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

RESPONSE: The highlighted steps have been updated to include all relevant details required to perform the step.

17. Is Figure 1 reprinted from a previous publication? If so, cite the figure appropriately in the Figure Legend, i.e. ³This figure has been modified from [citation].²

RESPONSE: Figure 1 is a reprint from "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)". The figure legend has been updated to include the phase “This figure has been modified from Rotoli et. al” with the proper citation.

18. Figure 1: Please change the time unit ³sec² to ³s² and include a space between the number and its time unit (10 s).

RESPONSE: Figure 1 has been updated by changing the sec to s, include a space between the number and its time unit (10 s), and include the unit of the numbers on the right.

19. Figure 2: Please include a space between the number and its temperature unit (98 °C).

RESPONSE: Figure 2 has been updated to include a space between the number and its temperature unit (98 °C).

Please indicate the unit of the numbers on the right.

RESPONSE: Figure 2 has been updated to include the unit of the numbers on the right.

20. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file. Please remove the title from the uploaded figures.

21. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

RESPONSE: The table has been updated to sort the items in alphabetical order.

22. References: Please do not abbreviate journal titles.

RESPONSE: Using the JoVE endnote format, we cannot alter the abbreviations of the journal titles.

>Reviewers' comments:
Reviewer #1:

This manuscript describes a method to analyze protein complexes in vivo (in live cells), using chemical cross-linking and non-reducing SDS-PAGE. The method and data presented are adapted from a previous publication from the same author published in 2018 entitled "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)".

Below are the criteria/guidelines listed by the Journal for this evaluation of the manuscript (in italics), and the comments on each one:

1. Are the title and abstract appropriate for this methods article?
The Title is appropriate. I think the phrase "in vivo" in the Abstract is misleading - it should be changed to "in living cells".

RESPONSE: This has been updated to in living cells throughout the manuscript.

2. Are there any other potential applications for the method/protocol the authors could

discuss?
I don't think so.

3. Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed e.g. pipettes.)
Yes. Note that "methanol" is misspelled as "methonal" in the Table.

RESPONSE: This has been updated to methanol in the table.

4. Do you think the steps listed in the procedure would lead to the described outcome?
Yes, I think so. However, it is not clear at all to me if the iodoacetamide blocking step for free cysteine described in lines 56-57 is sufficient to quantitatively block all free cysteine (which is an important part of the method). 2 minutes at room temperature with 0.1 mM iodoacetamide does not seem enough to me to accomplish this important part of the procedure - did the author do any experiments to address this concern, or to optimize this step? Or, did the author try another alkylating agent that is even more cell permeant, like N-ethylmaleimide (NEM), in place of the iodoacetamide?

RESPONSE: Iodoacetamine is a common reagent used to block free cysteine residues. Optimization of this step was done and can be reviewed in "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)". Briefly, a titration from 0.1 to 5 mM of iodoacetamide was added to the cell lysis buffer then the protein was extracted from the cells as described in the lysis procedure resulting is the same results as adding the reagent directly to cells. A criticism to this technique is that protein of interest may become oxidized by the lysis itself before the cysteines become modified by iodoacetamine. To avoid this we suggested to add the reagent directly to the cells prior to lysis.

The manuscript has been updated to include an optimization step as follows: ”Optimization of the iodoacetamide can be done using a variation of concentration and time. However, over exposure to this reagent will cause cell death.”

5. Are the steps listed in the procedure clearly explained?
Yes, but there is substantial redundancy - i.e., Section 12 repeats Section 6, and Section 13 repeats Section 7.

RESPONSE: We agree and have updated the manuscript.

Also, why are the cellular confluency levels of the U-2 OS cells different in lines 51 vs line 157? Is it important, or intended?

RESPONSE: The difference in the confluency levels of the U-2 OS cells were intentional. When fractionating for the nucleus we found there is the possibility for loss of material from each

extraction and centrifugation step. To compensate for that an increased amount of cells was used when compared to the total cell extraction demonstrated in figure 1.

Also, what are the sonicator settings used?

RESPONSE: The sonicator settings are 8 s while using the constant pulse mode at 40 %. This has been updated in the text.

6. Are any important steps missing from the procedure?
No. However, it is incorrect to state that the PVDF membranes are activated by methanol - they are wetted by methanol (lines 110 and 132).

RESPONSE: This has been updated to say "Wet the PVDF membrane by soaking it in 100% methanol for 30 s."

7. Are appropriate controls suggested?

I think using varying amounts of iodoacetamide or varying times of reaction with iodoacetamide is an important control experiment for the completeness of blocking free sulfhydryls, which is key to obtaining easily interpretable results using the method described. Another possible set of controls would be varying the amount or time of formaldehyde cross-linking, and examining the effects on results.

RESPONSE: We agree and both suggestions can be found in the discussion.

8. Are all the critical steps highlighted?
Yes.

9. Is there any additional information that would be useful to include?
Yes - Figure 2 is correctly identified as a modification of a previously published Figure (Figure 6A in "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)". However, Figure 1, which seems to be identical to already published Fig 1 in "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)", is not identified as such.

RESPONSE: Figure 1 is a reprint from "Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)". The figure legend has been updated to include the phase "This figure has been modified from Rotoli et. al" with the proper citation.

Also, the selected regions of the 10 second exposures of the Western blots shown in Figure 1A, B, and C are not identified with their molecular weights (presumably about 20 kDa), allowing the reader to easily identify which bands from the longer exposure blots above them are being shown.

RESPONSE: We used a commercially available marker that does not include a 20 kDa marker. The 10 second exposure is the monomer state of the dUTPase proteins which falls between the molecular weight markers 17 and 26 kDa. I agree it may be clearer however, we will not add a marker that was not in the standard.

In addition, there is a very central point that needs clarification regarding Figure 1 - Figure 1A is SDS-PAGE under non-reducing conditions if the dut enzymes are disulfide-linked dimers under normal physiological conditions, shouldn't the non-reduced immunoblot demonstrate only the disulfide linked dimer at about 40 kDa, rather than only a small amount of this dimer, compared to the very large amount of 20 kDa monomer (triplet) band seen in all lanes? Maybe I am missing something, or something is being left out of the description of the experiment or method? If the entire blot of Figure 1A would be shown at the 10 second exposure level, I suspect that the 40 kDa disulfide dimer band would not even be visible.

RESPONSE: Nuclear dUTPase is one of four reported isoforms present in humans. As noted on figure 1 the monomeric state of dUTPase is a combination of at least three of the isoforms of dUTPase (the mitochondria isoform, the nuclear isoform, and a truncated version notated at M24) all of which is annotated on the figure that is recognized by our polyclonal antibody. The nuclear isoform is the only isoform that forms a disulfide bond shifting its conformation to a dimer. Due to nuclear isoform being only a percentage of the monomeric state seen on the figure the western was exposed longer to demonstrate the dimeric state of that isoform.

The manuscript has been updated to the following to clarify this point. Of note, nuclear dUTPase is one of four isoforms present in humans. Three of the four isoforms have a unique amino-terminal domain while sharing a common catalytic core. As seen on the western blot the monomeric confirmation of dUTPase in human cells at the time of harvesting is a combination of at least three of the isoforms of dUTPase (the mitochondria isoform, the nuclear isoform, and a truncated version notated at M24) all of which are recognized by our polyclonal antibody. The nuclear isoform is the only isoform that contains a cysteine residue in its unique amino terminal domain. Due to the nuclear isoform being only a small percentage of the monomeric state of the proteins seen on the western, it was exposed longer to demonstrate the dimeric state of that isoform.

Are the anticipated results reasonable, and if so, are they useful to readers?
Yes.

Are any important references missing and are the included references useful?
The references are OK as is.

Additional comments and suggestions:
There are several typos:
Line 102 - "methonal"
Line 122 - "member"
Line 146 - "washers"
Line 195 - "uses"
Lines 196, 202 - "uL's"

RESPONSE: This has been updated.

Reviewer #2:
Manuscript Summary:

Thiol oxidation and reduction of cysteines is a reversible posttranslation modification where often cysteines engage in a sequence of consecutive and alternative modifications with intermediary states often difficult to detect as result of their short existence. Also, the specificity of such posttranslation modification remains an important question in the field.

Omission of the reducing agent in the sample buffer lead authors to an unplanned and fortunate discovery that cysteine residue (C3) on amino-terminus of human nuclear dUTP nucleotidohydrolase (nDUT) contributes to both dimerization and enzymatic activity. The paper describes how such an approach combined with formaldehyde cross-linking in vivo can be utilized to detect multimeric protein complexes in mammalian cell cultures. The human bone osteosarcoma cell line U-2 OS is used to validate the method. Even though the author is correct that this two-step method is beneficial due to its simplicity and low cost, there are several issues which need to be addressed in order to improve this manuscript.

Major Concerns:

1. The method lacks the appropriate controls such as amount of protein loaded in each lane. For nuclear extracts, controls such as Lamin B1 or HDAC1 are commonly used.

RESPONSE: This method is not intended to be quantitative comparing amounts of proteins from one lane to another but rather a simple yes or no as to if a larger complex was formed through a disulfide linkage in nonreducing conditions. However, we did extract proteins from each experiment starting with the same quantity of cells then loaded the same amount of extract (10 µL) per lane after extracting with 50 µL in figure 1 A and B. For the isolation of both mitochondria and nuclear extract we used the dUTPase isoforms found in each cellular compartment as an internal control of proper isolation.

2. Presence of multiple bands in Westerns can be an artifact of sometime too high

concentrations of primary and secondary antibodies, amount of protein loaded, or time of exposure. Authors should at least mention some of the troubleshooting that can be done in order to avoid artifacts or over interpretation of the data.

RESPONSE: We believe the multiple bands in the westerns are an artifact of the exposure time however, the main point of this figure is to demonstrate the appearance then disappearance of a band that correlates to the addition of the reducing agent BME when the western blots are exposed to x-ray film for the same amount of time which is clearly demonstrated.

Minor Concerns:

1. What is the origin of the doublet (dimer) in the Figure 2? Is there variation from prep to prep in regards to this band?

RESPONSE: We believe the doublet in figure 2 may be the other isoforms (variant 3 and/or 4) of dUTPase forming a complex with either themselves or with nuclear dUTPase. We have some preliminary data to confirm this however, we believe this is outside the scope of a methods paper.

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Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)

Shawna M. Rotoli, Julia L. Jones, Salvatore J. Caradonna

First published: 27 July 2018 | https://doi.org/10.1002/pro.3481

Description of supplementary materials: Experimental data supplementing and verifying methodology.

Abstract

dUTPase is an enzyme found in all organisms that have thymine as a constituent of DNA. Through evolution, humans have two major isoforms of dUTPase: a mitochondrial (mDut) and a nuclear (nDut) isoform. The nuclear isoform of dUTPase is a 164-amino-acids-long protein containing three cysteine residues. nDut's starting methionine is post-translationally cleaved, leaving four unique amino acids on its amino-terminus including one cysteine residue (C3). These are not present in the mitochondrial isoform (mDut). Using mass spectrometry analyses of recombinant dUTPase constructs, we have discovered an intermolecular disulfide bridge between cysteine-3 of each nDut monomer. We have found that these two residues stabilize a dimer configuration that is unique to the nDut isoform. We have also uncovered an intramolecular disulfide linkage between cysteine residues C78 and C134, stabilizing the monomeric state of the protein. Of note, both disulfide linkages are essential for nDut's enzymatic activity and dimeric formation can be augmented by the addition of the oxidizing agent, hydrogen peroxide to cells. Analyses of endogenous cellular dUTPase proteins confirm these differences between the two isoforms. We observed that mDut appears to be a mixture of monomer, dimer, and trimer conformations, as well as higher-order subunit interactions. In contrast, nDut appeared to exist only in monomeric and dimeric forms. Cysteine-based redox "switches" have recently emerged as a distinct class of post-translational modification. In light of this and our results, we propose that nDut possesses a redox switch whereby cysteine interactions regulate nDut's dUTP-hydrolyzing activity.

Metrics

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Details