invitrogen™

SILAC/™ Stem Cells Protein Identification (ID) and Quantitation Media Kit

For identifying and quantifying proteins from embryonic stem cells

Catalog no. MS10036

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User Manual

Table of Contents

SILAC Labeling Procedure for Experienced Users

Introduction

A brief procedure to perform SILAC labeling of human or mouse embryonic stem (ES) cells is described below.

For details on media preparation, ES cell culture, protein analysis, and MS analysis, refer to the detailed procedure in this manual.

<table>
<tr><th>Step</th><th>Procedure</th></tr>
<tr><td>Prepare cells and medium</td><td>1. Prepare Regular ES Cell and SILAC/™ Heavy and Light ES Cell Medium as follows:
<table>
<tr><th>Regular ES Cell Medium</th><th>SILAC/™ ES Cell Medium</th></tr>
<tr><td>KnockOut™ D-MEM
20% KnockOut™ Serum Replacement
2 mM L-GlutaMAX™-I Supplement
0.1 mM MEM Non-Essential Amino Acids Solution
1X (55 µM) 2-mercaptoethanol
4-8 ng/ml human bFGF (for human ES cells) or 1000 units/ml LIF (for mouse ES cells)</td><td>SILAC/™ KnockOut™ D-MEM
20% KnockOut™ Serum Replacement
100 mg/L L-Lysine (for light medium)
100 mg/L U-[$^{13}C_6$]-L-Lysine (*Lys) (for heavy medium)
100 mg/L L-Arginine
2 mM L-GlutaMAX™-I Supplement
0.1 mM MEM Non-Essential Amino Acids
1X (55 µM) 2-mercaptoethanol
4-8 ng/ml human bFGF (for human ES cells) or 1000 units/ml LIF (for mouse ES cells)
Optional: Phenol Red solution</td></tr>
</table>
2. Thaw ES cells and maintain the ES cells in MEF-conditioned Regular ES Cell Medium.
3. Harvest ES cells using Collagenase IV solution and resuspend the ES colonies in appropriate MEF-conditioned SILAC/™ Light or Heavy ES Cell Medium.</td></tr>
<tr><td>Perform SILAC labeling</td><td>1. Transfer the ES cells as follows into 35 mm MatriGel™ coated plates to obtain a seeding density of 5 x 10^4-1.5 x 10^5 cells/cm^2 cell culture plate:
• Light Cell Population: Transfer the ES cells in 4 ml MEF-conditioned SILAC/™ medium containing light lysine
• Heavy Cell Population: Transfer the ES cells in 4 ml MEF-conditioned SILAC/™ medium containing heavy lysine
2. Incubate in a 37°C incubator containing a humidified atmosphere of 5% CO_2 in air.
3. Grow the cells separately and change the medium every day using the appropriate MEF-conditioned SILAC/™ light or heavy ES Cell medium. Passage the cells at a ratio of 1:3 to 1:6 when cells reach 70-80% confluency (4-7 days).
4. Expand each cell population for at least six doubling times (usually is about 2 weeks) to achieve >90% incorporation of labeled amino acid into the proteins.
5. After six doublings, harvest a small aliquot of cells (~1 x 10^5 cells) from each cell population to determine the efficiency of incorporation. Store the cell pellet at -80°C.
6. At the end of six doublings, you will have ~6.4 x 10^6 cells when starting with 1 x 10^5 cells for each cell population. See page 15 for the approximate number of cells needed for each analysis.
7. Perform the appropriate cell treatment (page 21) and process samples (page 23).
8. Analyze tryptic peptides using MALDI-TOF MS or LC-MS. Perform protein identification using MS instrument software or Mascot software suite.
9. Quantitate protein using MS instrument software.</td></tr>
</table>

Kit Contents and Storage

Shipping and Storage

The shipping, and storage conditions for SILAC/™ Stem Cells Protein Identification (ID) and Quantitation Media Kit with [U-$^{13}C_6$]-L-Lysine (*Lys) and KnockOut™ D-MEM are listed below. For a detailed description of kit components, see page 4.

The kit includes appropriate media components and amino acids. Store all media protected from light.

Box	Component	Shipping	Storage
1	SILAC/™ KnockOut™ D-MEM	Room temperature	2-8°C
2	KnockOut™ Serum Replacement	Dry ice	-5 to -20°C
3	GlutaMAX™-I Supplement (100X)	Dry ice	-5 to -20°C
4	MEM Non-Essential Amino Acids Solution, 10 mM (100X), liquid	Gel ice	2-8°C
	SILAC/™ Phenol Red Solution (10 g/L)	Gel ice	2-8°C
	SILAC/™ L-Lysine HCl and L-Arginine	Gel ice	2-8°C
	SILAC/™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	Gel ice	2-8°C
	2-mercaptoethanol (1,000X), Liquid (55 mM) in Dulbecco's Phosphate Buffered Saline (D-PBS)	Gel ice	2-8ºC

Media Kit Components

The kit components for SILAC/™ Stem Cells Protein ID and Quantitation Media Kit with [U-$^{13}C_6$]-L-Lysine (*Lys) and KnockOut™ D-MEM are listed below.

Store all components at 2-8°C except KnockOut™ Serum Replacement and GlutaMAX™-I Supplement, which are stored at -5 to -20°C.

Component	Amount
SILAC/™ KnockOut™ D-MEM	2 x 1000 ml
KnockOut™ Serum Replacement	500 ml
GlutaMAX™-I Supplement	100 ml
MEM Non-Essential Amino Acids Solution, 10 mM (100X), liquid	100 ml
2-mercaptoethanol (1,000X)	50 ml
SILAC/™ Phenol Red Solution (10 g/L)	5 ml
SILAC/™ L-Lysine HCl	100 mg
SILAC/™ L-Arginine	2 x 100 mg
SILAC/™ [U-$^{13}C_6$]-L-Lysine HCl (*Lys)	100 mg

Accessory Products

SILAC/™ Kits

The table below lists additional SILAC/™ Kits available separately. For more information, visit www.invitrogen.com or call Technical Support (page 50).

Product	Quantity	Catalog no.
SILAC™ [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg)	100 mg	MS10009
SILAC™ [U-$^{13}C_6$]-L-Arginine (*Arg)	100 mg	MS10011
SILAC™ Protein Identification and Quantitation **Media** Kit		
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and D-MEM-Flex*	1 kit	MS10030
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI-Flex*	1 kit	MS10031
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and IMDM-Flex*	1 kit	MS10032
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and Advanced D-MEM/F-12-Flex*	1 kit	MS10033
SILAC™ **Phosphoprotein** Identification (ID) and Quantitation Kit		
*with [U-$^{13}C_6$]-L- Lysine (*Lys) and D-MEM*	1 kit	SP10001
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI 1640*	1 kit	SP10005
SILAC™ **Membrane** Protein Identification and Quantitation Kit		
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and D-MEM*	1 kit	SM10002
*with [U-$^{13}C_6$]-L-Lysine (*Lys) and RPMI 1640*	1 kit	SM10006

Media Components

The table below lists cell culture media products available separately. For more information, visit www.invitrogen.com or call Technical Support (page 50).

Product	Quantity	Catalog no.
KnockOut™ D-MEM	500 ml	10829-018
KnockOut™ Serum Replacement	500 ml	10828-028
GlutaMAX™-I Supplement	100 ml	35050-061
MEM Non-Essential Amino Acids 10 mM (100X), Liquid	100 ml	11140-050
2-mercaptoethanol (1,000X), liquid	50 ml	21985-023
D-MEM high glucose (1X), liquid	500 ml	11965-092
Fetal Bovine Serum (FBS), ES Cell-Qualified (US)	100 ml	16141-061
L-Glutamine (100X), 200 mM	20 ml	25030-149
Collagenase Type IV	1 g	17104-019
bFGF (basic Fibroblast Growth Factor), Human Recombinant	10 µg	13256-029
Trypan Blue Stain	100 ml	15250-061
Phosphate Buffered Saline (PBS), 1X	500 ml	10010-023
Recovery™ Cell Culture Freezing Medium	50 ml	12648-010
TrypLE™ Express Stable Trypsin Replacement Enzyme (1X) without Phenol Red	100 ml	12604-013
	500 ml	12604-021
TrypLE™ Express Stable Trypsin Replacement Enzyme (1X) with Phenol Red	100 ml	12605-010
	500 ml	12605-028

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Accessory Products, Continued

Additional Reagents

The table below lists additional products available separately from Invitrogen. For more information, visit www.invitrogen.com or call Technical Support (page 50).

Product	Quantity	Catalog no.
Recombinant Protein G Agarose	5 ml	15920-010
SimplyBlue™ SafeStain	1 L	LC6060
SilverQuest™ Silver Staining Kit	1 kit	LC6070
NuPAGE® Novex® 4-12% Bis-Tris Gel, 10 well, 1.0 mm	1 box of 10 gels	NP0321BOX
NuPAGE® MOPS SDS Running Buffer (20X)	500 ml	NP0001
NuPAGE® MES SDS Buffer (20X)	500 ml	NP0002
NuPAGE® Antioxidant	15 ml	NP0005
NuPAGE® LDS Sample Buffer (4X)	10 ml	NP0007
NuPAGE® Sample Reducing Agent (10X)	250 µl	NP0004
XCell *SureLock*™ Mini-Cell	1 unit	EI0001
Max Ion™ Peptide MALDI Matrix Kit	1 kit	MS10005
Invitrosol™ LC/MS Protein Solubilizer (5X)	5 ml	MS10007
Invitrosol™ MALDI Protein Solubilizer Kit	1 kit	MS10001
1-D PAGE Cleavable ICAT® Reagent Application Kit	1 kit	MS10012
Cleavable ICAT® (10 assay Kit)	1 kit	MS10022
Cleavable ICAT® Bulk Reagent Kit	1 kit	MS10010
iTRAQ™ Reagents Methods Development Kit	1 kit	MS10013
iTRAQ™ Reagents MultiPlex Kit	1 kit	MS10016
Trypsin	1 kit	MS10015

Antibodies

A large variety of high-quality antibodies against various proteins is available from Invitrogen. Visit our website for details or contact Technical Support (page 50).

Introduction

Overview

Introduction

The SILAC/™ (Stable Isotopic Labeling by Amino Acids in Cell Culture) Stem Cells Protein Identification (ID) and Quantitation Media Kit provides a simple, efficient, and reproducible method for quantitative analysis of differential protein expression in human or mouse embryonic stem (ES) cells. The kit is designed to allow efficient metabolic labeling of embryonic stem cells followed by sample preparation and analysis using mass spectrometry (MS).

SILAC Technology

The SILAC technology is a powerful tool for quantitative analysis of systems biology including post-translational modifications, low abundance proteins, phosphoproteins, and membrane proteins using mammalian cells. The SILAC/™ Protein ID and Quantitation Kits are based on the metabolic labeling technology developed by Brian Chait (Oda *et al.*, 1999) using isotopic nutrients (N^{15}) in cell culture media and performing comparative MS analysis. Chen and coworkers modified this method by using stable isotopes of amino acids instead of simple salts (Chen *et al.*, 2000). Because isotopic amino acids are incorporated into proteins in a sequence specific manner with Amino Acid Coded mass Tags (AACT), they were able to confirm the identity of proteins with higher confidence by comparing the sequence dependent mass shifts of an entire protein digest to the peptide mass fingerprint of the unlabeled protein. Residue specific mass alterations to efficiently detect protein modifications such as phosphorylation and oxidation were also demonstrated using isotopically labeled amino acids (Bae & Chen, 2004; Sechi & Oda, 2003; Zhu *et al.*, 2002).

The SILAC technology is a result of further developments of this method by Mathias Mann (Ong *et al.*, 2002) using stable isotopic amino acids as labels in cell culture, which when combined with global, differential MS analysis provides a tool to identify and quantitate proteins in complex biological samples.

In SILAC experiments, two ES cell populations are grown in identical cell culture media deficient in one or more essential amino acids. One cell population is grown in medium with heavy (isotopic) amino acid while the other cell population is grown in medium with light (normal) amino acid. The natural metabolic machinery of the cells is utilized to label all cellular proteins with the heavy amino acid (Amanchy *et al.*, 2005). After trypsin digestion, the peptides containing the light or heavy amino acids are chemically identical and can be processed together using any protein separation method eliminating quantitation errors due to unequal sampling. Because the peptides are isotopically distinct, they can be easily distinguished by mass using MS analysis. Based on the relative peak intensity of the isotopic peptide pairs, you can quantitate differential protein expression and identify differential post-translational modifications between samples (Liang *et al.*, 2006b) or in response to a stimulus (Liang *et al.*, 2006a).

For a system overview, see page 3. For details on light and heavy amino acids, see page 17.

Continued on next page

SILAC/™ Kit

The SILAC/™ Stem Cells Protein Identification and Quantitation Media Kit allows identification of key growth factors that regulate proliferation and differentiation as well as analysis of signal transduction. For detailed description on each kit component, see page 4.

Conventionally, ES cells are maintained on a layer of growth-arrested mouse embryonic fibroblasts (MEF). However, it has been shown that pluripotent ES cells can be maintained in defined medium that is conditioned by prior exposure to the MEF feeder layer (Xu *et al.*, 2001).

The SILAC/™ Stem Cell Kit is designed to provide >90% incorporation of labeled amino acids into ES cells maintained on MEF-conditioned medium (see **Note** below) and the labeling does not affect the growth or pluripotency of ES cells.

Note

We have routinely achieved >95% incorporation using protocols described in this manual with a variety of cell lines including HeLa, 293, and H1 human ES cells. Similar incorporation was also observed when labeling a wide variety of cells including yeast and prokaryotes (Beynon & Pratt, 2005).

However, lower incorporation efficiencies maybe due to unequal sampling or the introduction of exogenous light label amino acid into samples during the protocol. See page 19 for recommendations to avoid introducing exogenous light label amino acid into the sample.

Advantages

Using SILAC technology for quantitative proteomics in embryonic stem cells offers the following advantages:

- Simple, easy-to use labeling protocol designed for cell biologists and protein biochemists, and performed using standard laboratory equipment
- Produces >90% labeling efficiency as compared to other labeling methods currently available
- Allows specific sequence labeling of peptides since isotope labeled amino acid medium is used instead of isotopic nuclei labeled medium
- Generates uniformly labeled proteins to analyze several peptides for accurate results and increased sequence coverage
- Eliminates quantitation error due to unequal sample preparation and increases reproducibility as the two cell populations are mixed after treatment and treated as a single sample in all subsequent steps
- Provides flexibility in the choice of amino acid used for labeling and the types of treatment that can be applied to cells

Continued on next page

Applications

SILAC technology can be used to:

- Identify secreted growth factors
- Quantitatively analyze differential protein expression in the presence of growth factors or a stimulus
- Study signal transduction pathways (*i.e.*, changes in phosphorylation, lipidation, relocation, ubiquitination upon stimulation)

System Overview

To perform quantitative analysis of protein expression using SILAC technology, you will:

- Grow your human or mouse embryonic stem (ES) cells as two different populations.
- Metabolically label one ES cell population using non-radioactive isotopic labeled essential amino acid (heavy amino acid) while labeling the second ES cell population using normal essential amino acid (light amino acid) during cell culture.
- Harvest ES cells or medium from each population after the isotopic labeled amino acid is incorporated into the cellular proteins (usually complete incorporation is achieved within six doublings).
- Mix the cells or medium from each population.
- Process the samples using standard protein or peptide separation methods.
- Analyze tryptic peptides or phosphopeptides by MS analysis.
- Perform protein identification and quantification.

Important

The SILAC/™ Kits are designed for cell labeling experiments performed by cell biologists and protein biochemists while working with a proteomics core facility for sample processing and MS analysis. You need to identify a proteomics core facility capable of identifying proteins from Coomassie® or silver stained gel bands for MS analysis. Review the information on page 9 before starting the labeling experiments.

Purpose of the Manual

This manual provides the following information:

- Basic information for preparing cell culture media and growing cells
- Performing isotopic labeling of cells
- Guidelines for preparing cell lysates and processing the lysates for analysis
- Guidelines for MS analysis, protein identification, and quantitation
- Troubleshooting

Description of Kit Components

Kit Components

The SILAC/™ Stem Cells Protein ID and Quantitation Media Kit includes the following major components:

- GIBCO® KnockOut™ D-MEM for growth of human or mouse embryonic stem cells
- GIBCO® KnockOut™ Serum Replacement for efficient and reproducible ES cell growth without any interfering amino acids for SILAC
- SILAC/™ Normal (light) amino acids for supplementing the basal medium for cell culture
- SILAC/™ Isotope labeled (heavy) amino acids for performing isotope labeling in cell culture

SILAC/™ KnockOut™ D-MEM

The SILAC/™ KnockOut™ D-MEM is a basal medium optimized for growth of undifferentiated ES cells. The osmolarity is optimized to approximate that of the murine embryonic environment. The SILAC/™ KnockOut™ D-MEM is a basal medium that requires supplementation with amino acids, GlutaMAX™-I, and KnockOut™ Serum Replacement for cell culture. For feeder-free culture, the medium can be conditioned in the presence of MEF cells (see page 13 for preparing media).

The SILAC/™ KnockOut™ D-MEM is formulated without L-Arginine, L-Glutamine, L-Lysine, and phenol red.

KnockOut™ Serum Replacement

The Knockout™ Serum Replacement is a serum-free formulation optimized to grow and maintain undifferentiated ES cells in culture (Goldsborough *et al.*, 1998). It directly replaces FBS (fetal bovine serum) in existing protocols. The performance of Knockout™ Serum Replacement is enhanced when used with Knockout™ D-MEM.

Do not heat inactivate the Knockout™ Serum Replacement. **Do not** use regular FBS to perform SILAC labeling experiments. Trace amounts of amino acids present in regular FBS interfere with the incorporation of labeled amino acid and produce erroneous results.

GlutaMAX™-I Supplement

GlutaMAX™-I Supplement, L-alanyl-L-glutamine, is a dipeptide substitute for L-glutamine and is supplied as a 200 mM (100X), liquid stock in 0.85% NaCl. GlutaMAX™-I can be used as a direct substitute for L-glutamine at equimolar concentrations in cell cultures with minimal or no adaptation.

GlutaMAX™-I improves growth efficiency and performance of mammalian cell culture systems and eliminates problems associated with the spontaneous breakdown of L-glutamine during incubation. GlutaMAX™-I is highly soluble in aqueous solution and is heat stable.

Continued on next page

Description of Kit Components, Continued

MEM Non-Essential Amino Acids Solution

MEM Non-Essential Amino Acids Solution, 10 mM (100X) is prepared in distilled water and the non-essential amino acids in this solution are 100X the concentration in a MEM-alpha Medium. For detailed formulation, visit www.invitrogen.com.

SILAC/™ Amino Acids

SILAC/™ Amino Acids are highly pure, cell culture grade amino acids used for supplementing the basal media to prepare complete media. The SILAC/™ Amino Acids include the normal (light) and isotope labeled (heavy) amino acids.

SILAC/™ Light Amino Acids

The SILAC/™ Kits include L-Lysine HCl and L-Arginine as light (unlabeled) amino acids. These amino acids are normal, essential amino acids and do not contain any isotopic label. Use the light amino acids to prepare the light (unlabeled) medium as directed in the protocol (page 13).

SILAC/™ Heavy Amino Acid

The SILAC/™ Heavy Amino Acid includes the heavy (isotope labeled) amino acid, [U-$^{13}C_6$]-L-Lysine HCl (MW = 152.1259). The labeled *Lys is a stable isotope of [$^{12}C_6$]-L-Lysine (MW = 146.1055). The *Lys is 6 daltons heavier than the light L-Lysine. Use the heavy amino acid to prepare the heavy (labeled) medium as directed in the protocol (page 13).

Note

If you need maximal sequence coverage or need to monitor all possible phosphorylation sites, we recommend performing a double-labeling experiment wherein the proteins are labeled with [U-$^{13}C_6$]-L-Lysine and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine or [U-$^{13}C_6$]-L-Arginine. See page 17 for details.

[U-$^{13}C_6$, $^{15}N_4$]-L-Arginine and [U-$^{13}C_6$]-L-Arginine (available separately from Invitrogen, page vii) are stable isotopes of [$^{12}C_6$, $^{14}N_4$]-L-Arginine and [$^{12}C_6$]-L-Arginine, respectively. After trypsin digestion and MS analysis, you will observe peak pairs that are separated by 10 Da (for Arg and [U-$^{13}C_6$, $^{15}N_4$]-L-Arg pairs) or 6 Da (for Arg and [U-$^{13}C_6$]-L-Arg pairs).

Arg-containing peptides ionize better than Lys-containing peptides in MALDI-MS resulting in better sensitivity and sequence coverage. Using double labeling increases the number of informative peptides making the method more sensitive.

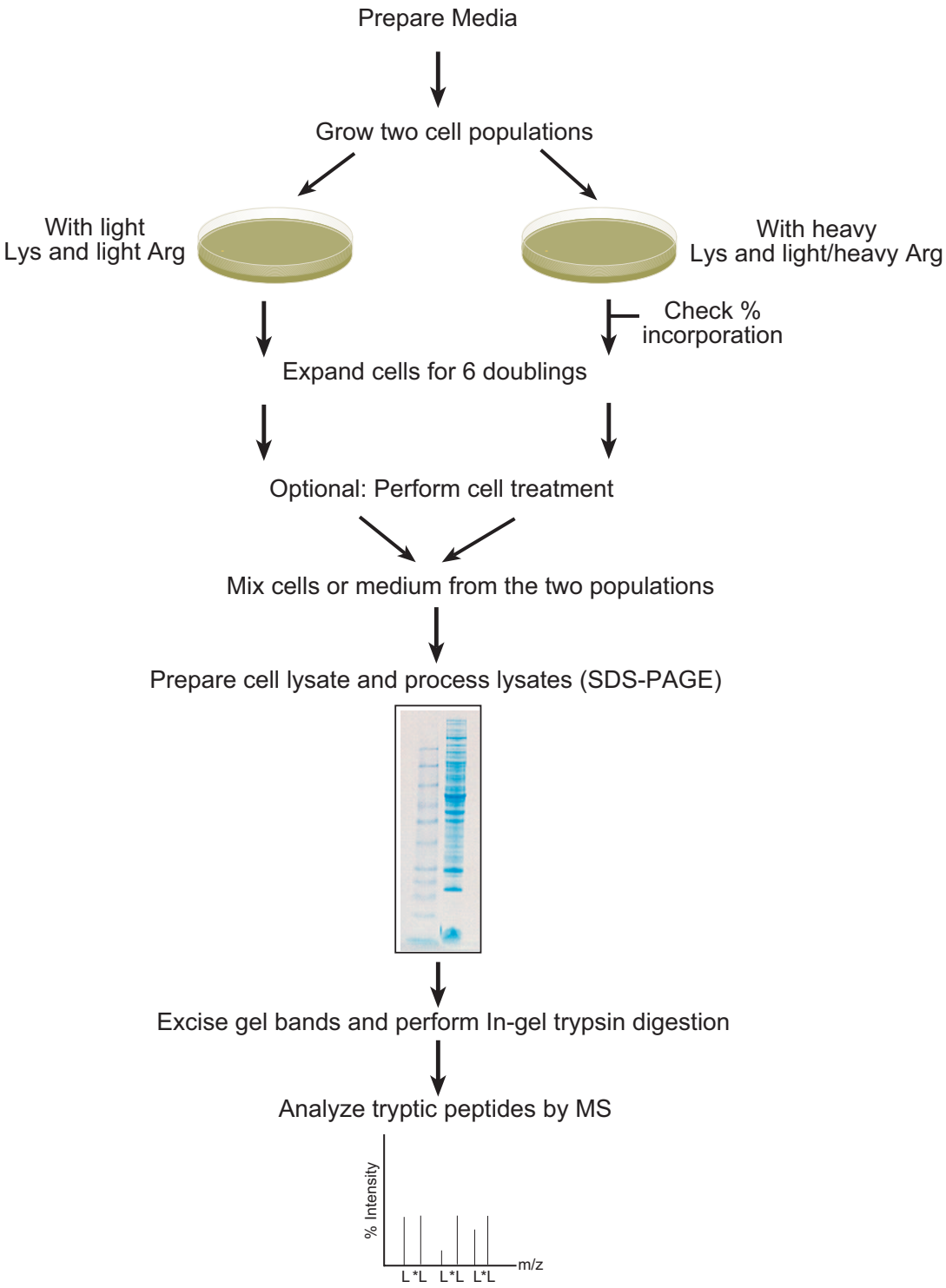
Use [U-$^{13}C_6$]-L-Arginine and [U-$^{13}C_6$]-L-Lysine for routine quantitative protein analysis.

Use [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine and [U-$^{13}C_6$]-L-Lysine for quantitative protein analysis when a higher level of confidence is required in the identification.

Experimental Overview

Flow Chart

The flow chart for the experimental outline using the SILAC/™ kit is shown below. See next page for an experimental outline.



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Experimental Overview, Continued

Experimental Outline

The experimental outline and general experimental timelines for using the SILAC™ kit are shown below. See next page for the experimental workflow.

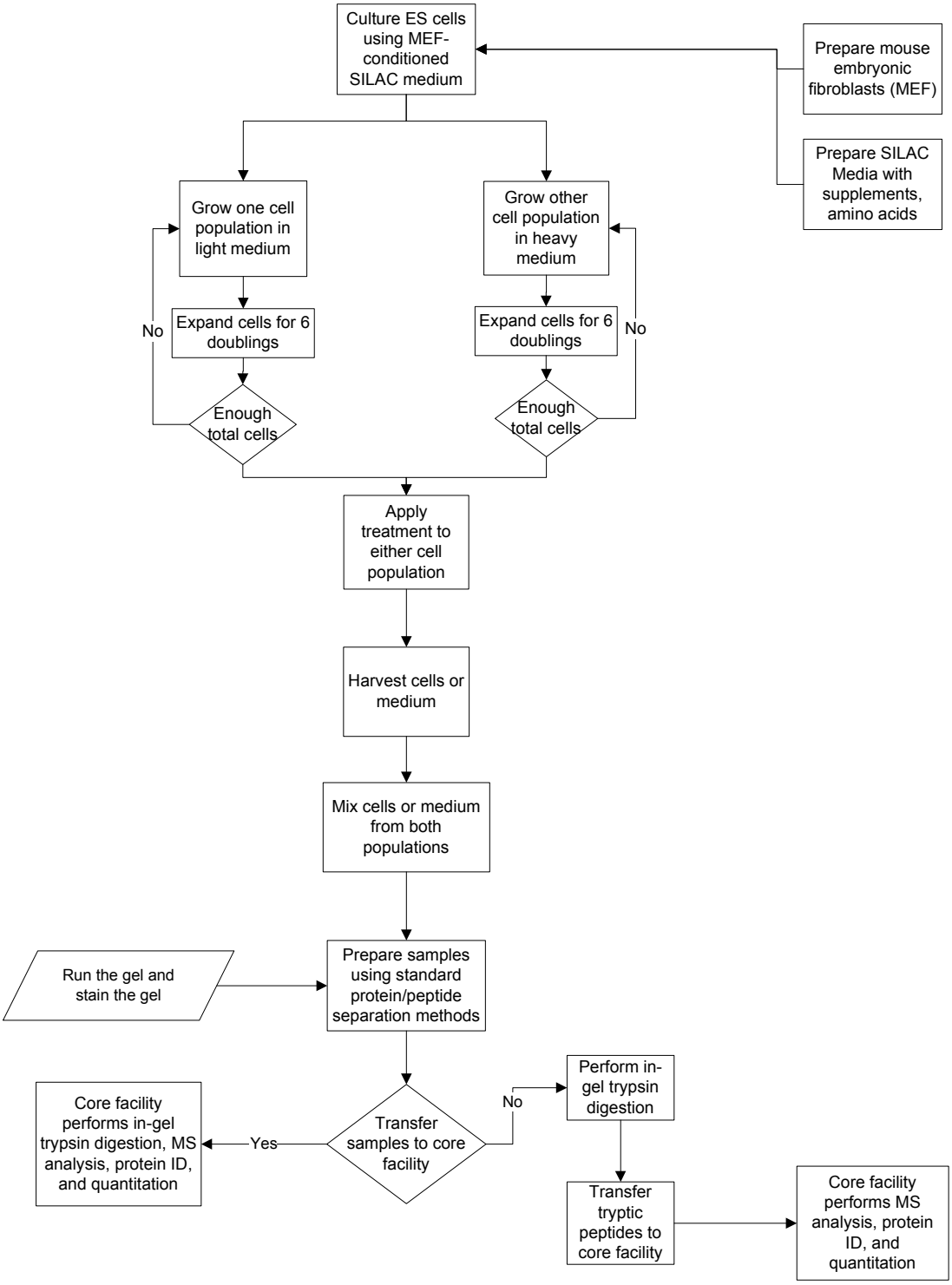
Step	Action	Page no.	Experimental Timeline
1	Initiate your ES cell line of interest for growth. Prepare mouse embryonic fibroblasts.	11 39	Varies
2	Prepare SILAC medium with supplements, and normal lysine or isotope labeled lysine.	13	1 day
3	Prepare MEF-conditioned SILAC medium.	41	1 day
4	Grow ES cells as two different populations; grow one ES cell population in MEF-conditioned SILAC medium containing light (normal) lysine and grow the other ES cell population in MEF-conditioned SILAC medium containing heavy (isotope labeled) lysine.	17	1 day
5	Expand the two ES cell populations for six doubling times to achieve complete incorporation of the labeled amino acid.	17	~2 weeks
6	Perform cell treatment, if needed.	21	3-7 days
7	Harvest culture media from each population and mix the media using equal protein amount to identify growth factors. **OR** Harvest cells from each population and mix the cells using a 1:1 ratio based on cell number to identify differential protein expression or unique cell surface markers.	23	1 day
8	Process the samples using standard protein or peptide separation methods.	23	3-5 days
9	Analyze tryptic peptides using MALDI-TOF MS or LC-MS.	27	2-6 days
10	Perform protein identification using MS instrument software or Mascot software suite.	30	7-10 days
11	Perform protein quantitation using instrument software such as GPS Explorer™.	32	

Continued on next page

Experimental Overview, Continued

Experimental Workflow

Below is the experimental workflow for using the SILAC™ Stem Cells Kit.



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Methods

Before Starting

Important

- Review the information in this section prior to starting your SILAC/™ experiments. You need to perform certain experiments and need to purchase some reagents before proceeding with isotope labeling experiments.
- You need to be familiar with standard stem cell culture techniques using mouse embryonic fibroblasts (MEF) to successfully use the SILAC/™ Kit. If you need help with human or mouse stem cell culture, preparation of MEF and conditioned media, general protein separation methods, refer to published references (Bodnar *et al.*, 2004; Xu *et al.*, 2001). Visit www.invitrogen.com/stemcell for more technical resources on stem cells.

Efficiency of Incorporation

To obtain easily interpretable results, it is important to obtain >90% incorporation of the isotope-labeled amino acid into proteins. You need to determine the efficiency of incorporation as described on page 21.

Based on the doubling time of your cell line, you can determine the efficiency of incorporation before starting the actual labeling experiment (if the doubling time of your cells is 16-18 hours) or along with your labeling experiment (if the doubling time of your cells is 24-48 hours).

Greater than 90% incorporation of the isotope labeled lysine into proteins is recommended for SILAC labeling experiments. We have routinely achieved >95% incorporation using the protocols described in this manual with a variety of cell lines including HeLa, 293, and H1 human ES cells. Similar incorporation was also observed when labeling a wide variety of cells including yeast and prokaryotes (Beynon & Pratt, 2005).

MS Core Facility

The SILAC/™ Kits are designed for use by cell biologists and protein biochemists to perform the labeling experiments and then coordinate with the proteomics core facility for sample processing and MS analysis.

Based on your expertise with certain protocols and the options provided by the core facility, you can transfer the samples to the core facility for MS analysis at various points as indicated in the protocols. As each core facility has specific requirements for sample preparation and handling, it is important that you consult with your core facility about the sample requirements prior to preparing the samples. You also need to work closely with the core facility to schedule time for the MS analysis when your samples are ready.

Recommended Core Facilities for SILAC

If you do not have access to a core facility or the core facility is not equipped to perform MS analysis for SILAC, contact Technical Support (page 50) for a list of recommended core facilities. We have identified and qualified some core facilities for performing MS analysis, protein identification, and quantitation for SILAC.

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Note

If you are an experienced user of MS, have access to various MS instruments, and are able to perform MALDI-MS or LC-MS analysis, you may chose to perform the MS analysis yourself without working with a core facility.

MS Instruments

SILAC experimental data can be analyzed using MALDI-TOF MS analysis for simple samples or using MS/MS analysis for complex samples.

SILAC/™ Kits were developed using the 4700 Proteomics Analyzer MALDI TOF/TOF equipped with GPS Explorer™ software (Applied Biosystems) and Q-Tof™ Platform (Waters) that allowed protein identification and quantitation after labeling. On page 30 we describe the procedures to set-up SILAC/™ quantitation with data collected on ABI/MDS Sciex Family of MALDI TOF/TOF™ equipped with GPS Explorer Software™ as an example.

SILAC/™ quantitation is now supported by software platforms running each of the major proteomic tandem MS instruments. See the table below for a list of tandem MS instrument and the corresponding software packages that support SILAC data analysis.

Instrument	Manufacturer
ABI/MDS Sciex Family of MALDI TOF/TOF™ (including the 4700 and 4800 Proteomics Analyzer platforms) equipped with GPS Explorer Software™ (versions 3.1 and higher)	Applied Biosystems
QSTAR® and Q TRAP® LC/MS/MS systems with ProteinPilot™ software	Applied Biosystems
Q-Tof™ Platform with ProteinLynx Global SERVER™ software (version 2.2.5 and higher)	Waters
LCQ™ and LTQ™ Traps as well as the Orbitrap and LTQ FT™ may be purchased with or supplemented by add-on of BioWorks™ software (version 3.3 and higher)	Thermo Fisher Scientific

Because specific software details may vary among instrument manufacturers, we recommend that you consult with your proteomics/MS core facility.

If you have other MS instruments, you can perform semi-automated analysis of SILAC raw data using the MS instrument for protein identification, but you need to perform protein quantitation using manual calculations (contact the instrument vendor).

Antibodies

A large variety of antibodies against various proteins is available from Invitrogen (page vii). Antibodies against specific epitope-tags such as 6X His-, V5-, Myc- are also available from Invitrogen. Visit our website at www.invitrogen.com for more information.

Preparing Cells and Media

Introduction

To perform SILAC experiments, you need a human or mouse ES cell line of choice.

General guidelines are included below for handling ES cells and preparing the media. If you are performing ES cell culture for the first time, refer to published protocols for more information (Bodnar *et al.*, 2004). For more technical resources on stem cells, visit www.invitrogen.com/stemcell.

ES Cells

The SILAC labeling does not affect the growth or pluripotency of ES cells.

The ES cell line of choice must be able to grow in KnockOut™ D-MEM supplemented with KnockOut™ Serum Replacement under the conditions used for labeling (see page 20 for details). If your ES cells require specific growth factors for growth, you may add the growth factors to the medium but **do not** add any additional amino acids to the growth medium.

Important

General guidelines for ES cell culture are described in this section.

If you have access to a stem cell core facility such as the University of California, San Francisco (UCSF) Stem Cell Facility, follow the recommended protocols described by the core facility. For details, visit UCSF web site at http://www.escells.ucsf.edu/researchers/protocols.asp).

For more technical resources on ES cells, visit www.invitrogen.com/stemcell.

If you have an optimized cell culture protocol for your ES cell line, use the optimized protocol.

General Guidelines

Follow the general guidelines below to grow and maintain ES cells.

- **All solutions and equipment that come in contact with the cells must be sterile.** Always use proper sterile technique and work in a laminar flow hood.
- Before starting the labeling experiments, be sure to have your ES cell line of interest established and have some frozen stocks on hand.
- Always use log phase cultures with >90% cell viability. Determine cell viability using the trypan blue dye exclusion method.
- Culture ES cells in tissue culture plates such as 6-, 24-, 48-, or 96-well plates rather than tissue culture flasks as the cells are difficult to scrape from flasks.
- Remove any differentiated cells from the plate prior to passaging the cells.
- Handle ES cells as potentially biohazardous material under the appropriate Biosafety Level as required by your institution.

- The MEM Non-Essential Amino Acids Solution, 10 mM (100X) is irritating to eyes, respiratory system, and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. See MSDS for details.
- The 2-Mercaptoethanol (1000X) is toxic. Do not breathe vapor and avoid contact with skin and eyes. Do not mouth pipet the solution. See MSDS for details.

Continued on next page

Materials Needed

You need the following items:

- Human or mouse ES cell lines (available from commercial sources or ATCC)
- Human bFGF (page vii) for human ES cells
- Murine Leukemia inhibitory factor (Millipore catalog no. ESG1106) for mouse ES cells
- Regular ES Cell Medium (see below)
- *Optional:* growth factors if needed for your cells
- Appropriate tissue culture dishes and media bottles
- 37°C incubator with a humidified atmosphere of 5% CO_2 in air
- Sterile centrifuge tubes
- Reagents to determine viable and total cell counts (page vii)
- 0.22 µm filtration unit to filter sterilize the medium
- *Optional:* [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine or [U-$^{13}C_6$]-L-Arginine for double labeling experiments (page vii)

Components Supplied in the Kit

You need the following items (supplied with the kit):

- SILAC/™ KnockOut™ D-MEM—deficient in lysine, arginine, glutamine, and phenol red
- KnockOut™ Serum Replacement, thaw and store at 4ºC until use or aliquot and freeze at -5 to -20ºC (thaw as needed)
- L-Lysine HCl
- L-Arginine
- GlutaMAX-I™ Supplement (100X), thaw and store at 4ºC until use
- SILAC™ Phenol Red Solution (10 g/L), optional
- 2-mercaptoethanol (1,000X)
- MEM Non-Essential Amino Acids Solution, 10 mM (100X)
- [U-$^{13}C_6$]-L-Lysine HCl (*Lys)

Media Types

To culture human or mouse ES cells, you need to prepare the following media:

- **SILAC/™ ES Cell Medium** (see next page for recipe)

  Components to prepare the SILAC™ ES Cell Medium are supplied with the SILAC/™ Stem Cells Kit. **Use this medium for labeling experiments only.** Do not use this medium for routine maintenance of ES cells.

- **Regular ES Cell Medium** (see page 14 for recipe)

  Components to prepare the Regular ES Cell Medium are not supplied with the kit. You need to purchase the media components separately from Invitrogen (page vii). Use this medium for routine maintenance of ES cells. **Do not use this medium for SILAC labeling experiments.**

Continued on next page

Preparing SILAC/™ ES Cell Medium

Prepare the SILAC/™ ES Cell Medium (100 ml each) using the components supplied in the kit as described below. Adjust the reagent volumes accordingly to prepare >100 ml medium. **To prepare Regular ES Cell Medium, see next page.** Perform all steps in a tissue culture hood under sterile conditions.

SILAC/™ KnockOut™ D-MEM
20% KnockOut™ Serum Replacement
100 mg/L L-Lysine
100 mg/L L-Arginine
2 mM L-GlutaMAX™-I Supplement
0.1 mM MEM Non-Essential Amino Acids Solution
1X (55 µM) 2-mercaptoethanol
4-8 ng/ml human bFGF (human ES cells) or 1000 units/ml LIF (mouse ES cells)
Optional: Phenol Red solution
Do not add any Penicillin-Streptomycin to human ES cells, as human ES cells are sensitive to antibiotics.

1. Resuspend 100 mg L-Lysine HCl and 100 mg [U-$^{13}C_6$]-L-Lysine (*Lys) each in 1 ml basal, unsupplemented SILAC/™ KnockOut™ D-MEM. Mix well until completely dissolved.
2. Resuspend 100 mg L-Arginine from each vial (2 vials are supplied in the kit) in 1 ml basal, unsupplemented SILAC/™ KnockOut™ D-MEM. Mix well until completely dissolved.

   Note: If you are using double labeled arginine (available separately from Invitrogen, see page vii), resuspend 100 mg [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) or 100 mg [U-$^{13}C_6$]-L-Arginine (*Arg) in 1 ml basal, unsupplemented SILAC/™ KnockOut™ D-MEM supplied with the kit. Mix well until completely dissolved.
3. Transfer 80 ml SILAC/™ KnockOut™ D-MEM to two separate 100 ml sterile filter unit with 0.22 µm membrane (includes membrane filter, funnel, and receiver bottle).
4. Add 20 ml KnockOut™ Serum Replacement to each filter unit.
5. To one filter device from Step 4, add 0.1 ml L-Lysine HCl (100 mg/ml) from Step 1 and 0.1 ml L-Arginine (100 mg/ml) from Step 2 to prepare **light** SILAC/™ ES Cell medium supplemented with light (normal) lysine and arginine. Mark the bottle appropriately. Store the remaining lysine and arginine solution at -20ºC.
6. To the second filter unit from Step 4, add 0.1 ml *Lys (100 mg/ml) from Step 1 and 0.1 ml L-Arginine (100 mg/ml) from Step 2 to prepare **heavy** SILAC/™ ES Cell **single** labeling medium supplemented with light arginine and heavy lysine. Mark the bottle appropriately. Store the remaining lysine and arginine solution at -20ºC.

   Optional: If you are preparing **double** labeled medium, add 0.1 ml *Lys (100 mg/ml) from Step 1 and 0.1 ml *Arg (100 mg/ml) from Step 2 to prepare **heavy** SILAC/™ ES Cell **double** labeling medium supplemented with heavy (isotope labeled) arginine and lysine. Mark the bottle appropriately. Store the remaining lysine and arginine solution at -20ºC.

Continued on next page

Preparing SILAC/™ ES Cell Medium, continued

7. To each filter unit, add 1 ml 100X L-GlutaMAX-I™, 1 ml 100X MEM Non-Essential Amino Acids Solution, and 0.1 ml 1,000X 2-mercaptoethanol.
8. *Optional:* You may supplement the medium with additional growth factors or cytokines, if needed for your ES cell line. Add 0.1 ml SILAC/™ Phenol Red Solution to each filter unit, except when you are planning to analyze secreted proteins avoid adding phenol red as phenol red binds to C18 columns.
9. Apply vacuum to each filter unit to filter sterilize each medium.
10. To culture ES cells, add the following growth factors to each 100 ml medium bottle:
    - Human ES cells: human bFGF to a final concentration of 4-8 ng/ml
    - Mouse ES cells: Murine LIF to a final concentration of 1000 units/ml
11. Store the medium at 2 to 8°C, protected from light until use. The medium is stable for 2 weeks when properly stored (avoid introducing any contamination into the medium).

Preparing Regular ES Cell Medium

Prepare the Regular ES Cell Medium (100 ml) as described below. Adjust the reagent volumes accordingly to prepare >100 ml medium. You need to purchase the media components separately from Invitrogen (page vii). **To prepare SILAC/™ES Cell Medium, see page 13.** Perform all steps in a tissue culture hood under sterile conditions.

KnockOut™ D-MEM
20% KnockOut™ Serum Replacement
2 mM L-GlutaMAX™-I Supplement
0.1 mM MEM Non-Essential Amino Acids Solution
1X (55 µM) 2-mercaptoethanol
4-8 ng/ml human bFGF (human ES cells) or 1000units/ml LIF (mouse ES cells)
Do not add any Penicillin-Streptomycin to human ES cells.

1. Transfer 80 ml KnockOut™ D-MEM to a 100 ml sterile filter unit with 0.22 µm membrane (includes membrane filter, funnel, and receiver bottle).
2. Add 20 ml KnockOut™ Serum Replacement to the filter unit.
3. To the filter unit, add 1 ml 100X L-GlutaMAX-I™, 1 ml 100X MEM Non-Essential Amino Acids Solution, 0.1 ml Phenol Red Solution, and 0.1 ml 1,000X 2-mercaptoethanol.
4. *Optional:* You may supplement the medium with additional growth factors or cytokines, if needed for your specific cell line.
5. Apply vacuum to the filter unit to filter sterilize each medium.
6. To culture ES cells, add the following growth factors to each 100 ml medium:
    - Human ES cells: human bFGF to a final concentration of 4-8 ng/ml
    - Mouse ES cells: murine LIF to a final concentration of 1000 units/ml
7. Store the medium at 2 to 8°C, protected from light until use. The medium is stable for 2 weeks when properly stored (avoid introducing any contamination into the medium).

Continued on next page

Culturing ES Cells

- After receiving the ES cells, follow the manufacturer's recommendations to thaw and subculture the human or mouse ES cells. For more technical resources on ES cells, visit www.invitrogen.com/stemcell. See the next page for examples of ES cell images when cultured on MEF-conditioned KnockOut™ D-MEM.
- Use the MEF-conditioned Regular ES Cell Medium prepared as described page 41 to culture the ES cells. **Do not** use the SILAC™ ES Cell Medium for routine ES cell culture.
- The protocols in this manual are designed for culturing ES cells on Matrigel™ coated plates. See page 42 for preparing Matrigel™ coated plates. If you have an optimized ES cell culture protocol using gelatin coated plates, use the protocol optimized for your ES cell line.
- The SILAC/™ Stem Cell Kit is designed for ES cell culture using MEF-conditioned medium. During SILAC labeling experiments, the ES cells are maintained on MEF-conditioned SILAC/™ heavy and light ES Cell Medium. See page 41 to prepare MEF and MEF-conditioned SILAC/™ heavy and light ES Cell Medium.
- Maintain ES cells at a relatively high density and passage ES cells when they are 70-80% confluent (*i.e.* tightly packed intermediate size colonies close to each other but not touching each other). Passage the ES cells at a ratio of 1:3 to 1:6 (4-7 days) but change the medium every day.
- After the ES cells are established, be sure to freeze an aliquot of cells for future use. Follow the manufacturer's recommendations for preparing the freezing medium and freezing cells.
- After thawing, allow the ES cells to undergo at least 4 doublings in MEF-conditioned Regular ES Cell Medium prior to using the ES cells for SILAC labeling. Keep the ES cell passage number low.

Once you have established the human or mouse ES cell culture on MEF-conditioned medium and frozen an aliquot of cells, you are ready to perform SILAC labeling as described on page 17.

See below for the recommended number of cells needed for labeling.

Cells for Labeling

You need log-phase ES cells with 90% viability to perform successful labeling.

Based on the type of analysis that you wish to perform, the approximate number of ES cells needed for each analysis from **each** cell population are listed below:

- ~60 x 10^6 or cells from 2 x 100 mm plates for identifying secreted growth factors
- ~30 x 10^6 or cells from 1 x 100 mm plate for studying differential protein expression
- ~10^7-10^8 or cells from 2 to 3 x 100 mm plate for studying signal transduction pathway
- 10^5 cells to determine the efficiency of labeling (for any analysis type)

Note: Since ES cells are usually passaged as colonies varying from 50-500 cells/colony, approximate cells/100 mm plate were used for cell number calculations.

Continued on next page

Images of ES Cells

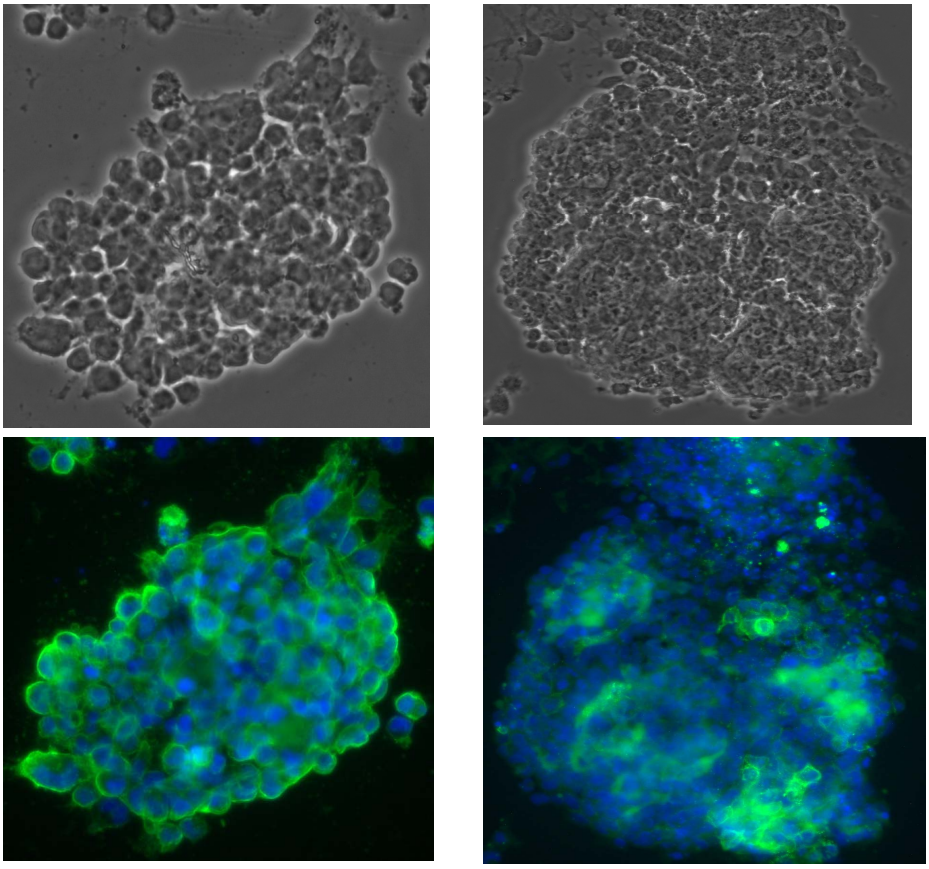
Images of undifferentiated and differentiated human ES cells grown in MEF-conditioned KnockOut™ D-MEM are shown below as examples. For more technical resources on ES cells including ES cell images, visit www.invitrogen.com/stemcell.

Colonies of human ES cell line were plated onto a synthetic matrix surface in MEF-conditioned KnockOut™ D-MEM containing Knockout™ Serum Replacement and 4 ng/ml bFGF. After 3 days of cultivation, the colonies were fixed in 4% formaldehyde, rinsed with D-PBS, immunostained with SSEA-4 (stage-specific embryonic antigen-4) antibody, fluorescently labeled with goat anti-mouse IgG_3 Alexa Fluor® 488, and stained with nuclear dye, DAPI. Colonies were visualized using a fluorescent microscope with 10x objective.

Results: The images for undifferentiated cells (panel A) illustrate good, compact morphology of individual cells, uniform colony "clump" with a well-defined edge (upper panel, and shows good staining for a known pluripotent state marker, SSEA-4 (lower panel).

The images for differentiated cells (panel B) illustrate breaking of the human ES colony as cells begin to migrate, distorting colony edge (upper panel), and inconsistent staining for pluripotency marker SSEA-4 (lower panel).

A: Undifferentiated hES cells **B: Differentiated hES cells**



Isotopic Labeling in Cell Culture

Introduction

Instructions for performing cell labeling are described in this section. At this point, you should have initiated your ES cell line of interest for growth and prepared any frozen stocks, if needed.

Isotopic Labeling

Metabolic labeling with stable isotope is performed using the SILAC technology. To obtain complete incorporation of the isotope labeled amino acid into the proteins, you need to adapt the cells to the medium containing the labeled amino acid. Complete incorporation is usually achieved within 6 doublings of cells in this medium.

Labeling with Isotopically Labeled Amino Acid

The SILAC™ Stem Cells Kit is supplied with [U-$^{13}C_6$]-L-Lysine HCl (MW = 152.1259) which is a stable isotope of [$^{12}C_6$]-L-Lysine (MW = 146.1055). The heavy *Lys is 6 daltons heavier than normal Lys.

For most of your experiments, performing single labeling with *Lys is sufficient to determine the relative expression of proteins.

Trypsin is the most widely used enzyme to generate peptides for MS analysis. Trypsin cleaves proteins at the C- terminus of arginine and lysine residues. Cells labeled with heavy *Lys and digested with trypsin yield peptides isotopically labeled with Lys. When these labeled peptides with C-terminal *Lys are mixed with nonlabeled peptides and MS analysis is performed, peptides are detected as "peak pairs" that are precisely 6.0204 Da apart. Using labeling with *Lys only, you detect peak pairs only for the subset of peptides with C-terminal Lys residues, while detecting the peptides with C-terminal Arg residues as superimposed singlet peaks.

If you need maximal sequence coverage or need to monitor all possible phosphorylation sites, we recommend performing a double-labeling experiment wherein the proteins are labeled with [U-$^{13}C_6$]-L-Lysine HCl and [U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (MW=184.1241). Arg-containing peptides ionize better in MALDI-MS than Lys-containing peptides, resulting in better sensitivity and sequence coverage. Using double labeling increases the number of informative peptides making the method more sensitive.

[U-$^{13}C_6$, $^{15}N_4$]-L-Arginine (*Arg) is available separately from Invitrogen (page vii) and is a stable isotope of [$^{12}C_6$, $^{14}N_4$]-L-Arginine (MW=174.1117). After trypsin digestion and MS analysis, you will observe peak pairs that are separated by 6.0204 Da (for Lys and *Lys pairs) and 10.0124 Da (Arg and *Arg pairs).

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Experimental Outline

1. Harvest ES cells and initiate two cultures. Grow one culture in the MEF-conditioned SILAC light (normal) supplemented medium and the other culture in MEF-conditioned SILAC heavy (isotope labeled) supplemented medium.
2. Grow the two ES cell populations for at least six doublings to allow complete incorporation of the labeled amino acid.
3. Perform the cell treatment (see below), if appropriate.

Treatment of Cells

You may induce cell differentiation using any growth factor or stimulant. Since the SILAC labeling experiments are performed in cell culture, various types of cell treatments can be performed to compare the effect of the treatment on protein expression.

The time for the treatment is highly variable from 5-30 minutes to several days depending on the treatment.

Materials Needed

- Human or mouse ES cells (see page 15 for the number of cells needed for labeling)
- MEF-conditioned SILAC/™ heavy and light ES Cell medium (page 41)
- Collagenase IV (page vii)
- *Optional:* growth factors if needed for your cells
- Appropriate tissue culture dishes and flasks
- Matrigel™ coated plates (page 42)
- 37°C incubator with a humidified atmosphere of 5% CO_2 in air
- Sterile centrifuge tubes
- Reagents to determine viable and total cell counts page vii)
- Appropriate reagents for cell treatment, if applicable

For determining the efficiency of incorporation, you also need:

- NuPAGE® LDS Sample Buffer (4X)
- NuPAGE® Sample Reducing Agent (10X)
- NuPAGE® Novex® Bis-Tris Gel
- NuPAGE® MES/MOPS SDS Running Buffer (20X)

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Note

Before performing the isotopic labeling experiments, be sure:

- You have the required number of ES cells actively growing with >90% viability.
- To keep some cells aside to measure the percentage of incorporation as directed in the protocol.
- To prevent introduction of exogenous light amino acids at specific steps, follow these guidelines:
  - Dilute Matrigel™ with SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) as described on page 42. Do not use SILAC/™ KnockOut™ D-MEM supplemented with Lys, Arg, Gln.
  - Prepare Collagenase IV solution in SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) as described on page 19.
  - Wash the MEF cells thoroughly with PBS to remove any traces of FBS that may contain amino acids. Perform a final wash with SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) prior to conditioning the medium as described on page 41.
  - Ensure the growth factors used for ES cell culture do not contain any exogenous amino acids.

Harvesting ES Cells

Once you have established the ES cell line and cells have undergone at least 6 doublings, use the ES cells for SILAC labeling.

1. Prepare 200 units/ml Collagenase IV solution in SILAC/™ KnockOut™ D-MEM without adding any supplements (deficient in Lys, Arg, Gln, and phenol red). Filter sterilize using 0.22 µm filtration device.
2. Remove the medium and wash the cells once with PBS. Add 1 ml 200 units/ml Collagenase IV to each well of a 6-well plate for each cell population. Use one 6-well plate for heavy and light labeling each.
3. Incubate at 37ºC for 5-10 minutes to detach the ES cells.

   Note: The incubation time varies and you need to monitor the detachment under the microscope. When majority of colonies are detached or the colony edges are rounded, you can proceed to the next step.
4. Add 1 ml of the appropriate MEF-conditioned SILAC/™ Light or Heavy ES Cell Medium to each well of a 6-well plate.
5. Transfer the ES cell suspension to sterile 15 ml centrifuge tubes and centrifuge at 200 x g for 5 minutes.
6. Remove the media and resuspend the colonies in the appropriate volume of MEF-conditioned SILAC/™ Light and Heavy ES Cell medium in two separate sterile 15 ml conical tubes.
7. Triturate the cells into small clusters of 50-500 cells but not to a single cell suspension.
8. Determine the viable and total cell count on an aliquot of ES cells using the trypan blue exclusion method.
9. Proceed immediately to **Labeling and Cell Culture**, next page.

Continued on next page

Isotopic Labeling in Cell Culture, Continued

Labeling and Cell Culture

Instructions for performing labeling with *Lys are described below.

1. Transfer the ES cells as follows into 35 mm MatriGel™ coated plates to obtain a **seeding density of 5 x 10^4-1.5 x 10^5 cells/cm^2** cell culture plate:
   - **Light Cell Population:** Transfer the ES cells in 4 ml MEF-conditioned SILAC/™ medium containing **light lysine** (prepared as described on pages 13 and 41)
   - **Heavy Cell Population:** Transfer the ES cells in 4 ml MEF-conditioned SILAC/™ medium containing **heavy lysine** and/**heavy arginine** (prepared as described on pages 13 and 41)
2. Incubate the dishes in a 37°C incubator containing a humidified atmosphere of 5% CO_2 in air.
3. The day after seeding, undifferentiated ES cells are seen as small colonies while single cells begin to differentiate.
4. Grow the cells separately and change the medium every day using the appropriate MEF-conditioned SILAC/™ light or heavy ES Cell medium. Passage the cells at a ratio of 1:3 to 1:6 when cells reach 70-80% confluency (4-7 days).

   Note: Cells grow at a similar rate in each media.
5. Expand each cell population for at least six doubling times (usually is about 2 weeks) to achieve >90% incorporation of labeled amino acid into the proteins.
6. After six doublings, harvest a small aliquot of cells (10^5 cells) from each cell population to determine the efficiency of incorporation. Store the cell pellet at -80°C until use. See next page for details on sample processing.
7. At the end of six doublings, you will have ~6.4 x 10^6 cells for each cell population. Based on the type of analysis that you wish to perform, the approximate number of cells needed for each analysis from **each** cell population are listed below:
   - ~60 x 10^6 or cells from 2 x 100 mm plates for identifying secreted growth factors
   - ~30 x 10^6 or cells from 1 x 100 mm plate for studying differential protein expression
   - ~10^7-10^8 or cells from 2 to 3 x 100 mm plate for studying signal transduction pathway

   Note: You may freeze the remaining cells or continue to maintain or expand the two cell populations in the light or heavy medium if you wish to repeat the experiment.
8. Proceed to **Performing the Cell Treatment** (next page, if needed) or **Processing Samples,** page 23.

Continued on next page

Performing Cell Treatment

After verifying that the you obtain >90% incorporation efficiency (see below), perform the cell treatment as described below. You may label the cells in light or heavy medium.

1. Determine the viable and total cell count using the trypan blue exclusion method.
2. Save an aliquot of cells as control prior to starting the treatment.
3. To either cell population, induce cell differentiation using growth factors or a stimulus.
4. Perform the treatment for the desired time (usually 5-30 minutes to several days depending on the treatment).
5. At the end of the treatment, proceed to **Processing Samples**, page 23.

Determining the Efficiency of Incorporation

To ensure >90% incorporation of the heavy amino acid into proteins, analyze small aliquots of cells (10^5) labeled with light or heavy amino acids and determine the efficiency of incorporation.

1. After six doublings, harvest a small aliquot of cells (~10^5 cells) from each cell population as described in Step 7, previous page.
2. Lyse each cell pellet separately in 180 µl 1X NuPAGE® LDS Sample Buffer and then add 20 µl NuPAGE® Reducing Agent (10X).
3. Heat the samples at 70°C for 8-10 minutes.
4. Load the samples from light and heavy medium side by side **without mixing** on a NuPAGE® Novex® 4-12% Bis-Tris Gel and perform electrophoresis using NuPAGE® Novex® MES or MOPS SDS Running Buffer. Be sure to load appropriate protein standards on the gel.

   Important: Avoid any sample cross contamination, and always wear laboratory gloves and work in a laminar hood.
5. Stain the gel with Coomassie® R-250 Stain.

   Note: You may transfer the gel to the core facility to perform trypsin digestion and MS analysis. For more information on proteomics core facilities that offer MS analysis for SILAC, see page 9.
6. Excise 3-4 side by side intense protein bands from each lane.
7. Perform in-gel trypsin digestion (page 49).
8. Perform MALDI-TOF MS analysis (page 27).

See next page for expected results. If you do not obtain >90% incorporation, see **Troubleshooting**, page 36.

Continued on next page

Isotopic Labeling in Cell Culture, Continued

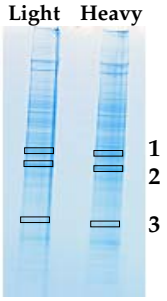
Example of Results

An example of results obtained after determining the efficiency of incorporation is shown below.

The MS analysis should show an increase in mass by 6 daltons for peptides labeled with *Lys when compared to peptides labeled with normal Lys (see figure below).

Note: If you have used double labeling with *Arg and *Lys, then the MS analysis should show an increase in mass by 6 and 10 daltons for peptides labeled with heavy *Lys and *[U-$^{13}C_6$, $^{15}N_4$]-Arg, respectively or 6 daltons for peptides labeled with heavy *Lys and *[U-$^{13}C_6$]-Arg, when compared to peptides labeled with normal (light) Lys and Arg.

SDS-PAGE Analysis

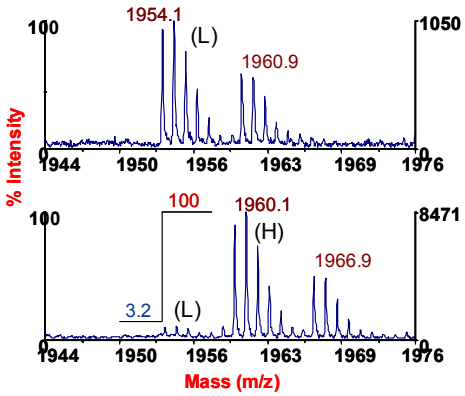


Samples were lysed and analyzed by SDS-PAGE using NuPAGE® Novex® 4-12% Bis-Tris Gel as described on the previous page and stained with a Coomassie® stain. Protein bands (1, 2, and 3) were excised from each side by side lane and subjected to in-gel trypsin digestion and MS analysis (see below).

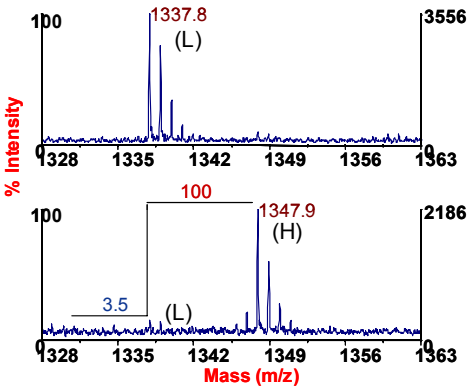
MS Analysis

MALDI-TOF MS analysis was performed on samples using the Voyager DE™-STR MALDI-TOF MS instrument.

(A) Lys-containing peptide



(B) Arg-containing peptide



L: Light Lys or Arg **H: [U-$^{13}C_6$] Lys or [U-$^{13}C_6$, $^{15}N_4$] Arg**

Processing Samples

Introduction

After performing cell labeling, harvest the cells or media and process the samples for MS analysis.

Sample processing guidelines and workflows are described below. Depending on the application that you wish to perform, choose the appropriate workflow and guidelines.

Note

- This section describes general workflows and guidelines to process the samples after labeling. If you have an established protocol to process samples for MS analysis for a specific application, use the established protocol.
- Recipes for lysis buffer and protocols for cell lysate to analyze phosphoproteins or study membrane proteins are described on page 43.
- General methods for protein analysis such as immunoprecipitation, analyzing protein complexes, and SDS-PAGE are described on page 47.

Applications

General guidelines and workflows for the following applications are described in this manual:

- Identifying growth or differentiation factors
- Analyzing differential protein expression or identifying unique cell surface markers upon differentiation
- Studying signal transduction pathway (*i.e.*, changes in phosphorylation, lipidation, relocation, ubiquitination upon stimulation)

Depending on the analysis that you wish to perform with the ES cell samples, choose the appropriate sample processing workflow and guidelines to obtain the best results.

Note: The SILAC/™ Stem Cells Kit is not limited only to the above listed applications. After labeling, the ES cell samples can also be used for other applications such as protein and /or peptide arrays.

Continued on next page

Identifying Growth or Differentiation Factors

The general workflow for identifying growth or differentiation factors using ES cell labeling medium is described below.

For this application, you need 60 x 10^6 cells or 2 x 100 mm plate of cells from each cell population.

1. Harvest light and heavy labeled culture media separately after stimulation (Step 4, page 21) by centrifugation. The volume of the medium is usually 20 ml, if using 2 x 100 mm culture plates.
2. Filter each media using a 0.45 µm filtration device to remove any cell debris.
3. Concentrate each media to about 0.5 ml using an ultrafiltration device with a 5000 Da molecular weight cut off membrane.
4. Determine the protein concentration in each sample.
5. Mix equal amount of proteins from media sample labeled with light and heavy amino acids.
6. Dry the media sample in a centrifugal vacuum concentrator (*e.g.,* Thermo Savant SpeedVac® centrifuge).
7. Resuspend the media sample in 60-200 µl 1X NuPAGE® LDS Sample Buffer containing 1X NuPAGE® Reducing Agent.
9. Heat the samples at 70°C for 8-10 minutes.
8. Separate the proteins by SDS-PAGE using NuPAGE® Novex® precast gels (page 47). Transfer stained gel to the proteomics core facility or proceed to the next step.
9. Perform in-gel trypsin digestion (page 49)
10. Proceed directly to MS analysis after trypsin digestion (page 27). Submit your tryptic peptides to the proteomics core facility for analysis.
11. After data analysis, perform crossover experiments to verify the growth factors identified (see **Important** below).

Important

We recommend that you verify the growth factors identified in the initial experiments by performing a crossover experiment.

Briefly, the crossover experiment involves repeating the experiment, except that you perform cell treatment to the other cell population (for example, if you stimulated the heavy labeled cells, then stimulate the light labeled cells to perform the crossover experiment).

Peptides derived from secreted proteins in a stimulus-specific response appear more abundant from the heavy labeled medium in the first experiment while in crossover experiment the peptides appear more abundant from the light labeled medium. Identified proteins that do not have a reciprocal response between the two experiments are more likely to be background proteins.

Continued on next page

Differential Protein Expression Analysis

The general workflow for analyzing differential protein expression or identifying unique cell surface markers upon differentiation using labeled ES cells is described below.

For this application, you need ~30 x 10^6 cells or 1 x 100 mm plate of cells from each cell population.

After performing the labeling for six doubling times and performing the cell treatment, if appropriate, harvest cells from each ES cell population as below.

1. Determine the viable and total cell count on an aliquot of cells using the trypan blue method.
2. Harvest the required number of ES cells from each population (page 21).
3. Resuspend each cell pellet in 1 ml chilled PBS.
4. Mix the cells grown in light (normal) medium and heavy (isotope labeled) medium in a 1:1 ratio based on the cell number.
5. Centrifuge the cells at 500 x g for 5 minutes at 4°C to remove PBS.
6. Resuspend the cell pellet in a hypotonic buffer to isolate plasma membrane or in a non-ionic detergent containing buffer to isolate cytosolic and membrane-associated proteins. Be sure to use appropriate protease inhibitors and phosphatase inhibitors in the buffer. See page 43 for buffer recipes and protocols to prepare cell lysates.
7. Mix well by pipetting up and down.
8. Centrifuge the lysate as follows:
   - For cytosolic and membrane-associated proteins, centrifuge at 100,000 x g for 20 minutes at 4°C. Collect the supernatant containing cytosolic and membrane associated proteins. Save the pellet at -80ºC, if you are interested in analysis of membrane proteins.
   - For membrane proteins, centrifuge at 100,000 x g for 60 minutes at 4°C. Collect the pellet containing membrane proteins. Save the supernatant at -80ºC if you are interested in analysis of cytosolic proteins.
9. Separate the proteins by SDS-PAGE using NuPAGE® Novex® precast gels (page 47). Transfer stained gel to the proteomics core facility or proceed to the next step.
10. Perform in-gel trypsin digestion (page 49)
11. Proceed directly to MS analysis after trypsin digestion (page 27). Submit your tryptic peptides to the proteomics core facility for analysis.
12. After data analysis, perform crossover experiments to verify differential expression (see **Important**, previous page).

Note

If you have performed any type of cell treatment, be sure to lyse the control cells (from Step 2, page 21) using the same lysis method used for treated cells.

Continued on next page

Studying Signal Transduction Pathway

The general workflow for studying signal transduction pathway using labeled ES cells is described below.

For this application, you need 10^7-10^8 cells or 2 to 3 x 100 mm plate of cells from each cell population.

After labeling for six doubling times and performing the cell treatment, if appropriate, harvest cells from each ES cell population as below.

1. Determine the viable and total cell count on an aliquot of cells using the trypan blue method.
2. Harvest the required number of ES cells from each population (page 21).
3. Resuspend each cell pellet in 1 ml chilled PBS.
4. Mix the cells grown in light (normal) medium and heavy (isotope labeled) medium in a 1:1 ratio based on the cell number.
5. Centrifuge the cells at 500 x g for 5 minutes at 4°C to remove PBS.
6. Resuspend the cell pellet and lyse the cells in a non-ionic detergent containing buffer. Be sure to use appropriate protease inhibitors in the buffer. See page 43 for lysis buffer recipes and protocols to prepare cell lysate.
7. Mix well by pipetting up and down.
8. Use the lysate to perform affinity enrichment of the signaling molecule or protein complex by immunoprecipitation (page 46).
9. Separate the immunoprecipitated proteins by SDS-PAGE using NuPAGE® Novex® precast gels (page 47). Transfer stained gel to the proteomics core facility or proceed to the next step.
10. Perform in-gel trypsin digestion (page 49)
11. Proceed directly to MS analysis after trypsin digestion (page 27). Submit your tryptic peptides to the proteomics core facility for analysis.

Mass Spectrometric Analysis

Introduction

General guidelines for performing MALDI-TOF MS and LC-MS analysis of tryptic digested peptides (page 49) are described in this section.

For details on the use of various MS instruments for analysis, refer to the manual supplied with the instruments.

Important

This section is designed for experienced users of MALDI-TOF and LC-MS analysis, especially core facility personnel that are familiar with standard techniques and instruments for MS analysis. General recommendations are included but detailed protocols for using the MS instruments are not included.

General Guidelines

Basic guidelines for sample preparation are given below. The choice of matrix and the amount of sample needed for mass spectrometry analysis depends on the technique used for analysis and the individual protein sample.

For more details on sample preparation, contact your proteomics core facility. For more information, refer to published protocols (Ausubel *et al.*, 1994; Coligan *et al.*, 1998; Peter, 2000; Simpson, 2003; Speicher, 2004).

- Sample concentration of 200-500 nM in a total volume of ~5 µl
- Prepare samples preferably in ultrapure water, methanol, or acetonitrile
- Sample must contain <10 mM buffer or salts
- Use Max Ion™ Peptide MALDI Kit for MALDI-TOF MS analysis (next page)

Recommended Methods for MS Analysis

The tryptic peptides (page 49) can be analyzed using the following MS analysis methods:

Important: For identifying and quantitating proteins using SILAC technology, it is important to perform MS analysis using tandem MS instruments that are capable of performing MS/MS analysis.

- For samples of low complexity, use MS fingerprinting techniques to identify and quantitate proteins.
- For samples of moderate complexity, use MALDI-TOF MS analysis. We routinely use 4700 Proteomics Analyzer (MALDI-TOF/TOF™ instrument) from Applied Biosystems. Other instruments such as Bruker Reflex III (Bruker Daltonics) or Voyager-DE™ STR MALDI TOF Workstation (Applied Biosystems) are also suitable.
- For complex samples, use on-line or off-line LC-MS/MS or two-dimensional LC-MS/MS. You may use Micromass Q-Tof Premier™ Mass Spectrometer (Waters) or QSTAR® Pulsar quadrupole TOF tandem MS (Applied Biosystems) equipped with a nanoelectrospray ion source or off-line separations followed by MALDI-TOF/TOF analysis.

  Some recommended gradients for LC-MS are listed on page 29.

Continued on next page

MS Reagents

A variety of reagents for MS analysis is available from Invitrogen (see page vii for ordering information).

Invitrosol™ LC/MS Protein Solubilizer

The Invitrosol™ LC/MS Protein Solubilizer is a novel surfactant blend that maintains a variety of hydrophobic proteins in solution, does not interfere with protease activity, and is compatible with reverse-phase high-pressure liquid chromatography (RP-HPLC) and LC-coupled electrospray ionization/mass spectrometry (ESI/MS) separations of the tryptic digested peptides. Use Invitrosol™ LC/MS Protein Solubilizer to remove incompatible buffer components prior to MS analysis or during in-gel trypsin digestion to improve the solubility of hydrophobic tryptic peptides.

Invitrosol™ MALDI Protein Solubilizer Kit

The Invitrosol™ MALDI Protein Solubilizer Kit is specifically designed for direct MALDI-TOF MS analysis of hydrophilic or hydrophobic intact proteins and peptides eliminating the need for solid phase extraction, acid hydrolysis, and matrix crystal washing. The Invitrosol™ MALDI Protein Solubilizer A and B are ready-to-use reagents composed of unique, proprietary detergent formulations that are designed to minimize suppression effects on the ionization of peptides/intact proteins and minimize cluster formation, and effectively solubilize hydrophobic proteins and improves sequence coverage of tryptic peptides in solution without affecting the sensitivity.

Max Ion™ Peptide MALDI Matrix Kit

The Max Ion™ Peptide MALDI Matrix Kit is designed for peptide analysis by using MALDI-TOF MS. The kit includes a proprietary resin that promotes the formation of homogeneous thin films of matrix crystals, improves the ionization of analyte species, and suppresses salt effects, resulting in improved spectral quality, enhanced signal-to-noise, and reduced matrix background.

Note

Depending on the type of MS instrument that you have, you may be able to:

- Perform fully automated analysis of SILAC raw data. This is supported through the MS instrument software for protein identification and quantitation (see page 10 for a list of instrument/software packages)

OR

- Perform semi-automated analysis of SILAC raw data. This is supported through the MS instrument software for protein identification but you need to perform protein quantitation using manual calculations (contact Technical Support, page 50) or consult the instrument vendor for detail.

Continued on next page

Recommended Gradients for LC-MS

If you are using LC-MS analysis, the following gradients are recommended. If you optimized the LC-MS analysis with specific gradients that are suitable for your analysis, use the optimized gradients for your analysis.

- For samples with less complexity, use a gradient of 5-45% (v/v) acetonitrile in 0.1% formic acid (or TFA) over 45 minutes and then use a gradient 45-95% acetonitrile in 0.1% formic acid (or TFA) over 5 minutes.

  Note: Use 0.1% formic acid solution on ESI based instruments and 0.1% TFA solution on off-line LC-MS/MS analysis using MALDI-TOF/TOF.

- For a complex sample, use a gradient of 5-45% (v/v) acetonitrile in 0.1% formic acid (or TFA) over 90 minutes or up to 120 minutes, and then use a gradient of 45-95% acetonitrile in 0.1% formic acid (or TFA) over 30 minutes or up to 60 minutes.

Protein Identification and Quantitation

Introduction

Once MS/MS analysis is complete, perform protein identification and quantitation as described in this section.

Important

This section is designed for experienced users of MALDI-TOF and LC-MS analysis, especially core facility personnel that are familiar with standard techniques and instruments for MS analysis.

Note

- Be sure to always compare the results with cells grown in the light and heavy medium containing each amino acid at the same concentration.
- The screen shots included in this section are provided as guidelines and may not represent the exact screen that you may view for the software, if the software has been upgraded. These screen shots were captured using GPS Explorer™ 3.x software.

Protein Identification

Protein identification is performed by searching the peptide fragments obtained after MS/MS analysis against non-redundant protein databases.

Most of the MS instruments are supplied with software that is capable of protein identification. You may use the instrument software to perform protein identification.

The protein identification method for SILAC/™ kits was developed by processing the raw MS data files from MS with Mascot Distiller (Matrix Science, London) and then searched the NCBI database using Mascot search algorithm. Our results have shown that using Mascot to identify proteins provides ~40% better results than compared to other protein identification methods.

Certain MS instruments contain software that perform protein identification using the Mascot search algorithm. For example, the GPS Explorer™ 3.0 software with AB/MDS Sciex Family of MALDI TOF/TOF™ Analyzers.

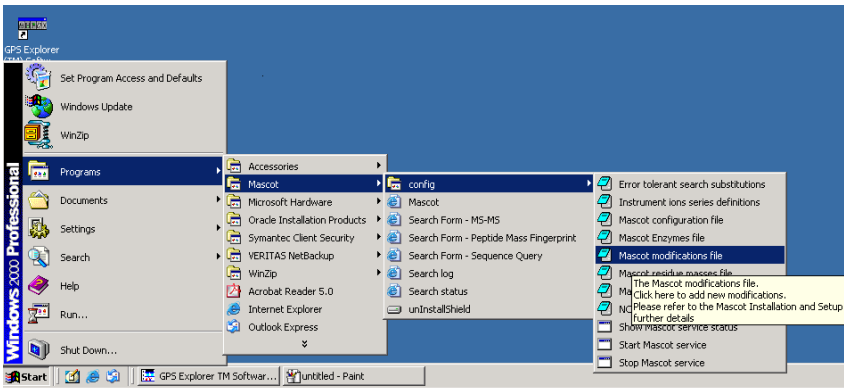
For more information on Mascot Distiller, visit www.matrixscience.com.

Continued on next page

Using Mascot for Protein Identification

Brief instructions are provided below to set up the Mascot server settings for protein identification using GPS Explorer™. Similar procedures apply to stand-alone versions of Mascot server. For installation, set up, and detailed instructions on using Mascot, visit www.matrixscience.com.

1. Start GPS Explorer™ software on your MS instrument (AB/MDS Sciex Family of MALDI TOF/TOF™ Analyzers).
2. Start Mascot server on your local computer and navigate to the Mascot Modification File screen (Mascot>Configuration>Mascot Modification Files).



3. Add the following text at the end of the Mascot Modification File to enable identification of proteins and isotopic peptide pairs for SILAC.

   "

   Title: Lys_light
   Residues: K 128.09497 128.1741
   "

   Title: Lys_heavy
   Residues: K 134.09497 134.1741
   "

   Title: Arg_light
   Residues: R 156.10112 156.1875
   "

   Title: Arg_heavy
   Residues: R 166.10112 166.1875
   "

   This will show isotope labeled lysine (heavy lysine) 6 Da larger than normal lysine (light lysine) and isotope labeled arginine (heavy arginine) 10 Da larger than normal arginine (light arginine).
4. Set the mass tolerance of the precursor peptide ion at 200 ppm and mass tolerance for the MS/MS fragment ions at 0.5 Da.
5. Select the variables modification in the setting for data analysis as follows depending on the type of labeling experiment:
   - For a single label experiment with *Lys, select a pair of light and heavy Lys as variables
   - For a double label experiment with *Lys and *Arg, select a pair of light and heavy Lys and a pair of light and heavy Arg

An example of the Mascot search result is shown on the next page.

Continued on next page

Protein Identification and Quantitation, Continued

Example of Mascot Search Result

The Mascot search result shows identities of proteins and the output shows peptides labeled with light or heavy Lys and/or Arg as shown below.

Peptide Summary Report

Switch to Protein Summary Report

To create a bookmark for this report, right click this link: Peptide Summary Report (SampleSetID: 343, AnalysisID: 818, Path=\Xiqu
Membrane\msilacexp3bqand12jrk\lysargmsms)

Select All | Select None | Search Selected | Error tolerant | Archive Report

1. gi|5453832 **Mass:** 111266 **Total score:** 729 **Peptides matched:** 37
oxygen regulated protein precursor; oxygen regulated protein (150kD) [Homo sapiens]
Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Rank	Peptide
23	1005.51	1004.50	1004.54	-0.05	0	(36)	1	YFQHLLGK
47	1011.54	1010.53	1010.54	-0.01	0	48	1	YFQHLLGK + Lys_heavy
48	1011.55	1010.54	1010.54	-0.00	0	(35)	1	YFQHLLGK + Lys_heavy
49	1012.54	1011.53	1011.55	-0.01	0	(41)	1	EVQYLLNK + Lys_heavy
50	1012.56	1011.55	1011.55	0.00	0	48	1	EVQYLLNK + Lys_heavy
131	1041.49	1040.48	1040.48	0.00	1	15	7	KYPDYESK + 2 Lys_heavy
161	1047.53	1046.53	1046.55	-0.03	0	(32)	1	LAGLFNEQR
162	1047.54	1046.53	1046.55	-0.02	0	(23)	1	LAGLFNEQR
174	1053.55	1052.54	1052.55	-0.01	0	70	1	LAGLFNEQR + Arg_heavy
175	1053.56	1052.55	1052.55	-0.00	0	(19)	9	LAGLFNEQR + Arg_heavy
176	1053.56	1052.55	1052.55	-0.00	0	(39)	1	LAGLFNEQR + Arg_heavy
178	1053.56	1052.56	1052.55	0.00	0	(9)	6	LAGLFNEQR + Arg_heavy
352	1124.58	1123.58	1123.58	-0.00	0	(29)	1	TLGGLEMELR + Arg_heavy
353	1124.59	1123.58	1123.58	-0.00	0	39	1	TLGGLEMELR + Arg_heavy
656	1250.60	1249.60	1249.59	0.01	0	79	1	FFGDSAASMAIK + Lys_heavy
708	1276.66	1275.65	1275.65	-0.00	0	39	1	EAGMQPQLQIR + Arg_heavy

Protein Quantitation

Once protein identification is complete using Mascot or other instrument specific software, perform quantitation for differential protein expression.

Protein quantitation is performed using the software available with your MS instrument such as GPS Explorer™ software. See page 10 for a list of MS instrument/software packages for SILAC data analysis.

GPS Explorer™

GPS Explorer™ 3.0 software is innovative applications software that supports many biological workflows such as traditional in-gel digestion, MDLC/MS/MS (LC MALDI), and PTM discovery all with intelligent results dependent analysis using RDA™ software feature.

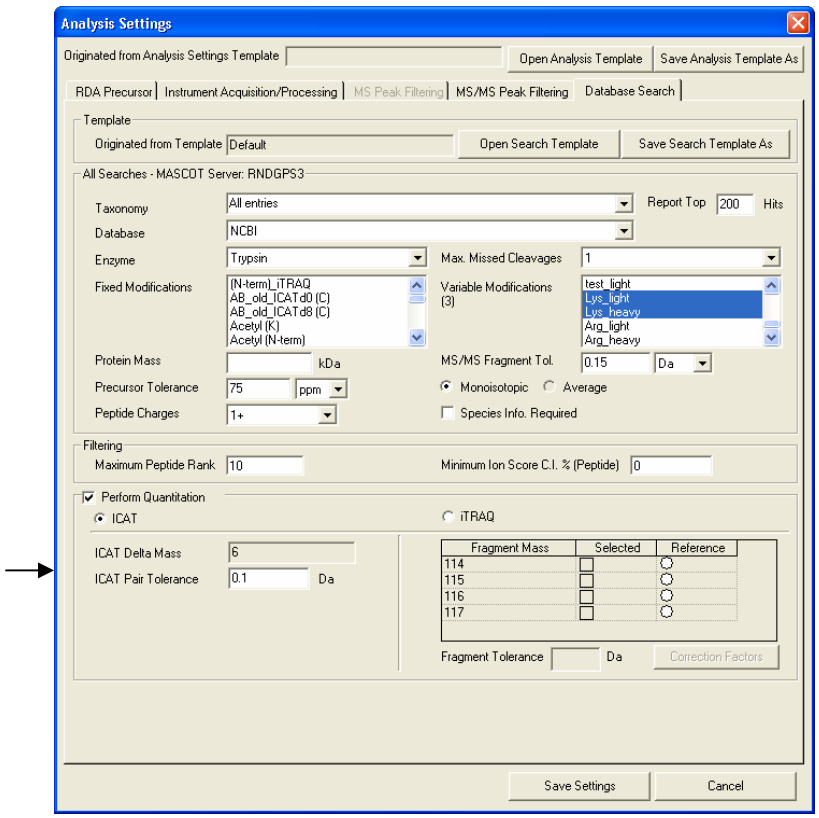
Currently, only one pair of light and heavy Lys or Arg at a time can be selected for quantitation using GPS Explorer™ software.

Continued on next page

Using GPS Explorer™ for Protein Quantitation

Brief instructions are provided below to set up the GPS Explorer™ software for SILAC data analysis. For details on using the software, follow the manufacturer's instructions.

1. Start GPS Explorer™ software on the MS instrument (AB/MDS Sciex Family of MALDI TOF/TOF™ Analyzers).
2. Navigate to the Data Analysis screen.
3. Select the following as variable modifications in the Analysis Settings Screen as shown in the figure below:
   - For a single label experiment with *Lys, select a pair of light and heavy Lys as variables. Ensure the mass difference of 6 Da shows up at the bottom of the screen in ICAT® Delta Mass under ICAT® Settings.
   - For a double label experiment with *Lys and *Arg, select a pair of light and heavy Lys or a pair of light and heavy Arg. Simultaneous selection of both labeled Lys and Arg does not work. Ensure the following mass difference shows up at the bottom of the screen in ICAT® Delta Mass under ICAT® Settings (indicated with an arrow in the figure below):
     - 6 Da mass difference when *Lys is selected **or**
     - 10 Da mass difference when *Arg is selected
4. Make sure the ICAT® Quantification box is checked and the ICAT® Pair Tolerance is set to 150 ppm under ICAT® Settings (see figure below).



An example of results using the GPS Explorer™ software is shown on the next page.

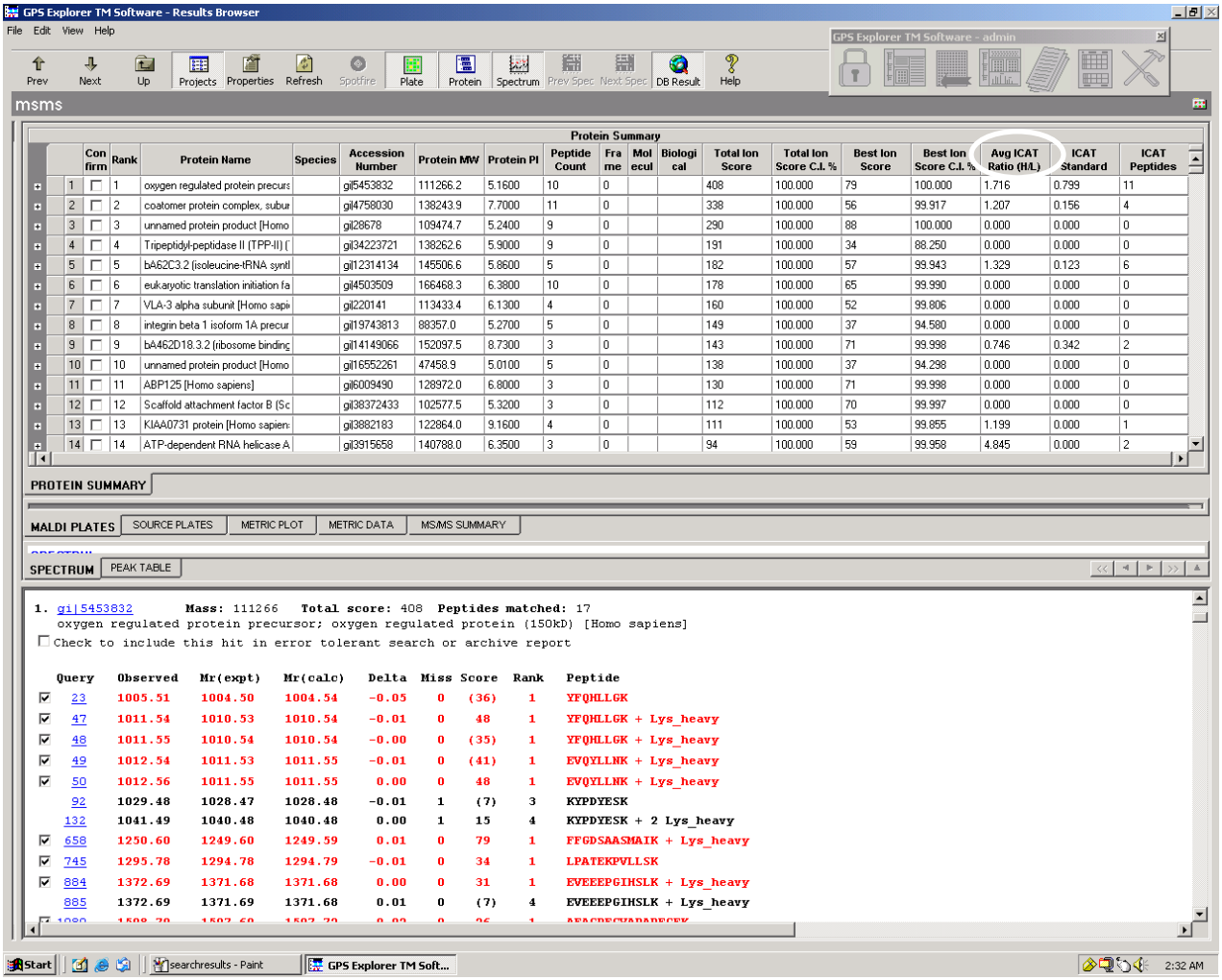
Continued on next page

Protein Identification and Quantitation, Continued

Example of GPS Explorer™ Analysis Results

An example of quantitation result based on a pair of light and heavy Lys peptides obtained after analysis using GPS Explorer™ software is shown below.

To view the quantitation results, review the data in the column Avg ICAT® Ratio (H/L) indicated with a circle in the figure below. For down regulated proteins, the ratio will be less than 1 and for up regulated proteins, the ratio will be greater than 1. See next page for details on interpreting the results.



Continued on next page

Interpreting the Results

To analyze differential protein expression results with SILAC experiments, review the data in the column, Avg ICAT® Ratio (H/L) as shown in the example of GPS Explorer™ results (indicated with a circle) on the previous page.

The ratio indicates up regulation or down regulation for various proteins analyzed. For example, the protein oxygen regulated protein precursor shows an Avg ICAT® Ratio (H/L) of 1.76 suggesting that this protein may be up regulated while the protein bA462D18.3.2 (ribosome binding protein) shows a ratio of 0.746 suggesting that this protein may be down regulated.

An Avg ICAT® Ratio (H/L) ratio of zero indicates the following:

- Only 1 peak from the peak pair was identified and therefore the software was unable to calculate a ratio. This happens when the signal for the peptide is very low (for low abundant peptides) and is sometimes occluded by background or if the ratio of peak pair relative abundance is very high (>10) or very low (<0.3). For such peptides, it is important to go back to the original raw data file and confirm manually.
- The cell treatment may have lowered the expression of the peptide in treated cells such that the software is unable to identify the signal as a significant signal.

The results are significant if the coefficient of variance (CV) is <30%. A significant variance amongst the peptides correlated to the same protein may indicate the following:

- The protein identification was incorrect
- Co-elution of an unrelated isobaric peptide distorted the peak profile
- Certain residues occurring in the peptide outlier are subject to metabolic interconversion (for example, Arg to Pro).

Under these conditions, check the profile manually and dismiss the peptide from the analysis, if appropriate.

Troubleshooting

Introduction

Review the table below to troubleshoot your experiments using SILAC/™ Stem Cells Protein Identification and Quantitation Kits.

For troubleshooting MS, refer to the manual supplied with the MS instrument or contact the core facility.

Problem	Cause	Solution
Protein ID scores are low or poor data quality after MS	Insufficient cells used	Use the recommended number of cells for each application as described on page 15. Use 5-fold more cells to obtain a good signal after MS for low abundant proteins.
	Improper MS analysis	• Ensure the MS instrument was properly tuned and calibrated prior to sample analysis. • Check that the correct database, organism taxonomy, peptide modifications, labeled amino acid, and enzyme were selected during data analysis.
	Loss of phosphopeptides after enrichment step	Follow the manufacturer's instructions to properly prepare the phosphopeptide column, and perform chromatography to avoid any loss of phosphopeptides.
	Loss of peptides after trypsin digestion	**Do not** concentrate the peptides to dryness after trypsin solution. If the peptides are concentrated to dryness, the peptides are difficult to resuspend resulting in loss of peptides.
Abundant proteins are well identified and quantitated, but the protein of interest is below background	Sample is too complex for the current analytical scheme	• Enrich the subcellular fraction of interest. • Enrich for specific peptides (phosphopeptides, glycopeptides, or N-terminal peptides) of interest. • Perform an additional dimension of LC or longer gradient on some protein bands of interest.
Peaks observed for unlabeled (light) amino acid for the protein	Incomplete incorporation of heavy amino acid	Perform the labeling for at least 6 doublings to ensure complete incorporation of the label. Be sure to use log-phase with >90% viability.
	Additional supplements added to the medium may contain amino acids	Always use KnockOut™ Serum Replacement to prepare the medium. **Do not** use regular FBS or use any other media supplements that may contain free amino acids.
	Amino acid prepared in complete medium	Prepare the amino acid using basal, unsupplemented KnockOut™ D-MEM supplied with the kit as described on page 13. Do not use any other complete medium to prepare the amino acids. See **Note** on page 19.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
The relative abundance between most of the heavy to light labeled proteins is not 1:1	Error in mixing cells or determining protein amount	• Count the cells prior to mixing and adjust the number of cells harvested to ensure the cells from two populations are mixed in a 1:1 ratio by cell number or equal protein amount. • Be sure to use log-phase cells with >90% viability. • Cell treatment may cause change in morphology resulting in an error in cell number. In this case, mix the cells using equal protein amount.
Poor amino acid incorporation (more apparent when labeling with lysine)	Arginine terminating peptides cause ionization suppression effects that impair the detection of lysine terminating peaks in MALDI	Perform analysis using LC-MS or include a simple fractionation step with a ZipTip® with C_{18} resin (use only a 30% elution step) prior to MALDI-MS analysis.
Sequence database search identifies keratin as the top candidate	Samples contaminated with keratin	• Always wear gloves while handling the gels and use ultrapure proteomics grade reagents for in-gel trypsin digestion. • Prepare fresh buffers. • Perform all gel manipulations in a clean dust-free environment away from a door or window and always use a fresh razor blade to excise the gel bands.
MS spectra contaminated with peaks at regular interval (*e.g.*, 44 Da repeats of polyethylene glycol)	Samples contaminated by polymer, NP-40, Triton X-100	• Be sure to use polypropylene microcentrifuge tubes and HPLC grade solvents. • Some manufacturers use mold release agents in manufacture of some plastics. Avoid using such plasticware.
The Coefficient of Variance (CV) for the protein quantitation within one experiment exceeds 30%	Improper MS analysis	• Ensure the MS instrument was properly tuned and calibrated prior to sample analysis. • Ensure the chromatographic separation was effective and the columns used for separation were free of contaminants from prior separations. Always run blanks between chromatographic separations to avoid any contaminations. Use clean MALDI plates for analysis.

Continued on next page

Problem	Cause	Solution
Observe 2 peaks for proline containing peptides	Conversion of arginine to proline	When proline containing peptides are used for quantitation, the correction factor in peak intensity for total heavy labeled peptides should be the sum of peak intensities of these mass channels (Liang *et al.*, 2006a).
Inconclusive identification and quantitation of phosphoproteins due to poor data quality	Loss of phosphorylation	• To avoid loss of phosphorylation, process the samples immediately after collection and perform all steps at 4°C. Do not store the samples for prolonged periods of time. • Use lysis buffer containing the tyrosine phosphatase inhibitor to prevent any loss of phosphorylation (page 43). • **Do not** concentrate the peptides to dryness after trypsin solution. If the peptides are concentrated to dryness, the peptides are difficult to resuspend resulting in loss of peptides.
	No phosphoprotein enrichment step performed	To enable proper data analysis and identification of phosphorylated proteins, always perform enrichment of phosphoproteins prior to analysis.
Lower incorporation efficiency	Incomplete incorporation of heavy amino acid	Perform labeling for at least 6 doublings to ensure complete incorporation of the label. Be sure to use log-phase cells with >90% viability. The SILAC/™ Kit is designed to provide >90% incorporation of labeled amino acids (page 2).
	Introduced exogenous sources of light amino acid that can reduce total labeling	Review the solutions below to prevent introduction of exogenous light amino acids at specific steps: • Dilute Matrigel™ with SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) as described on page 42. Do not use SILAC/™ KnockOut™ D-MEM supplemented with Lys, Arg, Gln. • Prepare Collagenase IV solution in SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) as described on page 19. • Wash the MEF cells thoroughly with PBS to remove any traces of FBS that may contain amino acids. Perform a final wash with SILAC/™ KnockOut™ D-MEM (without Lys, Arg, Gln) prior to conditioning the medium as described on page 41. • Ensure the growth factors used for ES cell culture do not contain any exogenous amino acids.

Appendix

Preparing Mouse Embryonic Fibroblasts (MEF)

Introduction

The SILAC™ Stem Cells kit is designed for culturing human ES cells maintained in a defined media that is conditioned using MEF cells.

Instructions to prepare MEF from mouse embryos and prepare MEF-conditioned SILAC/™ media are described in this section.

Important

The MEF preparation protocol described in this section is suitable to obtain MEF cells for conditioning SILAC/™ media. General guidelines and a brief protocol for preparing MEF is described below.

If you have access to a stem cell core facility such as the University of California, San Francisco (UCSF) Stem Cell Facility, follow the recommended protocols described by the core facility. For details, visit UCSF web site at http://www.escells.ucsf.edu/researchers/protocols.asp).

For more technical resources on ES cells, visit www.invitrogen.com\stemcell.

If you have an optimized MEF preparation protocol, use the optimized protocol.

Note

Follow the general recommendations and guidelines of the animal care committee for your local institution during animal dissection and disposal. Use approved methods for animal handling and dissection. Contact your local animal care committee for more information.

Experimental Outline

1. Dissect a 13-day pregnant mouse and remove the embryo.
2. Isolate MEF cells from the embryo using trypsin.
3. Triturate the MEF cells and culture the MEF cells in MEF medium.
4. Subculture the cells and freeze some MEF cells for future use.
5. Prepare the MEF-conditioned SILAC/™ light and heavy labeled media.

Materials Needed

- MEF medium (D-MEM, FBS, and L-glutamine are available from Invitrogen, page vii, medium recipe is on the next page)
- Light and heavy labeled SILAC/™ media (page 13)
- bFGF (page vii)
- Trypsin (page vii)
- Appropriate tissue culture plastic ware and culture dishes
- 37°C incubator with a humidified atmosphere of 5% CO_2 in air
- Sterile centrifuge tubes
- Reagents to determine viable and total cell counts (page vii)
- 13-day pregnant mouse
- 1X PBS (page vii)

Continued on next page

Preparing Mouse Embryonic Fibroblasts (MEF), Continued

Preparing MEF Medium

Prepare the MEF medium containing 10% FBS and supplemented with L-Glutamine using basal D-MEM as described below. Perform all steps in a tissue culture hood under sterile conditions.

1. Remove 100 ml D-MEM from 1 L D-MEM bottle and replace with 100 ml FBS.
2. To each 1 L medium bottle, add 10 ml 100X (200 mM) L-Glutamine.
3. *Optional:* Add 10 ml 100X Penicillin-Streptomycin (page vii), if needed (highly recommended).
4. Filter sterilize the medium using 0.22 μm filtration device.

Store the medium at 2 to 8°C, protected from light until use. The medium is stable for 6 months when properly stored (avoid introducing any contamination into the medium).

Harvesting MEF

Perform all steps in a tissue culture hood under sterile conditions.

1. Anesthetize a 13-day pregnant mouse using isoflurane or halothane and perform a cervical dislocation.
2. Remove the uterine horns from mouse, place in a 100 mm culture dish, and wash three times with sterile PBS.
3. Cut open embryonic sac with sterile scissors and transfer the embryos to the culture dish.
4. Remove the placenta from the embryos and transfer the embryos to a clean 35 mm culture dish.
5. Finely mince the embryo gently with scissors, add 2 ml trypsin/EDTA solution. Incubate for 5-10 minutes at 37ºC.
6. Add 5 ml MEF medium to inactivate trypsin and transfer the contents to a sterile 15 ml centrifuge tube.
7. Dissociate MEF cells by trituration. Allow large particle to settle to bottom of the tube and transfer supernatant to a T-75 culture flask containing 10-15 ml MEF medium.
8. Incubate the flask in a 37ºC incubator containing a humidified atmosphere of 5% CO_2 in air.
9. When cells are 80-85% confluent (~ 2days), passage the cells at a ratio of 1:2 every 3 days.
10. Once you have established the MEF, freeze a small aliquot of cells. Expand the MEF cells for a few passages but not beyond 5 passages.
11. Use MEF to prepare the MEF-conditioned SILAC™ medium, next page.

Continued on next page

Preparing Mouse Embryonic Fibroblasts (MEF), Continued

Preparing MEF-Conditioned Regular and SILAC/™ ES Cell Media

Once the MEF cells reach 80-85% confluency, use the cells as feeders to prepare MEF-conditioned media. Perform all steps in a tissue culture hood under sterile conditions.

1. Remove the MEF medium and wash cells with PBS. Perform a final wash with SILAC™ KnockOut™ D-MEM (without any supplements or amino acids).
2. Add 2 ml trypsin solution to the flask and incubate for 5 minutes.
3. Add 2 ml MEF medium and transfer the cells to a sterile 15 ml centrifuge tube and mix well.
4. Perform a cell count using Trypan Blue.
5. Irradiate MEF cells with 40 Gy or treat MEF cells with Mitomycin C (10 µg/ml for ~2 hours) to arrest the growth without killing the cells.
6. Centrifuge the cells at 800 x g for 5 minutes and resuspend the cells in MEF medium. Plate the cells in two T-25 flasks or 6-well plates at a cell density of 55,000 cells/cm^2.
7. The next morning, wash two flasks of MEF cells with PBS three times. Add 1-3 ml/well for a 6-well plate or 5 ml per T25 flask of the following medium
   - Light or heavy SILAC/™ ES cell media containing 4 ng/ml human bFGF or 1000 units/ml LIF (prepared as described on page 13) to the MEF cells
   - Regular ES Cell Medium containing 4 ng/ml human bFGF or 1000 units/ml LIF (prepared as described on page 14) to the MEF cells
8. Incubate the MEF cells in Regular or SILAC/™ light or heavy ES cell media in a 37ºC incubator containing a humidified atmosphere of 5% CO_2 in air overnight.
9. After overnight incubation, collect the respective MEF-conditioned ES cell media from feeder flask or plate. Supplement the conditioned medium with additional 4 ng/ml of human bFGF or 1000 units/ml LIF before using the conditioned medium to expand human ES cells.

Use the irradiated MEF for up to 6-7 days and collect the conditioned medium once every day.

Preparing Matrigel™ Coated Plates

Introduction

The ES cells are usually grown on Matrigel™ or gelatin coated plates. Matrigel™ is a basement membrane matrix that provides support for growth. Instructions to prepare Matrigel™ coated plates for growth of ES cells are described in this section.

If you have established protocols for growing ES cells using gelatin coated plates, use the established protocols.

- Maintain the Matrigel™ at 4ºC to prevent the gel from solidifying during pipetting
- Keep the tissue culture plates, pipettes, and medium cold

Materials Needed

- Matrigel™ Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free (Becton Dickinson catalog no. 356231)
- 6-well tissue culture plate and sterile pipettes
- SILAC/™ KnockOut™ D-MEM (without Arg, Lys, Gln, phenol red) supplied with the kit, chilled at 4ºC

Protocol

1. Thaw aliquots of Matrigel™ at 4ºC until the gel liquifies.
2. Dilute Matrigel™ 30-fold with chilled SILAC/™ KnockOut™ D-MEM (without Arg, Lys, Gln, phenol red).
3. Add 1 ml diluted Matrigel™ solution to coat a 6-well plate. Incubate for 1-2 hours at room temperature. Remove any non-polymerized Matrigel™ solution from the plate before use.
4. Use the Matrigel™ coated plates immediately for culture of ES cells or wrap the plates with a plastic wrap and store the plates at 4ºC. The Matrigel™ coated plates are stable for 1 week at 4ºC.

Buffer Recipes

Introduction

Recipes for recommended buffers for lysis of cells for phosphoprotein analysis or to solubilize membrane proteins are described below.

If you have an optimized buffer recipe for your application that produces efficient lysis, use the optimized buffer recipe.

Membrane Lysis Buffer

The Membrane Lysis Buffer is a hypotonic lysis buffer and is used with 1.25 M sucrose solution for cell lysis (Liang *et al.*, 2006b). This buffer is compatible with downstream applications such as immunoprecipitation and SDS-PAGE.

10 mM Tris-HCl, pH 8.0
1 mM Magnesium chloride
Protease inhibitors (0.5 mM AEBSF, 0.15 µM aprotonin, and 1 µM leupeptin)
Store buffer at 4ºC.

Before using for lysis, add 40 µl Benzonase® Nuclease to 50 ml Membrane Lysis Buffer and store on ice until use.

1. Resuspend the ES cell pellet in ~1.6 ml Membrane Lysis Buffer. Mix well by pipetting up and down.
2. Incubate on ice for 30 minutes.
3. Homogenize the lysate on ice using a Dounce homogenizer or equivalent for 30 strokes.
4. Add 0.4 ml 1.25 M sucrose solution to the lysate and mix well by pipetting up and down 5 times.
5. Centrifuge the lysate at 500 x g for 10 minutes at 4°C to remove nuclear fraction. Remove the supernatant and discard the nuclear pellet.
6. Centrifuge the supernatant at 100,000 x g for 1 hour at 4°C to obtain the membrane pellet. Carefully remove the supernatant and save the supernatant, if you are interested in analysis of cytosolic proteins.

Continued on next page

Phosphoprotein Lysis Buffer

The Phoshoprotein Lysis buffer contains strong detergents such as SDS for cell lysis and is mainly used for analysis of cytosolic and membrane-associated proteins (Liang *et al.*, 2006a). This buffer is compatible with downstream applications such as SDS-PAGE and immunoprecipitation. **Do not** use this buffer if you wish to precipitate protein complexes as the buffer includes SDS.

50 mM Tris-HCl, pH 8.0
1% Triton X-100
0.1% Sodium dodecyl sulfate (SDS)
0.5% Sodium deoxycholate
0.5 M NaCl
1 mM Sodium vanadate
10 mM Sodium fluoride
Protease inhibitors (0.5 mM AEBSF, 0.15 µM aprotonin, and 1 µM leupeptin)
Store buffer at 4ºC.

1. Resuspend the ES cell pellet in 8-10 ml Phosphoprotein Lysis Buffer.
2. Mix well by pipetting up and down.
3. Centrifuge at 100,000 x g for 20 minutes at 4°C.
4. Collect the supernatant (lysate) which contains the cytosolic and membrane-associated proteins. Save the pellet at -80°C, if you are interested in analysis of membrane proteins.

General Methods for Protein Analysis

Introduction

General methods for protein analysis after labeling your ES cells with SILAC/™ heavy and light media are described in this section. If you have established protein analysis methods for ES cells in your laboratory, use the established methods.

Experimental Outline

1. Perform immunoprecipitation, if needed.
2. Process the lysate or immunoprecipitated proteins using SDS-PAGE.
3. Stain the SDS-PAGE gel using Coomassie® or silver staining.
4. Excise the bands of interest from the gel or cut the gel into 40 equal pieces.
5. Perform in-gel trypsin digestion.

Note

To obtain the best results, we recommend using NuPAGE® Novex® Bis-Tris Gels. You may use Novex® 4-20% Tris-Glycine Gel or any other SDS/PAGE gel of choice for performing SDS/PAGE. Use an appropriate percentage of acrylamide gel that best resolves your proteins of interest.

Important

Due to the large variety of antibodies that can be used for immunoprecipitation, it is not possible to have a single immunoprecipitation protocol that is suitable for all antibodies. Use the immunoprecipitation procedure from this section as a starting protocol and based on your initial results, optimize the protocol by changing the antibody concentration, buffer formulation, and incubation time.

If you have an optimized immunoprecipitation protocol for a specific antibody, use the optimized protocol.

Materials Needed

You need the following items. Ordering information is on page vii.

- NuPAGE® Novex® Bis-Tris Gel
- NuPAGE® MES/MOPS SDS Running Buffer
- NuPAGE® Sample Reducing Agent (10X)
- NuPAGE® LDS Sample Buffer (4X) and NuPAGE® Antioxidant
- XCell *SureLock*™ Mini-Cell for electrophoresis of the gel
- Sterile tubes
- Antibody, Protein A or Protein G Agarose for immunoprecipitation, if needed
- Sequencing grade trypsin (10 ng/µl dissolved in 25 mM ammonium bicarbonate, pH 8.0, store on ice until use)
- 25 mM ammonium bicarbonate buffer, pH 8.0 for trypsin digestion
- 5% formic acid (FA)
- 100% and 70% (v/v) acetonitrile

Continued on next page

Immuno-precipitation

Immunoprecipitation protocol using Protein G Agarose is described below. You may use Protein A beads, if desired.

1. To the cell lysate prepared as described on pages 24-26, add 15 µl Protein G Agarose slurry (50% slurry in lysis buffer) per 1 ml lysate to pre-clear the lysate.
2. Rock the lysate at 4°C for 1 hour.
3. Centrifuge at 10,000 x g for 1 minute at 4°C.
4. Transfer the supernatant to a sterile tube and place on ice.
5. Add 50-100 µg of the antibody against the protein of interest.

   Note: You may optimize the amount of antibody used based on the initial results.
6. If the antibody is already coupled to Protein A or Protein G agarose, proceed to Step 8 directly.
7. Add 100 µl of the Protein G Agarose slurry to the supernatant.
8. Rock for 8-16 hours at 4°C.
9. Centrifuge at 10,000 x g for 5 minutes at 4°C. Remove the supernatant.
10. Wash the agarose pellet twice with a suitable buffer to remove any nonspecific proteins.
11. Resuspend the pellet in 50 µl 1X NuPAGE® LDS Sample Buffer. Add 5 µl NuPAGE® Sample Reducing Agent (10X).
12. Heat the sample at 70°C for 8-10 minutes.
13. Centrifuge the sample for 1 minute at 10,000 x g and load supernatant onto a NuPAGE® Novex® Bis-Tris Gel and analyze the protein immune complexes using SDS-PAGE, next page.

Continued on next page

Analyzing Protein Complexes

Instructions for analyzing protein complexes in solution using protein specific antibodies and Protein G Agarose are described below. You may use Protein A beads, if desired.

1. To the lysate prepared as described on pages 24-26, add 30-50 µg of the bait protein that allows binding to the protein complex.

   Note: You may optimize the amount of protein used based on the initial results.
2. Add 20-50 µl epitope-tagged resin such as GST agarose or Streptavidin agarose to precipitate the protein complex, if your protein of interest contains an expressed GST tag or a biotin tag.
3. Rock for 2-24 hours at 4°C.
4. Centrifuge at 10,000 x g for 5 minutes at 4°C. Remove supernatant.
5. Wash the pellet twice with a suitable buffer to remove nonspecific proteins.
6. Resuspend the pellet in 16-20 µl 1X NuPAGE® LDS Sample Buffer and add 2 µl of NuPAGE® Sample Reducing Agent (10X).
7. Heat the sample at 70°C for 8-10 minutes.
8. Centrifuge the sample for 1 minute at 10,000 x g and load supernatant onto a NuPAGE® Novex® Bis-Tris Gel and analyze the protein immune complexes using SDS-PAGE, below.

SDS-PAGE Analysis

The following procedure uses NuPAGE® Novex® Bis-Tris Gels with the XCell *SureLock*™ Mini-Cell. If you are using any other electrophoresis system, refer to the manufacturer's recommendations.

1. Assemble the gel cassette/Buffer Core sandwich as described in the XCell *SureLock*™ Mini-Cell manual (download the manual from www.invitrogen.com). If you are using only one gel, use the Buffer Dam to replace the second gel cassette.
2. Fill the Lower Buffer Chamber and Upper Buffer Chamber with the recommended volume of 1X NuPAGE® MES or MOPS SDS Running Buffer. Add 0.5 ml of NuPAGE® Antioxidant to the Upper Buffer Chamber.
3. Load the processed samples and load protein molecular weight standards in a different well.
4. Place the XCell *SureLock*™ Mini-Cell lid on the Buffer Core. With the power on the power supply turned off, connect the electrode cords to the power supply.
5. Perform SDS-PAGE at 200 V for 40-50 minutes for NuPAGE® Novex® Bis-Tris Gel.
6. At the end of electrophoresis, turn off the power and disassemble the gel cassette/Buffer Core sandwich assembly as described in the XCell *SureLock*™ Mini-Cell manual.
7. Proceed to gel staining, next page.

Continued on next page

Staining the Gel

After SDS-PAGE, stain the gel with a protein stain to visualize the protein bands. Use a Coomassie® stain such as SimplyBlue™ SafeStain for staining or silver stain such as SilverQuest™ Silver Staining Kit for staining low abundant proteins

SimplyBlue™ SafeStain is a ready-to-use, proprietary Coomassie® G-250 stain that is specially formulated for fast, sensitive detection and safe, non-hazardous disposal. Proteins stained using the SimplyBlue™ SafeStain are compatible with mass spectrometry analysis. Refer to the manual supplied with stain for protocol details. See page vii for ordering information.

SilverQuest™ Silver Staining Kit provides a rapid and easy method to silver stain proteins in polyacrylamide gels. This kit is specifically designed to provide sensitive silver staining compatible with mass spectrometry analysis. The SilverQuest™ Silver Staining Kit includes destaining solutions that effectively remove silver ions from protein bands in polyacrylamide gels. This improves trypsin digestion and subsequent mass spectrometry coverage of the protein, as silver ions are known to inhibit trypsin digestion of proteins (Chambers *et al.*, 1974). Refer to the manual supplied with stain for protocol details. See page vii for ordering information.

Note: If you are destaining the gel using the destaining solutions included in the SilverQuest™ Kit, wash the gel piece thoroughly with ultrapure water until the gel piece is completely destained, no yellow color is visible before trypsin digestion.

Note

After staining the gel, you may transfer the stained gel to the core facility to perform in-gel trypsin digestion and MS analysis as described in this manual.

If you wish to stain the gel and perform in-gel trypsin digestion, follow the protocol described on the next page.

For more information on proteomics core facilities that offer MS analysis for SILAC, see page 9.

Follow these guidelines for trypsin digestion to obtain the best results:

- Always use sequencing/proteomics grade trypsin for MS analysis (page vii)
- Always prepare the trypsin digestion buffer (25 mM ammonium bicarbonate buffer, pH 8.0) using ultra pure reagents and water
- Avoid touching the gel with bare hands to prevent contamination from keratin
- Be sure to use polypropylene microcentrifuge tubes and HPLC grade solvents to avoid any contamination from polymers

Continued on next page

In-gel Trypsin Digestion

A general protocol for in-gel trypsin digestion is provided below. You may use any method of choice or a method recommended by your proteomics core facility. For more information, refer to published reference sources (Coligan *et al.*, 1998; Helmann *et al.*, 1995).

Note: The digestion protocol given below is generally used for protein identification. If you need more sequence coverage, you may need to perform reduction and alkylation of peptides (Shevchenko *et al.*, 1996).

To avoid keratin contamination, perform the in-gel digestion in a laminar flow hood.

1. Rinse the stained gel in water for 10 minutes to remove any particulate material.
2. Excise the desired gel band from the stained gel. Mince the excised gel piece into smaller pieces (1 mm x 1 mm). Transfer the gel pieces to a clean microcentrifuge tube.
3. Add 500 µl 50% acetonitrile/25 mM ammonium bicarbonate, pH 8.0. Incubate at room temperature for 15-30 minutes for destaining the gel pieces. Vortex for 10 seconds and briefly centrifuge at maximum speed for 10 seconds. Discard the supernatant carefully without removing gel pieces.
4. Repeat Step 3 until the gel pieces are sufficiently destained.
5. Add 200 µl 100% acetonitrile to dehydrate the gel pieces.
6. Incubate for 5–10 minutes at room temperature. Vortex for 10 seconds and briefly centrifuge at maximum speed for 10 seconds. Discard the supernatant carefully without removing any gel pieces.
7. Dry the gel pieces in a centrifugal vacuum concentrator (*e.g.*, Thermo Savant SpeedVac® centrifuge).
8. Add enough cold trypsin solution (10 ng/µl in 25 mM ammonium bicarbonate, pH 8.0) to cover the gel pieces.
9. Incubate on ice for at least one hour to allow the trypsin solution to penetrate the gel pieces. The cold temperature helps to prevent autolysis of the trypsin.
10. Incubate overnight at 37°C.
11. Add 50 µl 5% formic acid (FA), and incubate for 30 minutes at room temperature.
12. Vortex for 30 seconds, centrifuge at 14,000 x g for 1 minute, and collect the supernatant.
13. Add 50 µl 5% FA, 50% acetonitrile, and incubate for 30 minutes at room temperature.
14. Vortex for 30 seconds, centrifuge at 14,000 x g for 1 minute, and collect the supernatant, pooling it with the supernatant from Step 12.
15. Concentrate the supernatant using a centrifugal vacuum concentrator to ~5 µl. **Do not allow the samples to dry out.**
16. Submit your tryptic peptides to the proteomics core facility for analysis.

Technical Support

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Corporate Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_service@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail: jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
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Introduction	The components of the SILAC/™ Stem Cells Protein ID and Quantitation Media Kit is qualified as described below. For details on the qualification, see the Certificate of Analysis shipped with the product or available at www.invitrogen.com.
SILAC/™ KnockOut™ D-MEM Media	The SILAC/™ media is tested for pH, osmolality, endotoxin, and is tested for the absence of bacterial or fungal contaminants. Product must meet the set specifications.
KnockOut™ Serum Replacement	Product performance is confirmed by evaluation of its ability to support the growth of undifferentiated D3 ES cell colonies on inactivated mouse embryonic fibroblasts. The product is also tested for pH, osmolality, endotoxin, and is tested for the absence of bacterial or fungal contaminants. Product must meet the set specifications.
GlutaMAX™-I Supplement	The GlutaMAX™-I Supplement is qualified by performing tests for pH, osmolality and is tested for the absence of bacterial and fungal contaminants. Product must meet the set specifications.
Amino Acids Solution	The MEM Non-Essential Amino Acids Solution is qualified by performing tests for pH and is tested for the absence of bacterial and fungal contaminants. Product must meet the set specifications.
2-mercaptoethanol	The 2-mercaptoethanol is qualified by performance testing, performing tests for pH, osmolality, and is tested for the absence of bacterial and fungal contaminants. Product must meet the set specifications.
SILAC/™ Amino Acids	The SILAC/™ Amino Acids (L-Lysine, isotope labeled L-Lysine, and L-Arginine) must meet the following specifications: L isomer: >99% ^{13}C atom: 97% Endotoxins: Low Sterility: Sterile with no bacterial or fungal contaminants In addition, the degree of incorporation achieved with each lot of amino acids is confirmed by performing the labeling experiment as described in this manual. After labeling the cells are lysed and the lysate is analyzed by SDS-PAGE. At least 3 intense protein bands are subjected to in-gel trypsin digestion followed by MALDI-TOF MS analysis. The observed peak pairs must be separated by the correct mass difference that is proportional to the theoretical isotopic shift, the intensity of the peak pairs is 1:1, and the incorporation of the heavy label is >90%.

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