NeuCode™ Amino Acids

Catalog Numbers A36755

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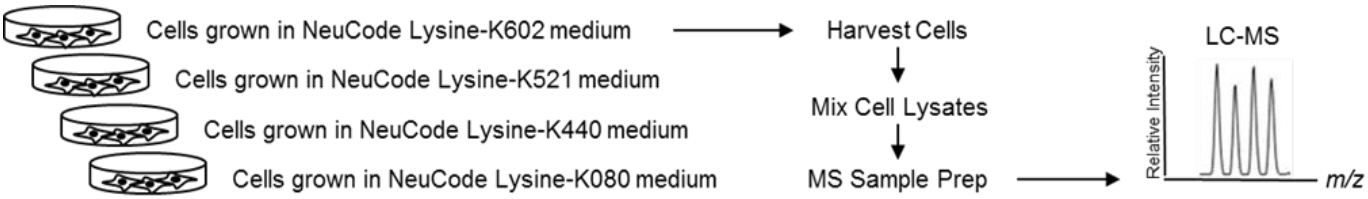
Contents

Product	Cat. No.	Contents	Storage
NeuCode™ Amino Acids	A36755	**NeuCode™ 4-Plex Lysine Bundle, 1 x 25 mg** Kit Contents: NeuCode™ Lysine - 602, 25 mg NeuCode™ Lysine - 521, 25 mg NeuCode™ Lysine - 440, 25 mg NeuCode™ Lysine - 080, 25 mg	Store at room temperature.

Product description

The Thermo Scientific™ NeuCode™ 4-Plex Lysine Bundle contains four NeuCode™ L-Lysine amino acids that can be used for isotope metabolic protein labeling enabling quantitation of protein expression levels from differentially treated cell populations. Traditional stable isotope labeling with amino acids in cell culture (SILAC) is a simple and accurate method to quantify differential changes in the proteome using "light" and "heavy" non-radioactive, stable isotope-labeled amino acids.[1] NeuCode™ isotopic labeling is a variation of SILAC in which two or more heavy amino acid isotopologs are used to compare relative protein expression among samples using high resolution mass spectrometry (MS).[2-4]

Traditional SILAC uses stable isotopes 4-10 Da heavier than natural amino acid isotopologs. This shifts the mass of labeled peptides into distinct peptide mass-to-charge (*m*/*z*) peaks in the MS spectrum enabling 2- or 3-plex experimental designs. However, these mass shifts result in increased MS spectral complexity, which ultimately reduces the number of proteins quantified in the combined sample. Like traditional SILAC, NeuCode™ SILAC enables quantification of peptides as the MS level by comparing relative peak area of peptides from multiple samples in the same liquid chromatography (LC)-MS analysis. However, NeuCode™ uses stable isotopes with smaller mass differences (12-36 mDa) that are not separated in normal lower resolution MS scans. Although this results in less complex MS spectra compared to traditional SILAC, the main advantage of the smaller spacing is that it enables higher sample multiplexing up to a 4-plex in a single isotopolog peak cluster (Figure 1). In addition, since only heavy amino acids are used for comparisons, NeuCode™ SILAC labeling can be used to measure changes in cells or other samples which may not be completely 100% labeled.



Important product infomation

- NeuCode™ lysine isotopologs are referenced as KXXX (K represents lysine, while XXX represents a designated number of 13C, 2H, or 15N stable isotopes).
- NeuCode™ amino acids are used to label and quantify lysine-containing peptides from LysC protein digests.
- High-resolution mass spectrometers (e.g., Thermo Scientific™ Orbitrap™ Elite™, Orbitrap™ Fusion™ Tribrid™, and Orbitrap™ Fusion™ Lumos™ Tribrid™ mass spectrometers) are required to fully resolve NeuCode™-labeled peptides.
- Stable isotope-labeled amino acids are biochemically identical to their natural analogs. Therefore, cell growth, morphology and signaling are not affected when incubated with heavy amino acids compared to cells grown in media containing an equivalent amount of light amino acids. Cells cultured using SILAC media supplemented with Dialyzed FBS (Product No. 26400036) may have some reduction in growth compared to cells grown in media supplemented with standard FBS.
- Addition of L-lysine-2HCl and/or L-arginine HCl amino acid salts to cell culture media may cause a temporary change in media color (red to yellow) upon dissolving. This effect is caused by a brief reduction in media pH that is reversed upon complete mixing and buffering of amino acids with the entire volume of media.
- The final concentration upon dissolving 25 mg of L-lysine-2HCl and 25 mg of L-arginine-HCl in 250 mL of medium is 0.46 mM and 0.47 mM, respectively. Some cell lines may require more lysine or arginine for optimal cell growth and should be supplemented accordingly.
- Media supplements, such as L-glutamine and antibiotics (e.g., penicillin, streptomycin), may be used to maintain media performance and sterility. Sterile-filtering supplemented media using a 0.2-45µm filter is recommended.
- To avoid contamination of MS samples, always wear gloves when handling samples and gels. Use ultrapure MS-grade reagents to prepare peptides and perform sample preparation in a cleaned work area.

Additional materials required

- Rapidly growing cell line adapted to DMEM, DMEM:F12 or RPMI 1640 media
- SILAC cell culture media such as DMEM for SILAC (Product No. 88364), RPMI 1640 media for SILAC (Product No. 88365), or DMEM:F12 for SILAC (Product No. 88370)
- Dialyzed FBS (Product No. 26400036)
- L-Arginine-HCl (Product No. 89989 or 88427)
- 500 mL sterile-filter flask with PES membrane (0.2-45 µm)
- Phosphate-buffered saline (PBS): 0.1M phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372)
- Cell lysis reagent such as Thermo Scientific™ RIPA Lysis and Extraction Buffer (Product No. 89901), M-PER™ Mammalian Protein Extraction Reagent (Product No. 78501) or Pierce™ IP Lysis Buffer (Product No. 87787)
- Protease and phosphatase inhibitors [Product No. 87786 and 78420, respectively or 78440 (combination)]
- Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Product No. 23227)
- Thermo Scientific™ Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (Product No. 84840)

Label stable isotopes

Supplement SILAC media with NeuCode™ Amino Acids

The following protocol is an example application for this product using 1 L (2 × 500 mL bottles) of SILAC media. Specific applications will require optimization.

1. To supplement 1 L of SILAC media with 10% serum, remove 100 mL of media and replace with 100 mL of thawed Dialyzed FBS.
2. Dissolve 100 mg of L-Arginine-HCl using 1 mL of media and add to 1 L of media containing Dialyzed FBS and mix thoroughly.
3. Dissolve 25 mg of each NeuCode™ lysine amino acid using 1 mL of media.
4. Add each dissolved NeuCode™ amino acid to a separate 250 mL of media containing Dialyzed FBS and L-Arginine.
5. Mix thoroughly and sterile-filter media into a separate flask and label accordingly.

   Note: After supplementing media with dialyzed serum, media stability is less than 6 months. Store media at 4°C protected from light.

Incorporate NeuCode™ Amino Acids

1. Depending on cell type, split 1-2 × 10^5 cells adapted to grow in DMEM, DMEM:F12 or RPMI 1640 media into tissue culture flasks or plates for each media supplemented with NeuCode™ amino acids.

   Note: For suspension cells grown in T-25 flasks, use 8 mL of each media. For adherent cells grown in 60 × 15 mm plates, use 4 mL of each media.
2. Passage both cell populations for at least 5 cell doublings by changing medium or splitting cells as appropriate every 2-3 days. Maintain density so that cells are actively growing in log phase (between 30-90% confluency).
3. After five cell doublings, the incorporation of heavy L-lysine should be > 95%.

   Note: Complete incorporation of NeuCode™ amino acids are not required for analysis but will increase quantifiable peptide identifications due to lower sample complexity.
4. Harvest 10^6 cells from each NeuCode™ amino acid-labeled sample and pellet cells by centrifuging for 5-10 minutes at 500 × *g*. Remove media, wash cells with 5 cell-pellet volumes of PBS, and pellet cells again.
5. Lyse cells on ice using an appropriate lysis buffer containing protease and phosphatase inhibitors. Lyse cells with minimal lysis buffer to obtain protein concentrations of 2-10 mg/mL. Centrifuge samples at 14,000 × *g* for 5 minutes to pellet the cell debris.
6. Digest proteins to MS-compatible peptides using the Pierce™ In-Gel Tryptic Digestion Kit, Pierce™ Mass Spec Sample Prep Kit for Cultured Cells or other suitable method.
7. Verify incorporation efficiency using MS analysis of peptides and database searching using a variable lysine variable modification (see Perform mass spectrometry and quantitation of NeuCode™-labeled peptides).
8. Once full isotope incorporation has been determined, continue to expand NeuCode™ amino acid-labeled cells to desired cell number required for subsequent cell treatment and lysis (see Perform mass spectrometry and quantitation of NeuCode™-labeled peptides).

   Note: Heavy-labeled cells can be frozen using the appropriate freezing medium (e.g., 10% DMSO in SILAC media).

Cell treatment and mixing

After verifying that the heavy isotope label was sufficiently incorporated, the remaining cells (prepared in step 3 of Incorporate NeuCode™ Amino Acids) are ready for treatment to alter protein abundance in one cell population. Types of treatment include cell differentiation induction, siRNA knockdown of target proteins, environmental stress or drug treatment.

1. After cell treatment, harvest and count NeuCode™ amino acid-labeled cells.
2. Pellet cells by centrifuging for 5-10 minutes at 500 × *g*. Remove media, wash cells with five cell-pellet volumes of PBS, and pellet cells again.
3. Lyse cells on ice using an appropriate lysis buffer containing protease and phosphatase inhibitors. Lyse cells with minimal lysis buffer to obtain protein concentrations of 2-10 mg/mL. Centrifuge samples at 14,000 × *g* for 5 minutes to pellet the cell debris.

   Note: The total number of cells to use for lysis depends on sample preparation enrichment and instrumentation sensitivity. To obtain 50-100 µg of total protein using whole cell lysis buffers, such as M-PER Reagent, RIPA or Pierce IP Lysis Buffer, 1-2 × 10^6 cells are typically required. When isolating membrane proteins using Thermo Scientific™ Mem-PER™ Reagent or nuclear proteins using Thermo Scientific™ NE-PER™ Reagent, use 1-2 × 10^7 cells. Greater than 2 × 10^8 cells may be needed to isolate low abundance proteins from organelles (mitochondria, lysosomes, etc.) or when using affinity enrichment strategies such as immunoprecipitation or post-translational modification (e.g. phosphorylation, glycosylation) capture.
4. Determine protein concentration of each sample in triplicate using the Pierce BCA Protein Assay Kit and a standard curve generated with bovine serum albumin (BSA).

5. Mix equal protein amounts of each cell lysate in a new tube.

   Note: Additional protein fractionation or enrichment techniques such as SDS-PAGE or immunoprecipitation (IP) may be performed on equally mixed lysates before MS sample prep.
6. Digest proteins to MS-compatible peptides using the Pierce™ In-Gel Tryptic Digestion Kit, Pierce™ Mass Spec Sample Prep Kit for Cultured Cells or other suitable method.

Perform mass spectrometry analysis and quantitation of NeuCode™-labeled peptides

Typical LC-MS/MS analysis of peptides is performed using a C18 reverse-phase column (e.g., Thermo Scientific™ EASY-Spray™ 50 cm LC Column, Product No. ES803) using a hybrid mass spectrometer with resolution >450 at *m/z* 200, such as Thermo Scientific™ Orbitrap™ Elite™, Thermo Scientific™ Orbitrap™ Fusion™ or Thermo Scientific™ Orbitrap™ Fusion™ Lumos™ is required to resolve NeuCode™-labeled peptide peaks.

1. Separate peptides using a 5-40% acetonitrile gradient containing 0.1% formic acid at 200 µL/minute for 1-3 hrs.
2. Acquire MS spectra using a low resolution scan (30K at m/z 400 for the Orbitrap™ Elite™ mass spectrometer, 60K at m/z 200 for the Orbitrap™ Fusion™/Lumos™ mass spectrometers) for peak selection for MS/MS followed by a high resolution scan (240k at m/z 400 for the Orbitrap™ Elite™ mass spectrometer, 500K at m/z 200 for the Orbitrap™ Fusion™/Lumos™ mass spectrometers) for quantitation. Alternatively, a single high-resolution MS scan can be acquired for both MS peak selection and quantitation followed by MS/MS for peptide identification.
3. Protein identification and quantification can be performed by searching the human IPI database or equivalent with the MaxQuant™ software (v1.6 or later).

Troubleshooting

Observation	Possible cause	Recommended action
Only light peptide spectra observed.	Did not use SILAC media and/or dialyzed serum.	Use Dialyzed FBS to supplement SILAC media.
	Incorrect modifications used for identification in software.	Verify Precursor Ions Quantifier method and isotope monoisotopic mass (see Additional Information).
	Improper mixing of samples.	Mix equal amounts (1:1) of NeuCode-labeled cell lysates.
		Lyse equal amounts of labeled cells and measure protein concentration to ensure equal amounts of protein lysate for mixing.
Peptides for protein of interest not detected/quantified.	Protein was in low abundance.	Increase amount of sample analyzed by MS.
		Fractionate or enrich for protein/peptide of interest before MS analysis.
	Peptide/protein identification score was low.	Verify database search criteria.
		Use more than one peptide for protein quantitation.
	Labeled peptides not completely resolved.	Analyze samples using resolution > 450k at 200 m/z
		Apply MS scan filters in method to exclude large (>1200 m/z) and/or higher charge state peptides (>+4) peptides
		Ensure MS instrument is calibrated correctly.

Additional information

Product Numbers	Product Name	Amino Acids	MW	Monotopic Mass Increase
A36754	NeuCode™ Lysine - 202	$^{13}C_2\,^{15}N_2$ L-Lysine-2HCl	223.08	4.00078
88437, 88438	NeuCode™ Lysine - 040	4,4,5,5-D_4 L-Lysine-2HCl	223.13	4.02511
A36751, 88209, 88432	NeuCode™ Lysine - 602	$^{13}C_6\,^{15}N_2$ L-Lysine-2HCl	227.05	8.01420
A36753	NeuCode™ Lysine - 521	1,2,3,4,5-13C5, 6,6-D2, ALHPA-15N L -Lysine-2HCl	227.08	8.02637
A36851	NeuCode™ Lysine - 341	3,4,5-13C3; 5,5,6,6-D4, EPSILON-15N L-Lysine-2HCl	227.11	8.03221
A36752	NeuCode™ Lysine - 440	3,4,5,6-13C4; 5,5,6,6-D4 L-Lysine-2HCl	227.11	8.03853
A36750, A33613, A33614	NeuCode™ Lysine - 080	3,3,4,4,5,5,6,6-D_4 L-Lysine-2HCl	227.16	8.05021

Note: Only NeuCode™ Lysine - 602, 521, 440 and 080 are part of A36755.

Additional information is available on our website.

- SILAC Protein Quantitation Kits FAQ
- Tech Tip #60: Prepare SILAC peptides using the In-Gel Tryptic Digestion Kit

Cited references

1. Ong, S.E., *et al*. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* **1(5):**376-86.
2. Rose, C.M., *et al*. (2013). Neutron encoded labeling for peptide identification. *Anal Chem* **85(10):**5129-37.
3. Merril, A.E., *et al*. (2014). NeuCode™ labels for relative protein quantification. *Mol Cell Proteomics* **13(9):**2503-12.
4. Baughman J.M., *et al*. (2016). NeuCode™ Proteomics Reveals Bap1 Regulation of Metabolism. *Cell Rep* **16(2):**583-95

Manufacturer: Pierce Biotechnology, Inc. | Thermo Fisher Scientific | 3747 N. Meridian Road | Rockford, Illinois 61101 USA

The information in this guide is subject to change without notice.