Identification of interacting proteins using PUP-IT

CURRENT STATUS: POSTED

Haopeng Wang
School of Life Science and Technology, ShanghaiTech University
wanghp@shanghaitech.edu.cn*Corresponding Author*

Min Zhuang
School of Life Science and Technology, ShanghaiTech University
zhuangmin@shanghaitech.edu.cn*Corresponding Author*

Qiang Liu
School of Life Science and Technology, ShanghaiTech University

Jun Zheng
School of Life Science and Technology, ShanghaiTech University

DOI:
10.1038/protex.2018.074

SUBJECT AREAS
Biochemistry

KEYWORDS
proximity labeling, PUP-IT

Abstract

Protein proximity labeling has been developed to identify protein-protein interactions. Here we report a tagging method termed PUP-IT (pupylation based interaction tagging) where a bacterial PUP ligase is fused to the bait protein and this chimeric protein mediates the covalent modification of prey protein with Pup protein. Pup is a small protein containing 64 amino acids. The N terminus of Pup can be fused to the bacteria-derived biotinylated BCCP domain. Therefore, any protein that modified by Pup can be enriched by streptavidin pull down under denaturing condition, which is often used by other types of sample preparation for mass spectrometry identification.

Here we describe two ways to achieve Pup labeling in cells. One is based on transient transfection and another based on the inducible cell line. After labeling in cells, the Pup labeled target proteins can be enriched by using the same streptavidin pull-down procedure.

Reagents

- RPMI 1640 (Gibco, cat # C22400500CP)
- FBS (Gibco, cat # 10099-141)
- 100× Penicillin-Streptomycin (Gibco, cat # 15140122)
- Opti-MEM (Life, cat # 31985070)
- 1M Tris pH 8.0, sterile (Sangon Biotech, cat # B548127-0500)
- NaCl (Sangon Biotech, cat # A501218-0001)
- EDTA(Sangon Biotech, cat # E0105-500g)
- Triton X-100 (Thermo, cat # 215682500)
- SDS (MDBio, cat # S001-100)
- Urea (Sangon Biotech, cat # UT0907)
- 100× protease inhibitor cocktail (Biotool, cat# B14001)
- Trypsin (Promega, cat # V5113)
- Doxycycline (SELLECK, cat # S4163)
- Iodoacetamide (SIGMA, cat # I1149-5G)
- Streptavidin magnetic beads (PIERCE, cat # 88816)

 DTT (MDBio, cat # D023-5G)

 Biotin (SCRC, cat # 67000260)

 Trifluoroacetic acid LC-MC Ultra (Sigma, cat # 14264-50ML)

 Formic acid (Sigma, cat # 94318-250ML)

 Acetonitrile (Merck Chemicals, cat # 1.00030.4008)

 Ammonium bicarbonate reagent plus(R) (Sigma, cat # A6141-500G)

 ZipTip C18 (Millipore, cat # ZTC18S096)

Reagent setup:

Lysis buffer (0.1 % SDS, 2 % triton X-100, 50 mM Tris 7.5, 200 mM NaCl)

Wash buffer 1: (8 M urea, 50 mM Tris 8.0, 200 mM NaCl, 0.2 % SDS)

Wash buffer 2: (8 M urea, 50 mM Tris 8.0, 200 mM NaCl, 2 % SDS)

Wash buffer 3: (8 M urea, 50 mM Tris 8.0, 200 mM NaCl)

Wash buffer 4: (50 mM Tris 8.0, 0.5 mM EDTA, 1 mM DTT)

Wash buffer 5: (50 mM NH_4HCO_3)

1000× doxycycline (2 mg/ml)

Equipment

• Standard molecular biology lab equipment

• Eppendorf mixer

• BioRad Gene Pulser electroporation system and cuvette

Procedure

Part 1. PUP-IT Labelling in Living Cells

1. Transient transfection based PUP-IT application

(1) Grow Jurkat cells in RPMI 1640 (with 10% FBS and 1×Penicillin-Streptomycin) at 37 °C with 5% CO_2, keep cell density between 0.1~1.0 million/ml.

(2) The day before transfection, harvest Jurkat cells by spinning at 800 g for 5 min at room temperature, then re-suspend in pre-warmed RPMI 1640 (with 10% FBS, no Penicillin-Streptomycin) at 0.4 million/ml.

(3) For each transfection, pre-warm 10 ml transfection recovery medium (RPMI 1640+10% FBS) in incubator 30 min before transfection.

(4) Harvest cells by spinning at 800 g for 5 min at room temperature.

(5) Re-suspend Jurkat cells at 40 million/ml in Opti-MEM.

(6) For each transfection, add 300 µl cells (12 million cells) in a 1.5 ml eppendorf tube, mix with 30 µl PUP-IT system DNA (10 µg CD28-PafA, 15 µg PupE and 5 µg pMAX-GFP), and gently tap to mix samples, incubate at room temperature for 15 min. *pMAX-GFP is used to examine the transfection efficiency.

(7) Gently transfer cells into a 0.4 cm Bio-Rad cuvette, avoid bubbles.

(8) Place the cuvette in the shockpod and run GenePulser program: exponential decay 1000 µF, voltage 250 V; 0.4 cm gap cuvette, expected time is between 35~40 ms.

(9) After the pulse, allow cells to recover in cuvette for 15 min at room temperature in the cell culture hood.

(10) Gently transfer cells to pre-warmed recovery media and maintain them in cell culture incubator.

(11) Around 12 hours after transfection, add 4 µM biotin to cell culture media (optional).

(12) Between 36~48 hours after transfection, check cell transfection efficiency by flow cytometer. The GFP positive cells are usually between 65-80% in living cells.

(13) Harvest cells by spinning at 800 g for 5 min at room temperature, then wash cells with 1 ml cold PBS for three times. Cells can be immediately lysed for sample preparation or stored in -80 °C until use.

2. Stable cell line based PUP-IT application

Generation of iPUP Jurkat cell line

(1) Bio-PupE-IRES-BFP was cloned into the expression plasmid of Tet-On 3G inducible expression system (Clontech cat#: 631168).

(2) Package the above inducible expression system plasmids (pTet3G and pTre3G) to generate lenti-virus, respectively.

(3) Co-infect Jurkat cells using these two viruses (1:1 ratio).

(4) 48 hours after transfection, add 2 μg/ml doxycycline to cell culture for another 24 hours.

(5) FACS sort BFP positive cells into 96 well plates with 1 cell/well for single clone selection.

(6) Three weeks after sorting, examine BFP expression in each clone by flow cytometer with or without doxycycline induction (2 μg/ml).

(7) Check the expression of bio-Pup(E) in BFP positive cells by western blot.

Generation of PUP-IT stable cell line

CD28, MUL1 and other baits were fused to PafA and cloned into pHR-EF1α-IRES-GFP vector and introduced into iPUP Jurkat cell line through lenti-virus transduction. GFP positive cells were sorted and amplified for future experiments.

Induction of PUP-IT

(1) Grow PUP-IT^{CD28} and other PUP-IT cell lines in RPMI 1640 (with 10% FBS) at 37 °C with 5% CO_2, keep cell density between 0.1~1.0 million/ml.

(2) For each sample, 10^7 cells (typically 20 ml resuspended cells at 0.5 million/ml) are aliquot to each 75 cm2 cell culture flask. Add 20 μl 1000× doxycycline (final concentration 2 μg/ml) to each sample. (Optional: Biotin can also been added to media at 4 μM to ensure the consistency of labeling in different cell culture media).

(3) About 24 hours after induction, harvest cells by spinning at 800 g for 5 min at room temperature, then wash cells once with 1ml cold PBS. Cells can be immediately lysed for sample preparation or stored in -80 °C until use.

Part 2. Streptavidin Pull Down of Bio-PUP(E) Modified Proteins

Day 1:

(1) Lyse 10~30 million cells in 1 ml lysis buffer supplemented with protease inhibitor cocktail on ice for one hour.

(2) Clarify the lysates by centrifuging at 13000 rpm for 10 min at 4 °C.

(3) Transfer 900 μl cell lysate to a new 1.5 ml centrifuge tube, add 576 mg urea powder to cell lysate (final volume is around 1.2 ml and the urea concentration is around 8 M). The dissolving of urea

absorbs heat and rotating the tube on a rotater at room temperature can accelerate this step. *NOTE: Take 10 µl cell lysate for western blot with streptavidin-HRP and anti-Myc blotting to confirm the expression and activity of PUP-IT.

(4) Add 12 µl of 1 M DTT (final concentration 10 mM) to cell lysate, shake at 600 rpm at 56 °C for 1 hour.

(5) Add 30 µl of 1 M iodoacetamide (final concentration 25 mM) to cell lysate and incubate in dark for 45 min.

(6) Add another 30 µl of 1 M DTT to quench iodoacetamide at room temperature for 30 min.

*NOTE: The purpose of steps 4~6 is to reduce and alkylate cysteine residues. These steps are optional.

(7) Add 50 µl streptavidin magnetic beads to each sample and incubate on a rotator at 4 °C overnight.

Day 2:

(1) Place sample tubes on a magnetic stand, wait till all the brown magnetic beads settle on the side of the centrifuge tube (about 1~2 minutes) and aspirate out lysate.

(2) Add 1 ml Wash Buffer 1 to each tube and shake at room temperature for 5 min on eppendorf mixer at 1000 rpm.

(3) Place sample tubes on magnetic stand and aspirate out Wash Buffer 1.

(4) Repeat another wash with Wash Buffer 1.

(5) Wash beads once with Wash Buffer 2. Add 1 ml Wash Buffer 2 to each tube and shake at room temperature for 5 min on eppendorf mixer at 1000 rpm. Place tubes on magnetic stand and aspirate out Wash Buffer 2.

(6) Wash beads with Wash Buffer 3. Add 1 ml Wash Buffer 3 to each tube and shake at room temperature for 5 min on eppendorf mixer at 1000 rpm. Place tubes on magnetic stand and aspirate out Wash Buffer 3.

(7) Repeat another wash with Wash Buffer 3.

(8) Wash beads once with Wash Buffer 4, then wash beads twice with Wash Buffer 5.

(9) Trypsin digest on beads. For each sample, dilute 6 µg trypsin in 125 µl buffer 5, add the diluted

trypsin to the beads. Incubate samples at 37 °C overnight.

Day 3:

(1) Transfer the supernatant to a new tube, wash beads twice with 30 µl Washing Buffer 5. Combine the supernatant with the washings.

(2) Concentrate the samples using SpeedVac concentrator to reduce sample volume to less than 40 µl.

(3) Adjust sample pH to lower than 3 by adding 5 µl 3% formic acid.

(4) Desalt the digested peptides with ZipTip.

(5) Dry samples in the SpeedVac concentrator. The samples can be stored in -80 °C and are ready to be dissolved for mass spectrometry analysis.

A proximity-tagging system to identify membrane protein–protein interactions

by Qiang Liu, Jun Zheng, Weiping Sun, +5

Nature Methods (14 August, 2018)