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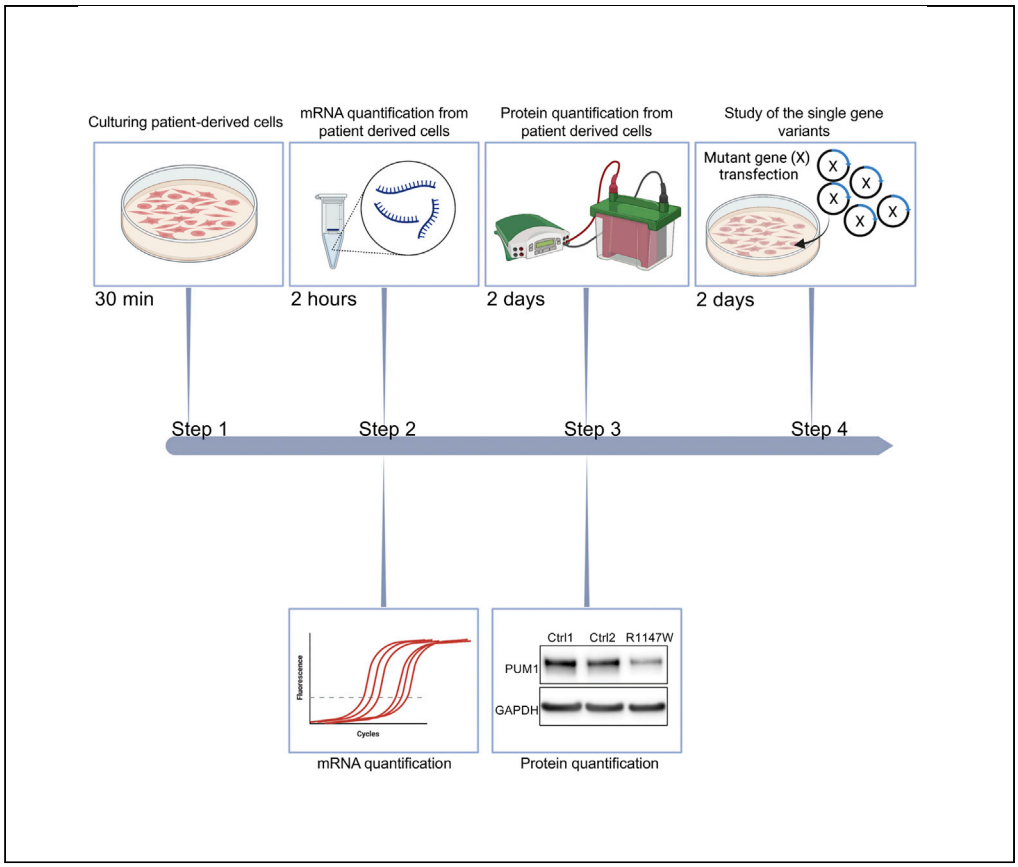
Determining the effects of loss of function mutations in human cell lines



Quantifying differences in the amount of protein and mRNA caused by missense mutations in a gene of interest can be challenging, especially when using patient-derived primary cells, which are intrinsically variable. In this protocol, we describe how to culture patient-derived lymphoblast and fibroblast cell lines for later mRNA and protein quantification. Here, we describe the steps to examine variants of PUM1 in HEK293T cells, but the protocol can also be applied to other proteins of interest.

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Highlights

Studying gene variants in patient-derived cell lines

Culturing primary fibroblasts after skin biopsy

Culturing lymphoblastoid cell lines

Transfection of mutant proteins in HEK293T cells

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Determining the effects of loss of function mutations in human cell lines

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SUMMARY

Quantifying differences in the amount of protein and mRNA caused by missense mutations in a gene of interest can be challenging, especially when using patient-derived primary cells, which are intrinsically variable. In this protocol, we describe how to culture patient-derived lymphoblast and fibroblast cell lines for later mRNA and protein quantification. We also describe the steps to examine variants of PUM1 in HEK293T cells, but the protocol can be applied to other proteins of interest.
For complete details on the use and execution of this protocol, please refer to Gennarino et al. (2018).

BEFORE YOU BEGIN

We begin with the assumption that the gene of interest has been found to produce a phenotype in the model organism, which in our case was a mouse. To then identify human patients and establish the pathogenicity of genetic variants will require a more comprehensive analysis and will take some time.

⚠ CRITICAL: When working with DNA and RNA derived from patient cells, you must strenuously avoid contamination from your environment—else you risk unreliable results and the loss of months of work, labor, and materials. Wear gloves, clean your bench with a 70% ethanol solution, use RNaseZap or other commercially available cleaning solutions when extracting RNA; use nuclease-free disposable plasticware and filtered tips. Refer to ''materials and equipment'' to prepare solutions, materials, and tools prior to the procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER (CAT#)
Expression vectors		
*PUM1*cDNA expression vector	OmickLink Expression Vector, GeneCopoeia	pEZ-M02, EX-E2337-M02-10
pEGFP-C1	Golenbock Lab	(Gennarino et al., 2018)
Empty expression vector	OmickLink Expression Vector, GeneCopoeia	EX-NEG-M02
OmickLink™Expression Vector pEZ-M02	GeneCopoeia	EX-E2337-M02-10

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REAGENT or RESOURCE	SOURCE	IDENTIFIER (CAT#)
OmickLink™Expression Vector	GeneCopoeia	EX-NEG-M02
Critical commercial assays/kits and instruments		
jetPRIME Transfection Reagent	Polyplus-transfection	114-07
QuikChange Lightning Multi Site- Directed Mutagenesis Kit	Agilent Technologies	210515
miRNeasy Mini kit	QIAGEN	217004
QuantiTect Reverse Transcription kit	QIAGEN	205311
PowerUP SYBR Green Master Mix	Thermo Fisher Scientific	A25743
NanoDrop 1000 V3.8	Thermo Fisher Scientific	ND-ONE-W
CFX96 Touch Real-Time PCR Detection System	Bio-Rad Laboratories	Model 855196
Nitrocellulose Blotting membrane 0.45 µm	Amersham Protran	10600002
Tissue culture		
Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (4.5 g/L D-glucose)	Thermo Fisher Scientific	11965084
RPMI 1640 medium	Thermo Fisher Scientific	11875093
L-glutamine 200 mM (100×)	GenDepot	CA009-010
Normocin	InvivoGen	ant-nr-2
Penicillin/Streptomycin	Thermo Fisher Scientific	21103049
Heat Inactivated-Fetal Bovine Serum (FBS)	Novus Biologicals	S11150H
Triton X-100	MilliporeSigma	T8787
NuPAGE 4%–12% Bis-Tris Gel	Thermo Fisher Scientific	NP0321PK2
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225
Trypsin-EDTA 1×	MilliporeSigma	59417C
Flat Bottom Cell Culture Plates (6-Well)	Corning	07-200-83
Tissue-culture treated dishes 10 cm	Corning	CLS430167
Cell Culture Flask 25 cm^2 vent cap	Alkali scientific	TVN0025
Chemicals and reagents		
Formamide	MilliporSigma	FX0420-6
Tris-HCl (pH 7.5, 1 M)	MilliporeSigma	15567027
NP-40	Thermo Fisher Scientific	85124
Sodium Deoxycholate	Thermo Fisher Scientific	89905
Tris Base	MilliporeSigma	10708976001
Sodium Azide	Fisher Scientific	BP922I500
Yeast Extract	MilliporeSigma	BP1422500
Tris-Acetate-EDTA buffer (TAE)	Boston BioProducts	BM-250
Bromophenol blue	MilliporeSigma	B0126-25G
Glycine	MilliporeSigma	410225-250G
Methanol	MilliporeSigma	322415-1L
HCl	MilliporeSigma	320331-500ML
NaOH	MilliporeSigma	221465
NaCl	MilliporeSigma	S3014
Polyethylene Glycol (PEG) 300	MilliporeSigma	807484100
Polyethylene Glycol (PEG) 400	MilliporeSigma	8074850050
Nonfat dry milk	Merck Millipore	M7409
NZ amine (casein hydrolysate)	Fisher Scientific	NC9874278
Experimental models: cell lines		
Human embryonic kidney immortalized 293 cells (HEK293T)	Zoghbi Lab	(Gennarino et al., 2018)
Software and database		
Primer3plus	https://primer3plus.com/cgi-bin/dev/primer3plus.cgi	Whitehead Institute (Untergasser et al., 2012)
QuikChange software	https://www.agilent.com/store/primerDesignProgram.jsp	Agilent

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER (CAT#)
Oligonucleotides		
Human *GAPDH:* Forward, 5′-CGACCACTTTGTCAAGCTCA-3′	n/a	(Gennarino et al., 2018)
Human *GAPDH:* Reverse, 5′-TTACTCCTTGGAGGCCATGT-3′	n/a	(Gennarino et al., 2018)
Human *PUM1:* Forward, 5′-GCCCCAGTCTTTGCAATTTA-3′	n/a	(Gennarino et al., 2018)
Human *PUM1:* Reverse, 5′ AATCACTCGGCAGCCATAAG-3′	n/a	(Gennarino et al., 2018)
Human *ATXN1:* Forward 5′-CCAGGTCAGCGTTGAAGTTT-3′	n/a	(Gennarino et al., 2018)
Human *ATXN1:* Reverse 5′-CAAAGAGCTGGCTGGTTCTC-3′	n/a	(Gennarino et al., 2018)
Human *E2F3:* Forward 5′-AAAGCCCCTCCAGAAACAAG-3′	n/a	(Gennarino et al., 2018)
Human *E2F3:* Forward 5′-AATGGGCCCTTGGGTACTT-3′	n/a	(Gennarino et al., 2018)
Subject 10: 5′-CATGACGATCTTCCACTGGCCTGGCTCC-3′	n/a	(Gennarino et al., 2018)
Subject 11: 5′-GCGATGTGGGGCCAGATCTTATGCAT GACGA-3′	n/a	(Gennarino et al., 2018)
Subject 12/13/14: 5′-GTACAAGCTGCTCTGAGTGCTGGT GAAGCTC-3′	n/a	(Gennarino et al., 2018)
Antibodies		
Goat polyclonal anti-PUM1	Bethyl Laboratories	A300-201A
Mouse monoclonal anti-GAPDH	MilliporeSigma	CB1001
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Fisher Scientific	G-21040
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Fisher Scientific	G-21234
Patient-derived cell lines		
Subject 11	Sydney Children's Hospital	(Gennarino et al., 2018)
Subject 12 and 13	Care4Rare Canada	(Gennarino et al., 2018)

MATERIALS AND EQUIPMENT

All essential buffers and solutions should be freshly made prior to sample preparation and used the same day to avoid degradation or loss of efficacy. Make sure that there is enough of all solutions that are required for tissue homogenization, removal of interfering chemicals, RNA extraction, cDNA synthesis, protein extraction and western blot.

Radio immunoprecipitation assay (RIPA) buffer		
Reagent	Final concentration	Amount
Tris-HCl (pH 7.5, 1 M)	25 mM	12.5 mL
NaCl	150 mM	4.4 g
NP-40	1%	50 mL
Sodium Deoxycholate	1%	5 g
Sodium dodecyl sulfate (SDS)	0.1%	0.5 g
H2O milliQ		Up to 500 mL

Note: the solution can be stored at 4°C or room temperature and completed by adding protease 1× and phosphatase inhibitors 1× just before starting the protein extraction.

⚠ CRITICAL: Adjust the pH to 7.5.

Transfer buffer 10×		
Reagent	Final concentration	Amount
Tris Base	250 mM	60.4 g
Glycine	1.92 M	288 g
H2O milliQ		Up to 2 L

Note: Store at 4°C.

Transfer buffer 1×		
Reagent	Final concentration	Amount
Transfer Buffer 10×	1×	100 mL
Methanol	15%	150 mL
H2O milliQ		Up to 1 L
Caution: Methanol is neurotoxic, use under chemical hood. Store at 4°C.		

Tris-buffered saline-tween (TBS-T) 20×		
Reagent	Final concentration	Amount
Tris Base	0.4 M	121.1 g
Tween20	0.1%	20 mL
H2O milliQ		Up to 1 L

Note: Store at room temperature.

⚠ CRITICAL: Adjust pH of the solution to 7.5.

Tris-buffered saline-tween (TBS-T) 1×		
Reagent	Final concentration	Amount
TBS-T 20×	1×	50 mL
H2O milliQ		Up to 1 L

Note: Store at room temperature.

TBS-T 1× – BSA- sodium azide		
Reagent	Final concentration	Amount
TBS-T 1×	1×	500 mL
BSA	3%	15 g
Sodium Azide	0.02%	10 mg

⚠ CRITICAL: Filter the solution with a 0.02 mm filter and store at 4°C. Sodium Azide is irritant, use under chemical hood.

Blocking solution for western blot		
Reagent	Final concentration	Amount
TBS-T 1×	1×	50 mL
Nonfat dry milk	5%	2.5 g

⚠ CRITICAL: Prepare fresh and store at 4°C between day 1 and day 2.

NZY^+ broth		
Reagent	Final concentration	Amount
NZ amine (casein hydrolysate)	–	10 g
NaCl	–	5 g
Yeast extract	–	5 g
H2O milliQ	–	Up to 1 L

STEP-BY-STEP METHOD DETAILS

Note: The steps below are only for the protocols we modified from the manufacturer's suggestion.

Culturing patient-derived fibroblast and lymphoblastoid cell lines

The acquisition of patient samples is not always easy, particularly in the case of children who have already been subjected to multiple stressful tests. The patient's clinician is in the best position to speak to the patient to see if they are willing to provide biological samples. Even so, we were able to procure fibroblasts or lymphoblastoid cell lines from only three unrelated patients carrying a single *de novo* variant in *PUM1* in addition to three age- and sex-matched healthy controls.

Note: The informed consent from the patient is always needed. If the sample derives from a third party such as a biobank and the subject is de-identified, it is likely that the consent form was already acquired by the third party. Regulations can differ between countries and institutions, so we recommend that you procure Institutional Review Board (IRB) approval before attempting to work with human specimens or clinical data.

Culturing patient-derived cell lines from frozen vials

Timing: 1 h

Note: Primary patient-derived cell lines can arrive either in frozen vials or in their culturing medium. To increase the chances of cell survival, we strongly suggest you re-culture the cells, as soon as you receive them, with fresh culturing medium.

Note: Fibroblasts and lymphoblastoid cells are cultured at 37°C in a humidified chamber with 5% CO_2.

1. Thaw the cells by placing the vial in a water bath set at 37°C for 2–5 min.
2. Transfer the contents of the vial to a 15 mL conical tube containing 5 mL of warm (~37°C) complete culturing medium.
3. Centrifuge at 500 g for 5 min to obtain a pellet of cells.
4. Aspirate the supernatant without disturbing the cell pellet.

5. Add 10 mL of warm (~37°C) complete medium and gently resuspend the pellet by pipetting up and down.
6. Transfer 10 mL of complete medium containing the cells to a T25 flask or 10 cm cell culture-treated dish.

Note: Since lymphoblastoid cells grow as suspension, we recommend using T25 flasks.

7. The next day replace the medium with fresh complete medium.

Culturing patient-derived cell lines from cells in culture

8. Aspirate the medium and add 10 mL of warm (~37°C) fresh complete medium.
9. Replace the medium the next day with fresh medium.

⚠ CRITICAL: Primary patient-derived cell lines are a precious resource and asking for new cells is usually not feasible. Therefore, before using the cells for any experiments, we recommend you expand the cells to have good number of vials as backup in liquid nitrogen for long storage. Having a backup will enable you to maintain the cells in low passage number (many experiments in human cells work better this way).

Expanding fibroblast cell lines

Timing: 15 min

Fibroblast culturing medium

Reagent	Final concentration	Amount
Dulbecco's Modified Eagle's Medium (DMEM)	–	450 mL
Heat-inactivated fetal bovine serum (FBS)	10%	50 mL
Penicillin/streptomycin	1%	5 mL
L-Glutamine	1%	5 mL
Normocin	–	1 mL
Total		500 mL

10. Once the fibroblasts are at 85%–90% of plate confluency: Aspirate the medium from the plate, trying to avoid disturbing the cells.
11. Add 5 mL of phosphate buffer (PBS) 1× to rinse the remaining medium from the plate.
12. Add 1 mL of warm (~37°C) Trypsin-EDTA 1× to the center of the plate.

Note: make sure the Trypsin-EDTA 1× contacts the entire plate surface.

13. Place the plate in the incubator for 5 min.
14. Using a P1000 pipette, mechanically detach the cells.
15. Add 4 mL of fibroblast culturing medium.
16. Transfer the volume in a 1:4 or 1:5 ratio to a new 10 cm cell culture treated dish.

⚠ CRITICAL: Fibroblasts grow as a monolayer and should be re-fed with fresh medium or split every 3–4 days at a ratio of 1:4 or 1:5, depending on how fast the lineage grows.

Expanding lymphoblastoid cell lines

Timing: 15 min

Lymphoblastoid culturing medium		
Reagent	Final concentration	Amount
RPMI-1640	–	450 mL
Heat-inactivated fetal bovine serum (FBS)	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
L-Glutamine	1%	5 mL
Normocin	–	1 mL
Total		500 mL

Note: After 3–4 days from the thawing, the cells should be sub-cultured. The culturing plateau level for lymphoblastoid cells is 1×10^6 cells/mL; at this confluency the medium will be acidic and the phenol red used as pH indicator will turn yellow.

17. Pipette up and down for 5 times using a 10 mL serological pipette.
18. Transfer the cells in a new 50 mL conical.
19. Take 10 µl of the volume and count the cells.
20. Seed 200,000–300,000 cells/mL in a new T25 flask.

⚠ CRITICAL: Lymphoblastoid cells grow in suspension with cells clumped in aggregates. They should be seeded at a concentration of 200,000–300,000 cells/mL and need subculture every 3–4 days.

mRNA extraction and quantification from patient-derived cell lines

Timing: 1–2 h

To extract total RNA from human patient or control fibroblast cell lines, cells were seeded in 6-well plates and harvested at 80% confluency (1×10^6 cells), whereas lymphoblastoid cell suspension cultures were collected at 6×10^6 confluence before proceeding for RNA extraction. Total RNA was obtained using the miRNeasy Mini kit (Qiagen).

⚠ CRITICAL: RNA is subject to degradation; even our hands contain RNase. It is therefore imperative to work in a clean environment: clean the work surface and the pipettes with RNase decontamination solutions such as RNaseZap, use only filtered pipette tips, clean your gloves with 70% alcohol after you put them on.

⚠ CRITICAL: QIAzol Lysis Reagent is a monophasic solution that consists of phenol and guanidine thiocyanate. Since phenol is toxic, the QIAzol solution should be handled with caution and always under a chemical fume hood. Laboratories working with phenol should use polyethylene glycol 300 or 400 (PEG-300 or PEG-400), rather than water, for immediate first aid treatment of dermal exposures. In addition, anyone using phenol should use appropriate personal protective equipment (PPE), including lab coat and nitrile gloves. If QIAzol contacts the gloves, it will penetrate the material within a couple of minutes, so immediately change your gloves. Similar precautions should be followed when working with chloroform.

21. Add 700 µL QIAzol Lysis Reagent to the cell pellet in a 1.7 mL tube and lyse the cells by pipetting rather vigorously, around 20 times up and down using a p200 pipette set at 200 µL.
22. Incubate at room temperature for 5 min.
23. Add 140 µL of chloroform (for each 700 µL of QIAzol) to the mix and shake vigorously for 15 s.

24. Incubate at room temperature for 3 min.
25. Centrifuge the lysate for 15 min, 12,000 g at 4°C. It is not necessary to use low brake for this step.
26. Carefully transfer the upper aqueous phase to a new collection tube and add 1.5 volumes of 100% ethanol. The phases should not be mixed.

Note: If you accidentally mix or disturb the phases, you must repeat from step 25.

⚠ CRITICAL: Perform steps 21–26 under chemical fume hood.

27. Load up to 700 µL of sample into a RNeasy Mini column placed in a 2 mL collection tube.
28. Centrifuge at 11,000 rpm for 15 s at room temperature, discarding the flow-through.
29. Add 350 µL buffer RWT to the RNAeasy Mini column and spin for 15 s at 11,000 rpm.
30. Discard the flow-through.
31. Add 10 µL DNAseI stock solution to 70 µL buffer RDD for each sample, mix gently by inverting the tube and spin briefly.
32. Add 80 µL of DNAseI reaction directly on the RNAeasy Mini column filter.
33. Incubate 15 min at RT.
34. Add 350 µL buffer RWT to the RNeasy spin column and centrifuge 15 s at 11,000 rpm.

Note: The DNAseI treatment purifies the RNA solution from genomic DNA contaminants. This treatment is optional, but we strongly recommend it if you plan to do qPCR; if you do not perform qPCR, you can skip steps 31–33.

35. Add 700 µL of RWT buffer to wash the column.
36. Centrifuge at 11,000 rpm for 30 s, discard the flow-through.
37. Add 500 µL of RPE wash buffer into the RNAeasy Mini column.
38. Centrifuge at 11,000 rpm for 30 s, discard the flow-through.
39. Add 500 µL of RPE wash buffer into the RNAeasy Mini column.
40. Centrifuge at 11,000 rpm for 2 min.
41. Place the RNeasy Mini column into a new 1.5 mL collection tube and centrifuge at full speed for 1 min.
42. Transfer the RNeasy Mini column to a new 1.5 mL collection tube.
43. Add pre-warmed (60°C) 30–50 µL RNease free water, directly into the column membrane.
44. After 5 min of incubation centrifuge at 11,000 rpm for 1 min.
45. Quantify RNA using NanoDrop 1000.
46. RNA can be stored at −20°C or −80°C for a longer preservation.

Note: Freezing and thawing the RNA could cause degradation. We recommend dividing the extracted RNA into small aliquots that can be thawed when needed.

RNA quality assessment

Note: RNA quality can be assessed by 1% agarose gel electrophoresis in TAE running buffer. The RNA quality assessment through the electrophoresis gives a different information compared to the information given by the 260/280 and 260/230 absorbance ratios. From the electrophoresis the operator will be able to see an overall RNA degradation. This information cannot be obtained from the 260/280 or 260/230 ratio since these analyses will measure protein and DNA contamination, respectively, regardless if the RNA is degraded or not.

⚠ CRITICAL: Prepare a 3× bromophenol blue loading dye by diluting the commercial 6× loading dye 1:1 in formamide. Rinse the gel chamber and the comb once with NaOH 0.4N and four to five times with abundant deionized water; let them dry before using them.

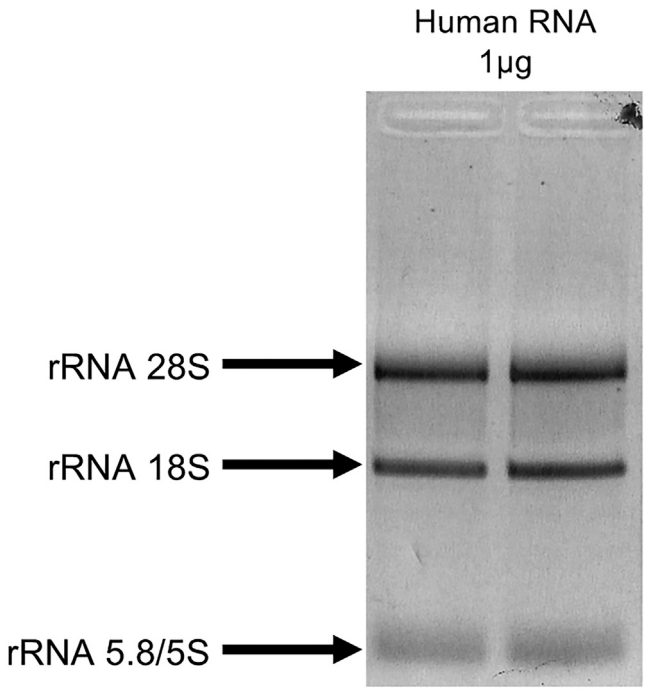

Figure 1. Agarose gel for RNA quality assessment
Representative image of agarose gel showing two RNA samples.
The three bands are the rRNA 28S, 18S and 5/5.8S.

Note: 150 ng to 1 µg of total RNA can be loaded on the agarose gel. 1 µg of RNA, when possible, will give you a better resolution.

47. Mix 3 µL of 3× formamide bromophenol blue loading dye with 1 µg of the extracted RNA and bring the mix volume up to 9 µL with nuclease-free water in order to obtain a 1× loading dye solution.
48. Mix gently without vortexing.
49. Spin down quickly and incubate at 60°C for 10 min.
50. Place on ice for 2 min.
51. Load 9 µL of RNA/dye solution on the 1% agarose TAE gel.
52. Run the gel at low speed until the loading blue runs over half of the gel.
53. Image the gel in a UV transilluminator. If the RNA quality is optimal, three bands will be visible corresponding to rRNA 28S, 18S, and 5-5.8S. If the RNA degrades, you will see a smear between those three bands (Figure 1).

Note: Alternatively, the quality of the RNA can be assessed using the Bioanalyzer System which performs a microfluidic-based resolution automated electrophoresis. This system can provide information on both RNA integrity and quality.

cDNA synthesis

Timing: 1 h

Thaw gDNA wipeout buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water on ice. Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then store on ice.

Note: The QuantiTect Reverse Transcription kit can retrotranscribe up to 1 μg of RNA (see key resources table).

54. Thaw previously frozen RNA on ice.
55. Prepare the genomic DNA elimination reaction on ice, adding 2 μL of gDNA wipeout buffer to a tube containing 1 μg of RNA. Bring the volume up to 12 μL with nuclease-free water.
56. Incubate for 8 min at 42°C. Then place immediately on ice.

⚠ CRITICAL: Do not incubate at 42°C for longer than 10 min.

57. Add to each reaction tube 1 μL of RT Primer Mix, 4 μL of Quantiscript RT Buffer and 1 μL of Quantiscript Reverse Transcriptase.
58. Incubate for 30 min at 42°C.
59. Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.
60. Store the cDNA at 4°C for short term storage or at −20°C for long term storage.

Quantitative RT-polymerase chain reaction (qRT-PCR)

In order to quantify mRNA level changes between the patient-derived cells and the control cell lines, perform quantitative RT-polymerase chain reaction (qRT-PCR). In our original study (Gennarino et al., 2018), we used the CFX96 Touch Real-Time PCR Detection System with PowerUP SYBR Green Master Mix to analyze *PUM1*, *ATXN1* and *E2F3* (see key resources table). Results were analyzed using the comparative Ct method normalized against the housekeeping gene *GAPDH* (Vandesompele et al., 2002). To ensure the efficacy of genomic DNA elimination, we ran negative control samples in the qRT-PCR that did not have reverse transcriptase (minus-RT) in the cDNA synthesis reaction.

Protein extraction and quantification in patient-derived cell lines

Timing: 2 days

To measure PUM1 quantities in patient-derived cells lines, we first tested different antibodies to find the one that gave the best signal with the lowest background. Since patient-derived cell lines are highly variable, we recommend repeating the experiment no less than 6–9 times after proper optimization.

⚠ CRITICAL: Patient-derived cell lines need to be compared with healthy donor cell lines that must be matched according to cell type, sex, and age. Again, given the variability of primary human cells, we suggest using at least 2 to 3 healthy donors as control comparisons for each patient-derived cell line.

61. Aspirate the medium from the plate without disturbing the cells.
62. Add 5 mL of phosphate buffer (PBS) 1× to rinse the plate from the remaining medium.
63. Add 1 mL of warm (~37°C) Trypsin-EDTA 1×.
64. Place the plate in the incubator for 5 min.
65. Using a P-1000 pipette, mechanically detach the cells by pipetting up and down.
66. Add 4 mL of fibroblast culturing medium.
67. Transfer the cells in a 15 mL conical and centrifuge at 500 g for 5 min.
68. Aspirate the medium without disturbing the pellet.
69. Add 300 μL of cold RIPA buffer completed with phosphatase and protease inhibitors 1×.
70. Using a P-1000 pipette dissolve the pellet pipetting up and down for 10–15 times.
71. Transfer the 300 μL of lysate in a 1.7 mL Eppendorf tube.
72. Place the tube in ice for 20 min.
73. Centrifuge for 20 min at 21,000 g at 4°C.
74. Transfer the supernatant in a new 1.7 mL Eppendorf tube and place it in ice.

75. Quantify the protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Note: To evaluate the protein concentration with this method, prepare a bovine serum albumin (BSA) standard curve with 2 μg/μL, 1 μg/μL, 0.5 μg/μL and a sample only containing the BCA mix.

a. mix reagent A and B in a ratio of 50:1 for a total volume of 40 μL per sample.
b. add 38 μL of BCA mix to a new 1.7 mL Eppendorf tube.
c. add 2 μL of protein extract.
d. add 2 μL of the BSA curve for each standard point.
e. vortex and spin the tubes briefly.
f. incubate for 30 min at 37°C.
g. analyze the absorbance at 562 nm using the NanoDrop OneC (Thermo Scientific) and based on the standard curve calculate the protein concentration (mg/mL).

76. Mix the extracted protein with NuPAGE Reducing Agent 10× (Thermo Fisher Scientific) and NuPAGE LDS Sample Buffer 4×.

Note: Consider a final volume of 30 μL–40 μL per well if using the 1.5 mm 10-wells gel or 20 μL–30 μL per well if using the 1.5 mm 15-wells gel.

77. Denature the proteins at 95°C for 10 min.
78. Pour 100–150 mL of running buffer to cover the bottom of the mini cell (Figure 2A).
79. Place a NuPAGE 4%–14% Bis-Tris Gel (10 wells 1.5 mm) in a mini gel cassette (Thermo Fisher Scientific) (Figure 2B).

Note: The wells of NuPAGE 4%–14% Bis-Tris Gel can sometimes contain residual part of gel, make sure of cleaning them by pipetting up and down 100–150 μL of running buffer into the wells.

80. Load the samples and run until the dye front reaches the bottom of the gel.

Note: Run at 70 V until the dye front surpasses the stacking gel (upper part of the gel), then increase to 120 V. To have well-shaped bands in the western blot, we do not recommend running the gel any faster than 120 V, particularly for proteins with a high molecular weight.

81. Prepare 1 L of transfer solution to transfer the proteins from the acrylamide gel to a nitrocellulose membrane.
82. Prepare the transfer sandwich (Figure 2C):

⚠ CRITICAL: Activate the nitrocellulose membrane in methanol or in transfer solution for 20–30 s.

a. Pour 500 mL of transfer buffer in the wide-set glass container and the remaining 500 mL in the transfer apparatus.
b. Place the sandwich with the black side facing down (Figure 2D).
c. Immerse for 10 s a sponge into the transfer buffer and place it over the black side of the sandwich (Figure 2D).
d. Open the gel support and with the help of a filter paper carefully detach the acrylamide gel from the plastic support (Figures 2E and 2F).
e. Place the filter paper and the acrylamide gel over the sponge with the gel facing up (Figure 2G).

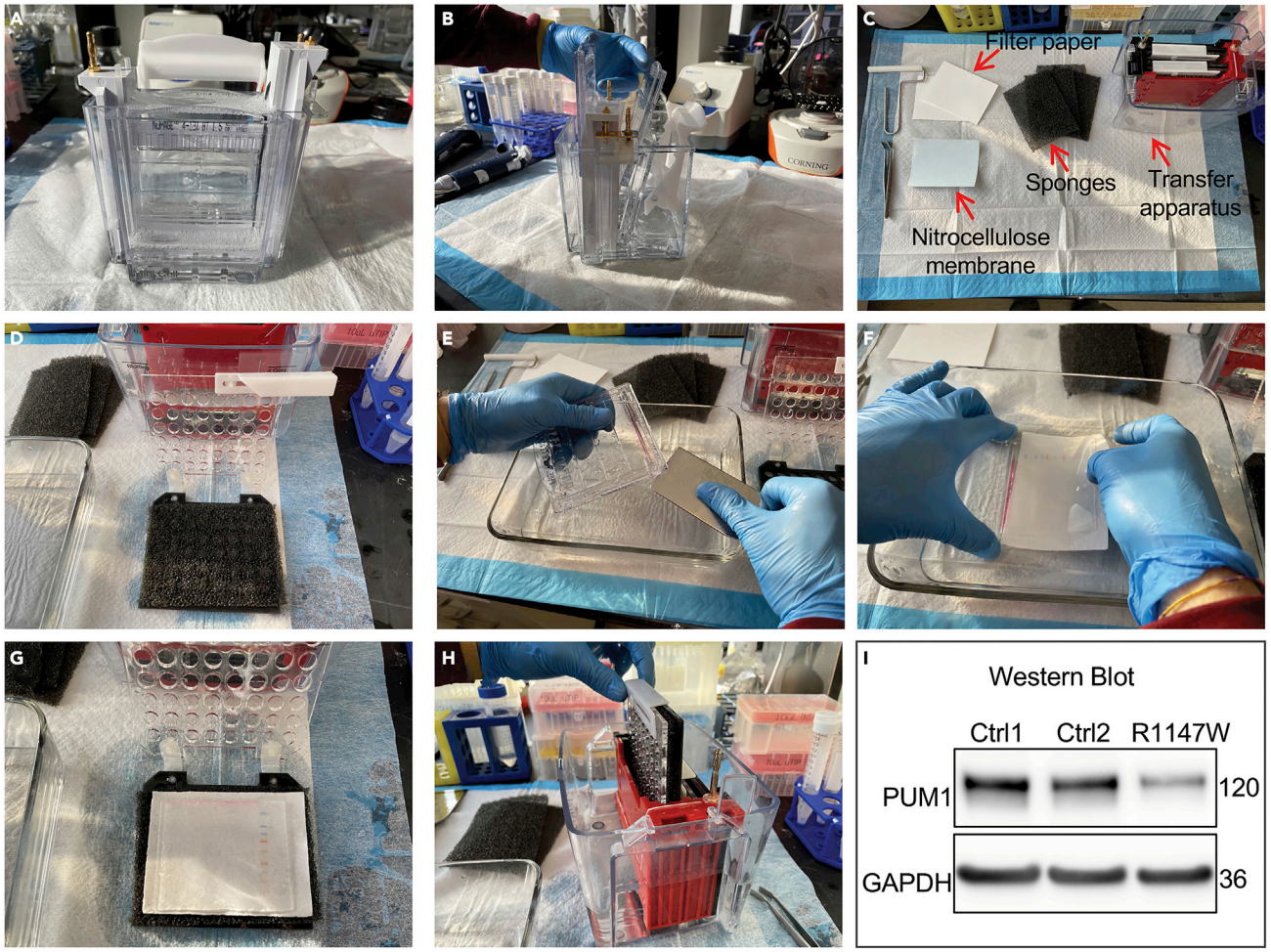

Figure 2. Western blot for the analysis of PUM1 levels in patient derived fibroblasts

(A) Picture of NuPage mini cell chamber containing running buffer to cover the bottom part.

(B) Lateral picture of the NuPage mini cell chamber loaded with a 1.5 mm 10 wells gel (asterisk) and a plastic adaptor on the opposite side.

(C) Components to assemble the transfer sandwich.

(D–H) Steps of the sandwich assembly (see text for details).

(I) PUM1 western blot redout from patient-derived cells. KDa: Kilodaltons; Ctrl1 and Ctrl2: age and sex match controls; S11: patient carrying the PUM1 *de novo* missense mutation (R1147W).

f. Gently put the nitrocellulose membrane over the acrylamide gel.
g. Immerse a filter paper for 5 s in the transfer buffer and put it over the nitrocellulose membrane.
h. Immerse the roller in the transfer buffer and gently roll over the chromatography paper to make sure no air-bubble between the acrylamide gel and the nitrocellulose membrane will remain.
i. Immerse for 10 s the sponge into the running buffer.
j. Put it on top of the chromatography paper and close the sandwich.
k. Place it into the apparatus with the black side facing the black side of the apparatus (Figure 2H).
l. Transfer the 500 mL from the wide-set glass container to the transfer apparatus.

Note: If transferring only one acrylamide gel, put two sponges wet of running buffer in the other sandwich.

83. Run the transfer applying a constant current of 340 mA for 90 min in cold room (4°C).
84. Prepare blocking solution dissolving 2.5 g of nonfat milk in 50 mL of TBS-T 1× to obtain a solution of milk at 5%.
85. Remove the nitrocellulose membrane, with the transferred proteins, from the transfer apparatus and incubate (blocking) with milk 5% in TBS-T 1× for at least 30 min at room temperature.
86. Rinse the membrane 1–2 times with TBS-T 1× to remove the excess of milk.
87. Incubate the membrane with the antibody of interest dissolved in TBS-T 1× with 3% BSA and 0.02% of Sodium Azide to a final concentration indicated in the manufacturer's data sheet overnight at 4°C.
88. Wash membranes with TBS-T 1× for 10 min each for three times.
89. Incubate the membrane with secondary antibody horseradish peroxidase, HRP-conjugated, dissolved in milk 5× at a final concentration of 1:10,000 for at least 1 h at room temperature.
90. To image the blot use ECL solution to reveal the images. All the blots are acquired on the G:BOX Chemi XX9 machine (Syngene; Frederick, MD) using GeneSys software 1.6.5.0. Gel exposures are determined by the software. (Figure 2I).

Note: To appreciate modest differences in protein expression, we found that several steps required optimization: i) dilution rates for the primary and secondary antibodies can vary according to your antibody, protein of interest, cell lines, and tissue. Run a few western blots starting from the highest dilution ratio (as recommended by the manufacturer) to the lowest ratio until you will be able to visualize a clear band in your gel; ii) while primary antibodies can be diluted in TBS-T 1×-BSA 3%, other antibodies will work better in TBS-T 1× in 5% non-fat milk. Because antibodies in milk cannot be re-used more than twice, and therefore can become rather expensive, run a western with the primary antibody diluted in both solutions to see which will give you a better resolution; iii) to minimize smearing, avoid running the gel faster than 120 V for proteins weighing more than 90 kDa; iv) to transfer as much protein as possible from your gel to your membrane to ensure a correct quantification, be aware of the influence of protein size. Many labs use a fast transfer technique, but proteins >90 kDa should be transferred at a power no greater than 340 mA for no less than 90 min but not more than 120 min; v) if your protein is highly expressed, use larger wells such as the 10-well gel at 1.5 mm. However, if you want to increase the signal for a protein that is expressed at low levels, use 15 wells at 1.5 mm. In the case of PUM1, 15-well gels did not show as clear a band as the10-well gels.

Study the single gene variant in human cell lines

HEK293T cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2. These are fast-growing cells that require high glucose DMEM (Invitrogen) medium to survive. Culturing media should contain broad-spectrum antibiotics such as 1% penicillin/streptomycin to reduce the chances of bacterial contamination. We also add 10% of heat-inactivated fetal bovine serum (FBS) to supplement the basal medium with nutrients derived from the serum (vitamins, amino acids, growth factors, etc.) and 1% L-Glutamine.

The purpose of this section is to guide the reader testing the effect of one or more mutations of the disease-driving gene. In the specific case of PUM1, the readout of the overexpression using PUM1 mutants was to address the protein and mRNA levels of the PUM1 targets ATXN1 and E2F3.

Transfection of mammalian gene expression vectors into HEK293T cells

Timing: 2 days

Note: To transfect HEK293T cells we used jetPRIME Transfection Reagent (see key resources table).

91. Seed HEK293T cells in 12 well-plates at a density of 0.1 × 10^6 cells/well.
92. On day two, cells should be at 70% confluence, ready to be transfected with either full or mutant cDNA of *PUM1* (3567 nt) cloned into a mammalian expression vector OmickLink™Expression Vector pEZ-M02.
93. Dilute the desired amount of DNA in 75 μL of jetPRIME buffer, vortex for 10 s and quickly spin down.
94. Add 1.6 μL of jetPRIME reagent, vortex briefly, spin down and incubate for 10 min at room temperature.
95. Add the transfection mix to each well and place the plate in the incubator for 48 h.
96. The day after transfection, aspirate the medium containing the transfection mix and replace with fresh complete culture medium.
97. After 48 h post-transfection, collect the cells and process for RNA or protein extraction.

Note: In our study, we transfected 0.5 μg, 1 μg and 2 μg of DNA to test the effect of different protein dosages on *PUM1* targets.

Mutagenesis

We created three *PUM1* mutants (R1139W, R1147W and T1035S) to insert into HEK293T cells by using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit. We designed the primers for mutagenesis using QuikChange software (key resources table).

98. Prepare the sample reaction mix as follow:

Reagents	Final concentration	Amount
Reaction Buffer 10×	1×	5 μL
Oligonucleotide Forward (10 μM)	0.2 μM	1 μL
Oligonucleotide Reverse (10 μM)	0.2 μM	1 μL
dNTP mix 10 μM	0.2 μM	1 μL
QuikSolution		3 μL
DNA plasmid		50 ng
PfuUltra HF DNA polymerase	2.5 U	1 μL
H_2O nuclease-free		Up to 50 μL

99. Run the PCR as follows:

PCR cycling setup			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	50 s	18 cycles
Annealing	60°C	50 s	
Extension	68°C	13 min (1 min/kb)	
Final extension	72°C	7 min	1
Hold	4°C	infinite	

100. Add 1 μL of the DpnI restriction enzyme (10 U/μL) directly to each reaction tube once the PCR is completed.
101. Gently but thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 min, then immediately incubate the reactions at 37°C for 1 h to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Note: It is important to adhere to the 18-cycle limit when cycling the mutagenesis reactions. More than 18 cycles can impair reaction efficiency.

102. Gently thaw the XL10-Gold ultracompetent cells on ice. For each control and sample reaction to be transformed, aliquot 45 μL of the ultracompetent cells to a prechilled 14-mL BD Falcon polypropylene round-bottom tube.
103. Add 2 μL of the β-ME mix.
104. Gently swirl the contents of the tube. Incubate the cells on ice for 10 min, swirling gently every 2 min.
105. Transfer 2 μL of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 min.
106. Preheat NZY+ broth in a 42°C water bath.
107. Heat-pulse the tubes in a 42°C water bath for 30 s. The duration of the heat pulse is critical for obtaining the highest efficiencies. Do not exceed 42°C.
108. Incubate the tubes on ice for 2 min.
109. Add 0.5 mL of preheated (42°C) NZY+ broth to each tube, then incubate the tubes at 37°C for 1 h with shaking at 225–250 rpm.
110. Plate the appropriate volume of each transformation reaction, as indicated in the table below, on agar plates containing the appropriate antibiotic for the plasmid vector.
111. Incubate the transformation plates at 37°C for >16 h.
112. Screen the positive colonies by enzymatic digestions and sequencing.

EXPECTED OUTCOMES

The RNA yield from patient-derived cell lines should be more than enough to generate and repeat all the qRT-PCRs in this protocol. In case of *de novo* missense mutations, the qPCR analysis should indicate if the mutation has an effect on mRNA stability and decay. In case of deletions, the qPCR will indicate if the wild- type allele is able to compensate for the absence of one allele.

Protein quantification from patient-derived cell lines needs to be assessed multiple times in high-quality manner in order to quantify a difference of 25% in protein expression. In order to understand which condition will give you the better resolution, we highly recommend to use multiple antibodies, different protein concentrations, and different sizes and number of wells for the NuPage protein gel before starting the quantification experiments.

LIMITATIONS

RNA degradation is common; work as cleanly as possible. Fortunately, mRNA levels of a heterozygous missense mutation will not change when compared to the expression levels of the same gene in heathy age-matched cells. If this happens, it is likely because a mutation changing one amino acid in the final protein product was not detected by the decay machinery.

To better analyze the effect of a particular point mutation, we recommend analyzing protein levels using western blot analysis (WB), extracting proteins from patient-derived cell lines with RIPA buffer as described above. In the case of PUM1, we recommend using the same antibody and setup conditions used in this protocol. For other proteins, you will likely need to test several antibodies from different companies, in isolation and in combination, and/or tweak the conditions to get the best results.

TROUBLESHOOTING

Problem 1

Patient-derived fibroblasts can sometimes be slow in the growth, due to the mutation they carry.

Potential solutions

Increase the amount of fetal bovine serum (FBS) from 10% to 20%, especially during the first days after thawing.

Problem 2

Ct variability of the housekeeping gene for qPCR normalization.

Potential solutions

Test more than one housekeeping genes such as *GAPDH*, *HPRT* and *ACTB* in order to understand which one has less Ct variability between the samples. Normalizing on a variable housekeeping, can give false positive or false negative results, introducing a confounding effect.

Problem 3

RNA degradation and contamination.

Potential solutions

When running total RNA in an agarose gel, the appearance of a smear indicates degradation. In this case, be sure all solutions and micropipettes are clean and nuclease-free. Also, use filter tips when dealing with RNA, to preserve its integrity, and keep the RNA on ice. For tissue, please take note that you will have a huge amount of RNA so if you take out only 20% of the supernatant after adding the chloroform will be enough to run all the planned qRT-PCRs. You should also control RNA purity by spectrophotometry. A 260/230 ratio below 2.0 might indicate solvent contamination, while a 260/280 ratio below 1.8 might indicate protein contamination.

Problem 4

Protein lysate can be sticky due to high amount of free nucleic acid.

Potential solutions

Before step 12 of protein quantification from patient-derived cell lines, aspirate the protein solution up and down 5–10 times using a 1 mL syringe with a 27G needle to break the long nucleic acid chains.

Problem 5

Low transformation efficiency of mutagenized plasmid.

Potential solutions

It is crucial to start from a good quality template DNA. Check the template plasmid DNA on agarose gel to ensure no degradation of the prep.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Vincenzo A. Gennarino (vag2138@cumc.columbia.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

No software was generated for this project. All softwares used in this study are publicly available and links are provided as appropriate in different sections of the methods and key resources table.

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AUTHOR CONTRIBUTIONS

V.A.G. devised the original protocol. N.dP. wrote the technical sections and re-optimized each step of the protocol. S.B., W.L, S.R., and A.C. contributed to the technical sections. N.dP. and V.A.G. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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