iST mNeonGreen-Trap Kit

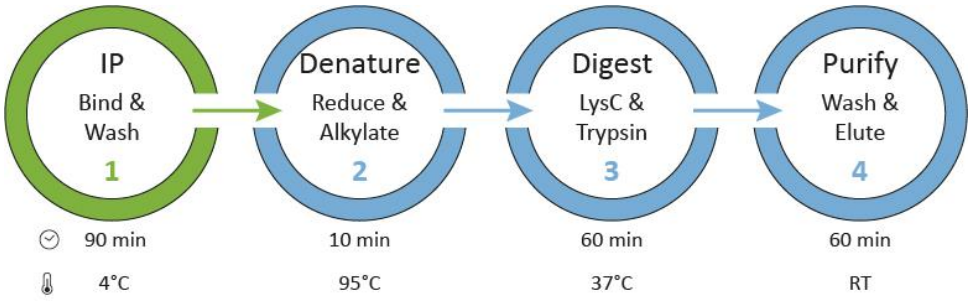
for AP-MS sample preparation of mNeonGreen-fusion proteins

PROTOCOL

INTRODUCTION

The PreOmics/ChromoTek iST mNeonGreen-Trap Kit enables efficient and fast immunoprecipitation of mNeonGreen-fusion proteins and their interaction factors, as well as the subsequent sample preparation for mass spectrometry-based proteomics. In first step, the kit's mNeonGreen-Trap utilizes small recombinant alpaca antibody fragments covalently coupled to agarose beads for pull-down. Then in a second step, this kit provides buffers, cartridges and enzymes in order to denature, reduce, alkylate, and digest mNeonGreen-fusion proteins and their interaction factors, and to perform a final peptide cleanup step in a streamlined manner. For sample-specific protocols and optimization contact us or visit our websites www.chromotek.com or www.preomics.com.

PROCEDURE



KIT CONTENTS

COMPONENT	CAP	QUANTITY	BUFFER PROPERTIES				DESCRIPTION	STORAGE
			Organic	Acidic	Basic	Volatile		
mNeonGreen-Trap Agarose	yellow/ white	0.25 mL slurry					anti-mNeonGreen single domain antibody coupled to agarose beads (nta-10; i.e. 8 reactions plus 2 controls).	**4°C**
DIGEST	red	1x					Enzyme *Trypsin*-mix to digest proteins.	**-20°C**
RESUSPEND	yellow	1x 1 mL				✓	Protease reconstitution buffer for enzymes.	RT
LYSE	brown	1x 1 mL			✓		Denature, reduce and alkylate proteins.	RT
STOP	black	1x 1 mL	✓	✓		✓	Stop the enzymatic activity.	RT
WASH 1	blue	1x 2 mL	✓	✓		✓	Clean up peptides from hydrophobic contaminants.	RT
WASH 2	green	1x 2 mL		✓		✓	Clean up peptides from hydrophilic contaminants.	RT
ELUTE	violet	1x 2 mL	✓		✓	✓	Elute the peptides from the cartridge.	RT
LC-LOAD	white	1x 1 mL		✓		✓	Load peptides on reversed-phase LC-MS column.	RT
CARTRIDGE		8x					Cartridge for 1 to 100 µg protein starting material.	RT
WASTE		8x					Tube for collecting waste after washing steps.	RT
COLLECTION		8x					Tube for collecting peptides after elution.	RT
ADAPTER		8x					Enables placing a cartridge into a tube.	RT
CAP		8x					Cap to optionally close the cartridge's bottom.	RT

The iST mNeonGreen-Trap Kit contains sufficient mNeonGreen-Trap Agarose beads for up to 10 immunoprecipitations. Out of these ten reactions, two shall be used for negative respectively positive control by immunoblot analysis. The iST mNeonGreen-Trap Kit can be used for up to 8 iST proteomic sample preparation reactions.

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mNEONGREEN-TRAP AGAROSE BEAD PROPERTIES

Bead size: ~ 90 µm (cross-linked 4% agarose beads)
Storage buffer: 20% EtOH

mNEONGREEN-TRAP AGAROSE STABILITY AND STORAGE

Shipped at ambient temperature. Upon receipt store at +4°C. Stable for 1 year. Do not freeze.

PRE-REQUISITES

Common lab equipment is required for the sample preparation:

EQUIPMENT	QUANTITY AND DESCRIPTION
PIPETTE	Careful sample handling and pipetting reduces contaminations and improves quantification.
SAMPLE	Lyophilized protein or pelleted cells. For other materials ask us for adapted protocols.
HEATING BLOCK	Heating shakers are recommended to help protein denaturation and during digestion.
CENTRIFUGE	Microcentrifuge for 1.5 ml reaction tubes is necessary for loading, washing and elution.
SPEED-VAC	Vacuum manifolds evaporate volatile buffers from the eluate before LC-MS.
ULTRASONIC BATH	Optional: can be used to resuspend peptides.
REACTION TUBES	1.5 mL plasticware reaction tubes, e.g. from Eppendorf. We recommend low-binding protein tubes.
ICE BUCKET	Keep required solutions and protein samples on ice.

REQUIRED SOLUTIONS (NOT INCLUDED)

Suggested buffer compositions for mammalian cells. For other cell types like yeast, plants, drosophila, etc. please use equivalent cell lysis buffer. We advise to use MS-grade chemicals to prepare all buffers.

IP LYSIS BUFFER	Selection of IP LYSIS Buffer: For cytoplasmic proteins, re-suspend cell pellet in 200 µl ice-cold **IP LYSIS buffer** (not included) by pipetting up and down. Supplement **IP LYSIS buffer** with protease inhibitor cocktail and 1 mM PMSF. Composition: 10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40 For nuclear and cytoplasmic proteins, resuspend cell pellet in 200 µl ice-cold **RIPA buffer** (not included) supplemented with DNaseI (f.c. 75-150 U/ml), $MgCl_2$ (f.c. 2.5 mM), protease inhibitor cocktail and PMSF (f.c. 1 mM). Composition: 10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.1 % SDS; 1 % Triton X-100; 1 % Deoxycholate
IP WASH I	10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.25% NP-40
IP WASH II	10 mM Tris/Cl pH 7.5; 150 mM NaCl
PBS	Phosphate-buffered saline: 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; adjust to a final pH of 7.4

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Immunoprecipitation using mNeonGreen-Trap Agarose

Keep solutions and protein samples on ice.

1. HARVEST

1.1. Use of ~10^6 - 10^7 mammalian cells (approx. one 10-cm dish) expressing the mNeonGreen-fusion protein of interest for one immunoprecipitation reaction. Aspirate growth medium, add 1 mL ice-cold PBS to cells and scrape cells from dish to harvest adherent cells.

1.2. Transfer cells to a pre-cooled 1.5 mL tube, spin at 500 rcf for 3 min at 4°C and discard supernatant.

1.3. Wash cell pellet twice with ice-cold **PBS**, gently resuspending the cells.

2. IP LYSIS

2.1. Resuspend cell pellet in 200 µL ice-cold **IP LYSIS buffer** supplemented with protease inhibitors and 1 mM PMSF by pipetting or using a syringe. See IP LYSIS buffer composition on page 2.

2.2. Place the tube on ice for 30 min with extensively pipetting every 10 min.

2.3. Centrifuge cell lysate at 20,000 rcf for 10 min at 4°C.

2.4. Transfer supernatant to a pre-cooled 1.5 mL tube. Add 300 µL **IP WASH II** buffer supplemented with protease inhibitors and 1 mM PMSF to lysate.

2.5. For negative/positive control save 50 µL diluted lysate for immunoblot analysis (input).

3. EQUILIBRATE BEADS

3.1. Resuspend **mNeonGreen-Trap Agarose beads** by pipetting and transfer 25 µL bead slurry into 500 µL ice-cold **IP WASH I buffer**.

3.2. Resuspend beads and pipette 25 µl bead slurry into 500 µl ice-cold **IP WASH I buffer**.

3.3. Centrifuge at 2,500 rcf for 2 min at +4°C. Discard supernatant and repeat wash once.

4. BIND PROTEINS

4.1. Add diluted lysate (step 2.4) to equilibrated **mNeonGreen-Trap Agarose beads** (step 3.3).

4.2. Tumble end-over-end for 1 hour at 4°C.

4.3. Centrifuge at 2,500 rcf for 2 min at +4°C. For negative/positive control save 50 µL supernatant for immunoblot analysis (non-bound/flow through). Discard remaining supernatant.

5. WASH BEADS

5.1. Resuspend **mNeonGreen-Trap Agarose beads** in 500 µl ice-cold **IP WASH I buffer**. Centrifuge at 2,500 rcf for 2 min at +4°C.

5.2. Discard supernatant and repeat this wash step once.
Optional: Increase salt concentration up to 500 mM for higher stringency washing.

5.3. Resuspend beads in 500 µl ice-cold **IP WASH II buffer**. Centrifuge at 2,500 rcf for 2 min at +4°C.

5.4. Discard supernatant, repeat this wash step three times. Transfer beads to a **fresh tube** at last wash step.
Optional: Increase salt concentration up to 500 mM for higher stringency washing. Do not include detergents.

5.5. For negative/positive control (bound protein) resuspend 25 µl beads in 100 µl 2x SDS-sample buffer. Boil resuspended beads for 10 min at 95°C to dissociate immunocomplexes from mNeonGreen-Trap Agarose beads. Beads can be collected by centrifugation at 2,500 rcf for 2 min at 4°C and SDS-PAGE is performed with the supernatant.

5.6. Discard supernatant and directly continue with the proteomic sample preparation using the iST part of kit as outlined on next page.

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Proteomic sample preparation using iST

6. DENATURE PROTEINS

6.1. Add 50 µL **LYSE** ● to the washed **beads** and place it in a pre-heated HEATING BLOCK (60 °C; 1,000 rpm; 10 min).

6.2. Optional: Spin down droplets (RT; max. 300 rcf; 10 sec).

7. DIGEST

7.1. Add 210 µL **RESUSPEND** ● to **DIGEST** ● (1 tube for 4 reactions), shake (RT; 500 rpm; 10 min), pipette up/down.

7.2. Add 50 µL **DIGEST** ● to **beads** and place it in a pre-heated HEATING BLOCK (37 °C; 500 rpm; 1-3 hours).

7.3. Add 100 µL **STOP** ● to **beads** (precipitation may happen), shake (RT; 500 rpm; 1 min), pipette up/down. *SP*

8. PURIFY

8.1. Use **ADAPTER** to place **CARTRIDGE** in **WASTE** tube. Label all tubes.

8.2. Centrifuge **beads** (RT; 2,500 rcf; 2 min).

8.3. Transfer the complete supernatant (combined LYSE/DIGEST/STOP buffers) to **CARTRIDGE**. Discard beads.

8.4. Spin **CARTRIDGE** in CENTRIFUGE (RT; 3,800 rcf; 1-3 min). If needed, adjust values to ensure complete flow-through.

8.5. Add 200 µL **WASH 1** ● to **CARTRIDGE**, repeat step 8.4., discard flow-through.

8.6. Add 200 µL **WASH 2** ● to **CARTRIDGE**, repeat step 8.4., discard flow-through. *SP*

8.7. Use **ADAPTER** to place **CARTRIDGE** in a fresh **COLLECTION** tube. Label all tubes.

8.8. Add 100 µL **ELUTE** ● to **CARTRIDGE**, repeat step 8.3., keep flow-through in **COLLECTION** tube.

8.9. Repeat step 8.8., keep flow-through in the same **COLLECTION** tube.

8.10. Remove **CARTRIDGE** and place **COLLECTION** tube in a SPEED-VAC (45 °C; until completely dry).

8.11. Add **LC-LOAD** ○ to **COLLECTION** tube. Aim for 1 g/L concentration (e.g. 50 µL to 50 µg pulled-down protein).

8.12. Sonicate **COLLECTION** tube in an ULTRASONIC BATH (5 min) or shake (RT; 500 rpm; 5 min). *SP*

SP - Storage Point: At this point, close the tube or **CARTRIDGE** (use a **CAP** for bottom). Lysates and peptides can be frozen at -20 °C. Storage of peptides should not exceed 2 weeks at -20 °C. For extended storage, finish the protocol and store at -80 °C.

Data Analysis

Consider the following as fixed modifications in your database search:

MODIFICATION	DESCRIPTION	COMPOSITION	SPECIFICITY	MASS	UNIMOD #
ALKYLATION	Carbamidomethyl on Cysteine	C_2H_3NO	[C]	+57 Da	4