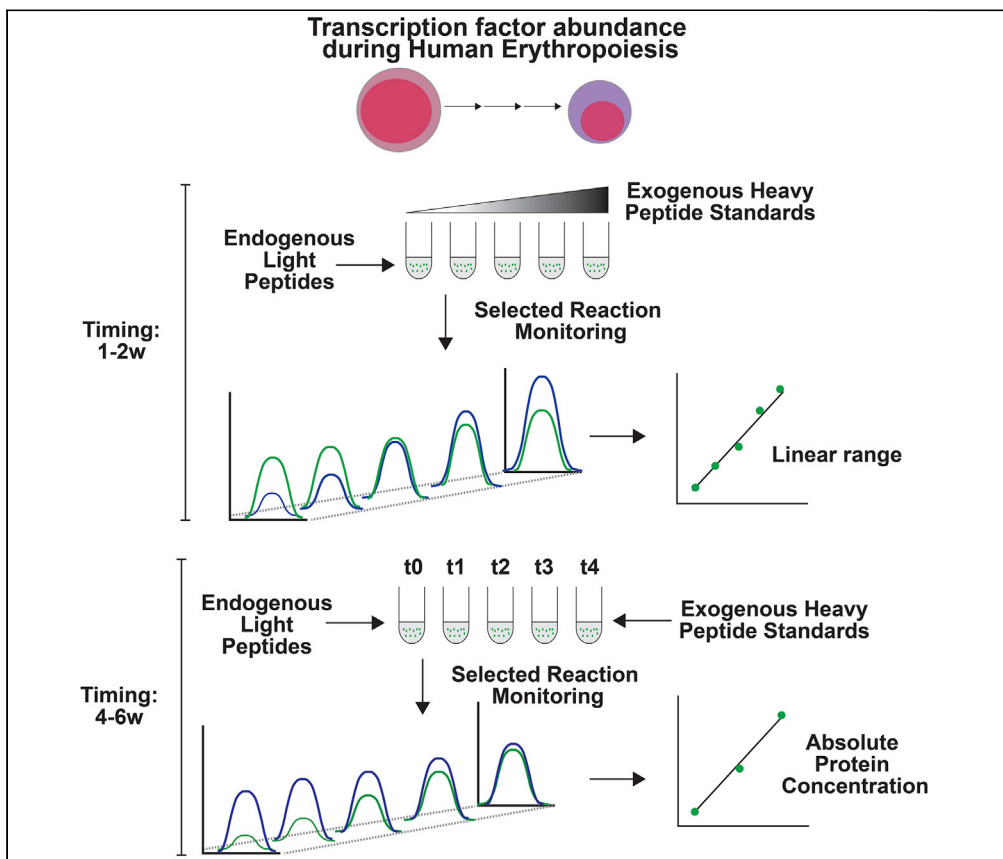


Protocol

Absolute quantification of transcription factors in human erythropoiesis using selected reaction monitoring mass spectrometry



Quantitative changes in transcription factor (TF) abundance regulate dynamic cellular processes, including cell fate decisions. Protein copy number provides information about the relative stoichiometry of TFs that can be used to determine how quantitative changes in TF abundance influence gene regulatory networks. In this protocol, we describe a targeted selected reaction monitoring (SRM)-based mass spectrometry method to systematically measure the absolute protein concentration of nuclear TFs as human hematopoietic stem and progenitor cells differentiate along the erythropoietic lineage.

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HIGHLIGHTS

Protocol for absolute quantification of TFs in human erythropoiesis

Selected reaction monitoring mass-spectrometry parameters for each peptide

Validated SRM assays corresponding to >100 TFs

Copy number reveals the relative stoichiometries of TFs during erythropoiesis

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Protocol

Absolute quantification of transcription factors in human erythropoiesis using selected reaction monitoring mass spectrometry

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SUMMARY

Quantitative changes in transcription factor (TF) abundance regulate dynamic cellular processes, including cell fate decisions. Protein copy number provides information about the relative stoichiometry of TFs that can be used to determine how quantitative changes in TF abundance influence gene regulatory networks. In this protocol, we describe a targeted selected reaction monitoring (SRM)-based mass-spectrometry method to systematically measure the absolute protein concentration of nuclear TFs as human hematopoietic stem and progenitor cells differentiate along the erythropoietic lineage. For complete details on the use and execution of this protocol, please refer to Gillespie et al. (2020).

BEFORE YOU BEGIN

Preparation of nuclear extracts

⌚ Timing: 1 day

Cord blood-derived hematopoietic stem and progenitor cells were induced to differentiate along the erythroid lineage as previously described (Palii et al., 2011). At multiple time points, aliquots of 15 million cells were cryopreserved in 10% DMSO.

1. Thaw cryopreserved hematopoietic and erythroid cells. Thaw one aliquot of 15 million cryopreserved cells per time point (days 0, 2, 4, 6, 7.5, 8, 8.5, 10, 10.5, 11, 11.5, 12, and 14) for a total of 13 tubes.
 - a. Remove cryovials from liquid nitrogen storage. Wipe the vials with 70% ethanol, quickly relieve pressure within the tube by loosening cap slightly, retighten, and immediately place the vials into a 37°C water bath without submerging the cap area.
 - b. Quickly thaw the cells (less than 1 min) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.



- c. Wipe the outside of the vials with 70% ethanol and transfer the vials into a laminar flow hood.
2. Transfer cells from each time point into separate 14 mL conical tubes and wash cells with 5 mL of 37°C pre-heated IMDM supplemented medium.
 - a. Using a 2 mL serological pipette, add 1 mL of 37°C pre-heated IMDM supplemented medium to the cryovial, gently aspirate the cell suspension and transfer into a 14 mL conical tube.
 - b. With the same pipet, rinse the cryovial three additional times using 1 mL of the same medium and transfer the suspension to the same 14 mL conical tube for a total of 5 mL cell suspension.
 - c. Take a sample of 20 μ L cell suspension for cell count and viability analysis with trypan blue.

△ CRITICAL: Record the number of viable cells. This number will be necessary for copy number determination at the end of this protocol.

- d. Centrifuge the cell suspension for 5 min at 199 \times g at 21°C.
- e. Carefully aspirate the supernatant without disturbing the cell pellet.
3. Wash cells in each tube separately with 5 mL of ice-cold PBS buffer.
 - a. Flick or tap the tube to loosen the cell pellet.
 - b. Resuspend cells gently in 5 mL ice-cold PBS buffer.
 - c. Centrifuge the cell suspension for 5 min at 199 \times g (4°C).
 - d. Carefully aspirate the supernatant without disturbing the cell pellet.
 - e. Make sure to remove as much PBS as possible from the cell pellet using a micropipette.
4. Extract nuclear proteins from hematopoietic and erythroid cells: Isolate nuclei
 - a. Resuspend each cell pellet into 120 μ L ice-cold Swelling Buffer and transfer into a low retention 1.5 mL Eppendorf tube.
 - b. Incubate on ice for 30 min while vortexing every 5 min to facilitate cell lysis.
 - c. Centrifuge for 5 min at 448 \times g (4°C) to pellet nuclei.
 - d. Carefully remove the supernatant by pipetting and discard.

Note: The supernatant is your cytoplasmic extract.

- e. Resuspend each nuclear pellet into 120 μ L ice-cold PBS buffer by pipetting.
- f. Centrifuge for 5 min at 448 \times g (4°C) to pellet nuclei.
- g. Carefully remove the supernatant by pipetting and discard.
5. Extract nuclear proteins
 - a. Resuspend each nuclear pellet into 60 μ L of 37°C pre-heated, freshly prepared Extraction Buffer 1.

Note: This buffer contains sodium deoxycholate, which is an ionic detergent. This buffer also contains Benzonase nuclease, which degrades nucleic acids and facilitates release of nuclear proteins.

△ CRITICAL: Extraction buffer 1 must be freshly prepared and pre-heated at 37°C to reduce viscosity.

△ CRITICAL: Optimal activity of Benzonase is at 37°C and requires the presence of MgCl₂ (1 mM).

- b. Incubate on a Thermomixer (1,400 rpm) at 37°C for 15 min.
- c. Assemble a 27.5 gauge needle onto a 1 mL syringe.
- d. Using the syringe and needle, aspirate and eject the nuclei suspension six times. Keep the syringe and needle as they will be used for step g.
- e. Add 60 μ L of 37°C pre-heated, freshly prepared Extraction Buffer 2.

△ **CRITICAL:** Extraction buffer 2 must be freshly prepared and pre-heated at 37°C to reduce viscosity. EDTA is necessary to chelate essential metal ions for reversible inhibition of Benzonase.

- f. Vortex and incubate at 70°C for 5 min to completely inactivate Benzonase.
- g. Extract proteins by six additional passages using the same 27.5 gauge needle and syringe used in step 5d.
- h. Incubate on a Thermomixer (1,400 rpm) at 40°C for 15 min.
- i. Centrifuge for 5 min at 17,000 × g (20°C–22°C) and pipet out 120 µL of the nuclear protein lysate into a new low retention Eppendorf 1.5 mL tube.

△ **CRITICAL:** Record the final volume of nuclear protein lysate. This number will be necessary for copy number determination at the end of this protocol.

6. Measure protein concentrations using BCA assay (Thermo Scientific).
 - a. Perform quantifications in triplicate.

▣ **Pause Point:** Nuclear protein extracts can be snap frozen in liquid N₂ and stored at –80°C for several years.

Enzymatic digestion of nuclear protein extracts

⌚ **Timing:** 2 days

7. Thaw nuclear protein lysates from the freezer.
8. Heat at 100°C for 5 min to ensure solubility.
9. Remove aliquots from each sample. Make all samples up to the same volume using a 1:1 mix of Extraction Buffers 1 and 2.
 - a. Protein amounts are detailed in the Step-by-step protocol.
10. Denature protein by boiling at 100°C for 5 min.
11. Cool samples to 37°C before proceeding to the next step.
12. Reduce the disulfide bonds with 10 mM dithiothreitol for 45 min at 56°C.
13. Cool samples to 21°C before proceeding to the next step.
14. Alkylate cysteine residues with 25 mM iodoacetamide for 30 min at 21°C. Protect samples from light.
 - a. Alkylation of cysteine sulfhydryl groups blocks the formation of disulfide bonds.
15. Quench the alkylation reaction by adding 10 mM dithiothreitol.
 - a. Incubate for 10 min at 20°C–22°C.
16. Digest the sample with endoproteinase Lys-C (1:200 wt/wt) for 3 h at 37°C.
 - a. Lys-C cleaves proteins C-terminal to lysine residues, regardless of whether they are followed by prolines.
 - b. Lys-C is also fully compatible with Trypsin digestion.
 - c. Lys-C retains activity in the presence of protein denaturants, and helps to further remove secondary structure to facilitate Trypsin digestion.
17. Dilute samples 5-fold with 50 mM Tris pH 8.0 or 50 mM Ammonium bicarbonate pH 8.0–8.5.

△ **CRITICAL:** Sodium deoxycholate must be diluted to 1% or lower in order for Trypsin to efficiently cleave proteins (Lin et al., 2008; Zhou et al., 2006).

18. Digest the sample with Trypsin (1:25 wt/wt) for 16–18 h at 37°C.
 - a. Trypsin cleaves proteins C-terminal to lysine and arginine residues, unless followed by a proline.

19. The next morning, add fresh Trypsin (1:75 wt/wt) and digest for 2 h at 37°C.
 - a. This step helps ensure digestion goes to completion.

▮ **Pause Point:** Digested peptide solutions can be stored at –20°C for up to 2 weeks, or –80°C for several months.

20. Add stable isotope labeled (SIL) peptides. The amount of each peptide will depend on which step of the protocol you are on. Refer to the Step-by-step protocol for further instructions.
21. Acid precipitate the sodium deoxycholate with 1 volume of cold 1% TFA for 30 min at 4°C.
 - a. Pellet the precipitate by centrifugation at 13,000 × g for 10 min at 20°C–22°C.

▴ **CRITICAL:** Peptides are in the supernatant. Do not discard.

Nuclear peptide purification

⌚ **Timing:** 3–4 h

Purify peptides by mixed cation exchange (MCX) solid phase extraction (SPE).

▴ **CRITICAL:** Sodium deoxycholate is not compatible with mass spectrometry. Acid precipitation will not remove all the sodium deoxycholate from your samples. We therefore strongly recommend you perform this peptide purification step to remove detergents from your samples.

Note: In theory, any 96-well plate vacuum manifold should be compatible with these MCX plates. We recommend testing this before purifying valuable samples.

22. Wet sorbent with 700 μL 100% acetonitrile
23. Equilibrate sorbent with 1.4 mL 0.1% trifluoroacetic acid (TFA).

Note: These columns hold a maximum volume of ~700 μL. Therefore, successive loadings will be required.

24. Load acidified sample twice (i.e., collect and re-load the flow through).
 - a. Use glass tubes inserted into a deep-well collection plate.
 - b. Save the final flow through until you confirm the peptides were recovered during elution.

▴ **CRITICAL:** Use caution when capping and uncapping glass tubes, as they are very brittle.

25. Wash bound sample with 700 μL 0.1% TFA.
26. Wash bound sample with 1.4 mL 80% acetonitrile, 0.1% TFA. This high organic wash will remove any detergents in the sample.
27. Wash the bound sample with 500 μL Milli-Q ddH₂O. This step is necessary to remove acid prior to elution with base.

▴ **CRITICAL:** This step must be performed quickly. Do not let the samples sit in water, as it will change the pH and promote peptide elution.

28. Elute with 500 μL MCX elution buffer.
 - a. Use glass tubes inserted into a deep-well collection plate.

▴ **CRITICAL:** Use caution when capping and uncapping glass tubes, as they are very brittle.

29. Dry peptides in a vacuum centrifuge at 19°C–22°C (i.e., SpeedVac). Do not use additional heat.

▮ **Pause Point:** Dried peptides can be capped and stored at –20°C for up to 2 weeks, or –80°C for several months.

△ **CRITICAL:** When thawing for use, allow glass vials to equilibrate to 20°C–22°C before carefully removing caps. Glass tubes are quite brittle.

Agilent 6490 triple quadrupole mass spectrometer (with chip cube) run parameters

⌚ **Timing:** 30 min

This step describes how to setup the mass spectrometer.

30. Setup the nano-flow pump gradient as indicated in the table below.
- Flow rate = 0.3 µL/min (micro flow).
 - Nano-flow Buffers A-B as indicated in [Materials and equipment](#)
 - Stop time = 88 min
 - Post time = 4 min

Time (min)	Buffer B (%)
0	3
2	3
62	25
74	40
75	50
78	50
80	80
85	80
87	3
88	3

31. Setup the capillary-flow pump as indicated below.

- Flow rate = 3 µL/min (micro flow)
- Constant Cap Buffer A
- Stop time = “as injector”
- Post time = none

32. Setup the chip cube timetable as shown below:

Time (min)	Parameter/position
0	Enrichment (trap flows to waste)
2	Analysis (trap inline with analytical)
87.01	Enrichment

33. Setup the QQQ parameters as detailed below:

Ion source	HPLC chip
Scan type	Dynamic MRM (i.e., scheduled)
Delta EMV (+)	200 V
Capillary Voltage (Positive)	1,800–1,820 V (start with lowest V)
Capillary Voltage (Negative)	1,700 V
Gas temp	150°C
Gas flow	11 L/min

34. Setup the Worklist on the mass spectrometer.

- Sample position is the position of the sample in the autosampler (i.e., P1-A1 for Plate 1 Position A1).
- Method is the method designed and saved above (steps 30-33). Transition acquisition details will be added in the Step-by-step protocol.
- Data File is the name and location where the mass spectrometer will save the acquired data.
- Inj Vol is the volume of sample you want to inject into the mass spectrometer.

Note: We recommend setting the Injection Volume here as opposed to within the method itself. This simplifies the process if you need to change this value.

- Once complete, start the acquisition cue. This is done by clicking the Start Worklist Run icon (multiple sample vials with an arrow picture).

△ CRITICAL: Do not click the Start Sample Run icon as this will initiate a different method that has not been properly setup in this protocol. This icon is often located next to the Start Worklist Run icon.

Note: When the mass spectrometer is running, the current line in the Worklist, along with the line below it, cannot be edited.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
IMDM medium	Sigma	Cat#I3390
Penicillin/streptomycin	Thermo Fisher	Cat#15140122
L-Glutamine	GIBCO	Cat#25030
Inositol	Sigma	Cat#I5125
Folic acid	Sigma	Cat#F7876
Monothioglycerol	Sigma	Cat#M6145
Ferrous nitrate	Sigma	Cat#8508
Ferrous sulfate	Sigma	Cat#F8633
Albumin-insulin-transferrin (BIT)	STEMCELL Technologies	Cat#9500
Benzonase	Millipore	Cat#70746
Acetonitrile (HPLC grade)	Fisher	Cat#A998
Formic Acid (LC-MS grade)	Sigma	Cat#5330020050
Methanol (HPLC grade)	Fisher	Cat#A452

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ammonium hydroxide solution (28%–30% NH ₃)	Sigma	Cat#338818
Protease inhibitor cocktail	Roche Diagnostics	Cat#05056489001
Software and algorithms		
Skyline	MacLean et al., 2010	https://skyline.ms/project/home/software/Skyline/begin.view
Other		
Oasis MCX 96-well uElution plate (2 mg sorbent/well; 30 μm)	Waters	Cat#186001830BA
Extraction plate manifold for Oasis 96-well plates	Waters	Cat#186001831
96-well deep-well plate	Waters	Cat#186001438
Quick load glass insert tubes for 96-well plates (700 μL)	Waters	Cat#186001437
Plastic caps for glass tubes	Waters	Cat#WAT078515
Large capacity HPLC chip column (160 nL trap; 150 mm × 75 μm analytical; Zorbax-SB C18 300A 5 μm beads)	Agilent	Cat#G4240-62010

MATERIALS AND EQUIPMENT

Recipes

IMDM supplemented media (serum free; store at 4°C)

Reagent	Final concentration
IMDM media	n/a
Penicillin/streptomycin	1%
L-Glutamine	4 mM
Inositol	40 μg/mL
Folic acid	10 μg/mL
Monothioglycerol	160 μM
Ferrous nitrate	90 ng/mL
Ferrous sulfate	900 ng/mL
Albumin-insulin-transferrin (BIT)	20%

Swelling buffer (make fresh)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
HEPES pH 7.9	10 mM
MgCl ₂	1.5 mM
KCl	10 mM
NP40	0.1%
Protease inhibitor cocktail	1 tablet for 10 mL extraction buffer

Extraction buffer 1 (make fresh)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
HEPES pH 7.9	50 mM
MgCl ₂	1 mM
NaCl	150 mM
Sodium deoxycholate	0.5%
Benzonase	50 ng/μL
Protease inhibitor cocktail	1 tablet for 10 mL extraction buffer

Extraction buffer 2 (make fresh)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
HEPES pH 7.9	50 mM
NaCl	150 mM
Sodium deoxycholate	9.5%
EDTA	1 mM
Protease inhibitor cocktail	1 tablet for 10 mL extraction buffer

MCX elution buffer (make fresh)

Reagent	Final concentration
NH ₄ OH	10% ^a
Methanol	90%

^aEquates to a final concentration of 2.8%–3% NH₃

SRM loading buffer (make fresh)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
Acetonitrile	3%
Formic acid	0.1%

HPLC nano-flow buffer A (store at 20°C–22°C)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
Formic acid	0.1%

HPLC nano-flow buffer B (Store at 20°C–22°C)

Reagent	Final concentration
Acetonitrile	100%
Formic acid	0.1%

HPLC capillary-flow buffer A (Store at 20°C–22°C)

Reagent	Final concentration
Milli-Q ddH ₂ O	n/a
Acetonitrile	3%
Formic acid	0.1%

STEP-BY-STEP METHOD DETAILS

Determining the linear range of quantification for selected peptides in erythroid nuclear extracts

⌚ Timing: 1–2 weeks (not including peptide synthesis)

This protocol details how one prepares to quantify the transcription factors quantified in Gillespie et al. by SRM. We provide peptide and SRM run parameter details in [Tables S1](#), [S2](#), and [S3](#) that can be directly used for this section of the protocol. We also detail the necessary step of determining analytical parameters for these assays using your laboratory setup.

1. Obtain at least two highly pure SIL peptides corresponding to the proteins you wish to measure from [Table S1](#).
 - a. SIL peptides must contain isotopically heavy Lysine or Arginine. This protocol employs peptides containing Lys-[13C6, 15N2] or Arg-[13C6, 15N4].
 - b. SIL peptides must be accurately quantified. Amino acid analysis can be used for peptide quantification.
 - c. SIL peptides should be >97% pure.

Note: AQUA peptides ([Gerber et al., 2003](#)) meet these criteria and can be commercially synthesized. Alternatively, SIL peptides can be synthesized and quantified in house.

⚠ **CRITICAL:** Aliquot SIL peptides to avoid multiple freeze-thaw cycles. Stocks should be stored at -80°C .

Note: Numerous resources are available should you wish to use additional peptides to quantify these TFs or to quantify additional TFs by SRM, including the SRM Atlas ([Kusebauch et al., 2016](#)), Peptide Atlas SRM Experiment Library (PASSEL) ([Farrah et al., 2012](#)), CPTAC Assay Portal ([Whiteaker et al., 2014](#)), Panorama Public ([Sharma et al., 2018](#)), MRMAssayDB ([Bhowmick et al., 2018](#)), and Prosit ([Gessulat et al., 2019](#)). We also provide a list of peptides and SRM run parameters for measuring hemoglobin expression in [Table S2](#), along with a list of peptides and their parameters that we could not successfully measure in [Table S3](#).

Note: A key step in SRM-based quantification of proteins is the initial selection of the peptides that will be used for quantification. While a number of resources are available to facilitate the selection of peptides for SRM analysis, it is often not possible to predict whether a peptide will be suitable for quantification in a particular biological matrix. Gillespie et al. describes an approach for selection of suitable peptides for quantification of TFs in nuclear extracts from erythroid cells.

2. Insert transition information for the peptides you wish to measure from [Table S1](#) into Skyline ([MacLean et al., 2010](#)).
 - a. Edit → Insert → Transition List.
 - b. Paste in relevant information. Do not include column headings.
 - c. Protein Description column is not required.
 - d. Check for errors and click insert.

Note: The MacCoss lab provides online tutorials, webinars, and courses detailing Skyline functionality (<https://skyline.ms/project/home/software/Skyline/begin.view>).

3. Export a SIL-peptide-only scheduled transition list from Skyline.

- a. Remove light transitions (Refine → Advanced). Select “light” from the dropdown menu.
- b. Set the retention time window. Navigate to Settings → Peptide Settings. In the Prediction tab, set the Time Window to 5 min.
- c. File → Export → Transition List.
- d. Use the below settings for export.

Instrument type	Agilent
Multiple methods	Checked
Max transitions per sample injection	200
Optimizing	None
Method type	Scheduled

△ CRITICAL: The Agilent 6490 mass spectrometer cannot measure more than 200 transitions at a given time. Depending on transition number and retention time, Skyline may export multiple transition lists.

- e. Update the retention time values to match those from [Table S1](#).
- f. Update the collision energy values to match those from [Table S1](#). These optimized values were obtained from the SRM Atlas ([Kusebauch et al., 2016](#)).

Note: Collision energy values need to be manually updated each time a transition file is exported.

- g. Update additional parameters in the transition list as follows:

Precursor m/z	Round to 1 decimal place (critical)
MS1 resolution	Wide
Product m/z	Round to 1 decimal place (critical)
MS2 resolution	Unit
Cell accelerator voltage	5 V

△ CRITICAL: We have experienced issues with mass incompatibilities between Skyline and Mass Hunter when including >1 decimal place. Do not use the “Decrease decimal” function in Excel, as this is only a visual change and does not change the actual number value.

Note: The Fragmentor voltage (380 V) is defaulted in the mass spectrometer and cannot be overwritten. Therefore, do not bother changing this value.

Note: When measuring SIL peptides, ensure the “ISTD?” column is TRUE.

4. Determine the linear range of quantification using dilutions of SIL peptides spiked into a constant amount of erythroid nuclear peptide extract. Use the below table to setup reactions.
 - a. The below table details reaction setup conditions to measure transitions exported from a single list. Scale reactions accordingly if multiple transition lists were exported in step 3.
 - b. Volumes in the table are adjusted by a factor of 3.5 to allow for triplicate injections. The exception to this is the 0 fmol/μL SIL blank, for which a single replicate is sufficient.
 - c. We recommend including extra samples to verify retention times. This is indicated with an asterisk in the below reaction setup table.
 - d. Prepare Lys-C-Trypsin-digested erythroid nuclear peptide samples, as described in [Before you begin](#).
 - e. Create an initial master stock containing 100 fmol/μL of each SIL peptide.

Final SIL amount (fmol)	SIL Master Mix (fmol/ μ L)	SIL volume \times 3.5 (μ L)	Erythroid peptide amount (μ g)
2,000	100	70	22.5
1,000	100	35	22.5
1,000*	100*	35*	22.5*
500	100	17.5	22.5
100	50	7	22.5
10	5	7	22.5
1	0.5	7	22.5
0.1	0.05	7	22.5
0.01	0.005	7	22.5
0	n/a	0	7.5

- Following Lys-C-Trypsin digestion of erythroid nuclear peptide samples, add SIL peptides.
- Acid precipitate the sodium deoxycholate with cold 1% TFA and purify peptides in supernatant by MCX SPE, as described in [Before you begin](#).
- Resuspend each peptide sample in 21 μ L SRM loading buffer.
- Inject 6 μ L per replicate into the mass spectrometer.
- Setup and run the mass spectrometer as outlined in [Before you begin](#).
 - Copy-paste the refined scheduled transition list exported in step 3 into the QQQ Acquisition tab. Click Apply.
 - At the bottom of the Acquisition window, set the cycle time for the mass spectrometer (i.e., 1,500–2,500 ms) such that the dwell time is between 15–20 ms. Click Apply.

△ CRITICAL: You must click Apply or else the settings will not update. Instrument software will not provide a warning for this.

Note: We do not recommend setting a dwell time below 12 ms. The difference between cycle and dwell time is described in Lange 2008 ([Lange et al., 2008](#)).

5. Import and analyze resulting data from step 4 in Skyline ([Figure 1](#)).

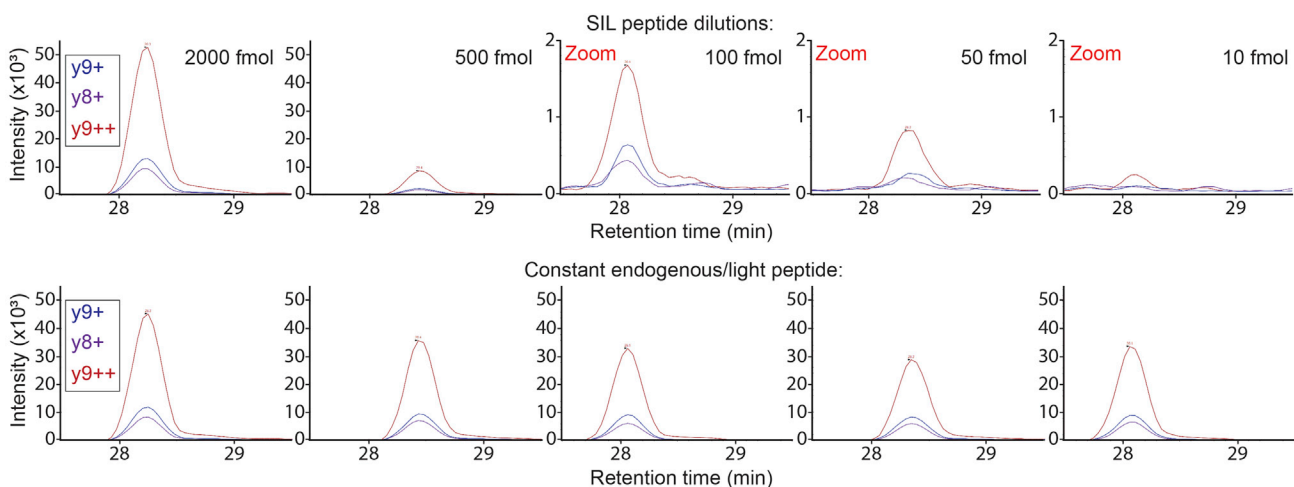


Figure 1. Extracted ion chromatograms of different SIL peptide dilutions spiked into an isotopically light biological sample

Note that transition rank order is preserved in all peaks, and retention times overlap between corresponding SIL and light peptides. For visibility, a different y axis was used for the last three SIL peptide dilutions.

- a. Import your mass spectrometry results (File → Import → Results) back into Skyline.
- b. Ensure the correct peaks are identified.
 - i. Transition intensities must exhibit a dose response.
 - ii. Transition intensity rank order should match between dilutions and replicates.
- c. Export a transition results report (File → Export → Report → Transition Results). The area column contains the background subtracted area-under-the-curve values for each transition peak.

Note: The Height column contains the background subtracted maximum intensity for each transition peak. If desired, these values can be used instead of the peak area values.

- d. Plot calibration curves and determine the linear range of quantification using regression.
- i. Using spreadsheet or data analysis software (i.e., Microsoft Excel, R), sum the two largest transition peak area values. This calculation should be performed separately for each replicate.
- ii. Log transform the resulting data, and plot vs. the log-transformed theoretical SIL peptide concentration (from step 4). The SIL peptide concentrations must be corrected for the purity of each peptide.
- iii. Create a regression line.
- iv. Determine the lower limit of quantification (LLOQ). This is the lowest concentration of SIL peptide that can be quantified with a coefficient of variation (CV) <20% (Kuzyk et al., 2013).
- v. Determine the upper LOQ (ULOQ), which is the highest SIL peptide concentration quantified that maintains a linear dose response.
- vi. The linear range of quantification is defined as the ULOQ-LLOQ.

Note: The limit of detection (LOD), which represents the lowest SIL peptide concentration that can be measured above background noise, can be calculated as the LLOQ/3 (Mani et al., 2012).

Absolute quantification of TF proteins in erythroid time course samples by SRM

⌚ Timing: 4–6 weeks

This protocol outlines the steps to determine the absolute concentration of endogenous transcription factors in erythroid nuclear extracts by SRM. We calculate these by comparing the transition ion intensities of endogenous, isotopically light, target peptides to the transition ion intensities of their isotopically heavy SIL peptide counterparts of known concentrations. We characterize these methods as peptide-based protein absolute quantifications.

6. Export a refined scheduled transition list to measure both SIL peptides and isotopically light peptides from your biological samples, as described in steps 3c and d.
 - a. Retention times were verified and updated in steps 4–5.
 - b. Prior to export, add back light transitions (Refine → Advanced). Click the “Add” box and select “light” from the dropdown menu.
 - c. Update the transition list(s) as described in steps 3f and g.
7. Digest erythroid nuclear protein extracts from all your time course samples with Lys-C and Trypsin, as described in [Before you begin](#). SIL peptides will be added in step 8.
 - a. Use 7.5 µg nuclear protein extract per sample and per technical replicate.
 - b. The number of samples required depends on the number of transition lists exported in step 6 and the number of replicates you wish to perform.

Note: It is not necessary to measure all biological replicates at the same time. The addition of SIL peptides will help account for sample preparation and machine variability.

Note: While technical replicates are ideal, they may not be feasible if sample is limited and/or costly.

8. Add concentration matched SIL peptides to each sample.
 - a. Concentration ranges are provided in [Table S1](#).
 - b. Peptides from the same protein should be added at the same concentration.

Note: If sample is not limiting, we recommend adding three different concentrations of SIL peptides to different samples. This will allow you to use a multi-point calibration curve to calculate the abundances of endogenous erythroid peptides and proteins.

9. Acid precipitate the sodium deoxycholate with cold 1% TFA and purify peptides in supernatant by MCX SPE, as described in [Before you begin](#).
10. Resuspend each sample in 7x μ L of SRM loading buffer, where x represents the number of technical replicates.
11. Inject 6 μ L per replicate into the mass spectrometer.
12. Setup and run the mass spectrometer as outlined in [Before you begin](#).
 - a. Copy-paste the refined scheduled transition list exported in step 6 into the QQQ Acquisition tab.

△ CRITICAL: We highly recommend leaving the retention time window set at 5 min. The composition of your samples can change significantly over the time course.

13. Import and analyze resulting data in Skyline, similar to step 5.

△ CRITICAL: Transitions that do not follow the below criteria are likely subject to interference and therefore not reliable for quantification. They should not be included in further analyses.

- a. Transition elution peak profiles should be approximately bell shaped.
- b. The transition intensity rank order for each peptide should be consistent between samples and replicates.
- c. The transition retention times for both the heavy and light isotope versions of the same peptide should be identical within a sample.
- d. Retention times may differ between samples, depending on the similarity of the samples.
- e. [Figure 2](#) illustrates examples of poor data quality.

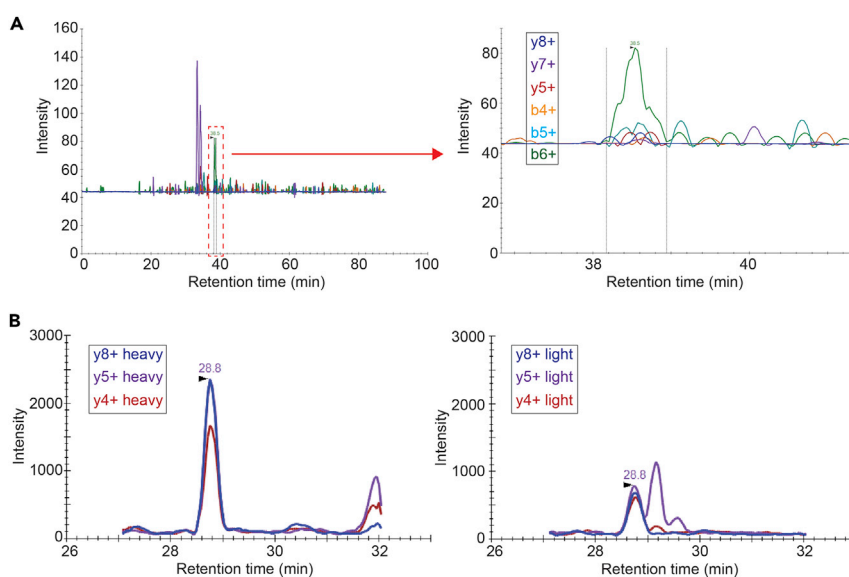


Figure 2. Extracted ion chromatograms showing examples of poor data quality

(A) Low abundance, noisy transitions with no clear peak or alignment of retention times
 (B) Transition rank order differs between measurements, and the y5+ peak is subject to interference.

14. Export transition results reports as described in step 5c.

EXPECTED OUTCOMES

This protocol will generate quantitative mass spectrometry data for endogenous and SIL peptides, as listed in [Table S1](#). This quantitative peptide information will be used to determine light-to-heavy (i.e., endogenous:SIL) ratios. Together with the known concentrations of highly pure SIL peptides, these ratios will be used to calculate the absolute concentration of your proteins of interest.

QUANTIFICATION AND STATISTICAL ANALYSIS

This section describes how to calculate the absolute concentration of your proteins using your acquired SRM data.

1. Using the transition results report exported in Step-by-step method details step 14, identify and sum the two transitions with the largest interference-free peak area values for each SIL and light (i.e., endogenous) peptide pair at each time point
 - a. These transitions should be the same for each SIL-light peptide pair.
 - b. Perform this calculation separately for each replicate.
 - c. If no SIL peptide signal is observed, we recommend not using that assay at that time point.
 - d. If signal is only observed for the SIL peptide, check the signal for the remaining light peptides for that protein.
 - i. If no signal is observed for any light peptides, then they are below the LOD of the mass spectrometer. This can often be confirmed by examining neighboring time points.
 - ii. If signal is observed for the other light peptides, it is likely the offending peptide represents a bad measurement. We recommend removing both the light and SIL peptide from the analysis for that time point.
 - e. Check if the difference between light and SIL peptide signals is greater than 1/100.
 - i. If this is true for all time points, then that peptide assay should not be used in the analysis. One could try to concentration match the peptides in a subsequent experiment if sample is not limiting.
 - ii. If only a single time point is affected, then note this, but we feel it is acceptable to use that data point for analysis (vs. excluding the data).
 - iii. This may be observed during the time course as protein expression increases from below LOD or decreased from peak expression to below LOD. In these instances, we recommend keeping the data as it accurately reflects the trajectory of protein expression.

2. Calculate the light-to-heavy (i.e., endogenous:SIL) ratio using the data derived from step 1.

△ CRITICAL: Do not use measurements that were outside the linear range of quantification determined in Step-by-step method details step 5d.

3. Calculate the fmol amount of the isotopically light peptides in your biological samples.
 - a. Use the light-to-heavy ratios calculated in step 2 and the SIL peptide fmol amounts from Step-by-step method details step 8, as detailed in the equation below. SIL peptides must be corrected for purity.

$$\text{Amount Erythroid Peptide (fmol)} = (\text{Light-to-Heavy ratio}) \times \text{Corrected Amount SIL Peptide (fmol)}$$

4. Calculate the concentration of the proteins you measured by SRM.
 - a. For each protein, average the peptide level fmol amounts for all its measured endogenous/light peptides.
 - b. To calculate protein concentration in fmol/ μg , take these protein amounts, in fmol, and divide by the starting amount of nuclear protein extracts (see [Step-by-step method details step 7](#)).

5. Obtain a single concentration for each protein at each time point by combining the fmol/ μg values obtained in the replicate measurements.
 - a. If any replicates have a valid measurement, take the average of their measurements.
 - b. If only one replicate has a valid measurement, it alone can be taken as the representative value.
 - c. If none of the replicates has a valid measurement, the value is marked as missing.
 - d. Missing values can be filled in by linear interpolation. For each missing value (i.e., on day Y), find the latest non-missing value prior (i.e., A on day X) and the earliest non-missing value later (i.e., B on day Z). Solve the equation below.

$$Y = A \times \frac{Z - Y}{Z - X} + B \times \frac{Y - X}{Z - X}.$$

Note: Interpolation may not be feasible if there are too many missing values and/or no clear trend in abundance is apparent.

6. To determine the protein concentration in copies per nucleus (i.e., copy number), perform the below calculations.
 - a. Calculate the total nuclear protein extracted per nucleus (P) for each time point. Assume there is one nucleus per cell and extraction efficiency was 100%. The answer will likely be on the picogram (pg) scale.

$$P_i = \frac{C_i V_i}{n_i},$$

where C = nuclear protein concentration, V = extraction volume, n = number of nuclei (i.e., number of cells), i = time point

- b. Average the protein pg/nucleus (P) values across all time points.

$$P_{avg} = \sum_{i=1}^m \frac{P_i}{m},$$

where m = total number of time points measured

- c. Convert fmol/ μg values from step 15 to fmol/nucleus

$$\frac{1 \text{ fmol}}{\mu\text{g}} \times \frac{10^6 \mu\text{g}}{1 \text{ g}} \times \frac{1 \text{ g}}{10^{-12} \text{ pg}} \times \frac{P_{avg} \text{ pg}}{\text{nucleus}} = \frac{P_{avg} \times 10^{-6} \text{ fmol}}{\text{nucleus}}.$$

- d. Convert fmol/nucleus to molecules/nucleus (i.e., copy number)

$$\frac{1 \text{ fmol}}{\text{nucleus}} \times \frac{1 \text{ mol}}{10^{15} \text{ fmol}} \times \frac{6.022 \times 10^{23} \text{ molecules}}{\text{mol}} = \frac{6.022 \times 10^8 \text{ molecules}}{\text{nucleus}}.$$

LIMITATIONS

The Agilent 6490 triple quadrupole mass spectrometer used in this protocol can measure a maximum of 200 concurrent transitions at any given retention time. Therefore, users are limited in the number of peptides that can be quantified in a single instrument run. This can impact both the protocol throughput and amount of biological sample required.

We expect a large percentage of these assays to be transferable between different biological samples from the same species. Two limitations that may be encountered when transferring assays between different samples are (1) retention time drift, and (2) ion m/z interference while measuring

transitions. Retention times can be normalized using iRT standards (Biognosys) (Escher et al., 2012). Ion m/z interference generally requires choosing and optimizing a different assay.

Peptide-based quantifications may not accurately represent the variability in protein abundances that may occur as a result of biological (i.e., different isoforms or post-translational modifications) or technical (i.e., inefficient enzymatic digestion) issues. One solution to alleviate this issue is to use multiple peptides to quantify any given protein. It is also possible to use synthetic peptides with specific post-translational modifications. Another method would be to use isotopically heavy recombinant protein standards corresponding to each protein of interest, which would be processed together with your biological samples.

TROUBLESHOOTING

Problem 1

Nuclear protein extracts are very viscous and difficult to work with.

Potential solution

Viscous extracts can benefit from heating. The addition of a small amount of SDS to heated extracts can help (i.e., 0.01%). SDS can be removed by MCX SPE. Only during the alkylation reaction must samples be at 20°C–22°C. Viscosity is usually reduced as the proteins are enzymatically digested into peptides.

Problem 2

Interference with ions that have similar m/z values prevents the acquisition of reliable data for a peptide.

Potential solution

Select and optimize additional transitions for that peptide.

Problem 3

Retention time drift prevents the full acquisition of a transition peak.

Potential solution

Ensure you are using a wide enough retention time window to account for drift between different biological samples. This value can be set differently for each transition. Note that we have never acquired data with a retention time window above 6 min. Retention times can be adjusted between samples if you notice a trend in drift. Retention times may also need to be optimized for each different biological sample you are measuring. Alternatively, iRT standards can be used as noted in [Limitations](#).

Problem 4

Significant degradation of SIL peptides is observed.

Potential solution

Try not to subject peptides to multiple rounds of freeze-thaw. Ensure peptide stocks are stored correctly (i.e., –80°C). We have noticed stability issues over time with peptides, especially crude SIL peptides. This is why it is important to repeat absolute quantification when significant time (i.e., 1 month) has passed since the last measurement.

Problem 5

Significant noise is observed for a peptide quantification.

Potential solution

Even with SIL peptide standards, it can be difficult to determine which peak correctly corresponds to your peptide of interest. In some cases, the noise may be so high, that we advise not using the peptide for quantification, even if it has a peak overlapping the SIL standard. We have observed cases where the noise is specific to a certain biological sample. In this case, all measurements can be made and interpolation can be used to replace the omitted noisy data.

Problem 6

Ion signals are dropping over time in the mass spectrometer.

Potential solution

HPLC columns have a finite life. In addition, capillary voltage will need to be increased over time. We recommend increasing in increments of 10 V.

Problem 7

Different peptides from the same protein differ significantly in the calculated absolute amounts.

Potential solution

This issue may arise due to biological or technical reasons. For example, peptides may span different spliced isoforms or may include unanticipated post-translational modifications. Alternatively, differences in digestion efficiency can contribute to this issue. One way to deal with this issue is to report the range of concentrations observed. One might also choose to use the highest measured peptide concentration as the concentration of the protein especially if it is supported by more than 1 peptide.

Problem 8

Peptide-based quantifications have high CV values.

Potential solution

High CV values (>35%) may be due to issues such as poor chromatography, a mass spectrometer requiring cleaning, and/or instability/insolubility of standard peptides. Solutions include replacing the HPLC column, cleaning and tuning the mass spectrometer, or obtaining a fresh aliquot of the standard peptides.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeff Ranish (jeff.ranish@isbscience.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data generated from this study has been deposited in the Peptide Atlas SRM Experiment Library (PASSEL) database under the accession PASS01454 and is available at the following URL: <http://www.peptideatlas.org/PASS/PASS01454>.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2020.100216>.

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AUTHOR CONTRIBUTIONS

T.J.P., J.A.R., and M.B. conceived and designed the study. C.G.P. performed protein extraction under M.B.'s supervision. M.A.G. designed, measured, and analyzed SRM assays under J.A.R.'s supervision. D.S.-T. and T.J.P. contributed to the quantification method. M.A.G. wrote the initial manuscript draft. M.A.G., C.G.P., T.J.P., M.B., and J.A.R. discussed, commented on, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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