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Title: Site-Specific Lysine Lactylation via Genetic Code Expansion in *E. coli* and Mammalian Cells

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**
- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
- 3. Filming location:** Will the filming need to take place in multiple locations? **No**
- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 12

Number of Shots: 26

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

Answers will be slightly edited in accordance with the journal guidelines

INTRODUCTION:

~~REQUIRED:~~ Summarize your research focus and the main questions it aims to answer.

- 1.1. **Shunyu Yao:** We develop a KlacRS/tRNA system to overcome mimicry and enzyme limitations, enabling precise lysine lactylation incorporation to study its biological roles.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Shunyu Yao:** This protocol can be applied to study tumor metabolism, enzyme functions and Klac sites.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Shunyu Yao:** This protocol enables investigation of site-specific lysine lactylation effects on protein function and facilitates measurement of resulting biological and functional outcomes.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Shunyu Yao:** Following this procedure, researchers can perform mechanistic studies, conduct high-throughput screening assays, and generate stable cell lines for extended functional analyses.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Shunyu Yao:** Our future studies aim to improve efficiency, enable stable integration, and develop Klac biosynthesis.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Transformation and Culturing *E. coli* Cells for Expression of Lactylated Protein

Demonstrator: Shihan Wu

2.1. To begin, co-transform 50 microliters of *Escherichia coli* DH-10-B chemically competent cells with approximately 100 nanograms each of the desired plasmids [1-TXT].

2.1.1. WIDE: Talent pipetting 100 nanograms each of plasmid to a tube with cells. **TXT: Plasmids: pBad-ENO1-K89TAG; pEvol-MmKlacRS1**

2.2. Incubate the transformation mixture on ice for 25 minutes [1], then heat-shock it at 42 degrees Celsius for 45 seconds [2]. Return the tube to ice for 3 minutes [3]. Add 350 microliters of Luria-Bertani medium [4] and shake the tube vigorously at 220 revolutions per minute for 1 hour at 37 degrees Celsius [5].

2.2.1. Talent placing the transformation tube on ice.

2.2.2. Talent transferring the tube into a 42 degrees Celsius water bath for heat shock.

2.2.3. Talent returning the tube to ice immediately after heat shock.

2.2.4. Talent pipetting 350 microliters of room temperature Luria-Bertani medium into the tube.

2.2.5. Talent placing the tube in a shaking incubator set to 220 revolutions per minute and 37 degrees Celsius.

2.3. Now, spread the transformed cell culture onto a Luria-Bertani agar plate supplemented with ampicillin and chloramphenicol [1-TXT]. Incubate the plate at 37 degrees Celsius for 12 to 16 hours [2].

2.3.1. Talent spreading the cell culture evenly across the surface of an Luria-Bertani agar plate containing ampicillin and chloramphenicol. **TXT: Ampicillin: 100 µg/mL; Chloramphenicol: 50 µg/mL**

2.3.2. Talent placing the agar plate in a 37 degrees Celsius incubator.

2.4. Then, pick a single colony from the plate to inoculate a 4-milliliter starter culture and incubate it overnight [1]. The next day, expand the overnight culture into 400 milliliters of Luria-Bertani medium containing ampicillin and chloramphenicol [2]. Shake the culture vigorously at 220 revolutions per minute and 37 degrees Celsius [3] until the optical density at 600 nanometers reaches between 0.6 and 0.8 [4].

- 2.4.1. Talent picking a single colony from the agar plate and transferring it into a tube containing 4 milliliters of medium.
- 2.4.2. Talent pouring the overnight culture into a flask containing 400 milliliters of Luria-Bertani medium with antibiotics.
- 2.4.3. Talent placing the flask in a shaking incubator and monitoring the optical density using a spectrophotometer.

Added shot: 2.4.4: Talent checking the optical density using a spectrophotometer.

Videographer's NOTE: 2.4.3 was split into 2 shots as follows:

2.4.3 Talent placing the flask in a shaking incubator.

2.4.4 Talent monitoring the optical density using a spectrophotometer.

VO is adjusted

- 2.5. Next, induce the culture with 0.2 percent L-arabinose and 1 millimolar lactyl-lysine [1], and incubate at 30 degrees Celsius and 220 revolutions per minute for 16 hours [2-TXT].
 - 2.5.1. Talent adding L-arabinose and Klac to the culture flask.
 - 2.5.2. Talent placing the flask back in a shaking incubator set to 30 degrees Celsius.

TXT: Purify and characterize the lactylated protein

3. Co-Transfection of HEK 293T Cells with KlacRS and sfGFP-149TAG Reporter

Demonstrator: Yinxun Lu

- 3.1. Seed cells in a 12-well plate at a density of 1×10^5 cells per well using 1 milliliter of DMEM [1]. Incubate for 24 hours until cell confluency reaches between 70 and 80 percent, then proceed to transfection [2].
 - 3.1.1. Talent pipetting the cell suspension into each well of a 12-well plate containing 1 milliliter of Dulbecco's Modified Eagle Medium.
 - 3.1.2. Talent placing the plate in an incubator.
- 3.2. To prepare the diluted DNA solution, add 0.75 micrograms of plasmid containing sfGFP reporter and pNeu-KlacRS (*P-new-K-lak-R-S*) in a 1 to 1 ratio into 38 microliters of serum-free DMEM with high glucose [1-TXT]. Gently pipette up and down or vortex briefly to mix [2].
 - 3.2.1. Talent adding 0.75 micrograms of each plasmid into 38 microliters of serum-free

Dulbecco's Modified Eagle Medium in a microcentrifuge tube. **TXT: pcDNA-sfGFP-N149TAG**

- 3.2.2. Talent mixing the DNA solution by pipetting up and down or briefly vortexing.
- 3.3. For the diluted transfection reagent, add 2.25 microliters of transfection reagent into 38 microliters of serum-free DMEM with high glucose [1]. Gently pipette up and down 3 to 4 times to mix thoroughly [2].
 - 3.3.1. Talent pipetting 2.25 microliters of transfection reagent into a fresh tube containing 38 microliters of serum-free Dulbecco's Modified Eagle Medium.
 - 3.3.2. Talent pipetting up and down multiple times to mix the diluted transfection reagent.
- 3.4. Immediately add the diluted transfection reagent to the diluted DNA solution all at once [1-TXT].
 - 3.4.1. Talent pouring the diluted transfection reagent into the DNA solution tube in a single motion. **TXT: Do not mix in the reverse order**
- 3.5. Now, briefly vortex to mix the contents [1] and incubate the solution at room temperature for 10 to 15 minutes to allow the transfection and DNA complexes to form [2].
 - 3.5.1. Talent mixing the combined solution by pipetting or briefly vortexing.
 - 3.5.2. Talent placing the tube on the benchtop at room temperature with a visible timer for incubation. **TXT: Use the complex within 20 minutes of preparation**
- 3.6. Add 50 microliters of the transfection and DNA complex dropwise onto the medium in each well [1] and gently swirl the plate to homogenize the mixture [2].
 - 3.6.1. Talent pipetting 50 microliters of the transfection and DNA solution dropwise into each well of the 12-well plate.
 - 3.6.2. Talent gently swirling the 12-well plate to distribute the transfection complex evenly.
- 3.7. Six hours after transfection, replace the medium with fresh DMEM containing 1 millimolar lactyl-lysine [1] and culture the cells for 24 to 48 hours [2-TXT].
 - 3.7.1. Talent aspirating the medium from each well and adding fresh Dulbecco's Modified Eagle Medium with 1 millimolar Klac.

- 3.7.2. Talent returning the plate to the incubator for continued culture. **TXT: Perform fluorescence microscopy and FACS to check the protein expression**

Results

4. Results

- 4.1. In *Escherichia coli*, full-length hENO1-89Klac (*H-eno1-89-K-lac*) was produced when 1 millimolar lactyl-lysine was added to the growth medium [1-TXT].
 - 4.1.1. LAB MEDIA: Figure 1A. *Video editor: Highlight the band in the -89Klac lane which is pointed by the arrowhead.* TXT: hENO1-89Klac: Human Enolase 1 containing Lysine Lactylation at position 89
- 4.2. Immunoblotting confirmed the presence of full-length hENO1-89Klac using anti-His tag [1] and anti-L-lactyl lysine antibodies [2].
 - 4.2.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the band in the 89Klac lane that is pointed by the arrowhead.*
 - 4.2.2. LAB MEDIA: Figure 1C. *Video editor: Highlight the strong band in the 89Klac lane that is pointed by the arrowhead.*
- 4.3. Electrospray ionization time-of-flight mass spectrometry revealed a dominant peak at 48178 Daltons, matching the expected mass of hENO1-89Klac lacking the initiating methionine [1].
 - 4.3.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the tallest peak at 48178.00 on the mass spectrum, labelled as hENO1-89Klac-Met+.*
- 4.4. Tandem mass spectrometry of the tryptic digest confirmed site-specific incorporation of K-lac (*K-lac*) at residue 89 through the presence of a complete b- and y-ion series mapping to the modified peptide [1].
 - 4.4.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the U in red font.*
- 4.5. In mammalian HEK293T cells, fluorescence microscopy showed strong sfGFP expression throughout the cells when Klac was added for 24 hours [1] and 48 hours [2], while no fluorescence was observed without Klac supplementation [3].
 - 4.5.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the green fluorescent cells in the +Klac panel at 24 hours.*
 - 4.5.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the green fluorescent cells in the +Klac panel at 48 hours.*
 - 4.5.3. LAB MEDIA: Figure 3A. *Video editor: Show the dark panels in the -Klac column.*

4.6. Flow cytometry revealed that approximately 31% of cells expressed sfGFP after 24 hours [1], increasing to about 37% after 48 hours of Klac supplementation [2].

4.6.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the boxed region showing 31.1% GFP+ cells in the +Klac 24-hour panel.*

4.6.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the boxed region showing 37.4% GFP+ cells in the +Klac 48-hour panel.*

1. Escherichia
Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia>
IPA: /ˌe-shə-ˈrik-ē-ə/
Phonetic Spelling: eh·shuh·rik·ee·uh
2. Chloramphenicol
Pronunciation link: <https://www.merriam-webster.com/dictionary/chloramphenicol>
IPA: /ˌklɔr-am-ˈfen-i-ˌkɒl/
Phonetic Spelling: klor·am·fen·ih·kawl
3. Ampicillin
Pronunciation link: <https://www.merriam-webster.com/dictionary/ampicillin>
IPA: /ˌam-pə-ˈsi-lən/
Phonetic Spelling: am·puh·sil·in
4. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /ˌspek-trō-fō-ˈtā-mə-tər/
Phonetic Spelling: spek·troh·foh·tom·uh·ter
5. Arabinose
Pronunciation link: <https://www.merriam-webster.com/dictionary/arabinose>
IPA: /ə-ˈra-bə-ˌnōs/
Phonetic Spelling: uh·rab·uh·nohs
6. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flō-ˈre-səns(t)s/
Phonetic Spelling: flor·ess·uhns
7. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /sī-ˈtā-mə-trē/
Phonetic Spelling: sigh·tom·uh·tree
8. Methionine
Pronunciation link: <https://www.merriam-webster.com/dictionary/methionine>

IPA: /mə-'thī-ə-,nēn/

Phonetic Spelling: muh·thigh·uh·neen

9. Immunoblotting

Pronunciation link: <https://www.merriam-webster.com/dictionary/immunoblot>

IPA: /ɪ,mju:nə'blɑ:t/

Phonetic Spelling: ih·myoon·uh·blot·ing

10. Electrospray

Pronunciation link: <https://www.merriam-webster.com/dictionary/electrospray>

IPA: /i-'lek-trō-,sprā/

Phonetic Spelling: ee·lek·troh·spray

11. Ionization

Pronunciation link: <https://www.merriam-webster.com/dictionary/ionization>

IPA: /ɪ-ə-nə-'zā-shən/

Phonetic Spelling: eye·uh·nuh·zay·shuhn

12. Spectrometry

Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometry>

IPA: /spek-'trä-mə-trē/

Phonetic Spelling: spek·trom·uh·tree

13. Tryptic

Pronunciation link: <https://www.merriam-webster.com/dictionary/tryptic>

IPA: /'trip-tik/

Phonetic Spelling: trip·tik