

Protein extraction, alkylation, and digestion for LC/MS of HEK-293 Version 3

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Abstract

Protocol for protein extraction, alkylation, and digestion to obtain LC/MS (liquid chromatographymass 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 NaHCO₃

Protocol

Protein Extraction

Step 1.

Wash approximately 10⁶ 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 NaHCO₃).

Protein Extraction

Step 2.

Resuspend the cells in 30 μ l of RIPA buffer (NACALAI, INC., Kyoto, Japan), passed in and out through 21G needles for destruction, and incubate on ice for 1 h.

O DURATION

01:00:00

Protein Extraction

Step 3.

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

O DURATION

00:10:00

Protein Extraction

Step 4.

Collect the supernatants.

Protein Extraction

Step 5.

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

Alkylation and Digestion

Step 6.

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

Alkylation and Digestion

Step 7.

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

Alkylation and Digestion

Step 8.

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

Alkylation and Digestion

Step 9.

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

Alkylation and Digestion

Step 10.

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

Alkylation and Digestion

Step 11.

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

Alkylation and Digestion

Step 12.

Dry the samples.

Alkylation and Digestion

Step 13.

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

Alkylation and Digestion

Step 14.

Reduce the samples for 2 h at 37°C.

O DURATION

02:00:00

Alkylation and Digestion

Step 15.

Alkylate the samples for 30 min at room temperature.

O DURATION

00:30:00

Alkylation and Digestion

Step 16.

Wash samples with ultrapure water. (1/5)

Alkylation and Digestion

Step 17.

Wash samples with ultrapure water. (2/5)

Alkylation and Digestion

Step 18.

Wash samples with ultrapure water. (3/5)

Alkylation and Digestion

Step 19.

Wash samples with ultrapure water. (4/5)

Alkylation and Digestion

Step 20.

Wash samples with ultrapure water. (5/5)

Alkylation and Digestion

Step 21.

Wash samples with destaining solution. (1/2)

Alkylation and Digestion

Step 22.

Wash samples with destaining solution. (2/2)

Alkylation and Digestion

Step 23.

Dry the samples.

Alkylation and Digestion

Step 24.

Trypsinize the resultant samples overnight at 35°C.

Alkylation and Digestion-Day2

Step 25.

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

Alkylation and Digestion-Day2

Step 26.

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

Alkylation and Digestion-Day2

Step 27.

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

Alkylation and Digestion-Day2

Step 28.

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

Alkylation and Digestion-Day2

Step 29.

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

Alkylation and Digestion-Day2

Step 30.

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

Alkylation and Digestion-Day2

Step 31.

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

Alkylation and Digestion-Day2

Step 32.

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

Alkylation and Digestion-Day2

Step 33.

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

Alkylation and Digestion-Day2

Step 34.

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

Alkylation and Digestion-Day2

Step 35.

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

Alkylation and Digestion-Day2

Step 36.

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

Alkylation and Digestion-Day2

Step 37.

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

Alkylation and Digestion-Day2

Step 38.

Vacuum dry the peptides.

Alkylation and Digestion-Day2

Step 39.

Store the finalized samples at -20°C.

LC/MS

Step 40.

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

LC/MS

Step 41.

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