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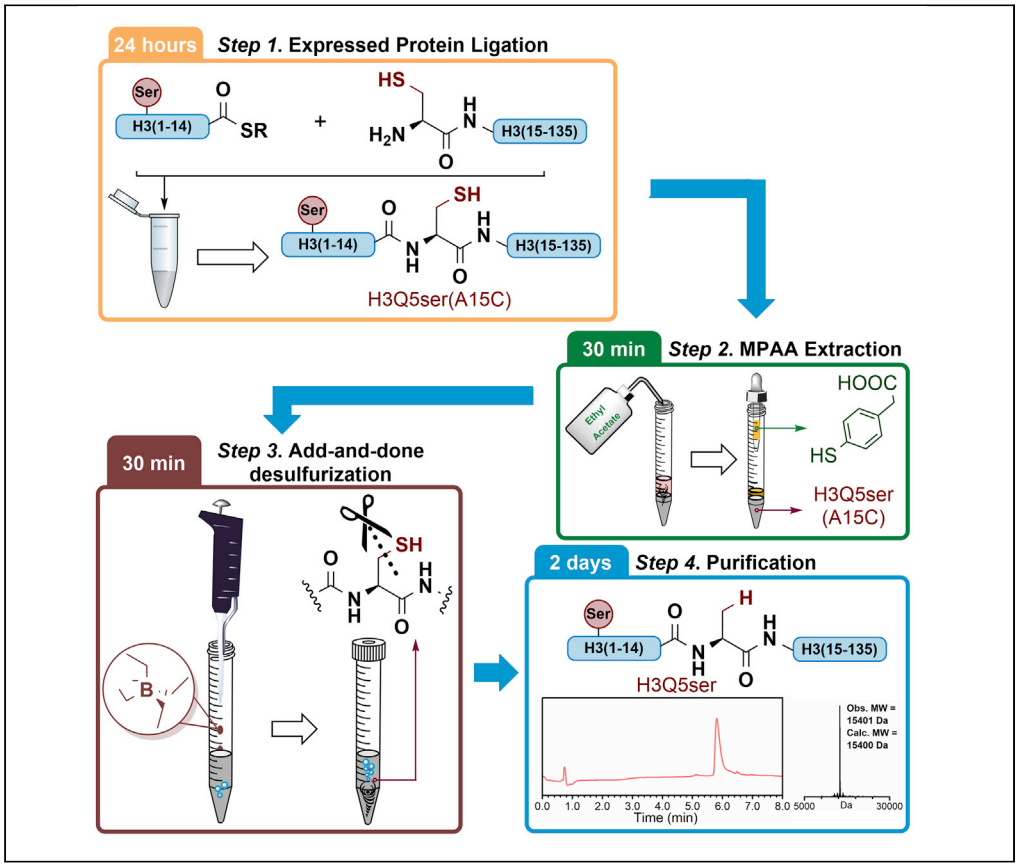
Protocol for semisynthesis of serotonylated histone H3 by rapid protein desulfurization in tandem with native chemical ligation



Here, we present a protocol of rapid protein desulfurization in tandem with native chemical ligation for facile syntheses of proteins with site-specific modifications. We describe using sodium tetraethylborate ($NaBEt_4$) to carry out this desulfurization in an add-and-done manner under ambient conditions without requirement of inert atmosphere protection, UV irradiation, heating, or exogenous thiol additives. Specifically, we detail the semisynthesis of serotonylated histone H3(H3Q5ser) *via* one-pot ligation desulfurization. This protocol can be applied to synthesize proteins of interest with homogenous post-translational modifications.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Add-and-done desulfurization (ADD) of protein by $NaBEt_4$ under ambient conditions

One-pot native chemical ligation-ADD facilitating semisynthesis of the H3Q5ser protein

Conveniently convert Cys to Ala residues in protein

Powerful tool for synthesizing proteins with homogenous post-translational modifications

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Protocol for semisynthesis of serotonylated histone H3 by rapid protein desulfurization in tandem with native chemical ligation

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SUMMARY

Here, we present a protocol of rapid protein desulfurization in tandem with native chemical ligation for facile syntheses of proteins with site-specific modifications. We describe using sodium tetraethylborate ($NaBEt_4$) to carry out this desulfurization in an add-and-done manner under ambient conditions without requirement of inert atmosphere protection, UV irradiation, heating, or exogenous thiol additives. Specifically, we detail the semisynthesis of serotonylated histone H3(H3Q5ser) *via* one-pot ligation desulfurization. This protocol can be applied to synthesize proteins of interest with homogenous post-translational modifications.

For complete information on the generation and use of this protocol, please refer to Sun et al. (2022).[1]

BEFORE YOU BEGIN

Chemical synthesis and modification of biomacromolecules with homogeneity at atomic-level precision can provide new opportunities to unveil fundamental chemical biology and to generate promising therapeutical modalities.[2–4] Many methods and strategies have been widely applied in this field, including solid-phase peptide synthesis (SPPS),[5] native chemical ligation (NCL),[6] desulfurization,[7,8] α-ketoacid-hydroxylamine (KAHA) ligation,[9,10] Ser/Thr ligation (STL),[11,12] and Cys/Pen ligation (CPL).[13] However, the development of highly chemoselective and operationally simple chemical transformations is still of continuous interest for the facile synthesis of peptides/proteins with more complicated functionalities.[14]

Based on a reagent called sodium tetraethylborate ($NaBEt_4$), we developed a superfast and easy-to-use desulfurization method for chemical protein synthesis and modification. Such a method can specifically convert cysteine or penicillamine residues of peptides/proteins into alanine or valine in an add-and-done manner. It can also be applied under ambient conditions, and no special apparatus is required. Combined with the existing SPPS, CPL, and NCL strategies, this tetraethylborate chemistry offers a new option for producing synthetic peptides/proteins with precise modifications.

The protocol below details the specific steps for the semi-synthesis of H3Q5ser *via* expressed protein ligation (EPL) and add-and-done desulfurization (ADD) in one pot. However, we have also used this $NaBEt_4$-based desulfurization method for desulfurization of short peptides, chemical synthesis of leukocyte-associated immunoglobulin-like receptor 1 (LAIR1) cytoplasmic domain, and chemoselective modification of commercially available proteins. For those applications, please refer to Sun et al.[1]

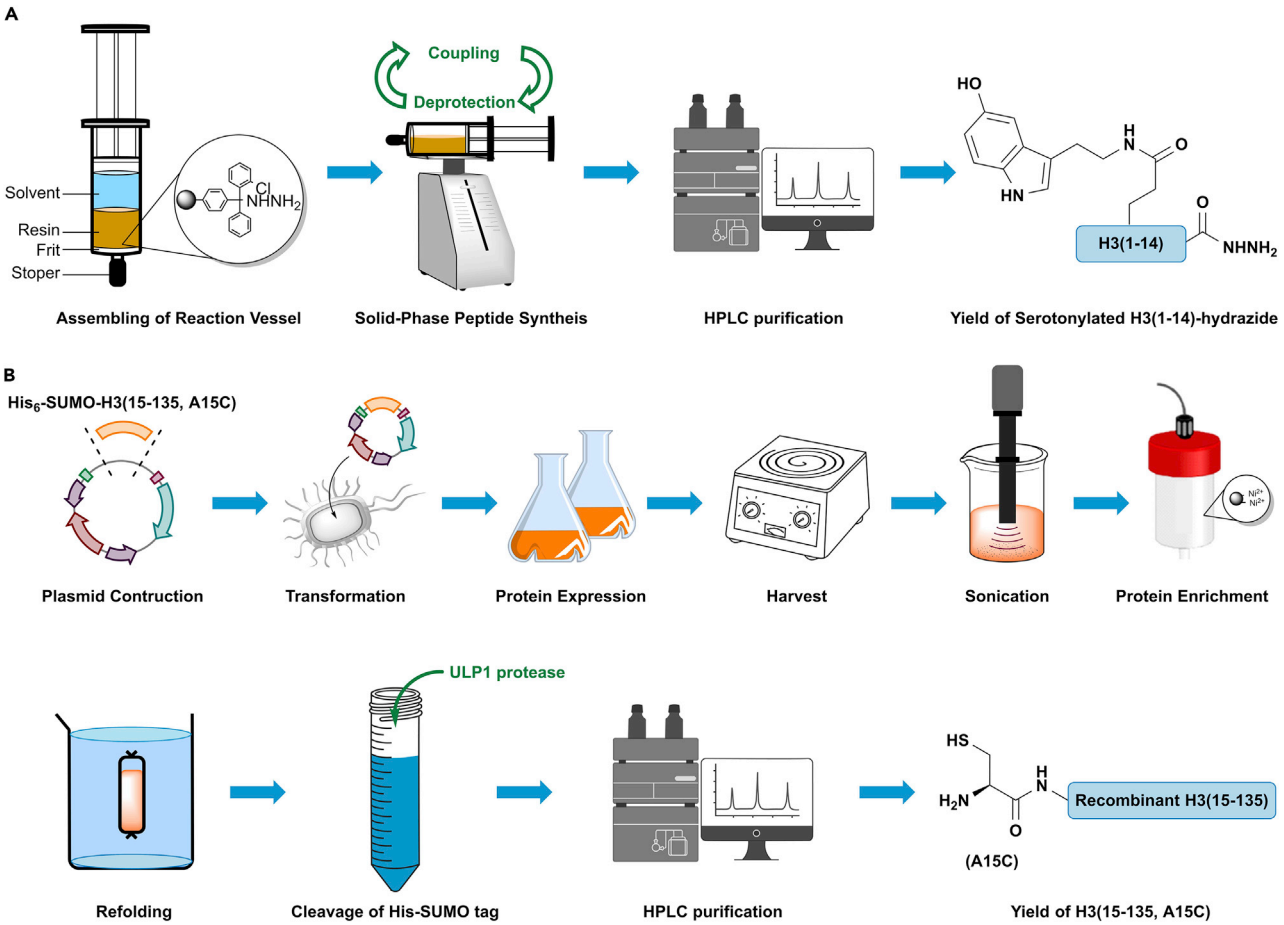

Figure 1. Preparation of materials for expressed protein ligation

(A) Synthesis of serotonylated H3(1-14)-$NHNH_2$ by SPPS. See step 1–4 in 'before you begin' section.

(B) Purification of truncated H3(15-135, A15C) from *E. coli*. See step 5–6 in 'before you begin' section.

Preparation of serotonylated H3(1–14)-$NHNH_2$

Timing: 1 week

1. Synthesis of fully protected H3(1-14) using standard Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS) on a 2-Cl-(Trt)-$NHNH_2$ resin (Figure 1A).
   a. Preparation of 2-Cl-(Trt)-$NHNH_2$ resin following reported protocol.[15]
      i. Weigh 200 mg 2-Cl-(Trt)-Cl resin (0.6 mmol/*g*) into a peptide synthesis vessel similar to one from Wei et al.[16]
      ii. Add 3 mL dimethylformamide (DMF) to the resin and drain it after 10 s.
      iii. Repeat step ii for two more times.
      iv. Add 3 mL dichloromethane (DCM) to the resin and drain it after 10 s.
      v. Repeat step iv for two more times.
      vi. Swell the resin in 3 mL 50% (vol/vol) DMF/DCM and drain it after 30 min.
      vii. Add 3 mL DMF containing 5% (vol/vol) $NH_2NH_2 \cdot H_2O$. Shake the resin for 30 min at room temperature (22°C–25°C) and then drain the solution.
      viii. Repeat step vii one more time.
      ix. Repeat steps ii-v to wash the resin thoroughly.

x. Add 3 mL DMF containing 5% (vol/vol) MeOH and shake the vessel for 10 min to cap the unreacted sites of resin.
xi. Wash the resin by following steps ii-v.

⚠ CRITICAL: The NH2NH2·H2O is a reactive nucleophile. Any remaining hydrazine may strongly interfere with the following coupling step, causing impure SPPS. It should be completely washed away from the activated resin.

Pause Point: After step xi, the resin was further washed by DCM for 3 more times as step iv-v. Then it was dried *in vacuo* for 15–30 min. The freshly prepared 2-Cl-(Trt)-$NHNH_2$ resin could be stored at −20°C for less than two weeks.

b. Coupling of amino acid to the 2-Cl-(Trt)-$NHNH_2$ resin.
   i. Weigh 0.5 mmol of corresponding Fmoc-protected amino acid (4.2 eq.) and 0.47 mmol of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 180 mg, 4.0 eq.) in a 5-mL tube.
   ii. Dissolve the solid by adding 3 mL DMF.
   iii. Add 1.0 mmol of N,N-Diisopropylethylamine (DIEA, 174 μL, 8.4 eq.) to the dissolved solution. Vortex the mixture for 30 s at room temperature (22°C–25°C).
   iv. Transfer the solution to the 2-Cl-(Trt)-$NHNH_2$ resin. Gently agitate the reaction vessel for 60 min at room temperature (22°C–25°C) in a shaker and drain the solution after that by vacuum.
   v. Repeat steps 1a.ii-v to wash the resin thoroughly.
c. Deprotection of Fmoc-group.
   i. Add 3 mL of 20% (vol/vol) 4-methyl piperidine in DMF to the resin in step b. Shake the resin for 2 min and then drain the solution.
   ii. Add 3 mL of 20% (vol/vol) 4-methyl piperidine in DMF to the resin in step b. Shake the resin for 15 min and then drain the solution.
   iii. Repeat steps 1a.ii-v to wash the resin thoroughly.
d. Repeat the coupling and deprotection cycle from steps b-c to finish the elongation of H3(1-14) as follows: Boc-Ala_1-$Arg(Pbf)_2$-$Thr(OtBu)_3$-$Lys(Boc)_4$-$Glu(OAll)_5$-$Thr(OtBu)_6$-Ala_7-$Arg(Pbf)_8$-$Lys(Boc)_9$-$Ser(OtBu)_{10}$-$Thr(OtBu)_{11}$-Gly_{12}-Gly_{13}-$Lys(Boc)_{14}$-NHNH-Resin (N→C).

⚠ CRITICAL: To enable the coupling of serotonin to the Glu_5, Fmoc-Glu(OAll)-OH with side-chain allyl protection should be incorporated at position 5 of the peptide sequence.

Pause Point: After step 1d, the resin was further washed by DCM for 3 more times as step iv-v. Then it was dried *in vacuo* for 15–30 min. The resin-bound peptide could be stored at −20°C for less than one month.

2. On-resin serotonylation of H3(1-14) modified from the previous protocol.[17]
   a. Deprotection of side-chain orthogonal protection at Glu_5.
      i. Weigh 0.024 mmol $Pd(PPh_3)_4$ (28 mg, 0.2 eq.) and 1.2 mmol N,N-dimethylbarbituric acid (190 mg, 10.0 eq.) in a 5-mL tube.
      ii. Dissolve the solid in the tube in 3 mL DCM.
      iii. Transfer the solution to the resin bound H3(1-14) prepared in the step 1. Gently agitate the reaction vessel for 60 min at room temperature (22°C–25°C) in a shaker and drain the solution after that by vacuum.
      iv. Repeat step iii one more time to ensure complete deallylation.
      v. Wash the resin extensively following steps 1a.ii-v to remove residual palladium.

Optional: The deallylation progress can be monitored by UPLC after an analytical cleavage of resin beads by 95% TFA, which is similar as the following step 3a–3k with 1/40 volume of reagents.

Note: The resin color may change to dark orange due to the residual Palladium, but it will not affect the later coupling, deprotection, and purification.

b. Serotonylation of Glu_5.
   i. Weigh 0.13 mmol PyAOP ((7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, 68 mg, 1.08 eq.) in a 5-mL tube and dissolve it with 3 mL DMF.
   ii. Transfer the DMF solution to the resin-bound peptide with side-chain free Glu_5 from step 2a.
   iii. Add 0.48 mmol DIEA (84 μL, 4.0 eq.) to the resin, and agitate the mixture for 30 s.
   iv. Add 0.24 mmol serotonin hydrochloride (51 mg) directly to the resin. Gently agitate the reaction vessel for 60 min at room temperature (22°C–25°C) in a shaker and drain the solution after that by vacuum.
   v. Repeat step 2b.i-iv one more time to ensure complete serotonylation.
   vi. Wash the resin following steps 1a.ii-v.
   vii. Wash the resin following steps 1a.iv-v and dry the resin *in vacuo* for 5 min.

Alternatives: The coupling time in step 2b.iv could be extended up to 16 h. The coupling progress can also be monitored by UPLC.

3. Global deprotection of serotonylated H3(1-14) from resin.
   a. Add 4 mL cleavage cocktail (TFA/EDT/TIPS/H_2O, 92.5/2.5/2.5/2.5, vol) to the dried resin. (TFA: Trifluoroacetic acid; EDT: Ethane-1,2-dithiol; TIPS: Triisopropylsilane).
   b. Agitate the mixture gently for 3 h at room temperature (22°C–25°C).
   c. Cool 45 mL diethyl ether to 0°C–4°C in a 50-mL centrifuge tube.
   d. Filter the deprotection solution from step 3.b into the cold ether from step 3.c.
   e. Triturate the crude peptide by shaking the centrifuge tube manually and then centrifuge the tube at 2,600 *g* for 5 min under room temperature (22°C–25°C).
   f. Decant the supernatant carefully.
   g. Add 45 mL precooled diethyl ether to the precipitation.
   h. Repeat step 3e-f.
   i. Repeat step 3g-h one more time.
   j. Dry the crude peptide by N_2 carefully for 10 min.
   k. Store the crude peptide at −20°C before further analysis and purification.

⚠ CRITICAL: 2.5% (vol/vol) EDT in the cleavage cocktail is crucial to prevent side reactions from serotonin during global deprotection. EDT has a strong odor, thus, this step should be handled in fumehood and use bleach to quench any spilled liquid.

Pause Point: Dried crude peptide can be stored at −20°C for at least one month.

4. Purification of serotonylated H3(1-14)-$NHNH_2$.
   a. Dissolve the crude peptide from step 3 in 10 mL 10% ACN/H_2O containing 0.1% TFA (vol/vol).
   b. Filter the solution through PTFE membrane filter (13 mm × 0.22 μm).
   c. Pipette 20 μL of the filtered solution and dilute it to 200 μL by adding 180 μL 10% ACN/H_2O containing 0.1% TFA (vol/vol).
   d. Inject 5.0 μL of the diluted sample to the UPLC-MS system for analysis of product purity with a linear gradient from 2% to 30% ACN/H_2O containing 0.1% TFA in 8 min (vol/vol).

Note: This LCMS conditions resolved the desired product very well. But for other peptides with a different sequence, the gradient and/or solvent composition may need to be optimized for analysis or purification.

e. Purify the crude peptide solution from step 4b by preparative HPLC equipped with a reversed-phases (RP) C18 column.
f. Collect fractions corresponding to the main peak in 15-mL centrifuge tubes.
g. Confirm pure fractions of the desired product by UPLC-MS analysis of collected fractions using the same gradient in step 4d.
h. Freeze those centrifuge tubes with pure product in liquid nitrogen for at least 15 min.
i. Lyophilize the frozen fractions under 40 mTorr at −78°C for 24 h.
j. Redissolve freeze-dried solid with 1–2 mL 50% (vol/vol) ACN/H_2O for each centrifuge tube. Combine all concentrated product solution to a weighed glass vial.
k. Re-lyophilize the combined product and then weigh the vial again to obtain the yield of serotonylated H3(1-14) with C-terminal hydrazide. (Typical yield: 45 mg, 23% isolated yield, purity >95%).

Pause Point: Purified peptide after lyophilization can be stored at −20°C for several months.

Preparation of truncated H3(15-135)

Timing: 2 weeks

5. Expression of truncated histone 3 (Figure 1B).
   a. Construct the pET28a plasmid encoding N-terminally His_6–SUMO tagged histone H3(15-135, A15C). The encoded protein sequence can be found at Sun et al.[1]
   b. Transform the plasmid into BL21 (DE3) cells.
   c. Culture a monoclone in Luria-Bertani (LB) medium at 37°C for overnight (8–12 h).
   d. Transfer the overnight culture into 1 L LB (1:100 inoculation).
   e. Culture the cells at 37°C until its OD600 reaches 0.8.
   f. Decrease the temperature to 25°C.
   g. Induce the protein expression by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1mM.
   h. Grow the culture at 25°C for 16 h.

Note: All media should contain 50 μg/mL Kanamycin.

6. Purification of H3(15-135, A15C) from *E. coli*.
   a. Harvest the cells by centrifugation at 13,000*g* under 4°C for 3 min.
   b. Suspend cell pellet in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 6M Urea, 20 mM Imidazole, pH: 7.5), 50 mL per liter of cell culture.
   c. Lyse the cell by sonication in an ice-water bath (4 s on:4 s off, on for 1 h, 40% amplitude).
   d. Clean the solution by centrifugation at 20,000*g* under 4°C for 45 min.
   e. Load the supernatant to a HisTrap HP column by vacuum.
   f. Wash the column with 50 mL lysis buffer.
   g. Elute the expressed SUMO-H3(15-135) with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 6 M Urea, 500 mM imidazole, pH: 7.5).
   h. dialyze the eluent at 4°C into refolding buffer (50 mM Tris-HCl, 500 mM NaCl, 2 M Urea, 5 mM DTT, pH: 7.5) for 16 h using the dialysis bag with 3 kDa cutoff .
   i. Add 1 mg ULP1 protease to the folded protein (around 50 mg) and incubate the solution for another 16 h to cleave the His_6–SUMO tag (solution will become cloudy).
   j. Centrifuge the solution at 20,000 g under 4°C for 10 min.
   k. Decant the supernatant and collect the histone pellet.
   l. Dissolve the histone pellet in the previous step with 6 M GdmCl solution.
   m. Filter the above solution through PTFE membrane filter (13 mm × 0.22 μm).

n. Purify truncated H3 by preparative HPLC equipped with a RP-C4 column.
o. Collect fractions corresponding to the main peak in 15-mL centrifuge tubes.
p. Confirm pure fractions of the desired product by UPLC-MS analysis of collected fractions.
q. Freeze those centrifuge tubes with pure product in liquid nitrogen for at least 15 min.
r. Lyophilize the frozen fractions under 40 mTorr at −78°C for 24 h.
s. Redissolve freeze-dried solid with 1–2 mL 50% (vol/vol) ACN/H_2O for each centrifuge tube. Combine all concentrated product solution to a weighed glass vial.
t. Re-lyophilize the combined product and then weigh the vial again to obtain the yield of H3(15-135, A15C).

Note: After ULP1 digestion and centrifugation, the supernatant mainly contains His_6-SUMO tag.

⚠ CRITICAL: DTT is critical for ULP1 cleavage as the N-terminal Cys from H3(15-135, A15C) may form a disulfide bond with glutathione during the expression and purification, which inhibits the cleavage by ULP1. Also, an excessive amount of DTT may form disulfide with H3 too. Treat the protein with 5 mM TCEP for a few min for a complete reduction of disulfide before UPLC-MS analysis or HPLC purification.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
BL21 (DE3) cells	TransGene Biotech	CD601-02
Chemicals, peptides, and recombinant proteins		
Sodium tetraethylborate	Sigma-Aldrich	CAT#481483
Ribonuclease A from bovine pancreas	Sigma-Aldrich	CAT#R6513
3,3′,3″-Phosphinetriyltripropanoic acid hydrochloride (TCEP·HCl)	Bidepharm	CAT#BD155793
Guanidine hydrochloride (GdmCl)	Bidepharm	CAT#BD105703
Citric acid	Bidepharm	CAT#BD34933
Sodium citrate	Bidepharm	CAT#BD105976
4-Mercaptophenylacetic acid (MPAA)	Sigma-Aldrich	CAT#653152
Acetylacetone	Sigma-Aldrich	CAT#P7754
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4·6H_2O$)	Bidepharm	CAT#BD309165
Sodium phosphate monobasic hydrate ($NaH_2PO_4·H2O$)	Bidepharm	CAT#BD01387304
2-Cl-(Trt)-Cl resin (0.6 mmol/g)	CS Bio	CAT#262001
Hydrazine hydrate ($NH_2NH_2·H_2O$)	TCI	CAT#H0172
HATU	CS Bio	CAT#311017
DIPEA	Energy Chemical	CAT#W320014
4-Methyl piperidine	Energy Chemical	CAT#E020655
Boc-Ala-OH	CS Bio	CAT#011202
Fmoc-Arg(Pbf)-OH	CS Bio	CAT#021107
Fmoc-Thr(OtBu)-OH	CS Bio	CAT#171108
Fmoc-Lys(Boc)-OH	CS Bio	CAT#121114
Fmoc-Glu(OAll)-OH	CS Bio	CAT#071106
Fmoc-Ala-OH	CS Bio	CAT#011101
Fmoc-Ser(OtBu)-OH	CS Bio	CAT#161108
Fmoc-Gly-OH	CS Bio	CAT#081101
Tetrakis(triphenylphosphine)-palladium $Pd(PPh_3)_4$	Energy Chemical	CAT#E060079
N,N-Dimethylbarbituric acid	Energy Chemical	CAT#A010336
PyAOP	CS Bio	CAT#311030

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TFA	Energy Chemical	CAT#W810133
EDT	Sigma-Aldrich	CAT#02390
TIPS	Energy Chemical	CAT#D020046
Serotonin hydrochloride	Energy Chemical	CAT#B010483
Isopropyl b-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	CAT#I6758
DTT	Sigma-Aldrich	CAT#D0632
N,N-Dimethylformamide (DMF)	AQA	Cat#DF-1161-2500
Dichloromethane (DCM)	Fisher Chemical	Cat#D/1852/17
Acetonitrile (ACN)	DUKSAN	Cat#2957
Diethyl ether	RCI Labscan	Cat#AR1044B
Luria-Bertani (LB) medium	Gibco	Cat#10855001
Tris-HCl	Invitrogen	Cat#15506017
Urea	Macklin	Cat#U820355
Imidazole	Macklin	Cat#I6122
Serotonylated H3(1-14) with N-terminal hydrazide	This study	N/A
Recombinant H3(15-135, A15C)	This study	N/A
Software and algorithms		
MassLynx	Waters	https://www.waters.com/waters/en_US/MassLynx-MS-Software/nav.htm?locale=en_US&cid=513662
UniDec	Marty et al.[18]	http://unidec.chem.ox.ac.uk/
Recombinant DNA		
pET28a plasmid encoding N-terminally His6–SUMO tagged histone H3	This study	Available upon request
Other		
LC-MS (ACQUITY UPLC H-Class PLUS System)	Waters	Cat#720003268en
Preparative HPLC	Waters	N/A
PTFE membrane filter (13 mm × 0.22 μm)	Cobetter	Cat#SFMPT-2013

MATERIALS AND EQUIPMENT

Solution preparation

0.5 *g*/mL $NaBEt_4$ solution

Reagent	Final concentration	Amount
Sodium tetraethylborate	0.5 *g*/mL	50 mg
ddH_2O	N/A	50 μL
Total	**N/A**	**100 μL**

Freshly preparation of this solution is recommended. Can also be stored at 25°C for 24 h or 4°C for 1–2 weeks.

⚠ CRITICAL: Sodium tetraethylborate is pyrophoric and hygroscopic in the presence of moisture and air. It should be dispensed carefully under an anaerobic atmosphere. Once dissolved concentratedly in water (around 0.5 *g*/mL), it becomes much more stable and can be handled under ambient conditions.

0.5 M citrate buffer with 6 M GdmCl, pH 4.5

Reagent	Final concentration	Amount
GdmCl	6 M	28.7 *g*
Citric acid	0.27 M	2.6 *g*
Sodium citrate dihydrate	0.23 M	3.4 *g*

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Continued		
Reagent	Final concentration	Amount
ddH_2O	N/A	10 mL
Total	**N/A**	**50 mL**

Can be store at 25°C up to one year.

Desulfurization buffer		
Reagent	Final concentration	Amount
0.5 M citrate buffer with 6 M GdmCl, pH 4.5	6 M	4.8 mL
TCEP	0.2 M	0.29 g
NaOH (6 M)	0.4 M	67 μL
Total	**N/A**	**5.0 mL**

Fresh preparation of this solution is recommended. Can also be stored at 25°C for 24 h or 4°C for 1–2 weeks.

Note: NaOH (6 M) is corrosive and irritant to the skin and eye, causing severe burns and damage. Wear goggles, lab coat, and gloves during the handling of such a solution. Once contacted, rinse cautiously with water for several min.

Note: It's recommended to check the pH after preparing the solution since TCEP generally acidifies aqueous buffers.

Alternatives: The citrate buffer here can be replaced by buffers with the same pH, such as acetate buffer.

0.2 M pH 3 phosphate buffer with 6 M GdmCl		
Reagent	Final concentration	Amount
GdmCl	6 M	28.7 g
$NaH_2PO_4 \cdot H_2O$	0.2 M	1.4 g
HCl (6 M)	N/A	N/A[a]
ddH_2O	N/A	10 mL
Total	**N/A**	**50 mL**

Can be stored at 25°C up to one year.

[a]Add 6 M HCl until the pH of the solution is around 3.0, checked by pH meter or pH paper.

Note: HCl (6 M) is corrosive and irritant to the skin and eye, causing severe burns and damage. Wear goggles, lab coat, and gloves during the handling of such solution. Once contacted, rinse cautiously with water for several min.

NCL buffer (0.2 M pH 6.8 phosphate buffer with 6 M GdmCl)		
Reagent	Final concentration	Amount
GdmCl	6 M	28.7 g
$NaH_2PO_4 \cdot H_2O$	0.12 M	0.58 g
$Na_2HPO_4 \cdot 6H_2O$	0.084 M	1.55 g
ddH_2O	N/A	10 mL
Total	**N/A**	**50 mL**

Can be stored at 25°C up to one year.

0.5 M Acetylacetone solution		
Reagent	Final concentration	Amount
Acetylacetone	0.5 M	51 μL
ddH_2O	N/A	950 μL
Total	**N/A**	**1.0 mL**
Can be stored at 4°C for up to two weeks.		

Lysis buffer, pH 7.5		
Reagent	Final concentration	Amount
1 M Tris (pH 7.5)	0.05 M	50 mL
5 M NaCl	0.5 M	100 mL
8 M Urea	6 M	750 mL
Imidazole	0.02 M	1.4 *g*
ddH_2O	N/A	100 mL
Total	**N/A**	**1.0 L**
Can be stored at 4°C for up to one month.		

Elution buffer, pH 7.5		
Reagent	Final concentration	Amount
1 M Tris (pH 7.5)	0.05 M	50 mL
5 M NaCl	0.5 M	100 mL
8 M Urea	6 M	750 mL
Imidazole	0.5 M	34 *g*
ddH_2O	N/A	50–100 mL
Total	**N/A**	**1.0 L**
Can be stored at 4°C for up to one month.		

Refolding buffer, pH 7.5		
Reagent	Final concentration	Amount
1 M Tris (pH 7.5)	0.05 M	50 mL
5 M NaCl	0.5 M	100 mL
8 M Urea	6 M	750 mL
1 M DTT	0.05 M	5.0 mL
ddH_2O	N/A	95 mL
Total	**N/A**	**1.0 L**
Can be stored at 4°C for up to one month.		

Equipment set up

UPLC and Preparative HPLC

Waters UPLC H-class system is applied for analytical purposes, which equipped with an ACQUITY UPLC photodiode array detector, a Waters SQ Detector 2 mass spectrometer, and a Waters ACQUITY BEH C18 or C4 column. Preparative HPLC is performed on Waters HPLC system, including a quaternary pump (Waters 2545), a UV/Vis detector (Waters 2489), and a Vydac 218TP C18 column. Detailed LC conditions are listed as below. Results will be collected and analyzed with Waters Masslynx. MS spectra are deconvoluted by UniDec.

Detailed conditions of UPLC and preparative HPLC		
	UPLC	Preparative HPLC
Column	Waters ACQUITY BEH C18 or C4 column (2.1 × 50 mm, 130 Å, 1.7 μm)	Vydac 214TP C4 column (22 × 250 mm, 300 Å, 10 μm)
Solvents	A: Acetonitrile (ACN) containing 0.1% (vol/vol) TFA; B: deionized H_2O containing 0.1% (vol/vol) TFA	A: Acetonitrile (ACN) containing 0.1% (vol/vol) TFA; B: deionized H_2O containing 0.1% (vol/vol) TFA

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	UPLC	Preparative HPLC
Flowrate	0.4 mL/min	10 mL/min
Run time	10 min	45 min
Gradient	5–15% of A for 2-min desalting, then 15%–65% of A for 8 min (vol/vol).	15–65% of A for 45 min (vol/vol).
Injection	10 μL (maximum)	10.0 mL (maximum)
Wavelength	190–400 nm	205 nm

STEP-BY-STEP METHOD DETAILS

Activation of peptidyl hydrazide

Timing: 5 h

This section describes the activation of peptidyl hydrazides to thioesters by acetylacetone (Figure 2), following a similar procedure described by Flood et al.[19]

1. Dissolve 3.0 μmol serotonylated H3(1–14)-$NHNH_2$ (5.0 mg) with 0.2 M pH 3 phosphate buffer with 6M GdmCl (600 μL) in a 1.5-mL Eppendorf (EP) tube.
2. Add 0.12 mmol MPAA (20 mg) to the solution above. The final concentration of MPAA is around 200 mM, and the concentration of peptidyl hydrazide is about 5 mM.

   Note: MPAA cannot fully dissolve in this buffer, and a yellowish slurry will be obtained. But the insoluble parts would not affect the thioester formation.

   Note: MPAA may have a strong odor/smell. Handle it in the fumehood and wear a mask, goggles and gloves. Spray bleach to distinct its odor once spilled or contacted.

3. Sonicate the solution to facilitate the dispersion of MPAA.
4. Add 4.8 μL 0.5 M acetylacetone (2.4 μmol, 0.8 eq.).

   ⚠ CRITICAL: The acetylacetone should be added less than 1 equivalent to the serotonylated H3(1–14)-$NHNH_2$. The remaining acetylacetone will react with the C-terminal Cys15 of truncated H3(15-135), causing uneven protein ligation and inseparable impurity in the end.

5. Incubate the mixture from step 4 at room temperature (22°C–25°C) for around 5 h.
6. Monitor the generation of MPAA-thioester by UPLC-MS.
7. After completion, the MPAA-thioester is subjected to the next step without purification.

   Optional: The MPAA-thioester of serotonylated H3(1-14) can be also purified by HPLC at this step.

   Note: See troubleshooting 1.

Expressed protein ligation

Timing: 18 h

This section describes the expressed protein ligation (EPL) between recombinant H3(15-135) and MPAA thioester of H3(1-14) with serotonylation (Figure 2).

8. Adjust the pH of the MPAA thioester solution above to around 6.0 by 6 M NaOH.

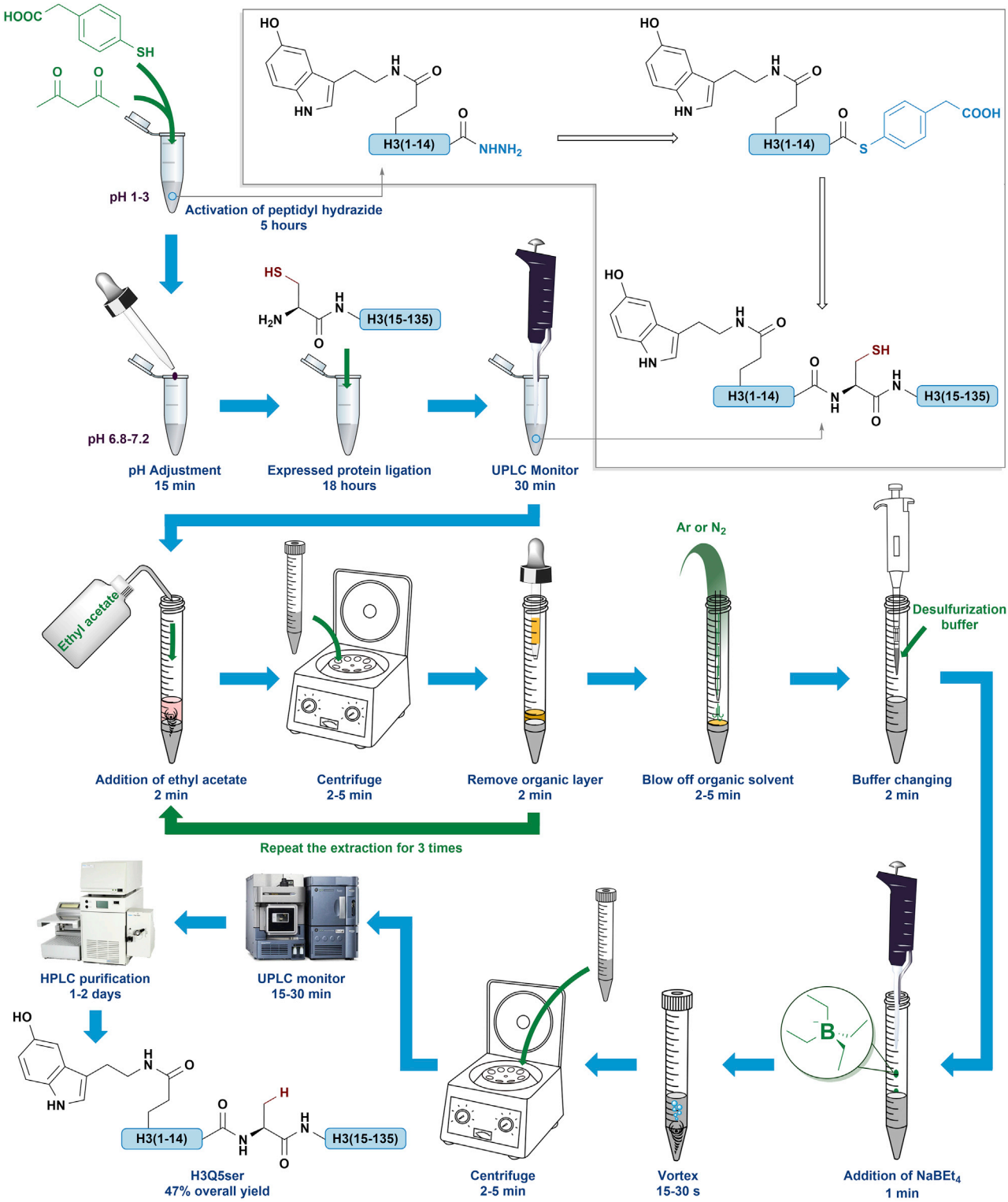

Figure 2. Concise protocol of H3Q5ser semisynthesis *via* EPL-ADD

See major step 1–42.

Note: 6 M NaOH aqueous solution is corrosive and irritative. Wear gloves, goggles, and a mask to avoid direct contact with skin and eyes.

9. Continue to refine the pH of the thioester solution from step 8 to 7.0 by 1 M NaOH carefully.

⚠ CRITICAL: Adjusting pH far above 7.0 may cause quantitative hydrolysis of MPAA thioester and self-cyclization. 1 M NaOH solution is recommended instead of the 6 M NaOH solution.

10. Dilute the solution with 1 mL NCL buffer to around 1.9 mM according to the serotonylated H3(1-14)-$NHNH_2$.
11. Pipette the MPAA thioester solution to another 2.0-mL eppendorf tube with recombinant H3(15-135) (1.6 mg, 0.11 μmol, 1.0 eq.).
12. Vortex the mixture until the expressed part is completely dissolved. The final concentration of H3(15-135) is around 1.0 mg/mL (72 μM).
13. Incubate the ligation at room temperature overnight (22°C–25°C, 8–12 h).
14. Pipette 20 μL of the step 13 reaction mixture and dilute it with 80 μL 100 mM TCEP solution.

Note: Without addition of TCEP solution, the UPLC-MS spectrum may look messy. See troubleshooting 2.

15. Using 1-mL syringe, pass the diluted aliquot through PTFE membrane filter (13 mm × 0.22 μm).
16. Check the ligation progress by injecting 5 μL of filtered sample into UPLC.

⚠ CRITICAL: Make sure all the recombinant part is converted to the ligated product. See troubleshooting 3.

Note: This EPL reaction can be left for 48 h under room temperature (22°C–25°C), but it is recommended to proceed to the next step after the completion of the reaction.

17. Once the EPL is completed, the reaction can be subjected to the next step directly.

Extraction of 4-Mercaptophenylacetic acid (MPAA)

Timing: 30 min

This section describes the MPAA extraction procedure to minimize the effect of MPAA in the following desulfurization. This step should be done in fumehood as volatile and flammable organic solvents will be involved (Figure 2).

18. Pipette the EPL solution to a 15-mL centrifuge tube.
19. Add 230 mg of TCEP·HCl in solid form.
20. Vortex the mixture until the salt is completely dissolved. The final concentration of TCEP is around 0.5 M.

Note: Some yellow precipitation from MPAA is normal, and it will be removed in the following extraction.

21. Adjust the solution pH to 3 by 6 M HCl.

Note: Acidic pH facilitates the extraction of MPAA by organic solvent.

22. Add 2 mL ethyl acetate.

Note: Ethyl acetate is a volatile and flammable liquid. Handle it in the fumehood and wear a mask, gloves, and goggles to avoid any direct contact.

23. Vortex the bi-layer mixture for a few sec.
24. Centrifuge at 2,600 *g* for 2–5 min.
25. Using a glass pipette, remove most of the upper organic layer carefully.
26. Repeat step 22–25 at least three times.

Note: According to our determination, the residual MPAA concentration is normally below 100 μM after such extraction treatment.

⚠ CRITICAL: Keep all insoluble precipitation during steps 22–26. See troubleshooting 4.

27. Use N_2 or Ar flow to blow off any residual ethyl acetate on the top of the aqueous solution.

Add-and-done desulfurization in one pot

Timing: 30 min

This section describes the one-pot add-and-done desulfurization procedure to generate H3Q5ser after the previous expressed protein ligation (Figure 2).

28. Dilute the solution above by adding 1.6 mL desulfurization buffer.
29. Adjust the pH to 4.5 by 6 M NaOH. The final concentration of protein is approximately 0.5 mg/mL (36 μM).

⚠ CRITICAL: The pH of the protein solution should be controlled between 4 and 5. See troubleshooting 5.

30. Add 48 μL 0.5 *g*/mL $NaBEt_4$ solution through a pipette. The final concentration of $NaBEt_4$ is around 50 mM.
31. Enclose the cap of the centrifuge tube and vortex the reaction mixture for 15–30 s.

Note: Intensive bubbling generation in this step is normal.

32. Centrifuge at 2,600 *g* for 1 min.
33. Check the desulfurization progress by UPLC as steps 14–16.

Note: The solution may be cloudy at first due to the formation of triethylborane. It will not affect the analysis and will gradually become clear after a few min.

Note: See troubleshooting 6.

Purification of H3Q5ser

Timing: 2 days

This section describes the purification of the final product H3Q5ser.

34. Dilute the reaction mixture from step 33 to 5.0 mL by 10% (vol/vol) ACN in H_2O.
35. Filter the solution through 0.22 μM PTFE filter to a 15-mL centrifuge tube.
36. Centrifuge the filtered solution at 2,600 *g* for 2 min at room temperature (22°C–25°C).
37. Set up HPLC parameters according to the table in the materials and equipment section.

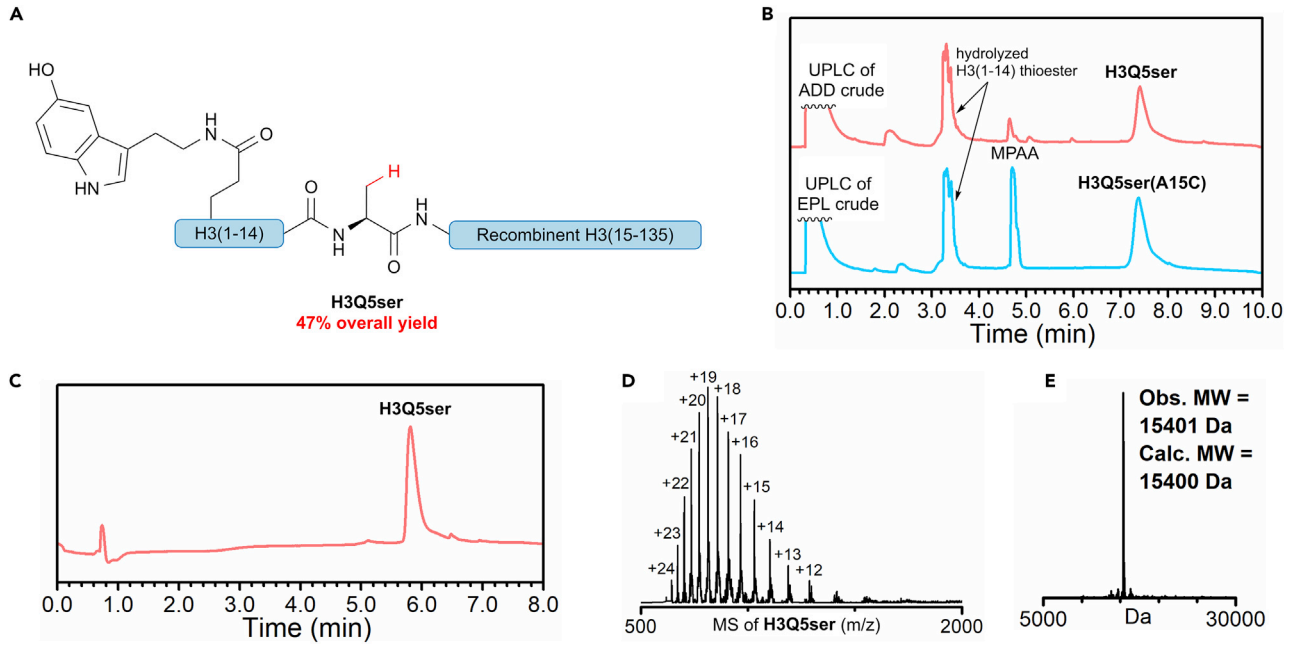

Figure 3. Characterization of H3Q5ser

(A) Isolated yield of H3Q5ser.

(B) UPLC trace of reaction crude before and after add-and-done desulfurization.

(C) UPLC trace of purified H3Q5ser at 280 nm.

(D) ESI + MS spectrum of H3Q5ser acquired by UPLC-MS. (ESI-MS calcd. for $C_{679}H_{1151}N_{216}O_{188}S_2$ $[M+13H]^{13+}$ m/z = 1185.6, found 1185.9, $C_{679}H_{1152}N_{216}O_{188}S_2$ $[M+14H]^{14+}$ m/z = 1101.0, found 1101.0, $C_{679}H_{1153}N_{216}O_{188}S_2$ $[M+15H]^{15+}$ m/z = 1027.7, found 1027.8, $C_{679}H_{1154}N_{216}O_{188}S_2$ $[M+16H]^{16+}$ m/z = 963.5, found 963.7, $C_{679}H_{1155}N_{216}O_{188}S_2$ $[M+17H]^{17+}$ m/z = 906.7, found 906.8, $C_{679}H_{1156}N_{216}O_{188}S_2$ $[M+18H]^{18+}$ m/z = 856.6, found 856.9, $C_{679}H_{1157}N_{216}O_{188}S_2$ $[M+19H]^{19+}$ m/z = 811.5, found 811.7, $C_{679}H_{1158}N_{216}O_{188}S_2$ $[M+20H]^{20+}$ m/z = 771.0, found 770.7, $C_{679}H_{1160}N_{216}O_{188}S_2$ $[M+22H]^{22+}$ m/z = 701.0, found 701.3; $C_{679}H_{1161}N_{216}O_{188}S_2$ $[M+23H]^{22+}$ m/z = 670.6, found 670.3).

(E) Deconvoluted MS spectrum of H3Q5ser by UniDec (Parameters: Bin Every: 1.0 Da; Sample Mass Every: 1.0 Da; Peak FWHM: 1.0 Th; Peak Detection Range: 1.0 Da; Peak Detection Threshold: 0.1). The figure is reprinted from permission from Sun et al.[1].

38. Inject 5.0 mL of sample from step 35 into the sample loop of a manual injector.
39. Turn the valve position from 'Load' to 'Inject' to trigger the HPLC UV acquisition.

Note: Waters HPLC was used at step 39 For other HPLC model, operation may be different and it should be referred to the official manual respectively.

40. Collect eluent with strong UV absorption.
41. Check fractions by UPLC to find out the desired product.
42. Lyophilize pure fractions to obtain the final product as a white powder.

EXPECTED OUTCOMES

A successful reproduction of this protocol should observe a complete consumption of H3(15–135) during the EPL followed by a clean desulfurization of the ligated product in dozens of sec. The final product H3Q5ser could be obtained as a white powder in high yield (40–47% yield over thioester formation, expressed protein ligation, and desulfurization). The UV and MS spectra of synthesized H3Q5ser should be consistent with that in Figure 3.

LIMITATIONS

The strategy of applying EPL-ADD in one pot provides a convenient way to produce peptides/proteins with tailor-made modification. However, there are still some key factors to be considered when it is used for syntheses of other targets. 1) Whether modifications of interest are compatible with the

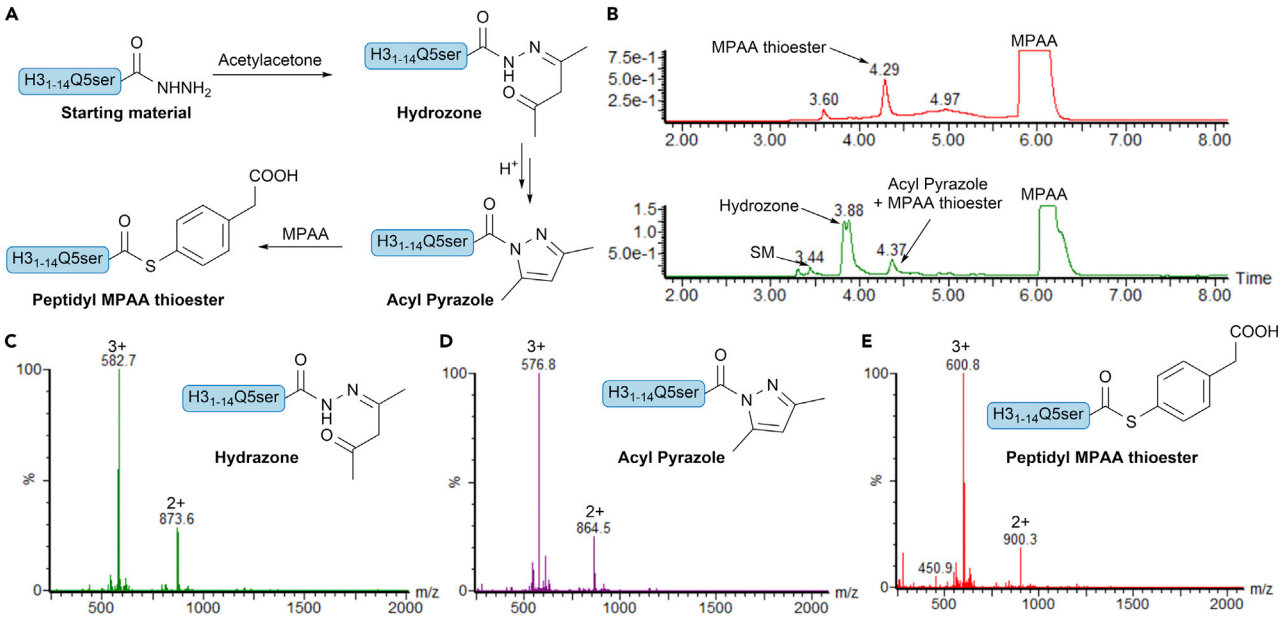

Figure 4. Comparison of MPAA thioester formation under different pH

(A) Concise scheme of peptidyl hydrazide activation to its MPAA thioester.

(B) UPLC trace of reaction crude under pH 1–2 and pH 4.5 (From top to bottom).

(C) ESI + MS spectrum of activation intermediate hydrazone. (ESI-MS calcd. for $C_{75}H_{131}N_{27}O_{21}$ $[M+2H]^{2+}$ m/z = 873.5, found 873.6, $C_{75}H_{132}N_{27}O_{21}$ $[M+3H]^{3+}$ m/z = 582.7, found 582.7).

(D) ESI + MS spectrum of activation intermediate hydrazone. (ESI-MS calcd. for $C_{75}H_{129}N_{27}O_{20}$ $[M+2H]^{2+}$ m/z = 864.5, found 864.5, $C_{75}H_{130}N_{27}O_{20}$ $[M+3H]^{3+}$ m/z = 576.7, found 576.8).

(E) ESI + MS spectrum of activation intermediate hydrazone. (ESI-MS calcd. for $C_{78}H_{129}N_{25}O_{22}S$ $[M+2H]^{2+}$ m/z = 900.5, found 900.3, $C_{78}H_{130}N_{25}O_{22}S$ $[M+3H]^{3+}$ m/z = 600.7, found 600.8).

conditions of EPL and ADD. For example, we found that alkyne and diazirine could not tolerate current free-radical based desulfurization methods. 2) If the reaction sites (e.g., thioester and N-terminal Cys in EPL; Cys residues in ADD) can be fully exposed to solvent. Some difficult peptides or membrane proteins are prone to aggregate even at 6 M GdmCl denatured buffer, causing incomplete ligation and desulfurization. 3) When some proteins form heavy froth with gas, it may cause incomplete desulfurization due to the fact that protein molecules inside the froth cannot contact desulfurization species. In these cases, elimination of froth by centrifugation and retreatment of $NaBEt_4$ solution may be required. Meanwhile, if other native Cys residues are present in the sequence, they will be desulfurized to Ala residues as well, which may afford wildtype sequence of protein after desulfurization. In that case, suitable orthogonal protection should be considered or developed prior to the NCL-ADD steps.

TROUBLESHOOTING

Problem 1

The conversion of serotonylated H3(1-14)-$NHNH_2$ to its MPAA thioester is too low.

Potential solution

Firstly, check what the major form of the peptide (Figure 4) is. 1) If it is mostly the starting material, that may suggest that the amount of acetylacetone is not accurate. Dispense the 0.5 M acetylacetone stock freshly and add another 4.8 μL of it as steps 4–6. 2) If hydrazone species is the major form (Figure 4C), it means the peptide substrate has reacted with acetylacetone, but the cyclization step is inefficient due to the low acidity in the buffer. Adjust the pH of the reaction mixture to 1–2 to

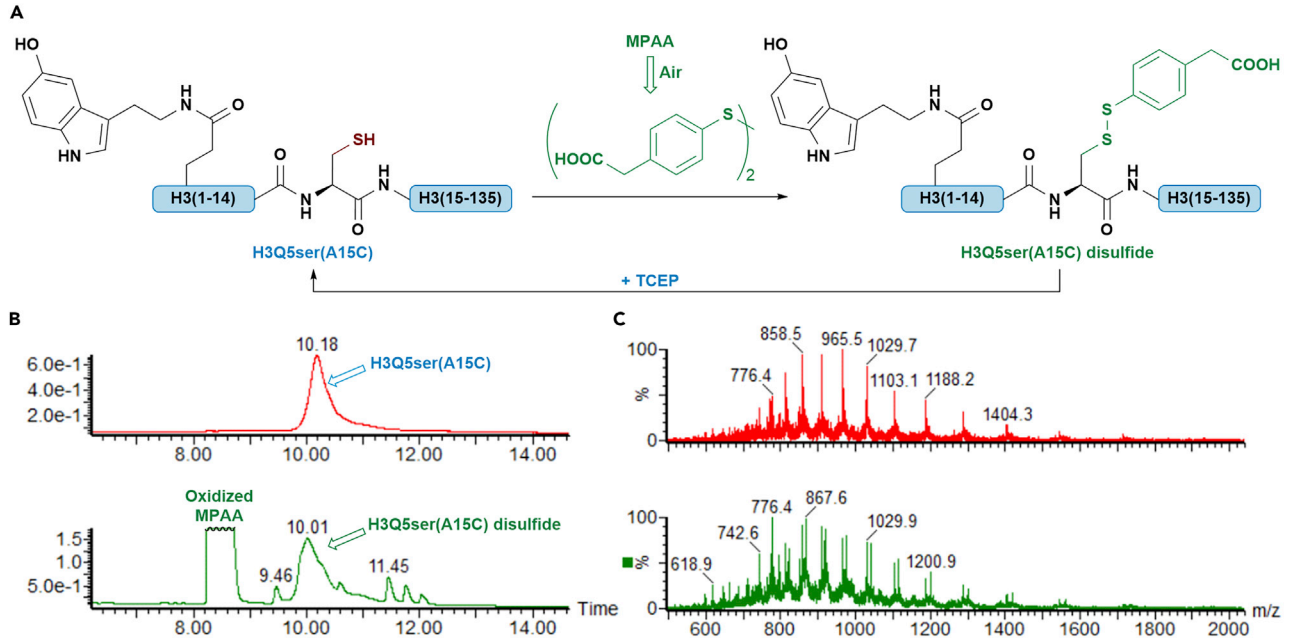

Figure 5. Analytical check of EPL reaction with or without TCEP treatment

(A) Scheme of disulfide bond formation and reduction of EPL product H3Q5ser(A15C). A 15-min UPLC run method was used here for better resolution of oxidized MPAA and ligated product.

(B) UPLC trace of EPL aliquots. Top: with TCEP treatment; Bottom: without TCEP treatment.

(C) ESI + MS spectrum of EPL product. Top: with TCEP treatment; Bottom: without TCEP treatment.

facilitate the acyl pyrrole formation and repeat steps 5–6. 3) If acyl pyrrole is present (Figure 4D), another 20 mg of MPAA can be added to accelerate the thioesterification and repeat steps 5–6.

Problem 2

The UPLC-MS spectrum of EPL is messy, containing side product mass equal to protein +166 Da.

Potential solution

The Cys residue of starting material or ligated product is oxidized to disulfide with MPAA after long-time incubation (Figure 5). Addition of TCEP to the reaction aliquot prior to the UPLC monitor is necessary for reducing the disulfide bond. Redo step 14 and incubate the reduction for at least 5 min.

Problem 3

The expressed protein ligation could not consume all the recombinant H3(15-135).

Potential solution

Carry out another batch of MPAA thioester preparation following major steps 1–10 and add it to the incomplete ligation mixture for another 16–24 h incubation. Make sure the ligation pH is 6.8–7.2 to decrease the hydrolysis rate of the MPAA thioester.

Problem 4

The protein concentration is significantly lower after the extraction of MPAA by ethyl acetate.

Potential solution

During the extraction, proteins may be precipitated by the organic solvent added to the system. After centrifugation, the precipitation is not at the bottom of the tube but in between the aqueous layer

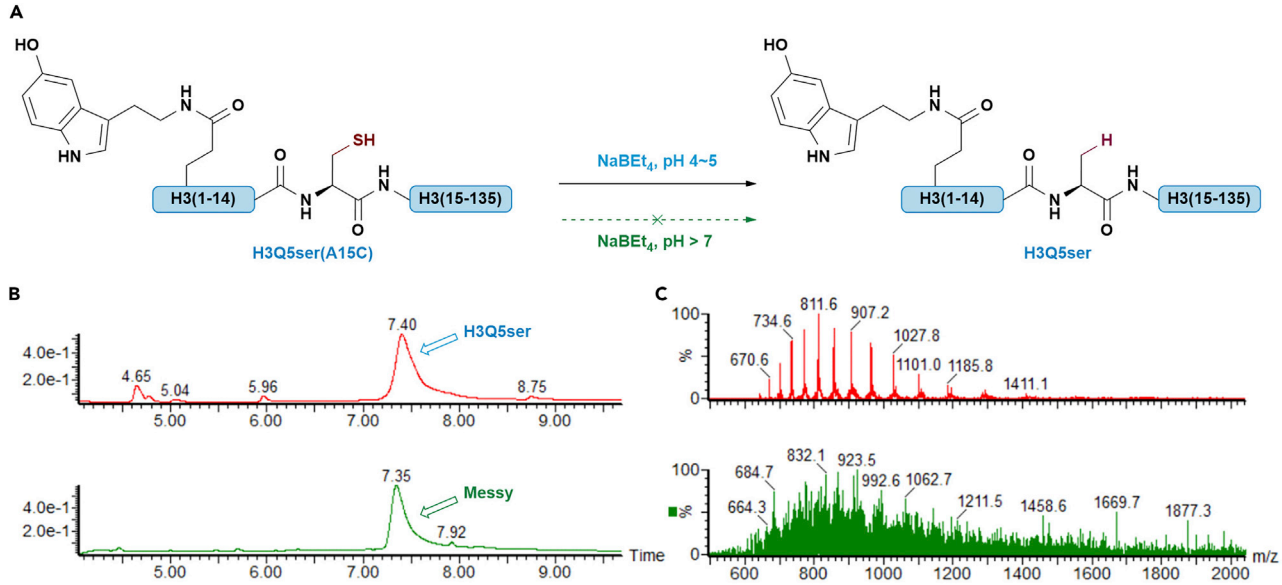

Figure 6. Analytical check of ADD reaction under different pHs

(A) Scheme of ADD.

(B) UPLC trace of ADD aliquots. Top: ADD in pH 4–5; Bottom: ADD in pH > 7.

(C) ESI + MS spectrum of ADD product. Top: ADD in pH 4–5; Bottom: ADD in pH > 7.

and organic layer. Discarding precipitation when pipetting out the organic layer may cause loss of protein materials. Keep a minimum amount of organic solvent at the top of the precipitation layer so that the pipetting will not disrupt it. After the removal of residual organic solvent in step 27, the solid can be redissolved in the aqueous buffer by vortex or sonication.

Problem 5

The UPLC-MS spectrum after desulfurization becomes messy.

Potential solution

The pH of the desulfurization mixture may be too high (pH > 7), causing undesired side reactions (Figure 6). Use 0.5 M citrate buffer for more buffer capability between pH 4.0–5.0. Check the pH before and after adding $NaBEt_4$ solution using a pH meter or pH paper with a standard buffer sample as a control. When pH is below 4.0, the desulfurization can also be done but $NaBEt_4$ decomposes faster due to its hydrolysis in very acidic pH.

Problem 6

The desulfurization is incomplete.

Potential solution

1) If there is a significant amount of MPAA (e.g., UV absorption larger than that of protein at 205 nm or over 0.5 mM by determination), repeat steps 22–27 again to remove residual MPAA. Then add another portion of $NaBEt_4$ to do the ADD again as steps 30–33. 2) If most of the MPAA has been removed, repeat steps 30–34 one more time. For H3Q5ser, typically no doping will be required. But this desulfurization buffer recipe has the redundancy for 5 times doping in total. 3) The buffer used may not fully denature the substrate. Dimethyl sulfoxide (DMSO), Hexafluoroisopropanol (HFIP), Trifluoroethanol (TFE), and ACN mixed with aqueous buffer are compatible with ADD, and they can be used for optimal solvent screening.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xuechen Li (xuechenl@hku.hk).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

No data and code were developed in this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, Z.S., X.L.; Methodology, Z.S.; Investigation, Z.S., T.W., Y.C.; Formal analysis, Z.S.; Visualization, Z.S.; Writing, Z.S., T.W., Y.C., X.L.; Supervision, X.L.; Funding acquisition, X.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Sun, Z., Ma, W., Cao, Y., Wei, T., Mo, X., Chow, H.Y., Tan, Y., Cheung, C.H., Liu, J., Lee, H.K., et al. (2022). Superfast desulfurization for protein chemical synthesis and modification. Chem *8*, 2542–2557. https://doi.org/10.1016/j.chempr.2022.07.017.

2. Agouridas, V., El Mahdi, O., Diemer, V., Cargoët, M., Monbaliu, J.C.M., and Melnyk, O. (2019). Native chemical ligation and extended methods: mechanisms, catalysis, scope, and limitations. Chem. Rev. *119*, 7328–7443. https://doi.org/10.1021/acs.chemrev.8b00712.

3. Thompson, R.E., and Muir, T.W. (2020). Chemoenzymatic semisynthesis of proteins. Chem. Rev. *120*, 3051–3126. https://doi.org/10.1021/acs.chemrev.9b00450.

4. Conibear, A.C. (2020). Deciphering protein post-translational modifications using chemical biology tools. Nat. Rev. Chem *4*, 674–695. https://doi.org/10.1038/s41570-020-00223-8.

5. Merrifield, R.B. (1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. *85*, 2149–2154. https://doi.org/10.1021/ja00897a025.

6. Dawson, P.E., Muir, T.W., Clark-Lewis, I., and Kent, S.B. (1994). Synthesis of proteins by native chemical ligation. Science *266*, 776–779. https://doi.org/10.1126/science.7973629.

7. Yan, L.Z., and Dawson, P.E. (2001). Synthesis of peptides and proteins without cysteine residues by native chemical ligation combined with desulfurization. J. Am. Chem. Soc. *123*, 526–533. https://doi.org/10.1021/ja003265m.

8. Wan, Q., and Danishefsky, S.J. (2007). Free-radical-based, specific desulfurization of cysteine: a powerful advance in the synthesis of polypeptides and glycopolypeptides. Angew. Chem. Int. Ed. Engl. *46*, 9248–9252. https://doi.org/10.1002/anie.200704195.

9. Bode, J.W., Fox, R.M., and Baucom, K.D. (2006). Chemoselective amide ligations by decarboxylative condensations of N-alkylhydroxylamines and ketoacids. Angew. Chem. Int. Ed. Engl. *45*, 1248–1252. https://doi.org/10.1002/anie.200503991.

10. Pattabiraman, V.R., Ogunkoya, A.O., and Bode, J.W. (2012). Chemical protein synthesis by chemoselective α-ketoacid-hydroxylamine (KAHA) ligations with 5-oxaproline. Angew. Chem. Int. Ed. Engl. *51*, 5114–5118. https://doi.org/10.1002/anie.201200907.

11. Li, X., Lam, H.Y., Zhang, Y., and Chan, C.K. (2010). Salicylaldehyde ester-induced chemoselective peptide ligations: enabling generation of natural peptidic linkages at the serine/threonine sites. Org. Lett. *12*, 1724–1727. https://doi.org/10.1021/ol1003109.

12. Zhang, Y., Xu, C., Lam, H.Y., Lee, C.L., and Li, X. (2013). Protein chemical synthesis by serine and threonine ligation. Proc. Natl. Acad. Sci. USA *110*, 6657–6662. https://doi.org/10.1073/pnas.1221012110.

13. Tan, Y., Li, J., Jin, K., Liu, J., Chen, Z., Yang, J., and Li, X. (2020). Cysteine/penicillamine ligation independent of terminal steric demands for chemical protein synthesis. Angew. Chem. Int. Ed. Engl. *59*, 12741–12745. https://doi.org/10.1002/anie.202003652.

14. Tan, Y., Wu, H., Wei, T., and Li, X. (2020). Chemical protein synthesis: advances, challenges, and outlooks. J. Am. Chem. Soc. *142*, 20288–20298. https://doi.org/10.1021/jacs.0c09664.

15. Zheng, J.S., Tang, S., Qi, Y.K., Wang, Z.P., and Liu, L. (2013). Chemical synthesis of proteins using peptide hydrazides as thioester surrogates. Nat. Protoc. *8*, 2483–2495. https://doi.org/10.1038/nprot.2013.152.

16. Wei, T., Liu, H., Wu, H., Pu, F., and Li, X. (2021). Serine/threonine ligation-assisted chemical synthesis of HMGA1a protein with site-specific post-translational modifications. STAR Protoc. *2*, 100777. https://doi.org/10.1016/j.xpro.2021.100777.

17. Farrelly, L.A., Thompson, R.E., Zhao, S., Lepack, A.E., Lyu, Y., Bhanu, N.V., Zhang, B., Loh, Y.H.E., Ramakrishnan, A., Vadodaria, K.C., et al. (2019). Histone serotonylation is a permissive modification that enhances TFIID binding to H3K4me3. Nature *567*, 535–539. https://doi.org/10.1038/s41586-019-1024-7.

18. Marty, M.T., Baldwin, A.J., Marklund, E.G., Hochberg, G.K.A., Benesch, J.L.P., and Robinson, C.V. (2015). Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles. Anal. Chem. *87*, 4370–4376. https://doi.org/10.1021/acs.analchem.5b00140.

19. Flood, D.T., Hintzen, J.C.J., Bird, M.J., Cistrone, P.A., Chen, J.S., and Dawson, P.E. (2018). Leveraging the knorr pyrazole synthesis for the facile generation of thioester surrogates for use in native chemical ligation. Angew. Chem. Int. Ed. Engl. *57*, 11634–11639. https://doi.org/10.1002/anie.201805191.