Early Winter 2008, vol. 3.3

NEB expressions

a scientific update from New England Biolabs

Welcome to the early winter edition of NEB expressions in which we introduce an innovative cellular imaging and analysis technology. SNAP- and CLIP-tag technologies are rapid and versatile methods for studying the function and localization of proteins in living and fixed cells. These novel reagents enable researchers to perform multiple experiments with just one genetic construct. An introduction to this technology written by one of its inventors, Dr. Kai Johnsson, is featured to the right.

As always, we invite your feedback on our products, services and corporate philosophy.

inside:

NEW ENGLAND BioLabs® Inc.
the leader in enzyme technology

SNAP-tag Technologies: Novel Tools to Study Protein Function

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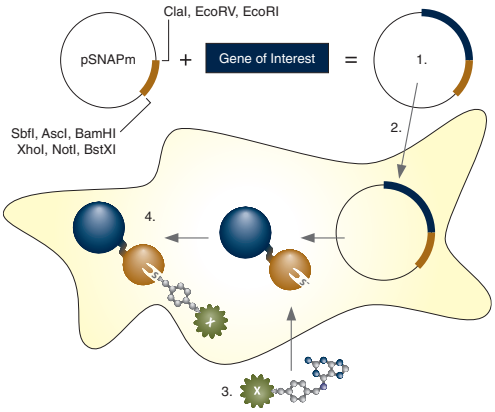
Ecole Polytechnique Fédérale de Lausanne

Scientists investigating the function of proteins in cells wrestle with rather basic questions: Where? When? With whom? Answering these questions for any given protein can be a challenging enterprise and, as most scientists are aware, there is often a painful discrepancy between the magnitude of the challenge and the tools available to address it. New England Biolabs now introduces a set of tools for the specific labeling of fusion proteins with synthetic probes that will help scientists illuminate various aspects of protein function.

Covalent Labeling of Fusion Proteins Using SNAP-, CLIP-, ACP- and MCP-tags

The expression of a protein of interest as a fusion protein with an additional polypeptide (tag) that aids in the characterization of the protein was first exploited by the group of Jon Beckwith in 1980 (1): β-galactosidase was fused to the cytoplasmic membrane protein MalF to facilitate its purification. Since then, an ever increasing number of tags have become popular in various branches of biology.



Imaging with SNAP-tag Technology: *1) Clone gene of interest into NEB expression vector. 2) Transfect plasmid fusion into cells, protein is expressed in cells. 3) Add label of interest. 4) Covalent modification occurs, labeling protein for visualization.*

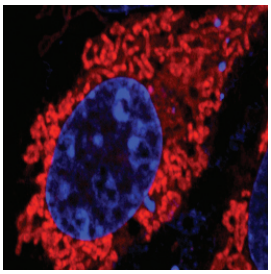
The practice of cell biology without the use of fluorescent proteins is unimaginable today, and the purification of recombinant proteins using affinity tags is standard practice.

(continued on page 2)

New SNAP-tag Starter Kits

cellular imaging & analysis products from NEB

NEB introduces you to this novel protein labeling system by offering several Starter Kits that include a plasmid encoding the tag, a control plasmid, two fluorophores and blocking agent. Starter Kits offer everything you need for complete experiments with endless downstream possibilities.



Live COS-7 expressing mitochondrial cytochrome oxidase 8A-SNAP (Cox8A-SNAP) were labeled with SNAP-Cell TMR-Star (red). Nuclei were counterstained with Hoechst 33342 (blue).

Introductory Offer

Try the **SNAP-Cell** or **SNAP-Surface Starter Kits**.
See your local distributor for details.

Advantages

- **Flexibility** – clone and express your protein once, then label your protein with a variety of substrates
- **Speed** – easy-to-use protocols enable rapid labeling with a single reagent
- **Precision** – label is covalently bound under biological conditions in a defined position with defined stoichiometry
- **Selection** – choose from the widest selection of commercial substrates, optimized for a range of imaging instrumentation

See page 4 for ordering information.

SNAP-tag Technologies...

(continued from page 1)

However, despite the ubiquitous role of fusion proteins in protein science, traditional tags face two major limitations: i. They are limited to properties that can be genetically encoded. For example, the spectroscopic properties of currently available fluorescent proteins are often inferior to synthetic fluorophores, but such fluorophores cannot be genetically encoded. ii. Traditional tags are tailor-made for a specific application and a single tag is often not suited to the study of different facets of protein function: expression, transport, interaction and degradation.

An approach that addresses these shortcomings relies on tags that can be specifically labeled with synthetic probes such as fluorophores or affinity labels. NEB now introduces a suite of novel tagging technologies for specifically labeling fusion proteins in cell biology and protein science applications.

The most versatile of these tags is the SNAP-tag, a 20 kDa mutant of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase that reacts specifically and rapidly with benzylguanine (BG) derivatives (Figure 1), leading to irreversible covalent labeling of the SNAP-tag with a synthetic probe (2). SNAP-tag has a number of features that make it ideal for a variety of applications in protein labeling. The rate of the reaction of SNAP-tag with BG derivatives is to a large extent independent of the nature of the synthetic probe attached to BG, permitting the labeling of SNAP fusion proteins with a wide variety of synthetic probes. Secondly, SNAP-tag has no restrictions with respect to cellular localization and expression host. Thirdly, SNAP-tag substrates are chemically inert towards other proteins, avoiding nonspecific labeling in cellular applications. Finally, many SNAP-tag substrates are cell permeable, permitting labeling of intracellular proteins in live cells.

The second tag introduced by NEB is a sibling of the SNAP-tag, the CLIP-tag (3). The CLIP-tag was created by engineering the substrate specificity of the SNAP-tag, permitting it to react specifically with O^2-benzylcytosine (BC) derivatives (Figure 1). Since the SNAP- and CLIP-tags specifically react with orthogonal substrates, SNAP and CLIP fusion proteins can be labeled simultaneously and specifically with different synthetic probes in living cells. The main application of the CLIP-tag is dual-labeling of fusion proteins in conjunction with the SNAP-tag.

A third method offered by NEB is conceptually different from the labeling of SNAP and CLIP fusion proteins as it is based on an enzyme-catalyzed post-translational modification. The protein of interest is fused to an acyl carrier protein (ACP) and the corresponding fusion protein is specifically labeled with CoA derivatives through a post-translational modification catalyzed by the phosphopantetheinyl transferase AcpS. (4) An interesting feature of the ACP-tag is its small size of 9 kDa. (5) In addition, a mutant of ACP, called MCP, is labeled by the phosphopantetheinyl transferase Sfp but not by AcpS, thereby permitting the selective labeling of ACP and MCP fusion proteins with different probes in one sample. In contrast to several of the substrates of the SNAP- and CLIP-tag, substrates of the ACP-tag are not cell permeable; therefore this approach is best suited for the labeling of cell surface proteins. In summary, NEB is offering four fully orthogonal labeling technologies (SNAP-tag, CLIP-tag, MCP-tag and ACP-tag) which can be used to selectively label corresponding fusion proteins with synthetic probes in both cell imaging and *in vitro* applications.

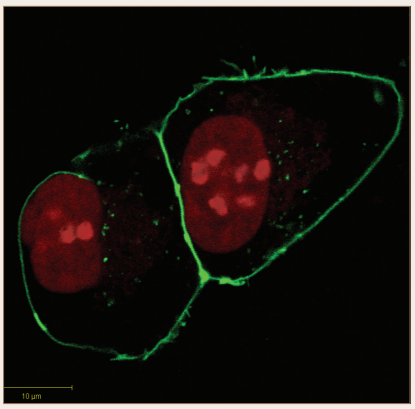


Figure 2: Simultaneous Labeling: *Live HEK293 expressing SNAP-Adrenergic Receptor b2 and Histone 2B-SNAP were labeled with SNAP-Surface Alexa Fluor® 488 (green) followed by SNAP-Cell TMR Star (red).*

Applications: Location, Timing and Interaction?

Location?

The proper localization and translocation of proteins is an integral aspect of cellular function. The labeling of SNAP and CLIP fusion proteins with synthetic fluorophores in living cells permits the determination of their cellular localization through fluorescence microscopy (Figure 2). As both the SNAP- and CLIP-tags can be labeled with different fluorophores, the simultaneous localization of more than one protein (via SNAP and CLIP fusions or in combination with fluorescent proteins) is possible. The labeling of SNAP and CLIP fusion proteins can be carried out either on fixed cells, as the tags retain their activity during fixation, or more commonly in living cells. Live cell imaging permits the dynamic, real-time study of protein translocation. A representative example is the monitoring of the nuclear redistribution of a

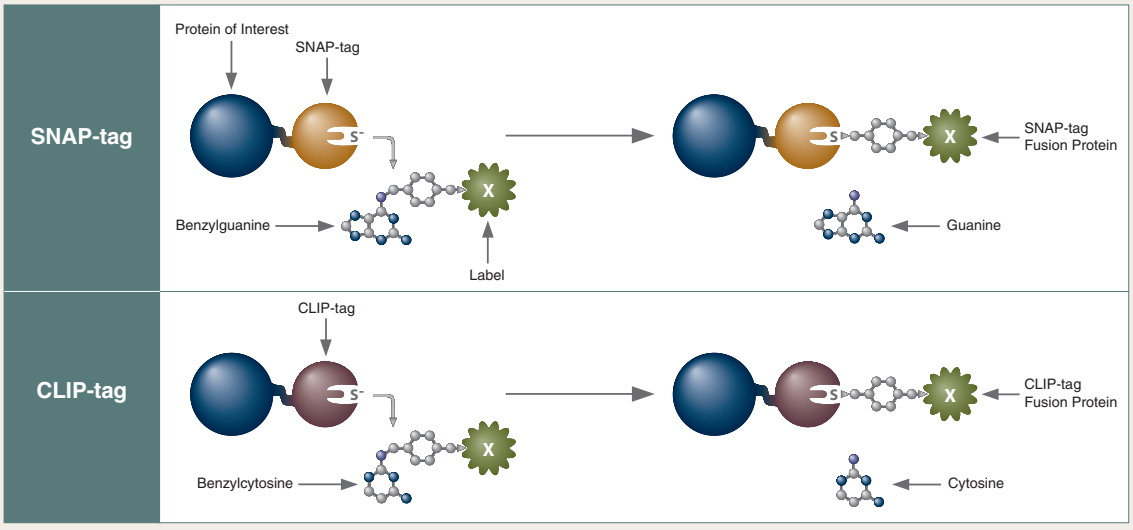


Figure 1: SNAP- or CLIP-tag Mechanism: *SNAP-tag or CLIP-tag fused to the protein of interest labels itself with "X" releasing guanine or cytosine.*

SNAP fusion of the human estrogen receptor α (ER) upon incubation of cells with the partial ER antagonist 4-hydroxytamoxifen (6,7). Another very attractive feature of the labeling of fusion proteins is that the labeling itself can be restricted to certain locations of a cell. For example, use of cell-impermeable substrates of SNAP-tag, or the cell surface-specific ACP/MCP system, enable the fraction of a plasma membrane protein present on the surface of the cell to be visualized through specific labeling(8). The approach thus permits the discrimination of different populations of a cell surface protein: those properly translocated to the plasma membrane from those retained in the secretory pathway or already internalized, e.g., upon ligand binding. The possibility to restrict experiments to sub-populations of G protein-coupled receptors (GPCRs) that are properly folded and functionally presented cell surfaces has been exploited in a number of important studies (9,10). It should also be noted that such discrimination cannot be easily achieved when using fluorescent proteins.

Timing?

Since the timing of labeling of a given fusion protein is under experimental control, questions about protein trafficking, protein turnover, organelle dynamics and macromolecular assembly become open to investigation. The stability of the covalent labeling brings the possibility of using different dyes at different time points during the experiment to distinguish between older and newly synthesized proteins in living cells. Such multicolor pulse chase labeling experiments generate distinct populations of otherwise identical proteins with discriminating features determined only by the time point of the respective labeling of each population. This approach was exploited to investigate phospholamban synthesis and turnover over time: multicolor pulse chase labeling of this protein over a 24 hour period allowed the distinction of older and newer copies, uncovering a potential new role in myoblast differentiation (11). Jansen et al. labeled proteins translated within a defined window of the cell cycle to probe the role of centromeric protein A (CENP-A), a histone H3 variant, in centromere determination and cell division (12). Unexpectedly, these experiments revealed that CENP-A is inserted into centromeric histones uniquely in the G1 phase. Recently, McMurray and Thorner used multicolor pulse chase labeling to differentiate old and new molecules of two septins from budding yeast, Cdc10 and Cdc12, to probe their stability and recycling during dynamic structural transitions in cell division and development (13). The labeling experiments revealed that mechanisms governing septin incorporation are specific to each subunit and to the developmental state of the cell. It should also be noted that a sequential labeling with more than two fluorophores is possible; such labeling experiments are particularly attractive to study biological structure formation as was shown for cell wall synthesis in budding yeast (14). Finally, the non-overlapping substrate specificity of SNAP-tag and CLIP-tag permit simultaneous pulse-chase experiments to visualize different generations of two different proteins in one cell, further increasing the potential of the approach (3).

Interaction?

Protein-protein interactions are at the heart of most biological processes and the chemical labeling of fusion proteins also offers multiple opportunities to characterize such interactions. Proteins can be labeled with fluorophores ideally suited for fluorescence energy transfer experiments. This has been exploited to study the homo- and hetero-dimerization of GPCRs (9,10). In addition to providing the ability to select pairs of fluorophores that are better suited for FRET experiments than fluorescent proteins, chemical labeling brings the extra benefit that immature or improperly folded proteins retained in the secretory pathway do not contribute to the observed signal. Sophisticated FRET experiments can also be complemented with more traditional biochemical approaches: magnetic beads derivatized with the SNAP-tag substrate BG can be used for efficient pull-down experiments to identify and characterize interaction partners of the protein of interest. Finally, SNAP-tag fusions are also attractive for high-throughput assays of protein-protein interactions *in vitro*; either FRET-based, employing surface-plasmon resonance (15) or through the creation of protein microarrays (16,17). The possibility to address a single question through a variety of different experimental approaches makes the SNAP-tag particularly attractive for the analysis of protein-protein interactions.

And what's next?

The aforementioned examples from the literature demonstrate the potential of specific chemical labeling of fusion proteins, in particular the SNAP- and CLIP-tags, to address central questions in cell biology and protein science. Innovation in chemistry via the synthesis of new labeling substrates with advantageous properties will open up completely new ways to study protein function: the recently introduced optical switches for increased sensitivity in FRET experiments (18) or the use of environmentally sensitive dyes (19) are good examples. The simplicity of the synthesis of such substrates and the availability of the necessary building blocks from NEB permit even those scientists with little background in chemistry to assemble their own substrates. Whereas advances in the development of new intrinsically fluorescent proteins force their users to go through continuous cycles of subcloning and characterization, users of the SNAP-tag and its relatives can be assured to directly benefit from such future inventions.

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New Cellular Imaging and Analysis Technologies

starter kits for a simple introduction

New England Biolabs is pleased to introduce an innovative technology for studying the function and localization of proteins in living and fixed cells. Protein labeling using SNAP-tag offers simplicity and versatility to cellular imaging of mammalian proteins, as well as the ability to capture proteins *in vitro*. The creation of a single genetic construct generates a fusion protein which, when covalently attached to a variety of flourophores, biotin or beads, provides a powerful tool for studying the role of proteins in living and fixed cells. For added flexibility, NEB offers two systems in which the protein is labeled through the generation of a fusion protein (SNAP-tag and CLIP-tag), as well as two systems in which the protein is labeled enzymatically (ACP-tag and MCP-tag).

With cell imaging technologies from NEB, one construct is all you need! Make one genetic construct and use it for multiple experiments saving valuable research time:

Applications of SNAP- and CLIP-tag Technologies

- Simultaneous protein labeling inside live cells
- Protein localization and translocation
- Pulse chase in living cells
- Receptor internalization
- Selective cell-surface labeling
- Protein pull-down assays
- Protein detection in SDS-PAGE
- Flow-cytometry
- High throughput binding assays in microtiter plates
- Biosensor interaction experiments

To help you get started, NEB offers several Starter Kits that include a plasmid encoding a tag, a control plasmid, two fluorophores and blocking agent (in most cases). Choose a Starter Kit to gain quick access to the technology and build experience with the techniques. Starter Kits are available for each labeling approach.

SNAP-Cell Starter Kit
#E9100
Label inside living cells, on cell surfaces or in vitro

SNAP-Surface Starter Kit
#E9120
Label on the surface of living cells or in vitro

CLIP-Cell Starter Kit
#E9200S
Label inside living cells, on cell surfaces or in solution

CLIP-Surface Starter Kit
#E9230S
Label on the surface of living cells or in vitro

ACP-Surface Starter Kit
#E9300S
Enzymatically label on cell surfaces or in vitro

Starter Kits

SNAP-, CLIP- and ACP-tags are easy to use and will provide extraordinary results

It's as Easy as Following These Steps:

1. Select the appropriate expression plasmid
2. Introduce the gene for the protein of interest into the plasmid
3. Transfect into the desired cell type
4. Add the appropriate substrate
5. Ask questions not possible with GFP or antibodies
6. Analyze the results

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Companion Products:

Transfection Reagents:

#M2557S	TransPass™ COS/293
#M2553S/L	TransPass™ D1
#M2554S/L	TransPass™ D2
#M2556S	TransPass™ HeLa
#M2558S	TransPass™ HUVEC

Substrates:

#S9110S	SNAP-Biotin
#S9103S	SNAP-Cell 505
#S9105S	SNAP-Cell TMR-Star
#S9106S	SNAP-Cell Block
#S9124S	SNAP-Surface 488
#S9112S	SNAP-Surface 549
#S9137S	SNAP-Surface 647
#S9143S	SNAP-Surface Block
#S9147S	SNAP-Vista Green
#S9221S	CLIP-Biotin
#S9217S	CLIP-Cell 505
#S9219S	CLIP-Cell TMR-Star
#S9220S	CLIP-Cell Block
#S9232S	CLIP-Surface 488
#S9233S	CLIP-Surface 547
#S9234S	CLIP-Surface 647
#S9235S	CLIP-Vista Green
#S9144S	SNAP-Capture Pull-Down Resin
#S9145S	SNAP-Capture Magnetic Beads
#S9351S	CoA-Biotin
#S9348S	CoA 488
#S9349S	CoA 547
#S9350S	CoA 647

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NEB FAQ Spotlight

cellular imaging and analysis technologies

Q How does SNAP-tag labeling differ from using GFP fusion proteins?

A GFP and SNAP-tags are both valuable technologies used to visualize proteins in live cells. GFP is an inherently fluorescent protein derived from *Aequorea victoria* while SNAP-tag is derived from hAGT, a human DNA repair enzyme. In contrast to GFP fusion proteins, SNAP-tag fusions can be readily turned on at will with the addition of a variety of fluorescent probes added directly to the culture media. Substituting different fluorophores or other functionality (biotin, magnetic beads, blocking chemistries) requires no new cloning or expression, merely the incubation of the appropriate substrate with cells, cell lysates or recombinant proteins.

Q What is the difference between SNAP-tag and CLIP-tag?

A SNAP-tag and CLIP-tag are both derived from O^6-alkylguanine-DNA-alkyltransferase (hAGT), a human DNA repair enzyme. SNAP-tag recognizes O^6-labeled benzylguanine substrates while CLIP-tag recognizes O^2-labeled benzylcytosine substrates. Each tag transfers the label from the nucleoside substrate to itself, resulting in efficient covalent labeling of the tag. In creating the tags, hAGT has been engineered to no longer interact with DNA, but rather with derivatives of the free nucleosides. The tags exhibit no crossreactivity with one another, enabling researchers to label fusion proteins containing SNAP- and CLIP-tags with different fluorophores in live cells.

Q Can I clone my protein as a fusion to the N- or C-terminus of the tags?

A Yes. SNAP- and CLIP-tags can be fused to either the N- or C-terminus of a protein of interest as they are self-labeling and more commonly used to visualize intracellular proteins rather than surface proteins with a range of cell permeable dyes. However, to label surface proteins on the outside of cells using ACP- or MCP-tag the tag must be cloned so that it is oriented to the extracellular surface of the plasma membrane. In this orientation, the tag is accessible to its dye-conjugated substrate, and in the case of the ACP and MCP tags, recombinant ACP or SFP synthases.

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New High Fidelity (HF) Enzymes – Effectively Eliminate Star Activity

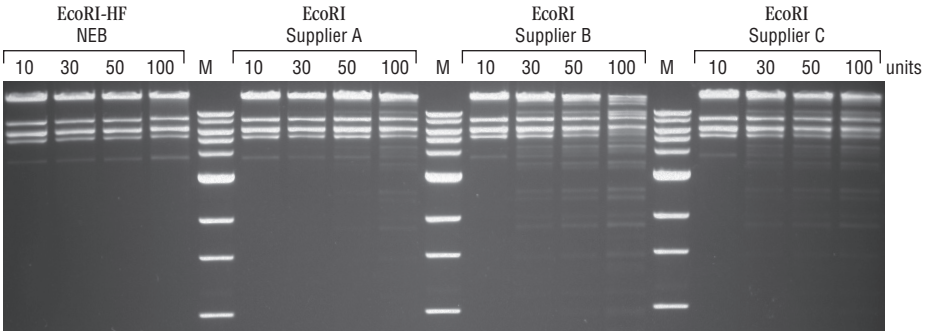
the latest innovation in restriction enzyme engineering

As part of our ongoing commitment to the study and improvement of restriction enzymes, NEB is pleased to introduce 10 new specificities to our line of High Fidelity (HF) restriction enzymes. These engineered enzymes have the same specificity as their established counterparts with the benefit of reduced star activity. Star activity, or relaxed specificity, is an intrinsic property of restriction enzymes. Most restriction enzymes will not exhibit star activity when used under recommended reaction conditions. However, for enzymes that have reported star activity, extra caution must be taken to set up reactions according to the recommended conditions to avoid unwanted cleavage.

Many techniques such as cloning, genotyping, mutational analysis, mapping, probe preparation, sequencing and methylation detection employ a wide range of reaction conditions and require the use of enzymes under suboptimal conditions. These new products with reduced star activity offer increased flexibility to reaction setup and help maximize results under a wide range of conditions.

In addition to reduced star activity, all of these engineered enzymes work optimally in NEBuffer 4, which has the highest level of enzyme compatibility, and will simplify double digest reactions. They are all Time-Saver™ Qualified, and digest substrate DNA in five minutes. In order to distinguish these engineered enzymes, the letters -HF™ have been added to the restriction enzyme name and they are packaged in unique purple-capped tubes.

Visit our website, www.neb.com to learn more about HF enzymes, the latest innovation in restriction enzyme technology from New England Biolabs.



EcoRI from various suppliers produces the correct banding pattern when 10 units are used, however, star activity is observed with larger amounts of enzyme. Star activity is not observed with EcoRI-HF, even at higher enzyme amounts. Reactions were set up according to recommended reaction conditions of each manufacturer. Reactions contained 1 µg Lambda DNA in a 50 µl reaction volume and were incubated overnight at 37°C. Marker M is the 1 kb DNA Ladder (NEB #N3232).

HF Enzymes FAQs

Q Why does the HF version of the enzyme have a different recommended buffer than the wild type enzyme?

A In many cases, changing the charged amino acids of a restriction endonuclease results in changes in buffer preference. These changes can be significant. For example, wild type SalI has a strict requirement for NEBuffer 3, a high ionic strength buffer. However, SalI-HF works well in NEBuffer 2 and NEBuffer 4, which are moderate ionic strength buffers.

Q Can the change in buffer be advantageous?

A Yes, as all HF enzymes work optimally in NEBuffer 4 and NEBuffer 4 shows the highest level of enzyme compatibility in the NEBuffer system, which is advantageous when designing double digests. (See page 8 for more information about our simplified buffer system.)

HF Enzymes and Their Wild Type Counterparts

PRODUCT NAME	PRODUCT NUMBER	BUFFER†	MAXIMUM UNITS WITH NO STAR ACTIVITY*	UNITS
BamHI-HF	#R3136	4	4,000	5,000
BamHI	#R0136	3 + BSA	32	10,000
EagI-HF	#R3505	4	500	500
EagI	#R0505	3	250	500
EcoRI-HF	#R3101	4	16,000	10,000
EcoRI	#R0101	U	250	10,000
EcoRV-HF	#R3195	4	64,000	4,000
EcoRV	#R0195	3 + BSA	1,000	4,000
MfeI-HF	#R3589	4	500	500
MfeI	#R0589	4	32	500

PRODUCT NAME	PRODUCT NUMBER	BUFFER†	MAXIMUM UNITS WITH NO STAR ACTIVITY*	UNITS
NcoI-HF	#R3193	4	16,000	1,000
NcoI	#R0193	3	120	1,000
NotI-HF	#R3189	4 + BSA	64,000	500
NotI	#R0189	3 + BSA	4,000	500
SacI-HF	#R3156	4 + BSA	4,000	2,000
SacI	#R0156	1 + BSA	120	2,000
SbfI-HF	#R3642	4	250	500
SbfI	#R0642	4	8	400 (500**)
SspI-HF	#R3132	4	500	1,000
SspI	#R0132	U	ND	1,000

†Wild type enzymes were tested in supplied buffer for comparisons.
*Wei, H. et al (2008) *Nucleic Acids Reseach* 36, e50.
**Changing to 500 units on January 1, 2009.

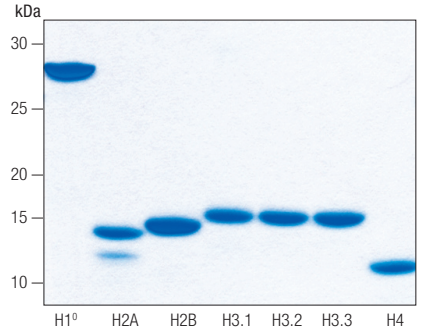
Recombinant Human Histones

enabling epigenetic research and drug discovery

New England Biolabs offers recombinant human histones as part of a growing line of modification enzymes and substrates to facilitate epigenetic research and drug discovery. Recombinant histones from NEB are highly purified and serve as ideal substrates for modification studies that can advance the understanding of gene expression.

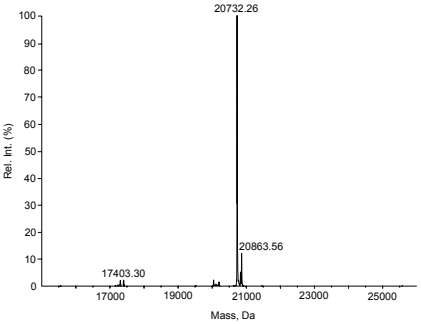
Advantages

- Free of post-translational modifications
- Ideal for high-throughput laboratories
- Highest purity

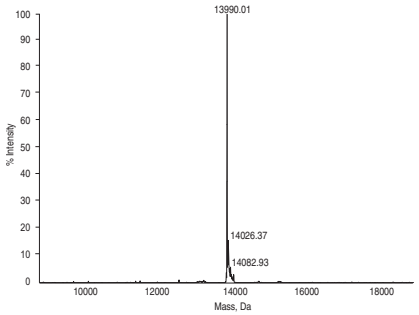


Experience the purity of Histones from NEB. *SDS-PAGE analysis of the seven histones available from NEB highlighting exceptional purity.*

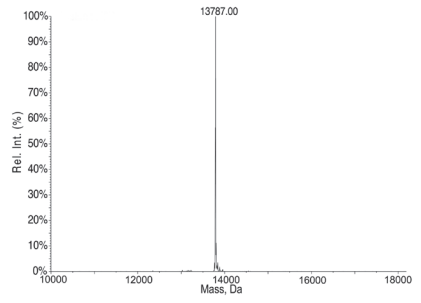
Histone H1⁰	
#M2501	100 µg
Histone H2A	
#M2502	100 µg
Histone H2B	
#M2505	100 µg
Histone H3.1	
#M2503	100 µg
Histone H3.2	
#M2506	100 µg
Histone H3.3	
#M2507	100 µg
Histone H4	
#M2504	100 µg



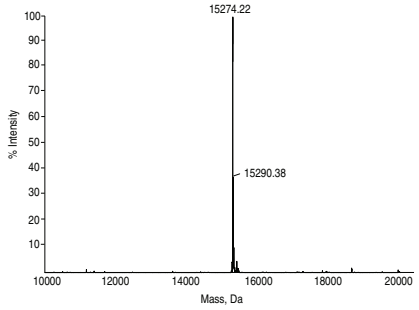
ESI-TOF Analysis of Histone H1⁰ Human, Recombinant.



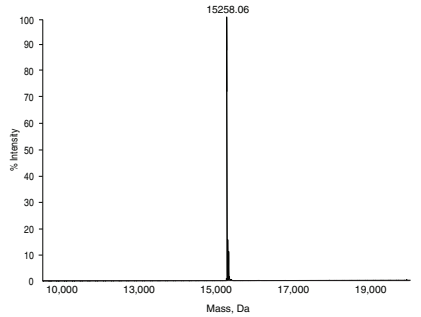
ESI-TOF Analysis of Histone H2A Human, Recombinant.



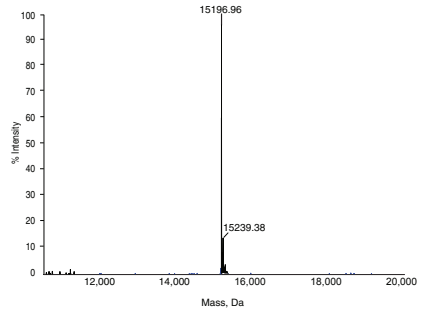
ESI-TOF Analysis of Histone H2B Human, Recombinant.



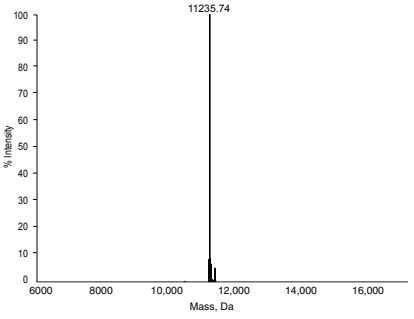
ESI-TOF Analysis of Histone H3.1 Human, Recombinant.



ESI-TOF Analysis of Histone H3.2 Human, Recombinant.



ESI-TOF Analysis of Histone H3.3 Human, Recombinant.



ESI-TOF Analysis of Histone H4 Human, Recombinant.

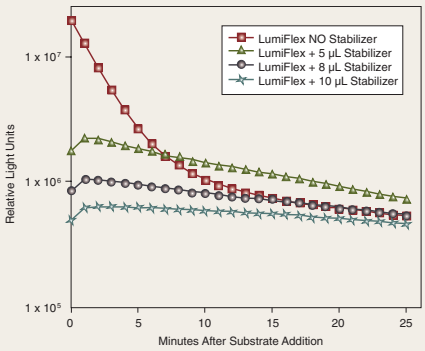
New LumiFlex GLuc Assay Kit

greater flexibility and sensitivity

Gaussia Luciferase is a highly sensitive secreted luciferase. This new kit for assaying *Gaussia* Luciferase (GLuc) activity, includes higher concentration coelenterazine substrate and an additional stabilizer of the light reaction, which allows the use of the assay in high throughput format without the requirement of an injector-equipped luminometer. LumiFlex GLuc Assay Kit can be used either with stabilizer for slow decay but less light intensity or without stabilizer for more light units but rapid decay bringing additional flexibility to your mammalian gene expression experiments.

Advantages:

- Flexible assay system
- Extreme sensitivity
- Very stable signal



Comparison of the GLuc light emission decay kinetics with the LumiFlex GLuc Kit using different amounts of stabilizer.

LumiFlex GLuc Assay Kit	
#E3308S	100 assays
#E3308L	1,000 assays
Gaussia Luciferase Assay Kit	
#E3300S	100 assays
#E3300L	1,000 assays

NEB 4

162 restriction enzymes now recommended for use in NEBuffer 4

Simplified Restriction Enzyme Buffer System

On November 1, 2008, the recommended buffer for 54 of our restriction enzymes changed to NEBuffer 4.

Advantages

- 162 enzymes can now be used in a single buffer
- Simplified double digests
- Use with confidence of 100% activity in either the previously recommended buffer or in the newly recommended NEBuffer 4

Use NEB's Double Digest Finder at www.neb.com to select double digest buffer and conditions.

54 Enzymes Now Supplied with NEBuffer 4:				
AcuI	BseWI	BstZ17I	MboII	SfoI
AflII	BsiEI	Cac8I	MnlI	SpeI
AluI	BseRI	CspCI	MseI	StuI
AsiSI	BsiHKAI	EaeI	MslI	TaqI
AvrII	BsmI	EarI	MspI	TfiI
BaeI	BsmAI	FauI	NaeI	TseI
BpuEI	BspCNI	HaeIII	NarI	XbaI
BsaI	BspQI	HinfI	PspGI	XhoI
BsaAI	BsrBI	HinP1I	PspXI	XmnI
BsaBI	BsrFI	KasI	RsaI	ZraI
BsaJI	BstUI	MboI	SfiI	

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