Protein extraction, alkylation, and digestion for LC/MS of HEK-293 Version 2

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Abstract

Protocol for protein extraction, alkylation, and digestion to obtain LC/MS (liquid chromatography-mass spectrometry) data of calculated protein signal intensities in HEK-293 cells to monitor them.

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Before start

Prepare a Krebs-Ringer-Buffer:

- 154 mM NaCl
- 5.6 mM KCl
- 5.5 mM glucose
- 20.1 mM HEPES pH 7.4
- 25 mM $NaHCO_3$

Materials

RIPA buffer 08714 by Nacalai Tesque
NaCl by Contributed by users
KCl by Contributed by users
Glucose by Contributed by users
HEPES pH 7.4 by Contributed by users
NaHCO3 by Contributed by users
Micro BCA Protein Assay Kit 23235 by Thermo Fisher Scientific
XL-Tryp Kit Direct Digestion YC - 3120 by APRO SCIENCE
In-Gel R-CAM Kit YC-5004 by APRO SCIENCE
ZipTipC18 by Merck Millipore

Pierce Quantitative Colorimetric Peptide Assay 23275 by Thermo Fisher Scientific

Protocol

Protein extraction

Step 1.

Extract the HEK-293 proteins using the standard protocol for the RIPA buffer (NACALAI, INC., Kyoto, Japan).

Alkylation and Digestion

Step 2.

Wash approximately 10^6 harvested cells once in Krebs-Ringer-Buffer (KRB; 154 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 20.1 mM HEPES pH 7.4, 25 mM $NaHCO_3$).

Alkylation and Digestion

Step 3.

Resuspend the cells in 30 µl of RIPA buffer, passed in and out through 21G needles for destruction, and incubate on ice for 1 h.

DURATION
01:00:00

Alkylation and Digestion

Step 4.

Centrifuge the cells at 10,000 *g* for 10 min at 4°C.

DURATION
00:10:00

Alkylation and Digestion

Step 5.

Collect the supernatants.

Alkylation and Digestion

Step 6.

Quantify the proteins by using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, U.S.A.).

Alkylation and Digestion

Step 7.

Solidify the samples in acrylamide gel. For further steps, use XL-Tryp Kit Direct Digestion (APRO SCIENCE, Naruto, Japan).

Alkylation and Digestion

Step 8.

Wash the samples in ultrapure water. (1/2)

Alkylation and Digestion

Step 9.

Wash the samples in ultrapure water. (2/2)

Alkylation and Digestion

Step 10.

Wash the samples in dehydration solution. (1/3)

Alkylation and Digestion

Step 11.

Wash the samples in dehydration solution. (2/3)

Alkylation and Digestion

Step 12.

Wash the samples in dehydration solution. (3/3)

Alkylation and Digestion

Step 13.

Dry the samples.

Alkylation and Digestion

Step 14.

Process the samples using an In-Gel R-CAM Kit (APRO SCIENCE, Naruto, Japan).

Alkylation and Digestion

Step 15.

Reduce the samples for 2 h at 37°C.

DURATION

02:00:00

Alkylation and Digestion

Step 16.

Alkylate the samples for 30 min at room temperature.

DURATION

00:30:00

Alkylation and Digestion

Step 17.

Wash samples with ultrapure water. (1/5)

Alkylation and Digestion

Step 18.

Wash samples with ultrapure water. (2/5)

Alkylation and Digestion

Step 19.

Wash samples with ultrapure water. (3/5)

Alkylation and Digestion

Step 20.

Wash samples with ultrapure water. (4/5)

Alkylation and Digestion

Step 21.

Wash samples with ultrapure water. (5/5)

Alkylation and Digestion

Step 22.

Wash samples with destaining solution. (1/2)

Alkylation and Digestion

Step 23.

Wash samples with destaining solution. (2/2)

Alkylation and Digestion

Step 24.

Dry the samples.

Alkylation and Digestion

Step 25.

Trypsinize the resultant samples overnight at 35°C.

Alkylation and Digestion-Day2

Step 26.

Collect the dissolved digested peptides by ZipTipC18 (Merck Millipore, Corp., Billerica, U.S.A.).

Alkylation and Digestion-Day2

Step 27.

Dampen the tips with acetonitrile x three tips. (1/2)

Alkylation and Digestion-Day2
Step 28.

Dampen the tips with acetonitrile x three tips. (2/2)

Alkylation and Digestion-Day2
Step 29.

Equilibrate tips with 0.1% trifluoroacetic acid x three tips. (1/2)

Alkylation and Digestion-Day2
Step 30.

Equilibrate tips with 0.1% trifluoroacetic acid x three tips. (2/2)

Alkylation and Digestion-Day2
Step 31.

Collect the peptides by 20 cycles of aspiration and dispensing x three tips.

Alkylation and Digestion-Day2
Step 32.

Wash the peptides with 0.1% trifluoroacetic acidx three tips. (1/2)

Alkylation and Digestion-Day2
Step 33.

Wash the peptides with 0.1% trifluoroacetic acid x three tips. (2/2)

Alkylation and Digestion-Day2
Step 34.

Elute by 0.1% trifluoroacetic acid /50% acetonitrile with aspiration and dispense x three tips. (1/5)

Alkylation and Digestion-Day2
Step 35.

Elute by 0.1% trifluoroacetic acid /50% acetonitrile with aspiration and dispense x three tips. (2/5)

Alkylation and Digestion-Day2
Step 36.

Elute by 0.1% trifluoroacetic acid /50% acetonitrile with aspiration and dispense x three tips. (3/5)

Alkylation and Digestion-Day2

Step 37.

Elute by 0.1% trifluoroacetic acid /50% acetonitrile with aspiration and dispense x three tips. (4/5)

Alkylation and Digestion-Day2

Step 38.

Elute by 0.1% trifluoroacetic acid /50% acetonitrile with aspiration and dispense x three tips. (5/5)

Alkylation and Digestion-Day2

Step 39.

Vacuum dry the peptides.

Alkylation and Digestion-Day2

Step 40.

Store the finalized samples at −20°C.

LC/MS

Step 41.

Before performing LC/MS, resuspend samples in 0.1% formic acid.

LC/MS

Step 42.

Quantify the amounts with the Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, Waltham, U.S.A.).