Promega

Discover Reliable Tools for Protein Analysis

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1 Cloning System and Protein Expression Vectors

Cloning System and Protein Expression Vectors

Functional protein analysis usually requires recombinant expression of the protein of interest. For this purpose, the protein coding sequence is cloned into a suitable expression vector and transferred into cells. Promega offers a wide range of tools to facilitate cloning into vectors for expression in prokaryotes, eukaryotes or cell-free expression systems. Additionally, in collaboration with the Kazusa DNA Research Institute, human ORF-clone gene sets and libraries are available. The ORF-clones in the library are extensively validated and are available as native ORFs and N-terminal HaloTag® fusions (HaloTag® ORF-clones). Furthermore, specialized expression vectors as well as competent cells for vector propagation can be found in this chapter.

Flexi® Cloning System

Flexi® Vector Systems are directional cloning systems that provide a method for transferring protein-coding sequences between different expression vectors without the need to resequence.

Description

Flexi® Vector Systems provide an efficient and high-fidelity method for transferring protein-encoding DNA into vectors capable of expressing native (non-tagged) protein or protein with an amino- (N-) or carboxy- (C-) terminal tag in bacterial, mammalian or cell-free expression systems. Once your protein-coding region is cloned into a Flexi® Vector, you can easily shuttle it into other Flexi® Vectors with different configurations without the need for resequencing **(Figure 1.1)**.

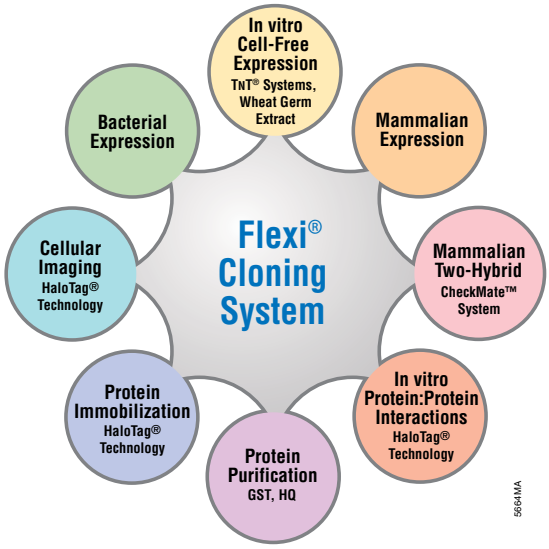


Figure 1.1. The Flexi® Vector Systems allow easy and efficient transfer of a protein-coding region between a wide variety of expression vectors without the need to resequence.

Principle

The Flexi® Vector System uses two rare-cutting restriction enzymes, SgfI and PmeI (both 8-cutters) in a simple, directional cloning method for protein-coding sequences. The desired protein-coding region is amplified by PCR before being cloned into one of the Flexi® Vectors **(Figure 1.2)**. An easy tool is available at the Promega website for primer design and to scan the nucleic acid sequence of the protein of interest sequence for SgfI and PmeI sites.

Flexi® Systems allow direct insertion into the type of vector suited to the experimental design. All Flexi® Vectors carry the lethal barnase gene, which is replaced by the DNA fragment of interest and acts as a negative selection marker for successful ligation of the PCR-amplified insert. To transfer the protein-coding region from one Flexi® Vector (donor) to another Flexi® Vector (acceptor) choose an appropriate acceptor vector with the desired expression and tag options **(Figure 1.3)**. The donor and acceptor vectors are digested with the Flexi® Enzyme Blend (SgfI and PmeI) prior to ligation of the insert, transformation and selection of cells. The PmeI site contains the stop codon for the protein-coding region and appends a single valine residue to the C-terminus of the protein.

C-terminal Flexi® Vectors allow expression of C-terminal-tagged proteins. While these vectors can act as acceptors of a protein-coding region flanked by SgfI and PmeI sites, they lack a PmeI site and contain a different blunt-end site, EcoICRI **(Figure 1.3, Panel B)**. When the blunt PmeI and EcoICRI ends are joined, the stop codon is not recreated, allowing readthrough into the C-terminal peptide sequence. However, this joined sequence cannot be cut by either PmeI or EcoICRI, so the protein-coding region cannot be removed from the C-terminal Flexi® Vectors and transferred to other Flexi® Vectors. In other words, transfer into C-terminal Flexi® Vectors is not reversible (i.e., it is a one-way exchange). By cloning the PCR fragment first into a native or N-terminal Flexi® Vector, the ability to transfer to any other Flexi® Vector is preserved **(Figure 1.3, Panel A)**.

Flexi® Cloning System (continued)

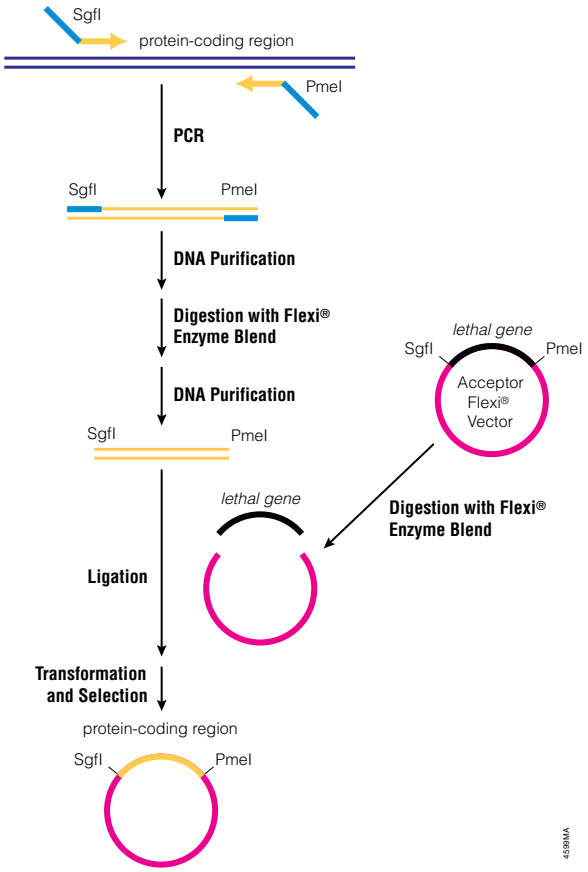
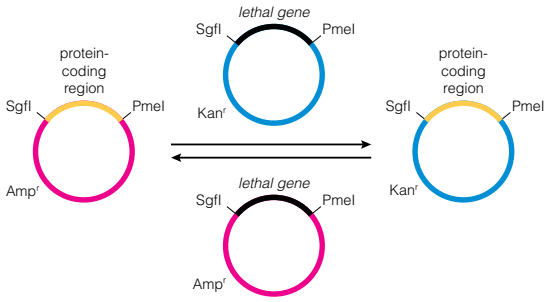


Figure 1.2. Cloning a protein-coding region into a Flexi® Vector. PCR primers are designed to append SgfI and PmeI sites onto the protein-coding region. The digested PCR product is ligated into the acceptor vector that has been digested with SgfI and PmeI. Following transformation, the cells are selected with an antibiotic appropriate Flexi® Vector used.

Getting started with Flexi® Vector Cloning

The Flexi® Vector Cloning System provides an easy way to get started with cloning and expression of genes of interest. For cloning there are many Flexi® Vectors from which to choose **(see Table 9.1)**. However, starting with C-terminal fusion vectors is not recommend since the protein-coding regions cannot be transferred into other vectors **(Figure 1.3, Panel B)**.

A. Transfer of a protein-coding region between N-terminal or native Flexi® Vectors.



B. Transfer of a protein-coding region into C-terminal Flexi® Vectors.

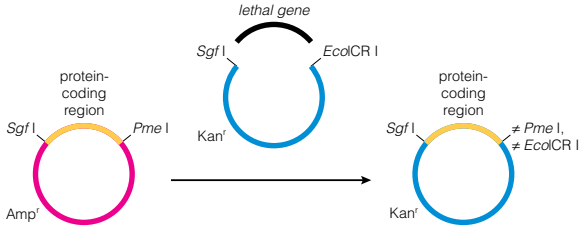


Figure 1.3. Transferring protein-coding regions in the Flexi® Vector Systems. Protein-coding region can be shuttled between vectors using two rare-cutting restriction endonucleases, SgfI and PmeI. The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. **Panel A**. Transfer between Flexi® Vectors for expression of native or N-terminal-tagged fusion proteins is reversible (i.e., is a two-way exchange) between native and N-terminal Flexi® Vector. **Panel B.** C-terminal Flexi® Vectors contain SgfI and EcoICRI sites and are designed to allow expression of C-terminal-tagged proteins. Joining PmeI and EcoICRI blunt ends eliminates the stop codon present in the PmeI site and allows readthrough to the C-terminal protein-coding sequences in the C-terminal Flexi® Vectors. Since both restriction sites are destroyed by joining, transfer into C-terminal Flexi® Vectors is not reversible (i.e., is a one-way exchange).

Ordering Information

Flexi® System, Entry/Transfer (Cat.# C8640)

Carboxy Flexi® System, Transfer (Cat.# C9320)

The Flexi® System, Transfer Kit (Cat.# C8820)

Human ORF-Clone Library

Human protein expression without cloning.

Description and Principle

The Promega Open-Reading-Frame (ORF-) clone library consists of more than 9,000 experimentally validated human clones for native and tagged protein expression. The tagged ORF-clones are fused to HaloTag®, a protein fusion tag that is used in multiple applications such as cellular imaging, protein purification and protein pull-down (see Chapters 4 & 6). HaloTag® ORF-clones (FHC-clones) are provided in the Flexi® Vector pFN21A, suitable for transient protein expression in mammalian cells. In FHC clones HaloTag® is fused to the N-terminus of the ORF sequence and expression of the protein of interest is under the control of a CMV promoter **(Figure 1.4)**. The native expression clones (FXC clones) are provided in Flexi® Vector pF1K **(Figure 1.5)**. All ORF-clones can be easily transferred into other Flexi® Vectors with different features **(see Table 9.1)**. For testing different expression strengths we offer vectors with a modified CMV immediate-early enhancer/promoter for constitutive expression in mammalian cells.

Features and Benefits

Experimentally validated ORF-clone library:

- Sequence validation of both the 5′- and 3′-end sequences by single-pass sequencing.
- Size confirmation through agarose gel electrophoresis.
- Expression validation by SDS-PAGE of expressed HaloTag® fusions in HEK293 cells (only for HaloTag® clones).
- Fluorescent microscopy demonstrating in situ labeling and visualization of HaloTag® fusions with HaloTag® TMR Ligand (only for HaloTag® clones) in HEK293 cells.

How to find and order an ORF-clone

Available ORF-clones can be found by using the online tool *Find My Gene*™ at: www.promega.com. *Find My Gene*™ allows the search by ORF-clone Name, Gene Symbol/ID/Name, Accession Number, or by blasting the protein or nucleic acid sequence of interest. Alignments of the sequence of interest with the offered ORF-clones can be performed within the online tool. The ORF-clones can be ordered online, using FAX or email with respective catalog/clone number.

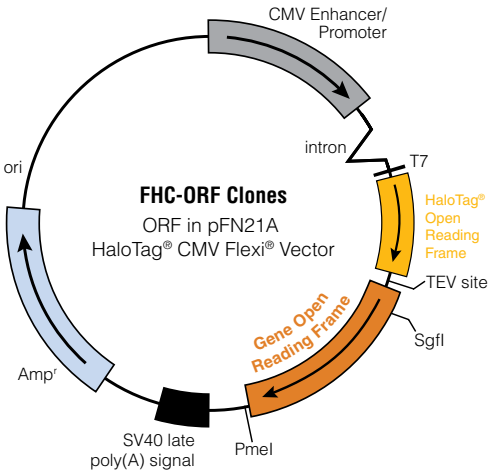


Figure 1.4. Vector map of HaloTag® ORF-clones.

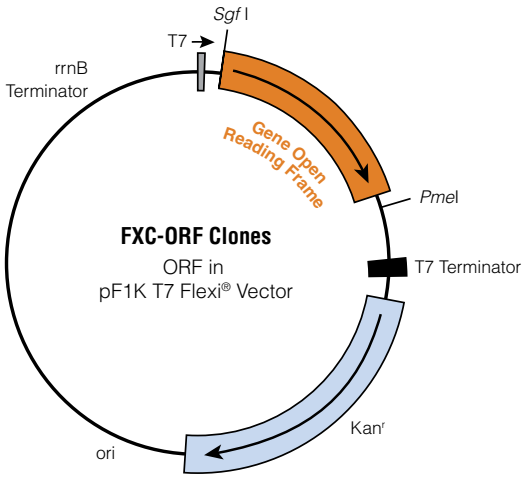


Figure 1.5. Vector map of native ORF-clones.

Human ORF-Clone Library (continued)

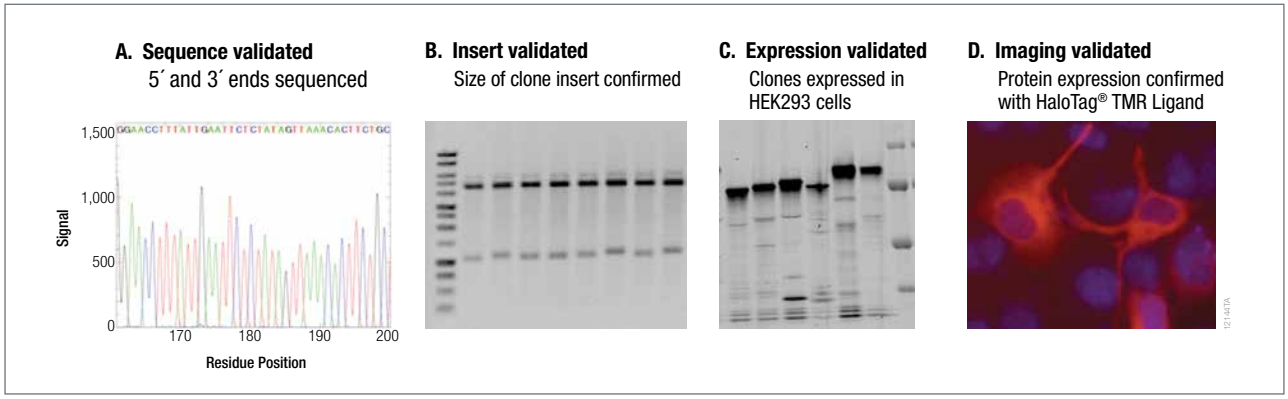


Figure 1.6. Experimental validation of ORF-clones. All ORFs clone are sequence validated (5'- and 3'-ends sequenced; **(Panel A)** and insert validated to confirm the size of the cloned insert **(Panel B)**. The HaloTag® ORFs are also expression validated by SDS-PAGE **(Panel C)** and imaging validated by fluorescent microscopy using HaloTag® TMR Ligand **(Panel D)**.

Table 1.1. Overview of the Human ORF-clone Collection.

Features	HaloTag® Collection	Native Collection
Size of Collection	>9,000	>6,300
Fusion Tag	HaloTag® for protein purification, imaging, pull-downs and NanoBRET™.	Native
Validated Clones		
Sequence Validated	✓ 100% clones	✓ 100 % clones
Insert Validated	✓ 99% clones	✓ 97%
Expression Validated	✓ 99% clones	(–)
Imaging Validated	✓ 78% clones	(–)
Format	DNA	DNA

Additional Information

The ORF-clones are provided in 100ng of purified plasmid DNA in TE buffer. We recommend that you transform your ORF-clone in competent cells, and create a bacterial glycerol stock. Upon request, the generation of other ORF-clones is offered via Promega Custom Ordering (www.promega.com/products/manufacturing-and-custom-capabilities/). The Arabidopsis Biological Resource Center at the Ohio State University distributes HaloTag® ORF-clones from Arabidopsis. For more information visit: www.arabidopsis.org/abrc/halo_tagged_orf_clones.jsp

Regulated Mammalian Expression System

Inducible expression in mammalian cells.

Description

The Regulated Mammalian Expression System features low basal levels, robust and rapid induction, and downregulation of gene expression in mammalian cells. The Regulated Mammalian Expression System is based on a novel on/off switch that relies on the rapid and sensitive modulation by coumermycin-related compounds of a chimeric transactivator protein. The levels of protein expression can be regulated by adjusting the coumermycin concentration. More significantly, this expression can be promptly and effectively switched off by adding novobiocin **(Figure 1.7)**.

Features and Benefits

- **Enhanced Data:** High level of controlled induction combined with low basal protein expression.
- **Regulated Expression:** Dose-response induction of protein expression; rapid and sensitive on/off switch for protein expression.
- **Versatility:** Compatible with other Flexi® Vectors.

References

Zhao, H-F. *et al.* (2003) A coumermycin/novobiocin-regulated gene expression system. *Hum. Gene Ther.* **14**(47), 1619–29.

Principle

The protein coding region of interest is cloned into either the pF12A RM Flexi® Vector or pF12K RM Flexi® Vector, both of which are specially designed for Regulated Mammalian (RM) protein expression. These vectors incorporate regulatory promoter sequences upstream of the protein-coding region and are compatible with the Flexi® Vector System. In transient transfection paradigms, the pF12A or pF12K RM Flexi® Vector containing the protein-coding region of interest is co-transfected into mammalian cells together with the pReg neo Vector. The pReg neo Vector is designed to express a chimeric transactivator protein that interacts with the regulatory promoter region in the pF12A and pF12K RM Flexi® Vectors in a regulated fashion in response to coumermycin and novobiocin. Additionally, the pReg neo Vector encodes a neomycin phosphotransferase gene that allows stable cell selection and generation with the antibiotic G-418.

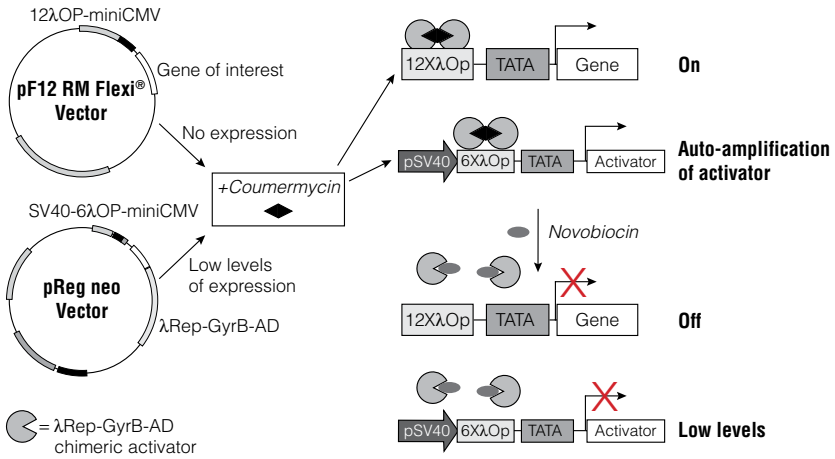


Figure 1.7. Diagram of the coumermycin-regulated mammalian expression system.

Ordering Information

Regulated Mammalian Expression System (Cat.# C9470)

pTARGET™ Mammalian Expression Vector System

PCR products can be cloned directly into the T-overhang of the pTARGET™ Vector and used for protein expression in mammalian cells under a CMV promoter.

Description

The pTARGET™ Mammalian Expression Vector System is a convenient system for cloning PCR products and for expressing cloned PCR products in mammalian cells. The pTARGET™ Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. For cloning of ampllifed PCR products in general, the pGEM®-T Vector and pGEM®-T Easy Vector Systems are recommended.

Principle

The vector is prepared by digestion with EcoRV followed by addition of a 3´-terminal thymidine to each end. These single 3´T-overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid in two ways. First, the overhangs prevent recircularization of the vector; second, they provide a compatible overhang for PCR products, as thermostable polymerases add a single deoxyadenosine, in a template-independent fashion, to the 3´-ends of amplified fragments.

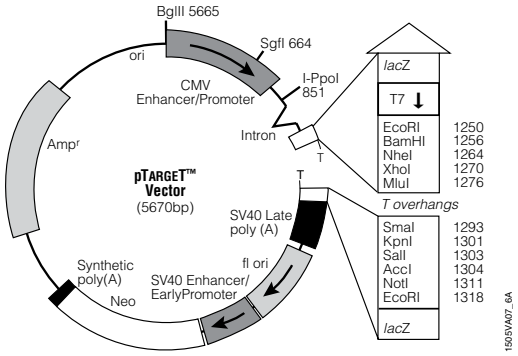


Figure 1.8. pTARGET™ Mammalian Expression Vectors for transient and stable protein expression.

Features and Benefits

- **Simple PCR Cloning:** T-overhangs permit direct ligation of PCR products. Note: If amplifying long fragments, use GoTaq® Long PCR Master Mix, which produces A-overhangs.
- **Strong, Constitutive Expression:** The CMV enhancer/promoter region allows strong, constitutive expression in many cell types.
- **Blue/White Screening:** Easy identification of recombinant clones. A single digest removes the insert DNA.
- **Stable Transfectants:** Select for stable transfectants using the antibiotic G-418.

References

Dastidar, S.G. *et.al.* (2011) FoxG1 promotes the survival of postmitotic neurons. *J. Neurosci.* **31**(2), 402–13.

Carpenter, J.E. *et al.* (2011) Autophagosome formation during varicella-zoster virus infection following endoplasmic reticulum stress and the unfolded protein response. *J. Virol.* **85**(18), 9414–24.

Ordering Information

pTARGET™ Mammalian Expression Vector (Cat.# A1410)

pGEM®-T Vector (Cat.# A3600)

pGEM®-T Easy Vector Systems (Cat.# A1360, A1380)

JM109 Competent Cells

Competent cells for high-efficiency transformation of vectors for cloning purposes.

Description and Principle

JM109 Competent Cells are derived from an *E.coli* K strain that is *recA*– and *endA*– to minimize recombination and improve the quality of plasmid DNA. In addition, the cells carry the F´ episome, which allows blue/white screening. The Competent Cells are available for convenient transformation in two efficiencies: at greater than 10^8cfu/µg and at greater than 10^7cfu/µg. In addition, single-use sizes are supplied for maximal ease-of-use.

Features and Benefits

- **Convenient:** Ready-to-use; no preparation time necessary, blue/white screening.
- **Reliable:** Transformation efficiencies guaranteed.
- **Safe:** The *recA*– mutation prevents undesirable recombination events, and the *endA*– mutation in JM109 cells prevents carryover nuclease in miniprep DNA.

Additional Information

JM109 Genotype: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (rk–, mk+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´*tra*D36, *pro*AB, *laq*I^{q}ZΔM15].

Ordering Information

Single-Use JM109 Competent Cells >10^8cfu/µg (Cat.# L2005)

JM109 Competent Cells >10^8cfu/µg (Cat.# L2001)

JM109 Competent Cells >10^7cfu/µg (Cat.# L1001)

Pro 5-alpha Competent Cells

Competent cells for maximal efficiency transformation of vectors for difficult cloning experiments.

Description and Principle

Single-Use Pro 5-alpha Competent Cells are an *E.coli* strain that can be used for the efficient transformation of unmethylated DNA derived from PCR, cDNA and many other sources. The elimination of nonspecific endonuclease I (endA1) enables the highest quality plasmid preparations. The strain is resistant to phage T1 (fhuA2) and suitable for blue/white screening by α-complementation of the β-galactosidase gene.

Features and Benefits

- **Convenient:** Ready-to-use; no preparation time necessary, blue/white screening.
- **Reliable:** Transformation efficiencies guaranteed.
- **Safe:** The *recA*– mutation prevents undesirable recombination events.

Additional Information

Pro 5-alpha Genotype: *fhu*A2, Δ(argF-*lacZ*), U169, *pho*A, *gln*V44, φ 80, Δ(*lacZ*)M15, *gyr*A96, *rec*A1, *rel*A1, *end*A1, *thi*-1, *hsd*R17.

Ordering Information

Single-Use Pro 5-alpha Competent Cells >10^9cfu/µg (Cat.# L1221)

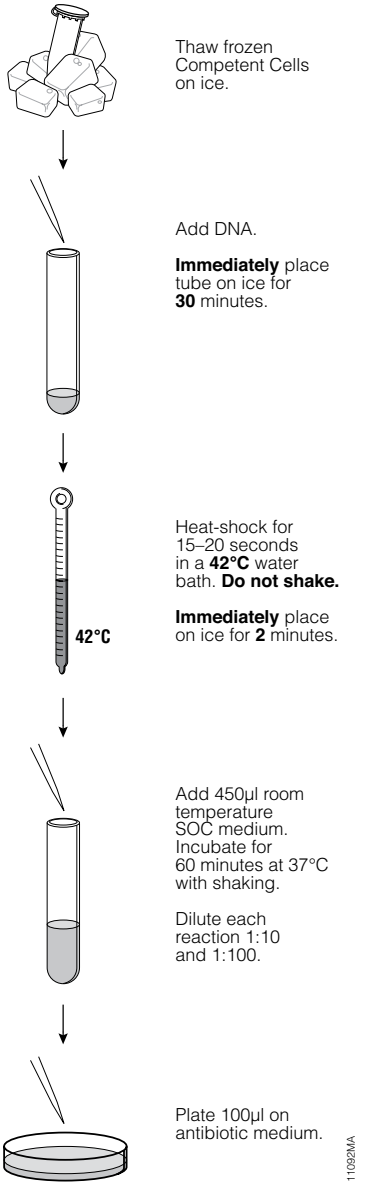


Figure 1.9. Standard transformation protocol using Single-Use Competent Cells.

2 Bacterial Strains for Protein Expression

Bacterial Strains for Protein Expression

Protein expression in *Escherichia coli (E. coli)* has been a popular means of producing recombinant proteins for several decades. *E. coli* is a well-established host that offers easy genetic manipulation, short and inexpensive culture. Additionally, *E. coli* has a long history of being able to produce many different types of proteins.

The T7 RNA Polymerase System is the most popular approach for producing proteins in *E. coli*. In this system, an expression vector containing a gene of interest, cloned downstream of the T7 promoter, is introduced into a T7 expression host. T7 expression hosts such as DE3 strains have a chromosomal copy of the phage T7 RNA polymerase gene. When an inducer such as IPTG or rhamnose is added to the culture, T7 RNA polymerase is expressed and transcribes the gene of interest, followed by translation of the desired protein by endogenous protein translation machinery.

Promega offers ready–to–use competent cells for expression of recombinant proteins in *E. coli*.

Single-Step (KRX) Competent Cells

Tightly-controlled protein expression in *E. coli* based on T7 RNA polymerase rhamnose-inducible system.

Description

The Single-Step (KRX) Competent Cells are an *E. coli* K strain that is designed for both efficient transformation (>10^8 cfu/µg) and tightly-controlled protein expression. The stringent control provided by the rhamnose-driven T7 RNA polymerase may allow cloning of proteins toxic to *E. coli.* The KRX Single-Step Competent Cells are available in convenient single transformation size (50µl aliquots).

Principle

Single-Step (KRX) Competent Cells contain a chromosomal copy of the T7 RNA polymerase driven by a rhamnose promoter (rhaBAD) that provides tight control of the proteins expressed via a T7 promoter **(Figure 2.1)**. Addition of rhamnose induces the expression of the T7 RNA polymerase, which in turn transcribes the gene of interest under control of a T7 promoter. Protein expression level in KRX cells are as high or higher than levels expressed in BL21(DE3)-derived strains. However, pre-induction protein expression levels in Single-Step (KRX) Competent Cells are significantly lower than those of BL21(DE3)-derived strains **(Figure 2.2)** and therefore recommended for the expression of protein toxic to *E. coli*.

Features and Benefits

- **Save Time:** Clone and express your vector in one step.
- **Controlled Protein Expression:** Highly regulatable protein expression.
- **Achieve High Yields:** Protein expression as high or higher than levels expressed in BL21(DE3)-derived strains.
- **Blue/White Screening:** Convenient method for detecting recombinant clones.

Additional Information:

KRX Genotype: [F′, *tra*D36, Δ*omp*P, *pro*A$^+$B$^+$, lacI^q, Δ(*lacZ*) M15] Δ*omp*T, *end*A1, *rec*A1, *gyr*A96 (Nal^r), *thi*-1, *hsd*R17 (r_k^-, m_k^+), e14$^-$ (McrA$^-$), *rel*A1, *sup*E44, Δ(*lac-pro*AB), Δ(*rha*BAD)::T7 RNA polymerase.

Ordering Information

Single-Step (KRX) Competent Cells (Cat.# L3002)

References

Malu, B *et al.* (2013) A nondiscriminating glutamyl-tRNA synthetase in the plasmodium apicoplast: the first enzyme in an indirect aminoacylation pathway. *J. Biol. Chem.* **288**(45), 32539–52.

Barquilla, A *et al.* (2012) Third target of rapamycin complex negatively regulates development of quiescence in Trypanosoma brucei. *Proc.Natl. Acad. Sci*, **109**(36), 14399–404.

Single-Step (KRX) Competent Cells (continued)

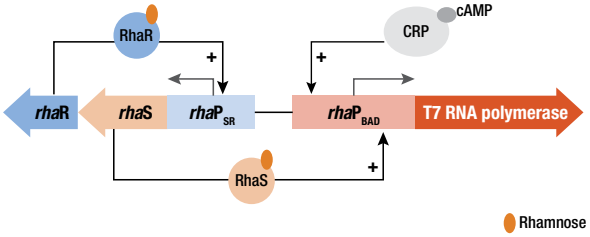


Figure 2.1. Tightly controlled inducible expression with L-Rhamnose in KRX *E. coli*. T7 RNA polymerase expression is under the control of the $rhaP_{BAD}$ promoter in the KRX strain. This promoter is subject to multiple levels of control. In the presence of preferred carbon sources, such as glucose, cyclic AMP (cAMP) concentrations are low and the cAMP receptor protein (CRP) does not activate transcription. Upon depletion of glucose, cAMP levels rise and CRP can activate transcription at $rhaP_{BAD}$. In addition, L-rhamnose can bind to RhaR, which binds the rhaPSR promoter, resulting in the production of active RhaS and more RhaR. RhaS also binds rhamnose, which then binds the $rhaP_{BAD}$ promoter, resulting in the production of high levels of T7 RNA polymerase. The T7 RNA polymerase in turn transcribes the gene of interest.

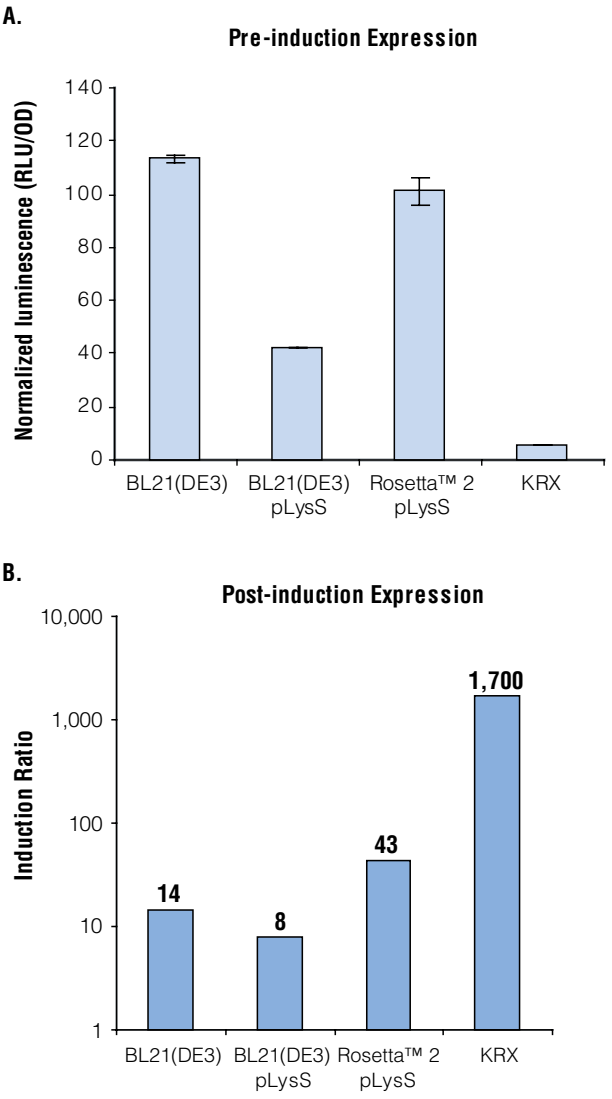


Figure 2.2. Pre-induction and post-induction expression levels of firefly luciferase. KRX shows only very low pre-induction **(Panel A)** and very high post-induction levels **(Panel B)** compared to other strains such as BL21(DE3).

BL21 Competent Protein Expression Cells

Inducible recombinant protein expression *in E. coli.*

Description

BL21(DE3)pLysS Competent Cells and Single-Use BL21(DE3)pLysS Competent Cells allow high-efficiency protein expression of any gene that is under the control of a T7 promoter. The strain carries both the DE3 lysogen and the plasmid pLysS. pLysS constitutively expresses low levels of T7 lysozyme, which reduce basal expression of recombinant genes by inhibiting basal levels of T7 RNA polymerase. High protein expression is achieved by IPTG addition. Competent cells are available in standard format (200µl aliquots) as well as in 50µl aliquots.

Principle

BL21(DE3)pLysS is a derivative of BL21 that has the T7 RNA polymerase gene under the control of the lacUV5 promoter. This arrangement is on a phage genome, called λDE3. λDE3 is inserted into the chromosome of BL21 to make BL21(DE3). pLysS is a plasmid that contains the T7 lysozyme gene (LysS). The T7 lysozyme binds to T7 RNA polymerase causing inhibition until induction by the addition of IPTG. When IPTG is added, the amount of T7 RNA polymerase increases and overcomes the inhibition by LysS.

Features and Benefits

- **T7 RNA Polymerase under the Control of the lac UV5 Promoter:** Inducible protein expression.
- **Deficient in Proteases lon and OmpT:** Increased stability of expressed protein.
- **pLysS Plasmid:** Lower background expression of target genes.

Additional Information:

Genotype: F–, *ompT*, *hsdSB* (r_B–, m_B–), *dcm*, *gal*, λ(DE3), pLysS, Cm^r.

References

Firdaus, M. *et al.* (2013) The pH sensitivity of murine heat shock protein 47 (HSP47) binding to collagen is affected by mutations in the breach histidine cluster. *J. Biol. Chem.* **288**(6), 4452–61

Otsu, W. *et al.* (2013) A new class of endoplasmic reticulum export signal PhiXPhiXPhi for transmembrane proteins and its selective interaction with Sec24C. *J. Biol. Chem*. **288**(25), 18521–61

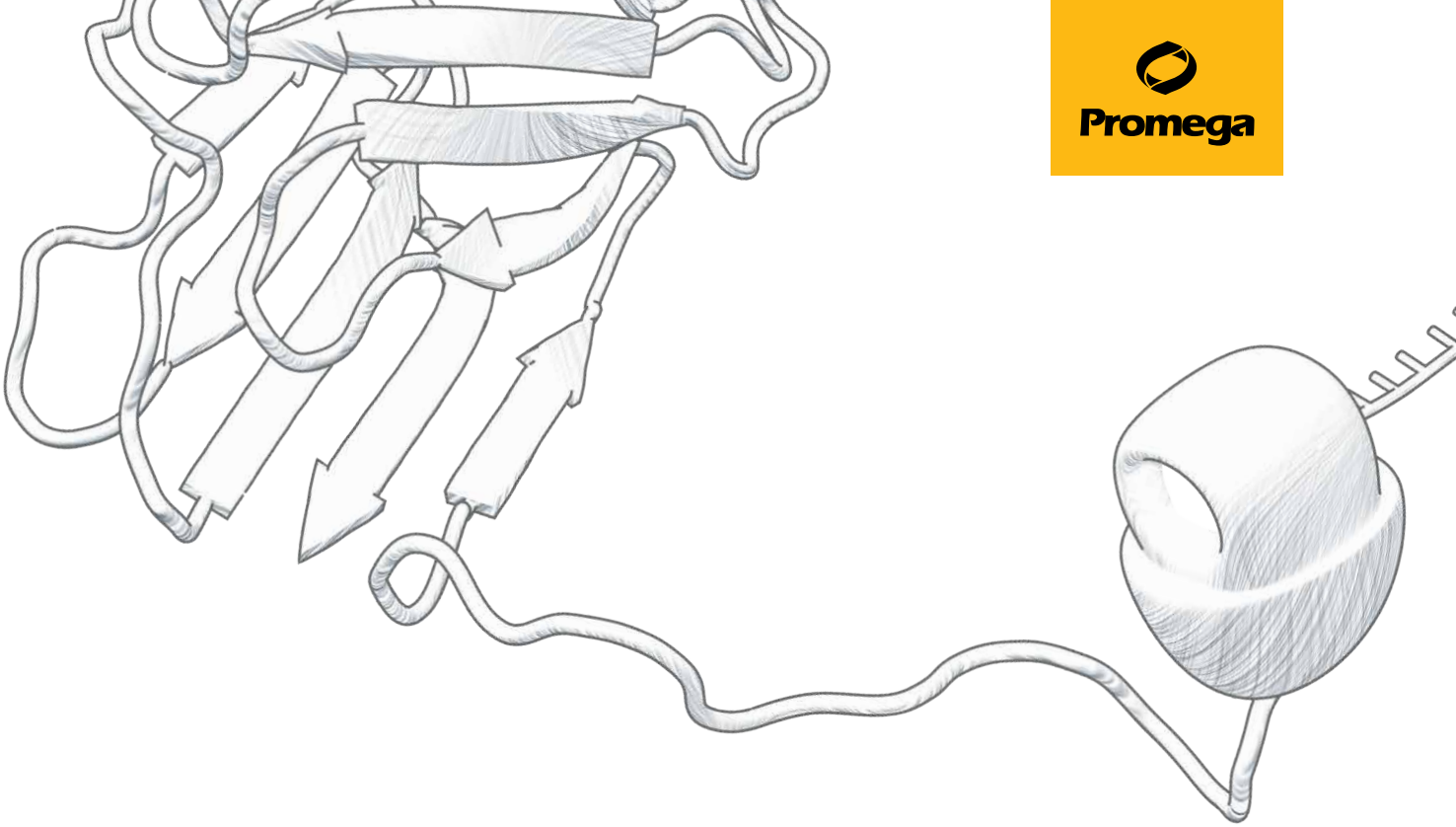
Yamazaki, D. *et al.* (2013) srGAP1 regulates lamellipodial dynamics and cell migratory behavior by modulating Rac1 activity. *Mol.Biol. Cell.* **24**(21), 3393–05

Ordering Information

BL21(DE3)pLysS Competent Cells (Cat.# L1191)

Single-Use BL21(DE3)pLysS Competent Cells (Cat.# L1195)

3 Cell-Free Protein Expression Systems



Introduction

Cell-free protein synthesis is an important tool for molecular biologists in basic and applied sciences. It is increasingly being used in high-throughput functional genomics and proteomics, with significant advantages compared to protein expression in live cells. Cell-free protein synthesis is essential for the generation of protein arrays, such as nucleic acid programmable protein array (NAPPA) and enzyme engineering using display technologies. The cell-free approach provides the fastest way to correlate phenotype (function of expressed protein) to genotype. Protein synthesis can be performed in a few hours using either mRNA template in translational systems or DNA template (plasmid DNA or PCR fragments) in coupled transcription and translation systems. Furthermore, cell-free protein expression systems are indispensable for the expression of toxic proteins, membrane proteins, viral proteins and for proteins that undergo rapid proteolytic degradation by intracellular proteases.

Chapter 3 Overview (continued)

Origins of Cell-Free Expression Systems

Cell-free protein expression lysates are generated from cells engaged in a high rate of protein synthesis, such as immature red blood cells (reticulocytes). The most frequently used cell-free expression systems originate from rabbit reticulocytes, wheat germ and *E. coli*. There are two types of cell-free expression systems: Translation Systems and Coupled Translation and Transcription (TNT®) Systems **(Figure 3.1).** Both types of systems provide the macromolecular components required for translation, such as ribosomes, tRNAs, aminoacyl-tRNA synthetases, initiation, elongation and termination factors. To ensure efficient translation, each extract has to be supplemented with amino acids, energy sources (ATP, GTP), energy regenerating systems and salts (Mg^{2+}, K^{+}, etc.). For eukaryotic systems creatine phosphate and creatine phosphokinase serve as energy regenerating system, whereas prokaryotic systems are supplemented with phosphoenol pyruvate and pyruvate kinase. Coupled transcription and translation systems are supplemented with phage-derived RNA polymerase (T7, T3 or SP6) allowing the expression of genes cloned downstream of a T7, T3 or SP6 promoter.

Selection of Cell-Free Protein Expression

Many different cell-free expression systems derived from prokaryotic and eukaryotic source are available. The choice of the system is dependent on several factors, including the origin of the template RNA and DNA, protein yield or whether the protein of interest requires post-translational modification (e.g., core glycosylation). We offer translation systems (mRNA-based) and coupled transcription/translation systems (DNA-based) from prokaryotic and eukaryotic sources. **Table 3.2** provides an overview of translational systems and **Table 3.3** provides an overview of coupled translation/transcription systems.

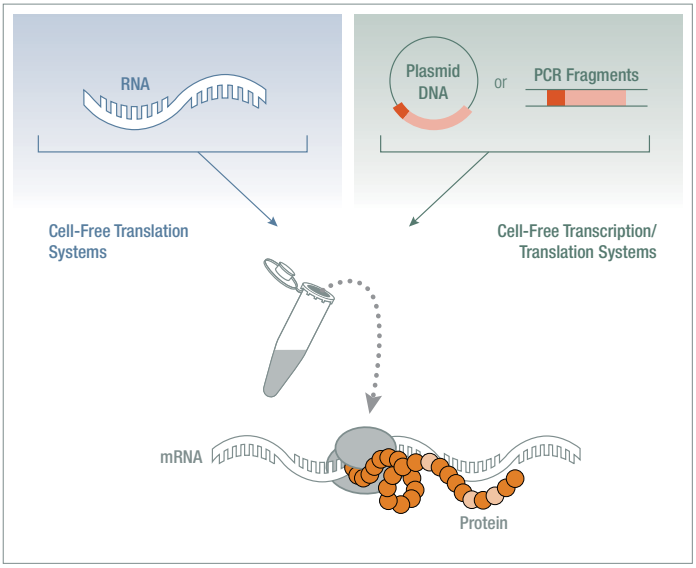


Figure 3.1. Cell-free protein expression systems are divided into mRNA-based translation systems and in DNA-based transcription/translation systems.

Chapter 3 Overview (continued)

Table 3.1. Applications of Cell-Free Protein Synthesis

Functional Genome/Proteome Analysis

- Gene mutation/deletion analysis (e.g., enzyme kinetics)
- Protein domain mapping
- Characterization of protein interactions
- Gel Shift EMSA
- Generation of protein arrays

Expression of Difficult-to-Express Proteins

- Cell-toxic proteins, membrane protein, viral proteins, kinases

Protein Evolution/Enzyme Engineering

- Display technologies (e.g., ribosome, mRNA display, in vitro compartmentalization)
- Evolution of antibodies in vitro by ribosome display

Analysis of Transcriptional/Translational Regulation

- Structural RNA analysis such as characterization of regulatory elements for translation (e.g., UTRs, Capping, IRES)
- RNA virus characterization

Screenings

- Screening of chemical libraries for effect on translation
- Drug screening (e.g., antibiotics)

Protein Labeling

- Open systems allow the incorporation of labeled amino acids

3.1 Translation Systems: mRNA-based

OVERVIEW

Cell-free translation systems are used for protein expression of either in vitro transcribed mRNA or mRNA isolated from tissues or cells. These systems are used to express single proteins as well as multiple proteins in high-throughput applications such as display technologies. Furthermore, cell-free translation systems are useful for functional and structural RNA analysis, or to study aspects of the translational machinery. Eukaryotic translation systems originate from either rabbit reticulocyte lysates (RRL) or wheat germ extracts (WGE). We offer three mRNA-based translation systems. The extracts are treated with microccal nuclease to destroy endogenous mRNA and thus reduce background translation to a minimum **(Table 3.2)**.

The *Flexi® Rabbit Reticulocyte Lysate System* offers greater flexibility in reaction conditions by allowing translation reactions to be optimized for a wide range of parameters, including Mg^{2+} and K^{+} concentrations. The Wheat Germ Extract is a useful alternative to the RRL systems for expressing small proteins or for expressing proteins known to be abundant in RRL. Researchers expressing proteins from plants or yeasts or other fungi also may find WGE preferable to RRL.

Table 3.2. Overview of Cell-FreeTranslation Systems that use mRNA as a Template.

Translation System	Nuclease-Treated	Signal Cleavage & Core Glycosylation with CMM*	Labeling Options**	Luciferase Control RNA	Protein Yield
Rabbit Reticulocyte Lysate System, Nuclease-Treated (Cat.# L4960)	+	+	Met,Cys,Leu, FluoroTect™; Transcend™	+	1–4 µg/ml
Flexi® Rabbit Reticulocyte Lysate (Cat.# L4540) ***	+	+	Met, Cys, Leu, FluoroTect™; Transcend™	+	1–4 µg/ml
Wheat Germ Extract (Cat.# L4380)	+	-	Met, Cys, Leu, FluoroTect™; Transcend™	+	0.6–3 µg/ml

* CMM: Canine Microsomal Membranes

** The lysates are provided with three Amino Acid Mixtures for the incorporation of labeled amino acids like methionine, cysteine & leucine. Transcend™ tRNA (Cat.# **L5070**; **L5080**) and FluoroTect™ (Cat.# **L5001**) can be used to incorporate biotinylated or fluorescently labeled lysine residues.

*** The system provides greater flexibility of reaction conditions than standard rabbit reticulocyte lysate systems. The Flexi® Rabbit Reticulocyte Lysate System allows translation reactions to be optimized for a wide range of parameters, including Mg^{2+} and K^{+} concentrations and the option to add DTT.

Rabbit Reticulocyte Lysate System, Nuclease-Treated

In vitro protein synthesis starting from mRNA.

Description

Rabbit Reticulocyte Lysate (RRL), Nuclease-Treated, is optimized for mRNA translation by the addition of several supplements. These include hemin, which prevents activation of the heme-regulated eIF-2a kinase; an energy-generating system consisting of phosphocreatine kinase and phosphocreatine; and calf liver tRNAs to balance the accepting tRNA populations, thus optimizing codon usage and expanding the range of mRNAs that can be translated efficiently. In addition, the lysates are treated with micrococcal nuclease to eliminate endogenous mRNA. RRLs post-translationally modify proteins via phosphorylation, acetylation and isoprenylation. Signal peptide cleavage and core glycosylation also can be achieved by the addition of Canine Pancreatic Microsomal Membranes. See **Table 3.1** for additional applications.

Principle

In RRL translation reactions, mRNA is used as template for translation. In general, optimal results will be achieved after an incubation time of 1.5 hours at 30°C. However, many template-related factors affect translation efficiency of specific mRNAs in the RRL system and should be considered when designing in vitro translation experiments. The optimal mRNA concentration will vary for particular transcripts and should be determined empirically. In addition, the presence of certain nucleic acid sequence elements can have profound effects on initiation fidelity and translation efficiency. Poly(A)+ tails, 5´-caps, 5´-untranslated regions and the sequence context around the AUG start (or secondary AUGs in the sequence) all may affect translation of a given mRNA.

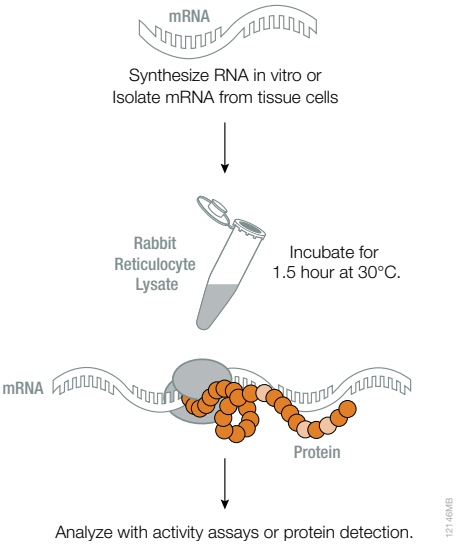


Figure 3.2. Flow chart of **in vitro** translation procedure using Rabbit Reticulocyte Lysate.

Features and Benefits

- **Consistent:** Reliable and consistent translation with each lot.
- **Optimized and Ready-to-Use:** The treated Rabbit Reticulocyte Lysate is optimized for translation.
- **Convenient:** Luciferase Control RNA included.

Ordering Information

Rabbit Reticulocyte Lysate (RRL), Nuclease-Treated (Cat.# L4960)

Flexi® Rabbit Reticulocyte Lysate System

In vitro protein synthesis starting from mRNA. Optimize translation for low-expressing mRNA.

Description

The Flexi® Rabbit Reticulocyte Lysate System is widely used to identify mRNA species and characterize their products. It provides greater flexibility of reaction conditions than the Rabbit Reticulocyte Lysate, Nuclease-Treated, by allowing translation reactions to be optimized for a wide range of parameters, including Mg^{2+} and K^{+} concentrations. See **Table 3.1** for additional applications.

Principle

As with the standard Rabbit Reticulocyte Lysate, the Flexi® Rabbit Reticulocyte Lysate System is optimized for translation by addition of the following supplements: hemin, to prevent inhibition of initiation factor eIF-2α; an energy-generating system consisting of pretested phosphocreatine kinase and phosphocreatine; calf liver tRNAs to balance the accepting tRNA populations, thus optimizing codon usage and expanding the range of mRNAs that can be translated efficiently; and micrococcal nuclease to eliminate endogenous mRNA, thus reducing background translation. This eukaryotic system has been reported to post-translationally modify proteins via phosphorylation, acetylation and isoprenylation. With the addition of Canine Pancreatic Microsomal Membranes signal peptide cleavage and core glycosylation can occur. The Flexi® Rabbit Reticulocyte Lysate System provides greater flexibility of reaction conditions than standard RRL systems.

Features and Benefits

- **Consistent:** Reliable and consistent translation with each lot.
- **Easy Optimization:** To aid in optimizing magnesium concentrations, the endogenous magnesium concentration is provided for each lot of Flexi® Lysate.
- **Convenient:** Luciferase Control RNA and detection reagent included.

Ordering Information

Flexi® Rabbit Reticulocyte Lysate System (Cat.# L4540)

Wheat Germ Extract

In vitro protein synthesis starting from mRNA.

Description

Wheat Germ Extract (WGE) is a well-defined processed and optimized extract from wheat germ. It contains the cellular components necessary for protein synthesis (tRNA, ribosomes and initiation, elongation and termination factors). The extract is supplemented with an energy-generating system (phosphocreatine/phosphocreatine kinase), and with spermidine to stimulate the efficiency of chain elongation. Only exogenous amino acids and mRNA are needed to initiate translation. Potassium acetate can be used to optimize translation for a wide range of mRNAs. See **Table 3.1** for additional applications.

Principle

Wheat Germ Extract is a useful alternative to the Rabbit Reticulocyte Lysate (RRL) systems for expressing small proteins or for expressing proteins expected to be abundant in RRL. Researchers expressing proteins from plants, yeast or other fungi also may find Wheat Germ Extract preferable to RRL.

Features and Benefits

- **Optimized Extract:** Assists in expression of eukaryotic messages that do not express well in RRL.
- **Flexible:** Three Amino Acid Mixtures are provided, which enable different radioisotope choices.
- **Robust:** Potassium Acetate is provided to enhance translation for a wide range of mRNAs.
- **Convenient:** Luciferase Control RNA included.

Ordering Information

Wheat Germ Extract (Cat.# L4380)

3.2 Transcription and Translation Systems: DNA-based

OVERVIEW

Coupled transcription and translation (TnT®) systems offer researchers time-saving alternatives for eukaryotic in vitro transcription and translation, by coupling these processes in a single tube format. TnT® Systems are used for a variety of applications in low- to high-throughput functional genome and proteome analyses, as summarized in **Table 3.1**. TnT® Systems are supplemented with T7, T3 or SP6 RNA polymerases, allowing protein expression from DNA cloned downstream of a T7, T3 or SP6 promoter.

We offer TnT® Systems originating from eukaryotic sources such as rabbit reticulocyte, wheat germ and insect cells as well as from prokaryotic *E. coli* extracts **(Table 3.3)**.

The highest production rates are normally achieved with *E. coli* extracts. However, eukaryotic systems often produce eukaryotic proteins with higher activity. Therefore, the origin of the protein of interest should be considered when selecting a cell-free expression system.

DNA Template Consideration: Plasmids and PCR-Fragments

The performance of cell-free systems depends on the DNA template used. Basically, any vector containing T7, SP6 or T3 promoters can be used with TnT® Systems. However, there are several points to consider when engineering a DNA fragment or plasmid for optimal expression in a eukaryotic system: **(i)** the ATG start codon in the sequence should be the first ATG encountered following the transcription start site; **(ii)** ideally, following the promoter, the ATG is included in a Kozak consensus sequence; **(iii)** a stop codon should be included at the 3′- terminus of the sequence; and **(iv)** a synthetic poly(A) tail should be included following the stop codon. Additionally, vectors used in the TnT® T7 Coupled Wheat Germ System should either be linearized or have a T7 transcription terminator in a circular template.

In prokaryotic systems, the selection of a start codon generally depends on the presence of a ribosomal binding site (RBS; Shine-Dalgarno sequence), which contains a signal that marks the start of the reading frame. The presence of an optimal RBS can greatly increase expression in prokaryotic systems. The prokaryotic system does not recognize ATGs upstream of the ATG start codon unless they contain a properly positioned RBS.

Promega vectors approved for use with TnT® Systems can be found in **Table 9.1**.

The template considerations mentioned above are also valid for using PCR fragments as templates for the TnT® reaction. For the generation of the PCR fragments for protein expression in eukaryotic systems, the integration of a Kozak sequence downstream of a T7 or SP6 promoter is recommended **(Figure 3.4)**.

Labeling of Proteins during in vitro Synthesis

All TnT® Systems are provided with three different Amino Acid Mixtures for the incorporation of radiolabeled amino acids like methionine, cysteine and leucine. Transcend™ tRNA and FluoroTect™ Systems can be used to incorporate biotinylated or fluorescently-labeled lysine residues (see Section 3.3).

Signal Peptide Cleavage and Core Glycosylation

Rabbit reticulocyte lysate has been reported to post-translationally modify proteins via phosphorylation, acetylation and isoprenylation. However, the addition of Canine Pancreatic Microsomal Membranes (CMM), to RRLs is required to achieve signal peptide cleavage and core glycosylation of the translation product.

Chapter 3.2 Overview (continued)

Table 3.3. Overview of Transcription and Translation Systems

	System	Plasmid DNA (Circular or Linearized)	PCR-generated DNA	RBS Required[1]	Kozak Preferred[2]	Labeling Options	Signal cleavage & glycosylation with CMM[3]	Control DNA & Detection Reagent[4]	Yield
Rabbit	TnT® Coupled Reticulocyte Lysate System (T7, T3, or SP6 RNA Polymerase; Cat.# L4610, L4950, L4600)[5]	+[6]	+[7]	–	+	Met, Cys, Leu, FluoroTect™, Transcend™	+	+	3–6µg/ml
	TnT® Quick Coupled Transcription/ Translation (T7 or SP6 RNA Polymerase; Cat.# L1170, L2080)	+[6]	+[7]	–	+	Met, FluoroTect™, Transcend™	+	+	3–6µg/ml
	TnT® T7 Quick for PCR DNA (Cat.# L5540)	NR	+	–	+	Met, FluoroTect™, Transcend™	+	–	3–6µg/ml
Wheat Germ	TnT® Coupled Wheat Germ (T7 or SP6 RNA Polymerase) (Cat.# L4130, L4140)[4]	+[8]	+[7]	–	+	Met, Cys, Leu, FluoroTect™, Transcend™	–	+	3–6µg/ml
	TnT® SP6 High-Yield Wheat Germ Protein Expression System (Cat.# L3260)	+	+	–	+	Met, Cys, Leu, FluoroTect™, Transcend™	–	–	10–100µg/ml
Insect	TnT® Insect Cell Extract Protein Expression System (Cat.# L1101)	+	NR	–	+	Met, Cys, Leu, FluoroTect™, Transcend™	–	Control DNA	15–75µg/ml
E. coli	*E coli* S30 for Linear DNA (Cat.# L1030) relies on endogenous RNA polymerases	+[9]	+	+	–	Met, Cys, Leu, FluoroTect™, Transcend™	–	+	1–5µg/ml
	S30 T7 High-Yield Protein Expression System (Cat.# L1110)	+	NR	+	–	Met, Cys, Leu, FluoroTect™, Transcend™	–	Control DNA	200–500µg/ml

NR: Not Recommended

[1] DNA templates for TnT® *E.coli* Systems requires the Shine Dalgarno ribosomal binding site (RBS).

[2] DNA templates for eukaryotic TnT® Systems should preferably contain the Kozak consensus sequence for translation initiation.

[3] CMM: Canine Microsomal Membranes.

[4] Control DNA contains the firefly luciferase gene. Luciferase activity is detected by the Luciferase Assay Reagent (Cat.# E1500).

[5] Translation reactions can be further optimized by adding Mg^{2+} and K^{+}.

[6] SP6 circular plasmids give higher yields than T7 or T3 circular plasmids; T7 or T3 linearized plasmid may be considered as templates; SP6 linearized plasmids are not recommended.

[7] Not recommended for SP6 containing template.

[8] For T7 circular plasmids include the T7 terminator sequence; otherwise linearized plasmids are preferred; for SP6 templates only circular plasmids.

[9] Only linearized templates.

TnT® Coupled Reticulocyte Lysate Systems

Robust eukaryotic cell-free expression systems for the expression of functional mammalian proteins in a simple one-step procedure.

Description and Principle

We offer two types of Rabbit Reticulocyte Lysate Transcription and Translation (TnT®) Systems: The TnT® Coupled (T7, T3, SP6) System and the TnT® Quick Coupled (T7, SP6) System. The main difference between these systems is that the TnT® Quick Coupled System provides a master mix containing all the reaction components required in one tube, whereas the TnT® Coupled System has all the reaction components provided in separate tubes **(Figure 3.3)**. TnT® T7 Quick for PCR DNA is a rapid and convenient coupled TnT® System designed for expression of PCR-generated DNA templates. The system is robust and able to express a variety of proteins ranging in size from 10–150kDa. The lysates are supplied with all reagents needed for TnT® reactions including RNA polymerases. To use these systems, DNA is added directly to TnT® Lysate and incubated in a 50µl reaction for 60–90 minutes at 30°C. See **Table 3.1** for additional applications.

Features and Benefits

- **Use in Multiple Applications:** The TnT® Systems are widely used for functional genomics and proteomics analyses.
- **Save Time:** The TnT® Reaction is completed in a maximum of 1.5 hours.
- **Complete System:** All reagents for the TnT® Reaction are provided (except for labeled amino acids).
- **Reliable:** Can eliminate solubility issues by using an in vitro mammalian system.

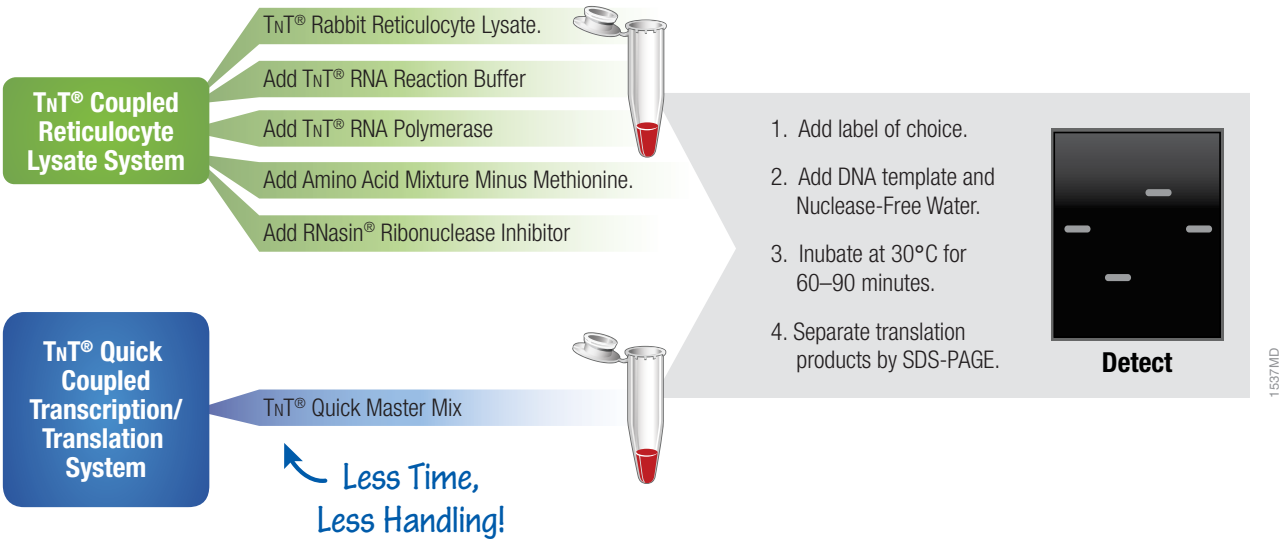


Figure 3.3. Comparison of the TnT® Coupled Reticulocyte Lysate System and the TnT® Quick Coupled Transcription/Translation System protocols.

TnT® Coupled Reticulocyte Lysate Systems (continued)

Eukaryotes

SP6

5'$(N)_{6–10}$ - TATTTAGGTGACACTATAG$(N)_{3–6}$ - CCACC**ATG**G - $(N)_{17–22}$ -3'

SP6 Promoter | Kozak region | Sequence-specific Nucleotides

T7

5'$(N)_{6–10}$ - TAATACGACTCACTATAGGG$(N)_{3–6}$ - CCACC**ATG**G - $(N)_{17–22}$ -3'

T7 Promoter | Kozak region | Sequence-specific Nucleotides

Figure 3.4. Forward primers used to generate PCR fragments for protein expression in TnT® Systems.

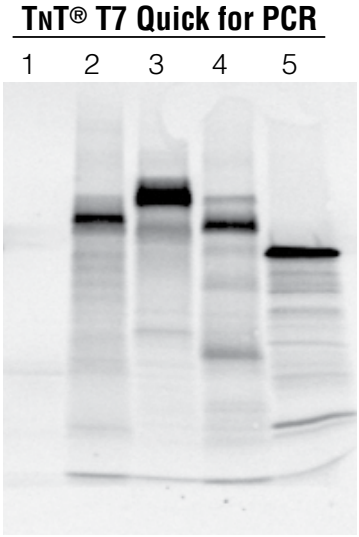


Figure 3.5. TnT® T7 Quick for PCR was used to express variants of the APC gene and BRCA1 gene. PCR fragments were used as starting material for the TnT® reaction. Transcend™ tRNA was included in the reaction for the incorporation of biotinylated lysine residues. **Lane 1** contains the no DNA controls; **lane 2**, APC Seg 2 PCR fragment; **lane 3**, APC Seg 3 PCR DNA fragment; **lane 4**, BRCA1 Seg 3 PCR fragment; **lane 5**, the Luciferase T7 Control DNA.

Ordering Information

TnT® Coupled Reticulocyte Lysate Systems:

TnT® SP6 Coupled Reticulocyte Lysate System (Cat.# L4600)

TnT® T7 Coupled Reticulocyte Lysate System (Cat.# L4610)

TnT® T3 Coupled Reticulocyte Lysate System (Cat.# L4950)

TnT® Quick Coupled Transcription/ Translation Systems:

TnT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170)

TnT® SP6 Quick Coupled Transcription/Translation System (Cat.# L2080)

TnT® T7 Quick for PCR DNA (Cat.# L5540)

TnT® SP6 High-Yield Wheat Germ Protein Expression System

In vitro protein synthesis starting from DNA.

Description

The TnT® SP6 High-Yield Wheat Germ Protein Expression System is a convenient, quick, single-tube, coupled transcription/translation system designed to express up to 100µg/ml of protein. The TnT® SP6 High-Yield Wheat Germ Protein Expression System expresses genes cloned downstream of an SP6 RNA polymerase promoter. This cell-free expression system is prepared from an optimized wheat germ extract and contains all the components (tRNA, ribosomes, amino acids, SP6 RNA polymerase, and translation initiation, elongation and termination factors) necessary for protein synthesis directly from DNA templates. See **Table 3.1** for additional applications.

Principle

The TnT® SP6 High-Yield Wheat Germ Protein Expression System can be used with standard plasmid DNA or PCR-generated templates containing the SP6 promoter. However, to achieve optimal yield, specialized vectors designed for Wheat Germ Extracts such as pF3A WG (BYDV) Flexi® Vector or pF3K WG (BYDV) Flexi® Vector are recommended. DNA templates are directly added to the SP6 High Yield Master Mix and incubated in a 50µl reaction for 2 hours at 25°C. Expressed proteins can be used directly or purified for related applications.

Features and Benefits

- **Save Time:** Generate proteins in two hours, compared to days when using cell-based (*E. coli*) systems.
- **Choose Your Format:** Use plasmid- or PCR-generated templates.
- **Generate Full-Length Protein:** Generate soluble, full-length protein and avoid problems associated with *E. coli* systems.

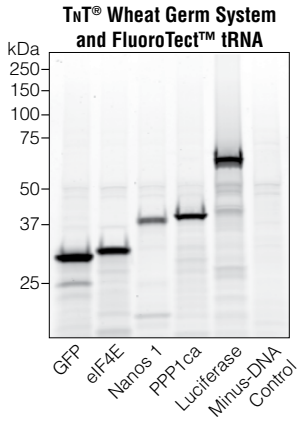


Figure 3.6. Proteins of different size and origin were expressed using TnT® SP6 High-Yield Wheat Germ Protein Expression System in the presence of FluoroTect™ tRNA for lysine residue labeling. Samples were separated by SDS-PAGE and imaged using a fluorescence scanner.

Ordering Information

TnT® SP6 High-Yield Wheat Germ Protein Expression System (Cat.# L3260, L3261)

TnT® T7 Insect Cell Extract Protein Expression System

In vitro protein synthesis starting from a DNA template.

Description

The TnT® T7 Insect Cell Extract Protein Expression System is a convenient, quick, single-tube, coupled transcription and translation system for the cell-free expression of proteins. See **Table 3.1** for additional applications.

Principle

The extract is made from the commonly used *Spodoptera frugiperda* Sf21 cell line. All components necessary for transcription and translation are present in the TnT® T7 Insect Cell Extract (ICE) Master Mix. Proteins are expressed from genes cloned downstream of the T7 promoter in ICE vectors such as pF25A or pF25K ICE T7 Flexi® Vector **(Table 9.1)**. These vectors contain 5´- and 3´-untranslated (UTR) sequences from the baculovirus polyhedrin gene to enhance translation efficiency. After addition of the DNA template, protein synthesis is initiated. The reactions are incubated at 28–30°C and are complete within 4 hours. Approximately 15–75µg/ml of functional protein can be produced using the TnT® T7 Insect Cell Extract Protein Expression System.

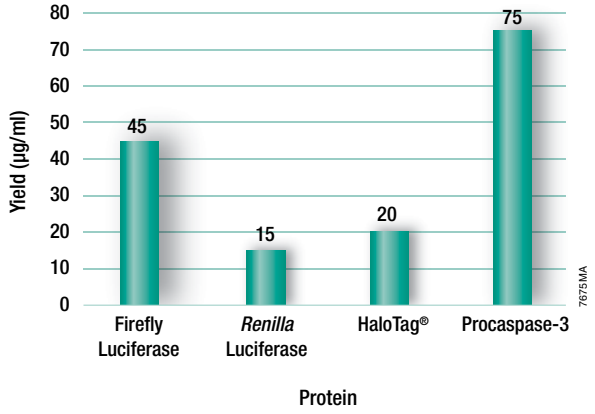


Figure 3.7. Typical protein yields using the TnT® T7 Insect Cell Extract Protein Expression System.

Features and Benefits

- **Obtain Data Faster:** Protein is expressed in only 4 hours.
- **Achieve High Protein Yields:** Express up to 75µg/ml of protein.
- **Convenient:** Luciferase Control DNA included.

Ordering Information

TnT® T7 Insect Cell Extract Protein Expression System (Cat.# L1102, L1101)

E. coli S30 Extract System for Linear Templates

In vitro protein synthesis starting from DNA.

Description

The *E. coli* S30 Extract System for Linear Templates allows successful transcription/translation of linear DNA templates. You need only to provide linear DNA containing a prokaryotic *E. coli*-like promoter (such as *lac*UV5, *tac*, λPL (con) and λ-P_R). A ribosome binding site is required to direct the synthesis of proteins in vitro. In vitro-generated RNA from DNA templates lacking an *E. coli* promoter may also be used in this system, but protein yields produced from linear DNA templates will be decreased 1–10%.

Principle

The S30 Extract for Linear Templates is prepared from an *E. coli* B strain (SL119), which is deficient in *Omp*T endoproteinase, Ion protease and exonuclease V (*rec*BCD). The absence of protease activity results in greater stability of expressed proteins. The *rec*D mutation of the SL119 strain produces a more active S30 Extract for Linear DNA than the previously described nuclease-deficient, *rec*BC-derived S30 extracts. However, the S30 Extract for Linear Templates is less active than the S30 Extract System for Circular DNA. An easy-to-perform, nonradioactive positive control reaction using the Luciferase Assay Reagent provided, allows detection of luciferase gene expression in the *E. coli* S30 System for linear templates. The control reaction produces high light output for several minutes, allowing the researcher to choose from several detection methods, including simple visual observation of luminescence.

Features and Benefits

- **Flexible:** Various templates can be used: DNA fragments, PCR-synthesized DNA, ligated overlapping oligonucleotides, in vitro-generated RNA and prokaryotic RNA.
- **Complete:** Contains all necessary components for coupled transcription/translation.
- **Optimized:** Premix is optimized for each lot of S30 Extract.
- **Control DNA:** Easy detection of firefly luciferase expression using (included) Luciferase Assay Reagent.

Ordering Information

E. coli S30 Extract System for Linear Templates (Cat.# L1030)

E. coli S30 T7 High-Yield Protein Expression System

In vitro protein synthesis starting from DNA.

Description

The S30 T7 High-Yield Protein Expression System is an *E. coli* extract-based protein synthesis system. It simplifies the transcription and translation of DNA sequences cloned in plasmid or lambda vectors containing a T7 promoter, by providing an extract that contains T7 RNA polymerase for transcription and all necessary components for translation.

Principle

The *E. coli* S30 T7 High-Yield Protein Expression System is designed to express up to 500µg/ml of protein in one hour from plasmid vectors containing a T7 promoter and a ribosome binding site. The protein expression system provides an extract that contains T7 RNA polymerase for transcription and is deficient in OmpT endoproteinase and Ion protease activity. All other necessary components in the system are optimized for protein expression. This results in greater stability and enhanced expression of target proteins. Control DNA expression results in production of *Renilla* luciferase, which can be detected by Coomassie® Blue staining following SDS-PAGE or assayed with *Renilla* Luciferase Assay System (Cat.# **E2810**).

Features and Benefits

- **Obtain Data Faster:** Protein expression in only one hour.
- **Achieve High Protein Expression:** Express up to 500µg/ml of protein for multiple applications.
- **Scalable:** Convenient screening protocol for high-throughput protein expression.

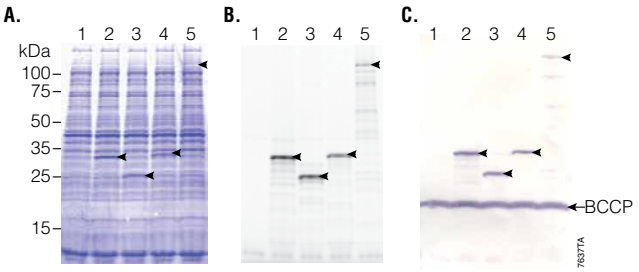


Figure 3.8. Coupled **in vitro** transcription/translation of circular DNA templates using the S30 T7 High-Yield Protein Expression System. The protein-coding sequences cloned into pFN6A (HQ) Flexi® Vector were expressed as described in the *S30 T7 High-Yield Protein Expression System Technical Manual* #TM306, resolved by SDS-PAGE (4–20% Tris-glycine) and visualized by Coomassie® blue staining **(Panel A)**, fluorescence scanning **(Panel B)**, or transferred to PVDF membrane, treated with Streptavidin Alkaline Phosphatase (Cat.# V5591) and stained with Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat..# S3841; **Panel C**). For each gel: lane 1, no DNA; lane 2, *Renilla* luciferase; lane 3, Monster Green® Fluorescent Protein; lane 4, HaloTag® protein; lane 5, α-galactosidase (BCCP = *E. coli* biotin carboxyl carrier protein).

Ordering Information

S30 T7 High-Yield Protein Expression System (Cat.# **L1110, L1115**)

3.3 Cell-Free Protein Labeling Reagents

OVERVIEW

Labeling and detection of proteins expressed using cell-free systems is necessary for most applications such as protein:protein interaction and protein:nucleic acid interaction studies. FluoroTect™ Detection and Transcend™ Detection Systems were developed for non-radioactive protein labeling during cell-free protein synthesis. Both labeling products are based on the incorporation of labeled lysine residues into the polypeptide chain. The labeled protein products can be easily detected either by fluorescent imaging after SDS-PAGE or by western blotting using streptavidin conjugates either to horseradish peroxidase (Strep-HRP) or Alkaline Phosphatase (Strep-AP).

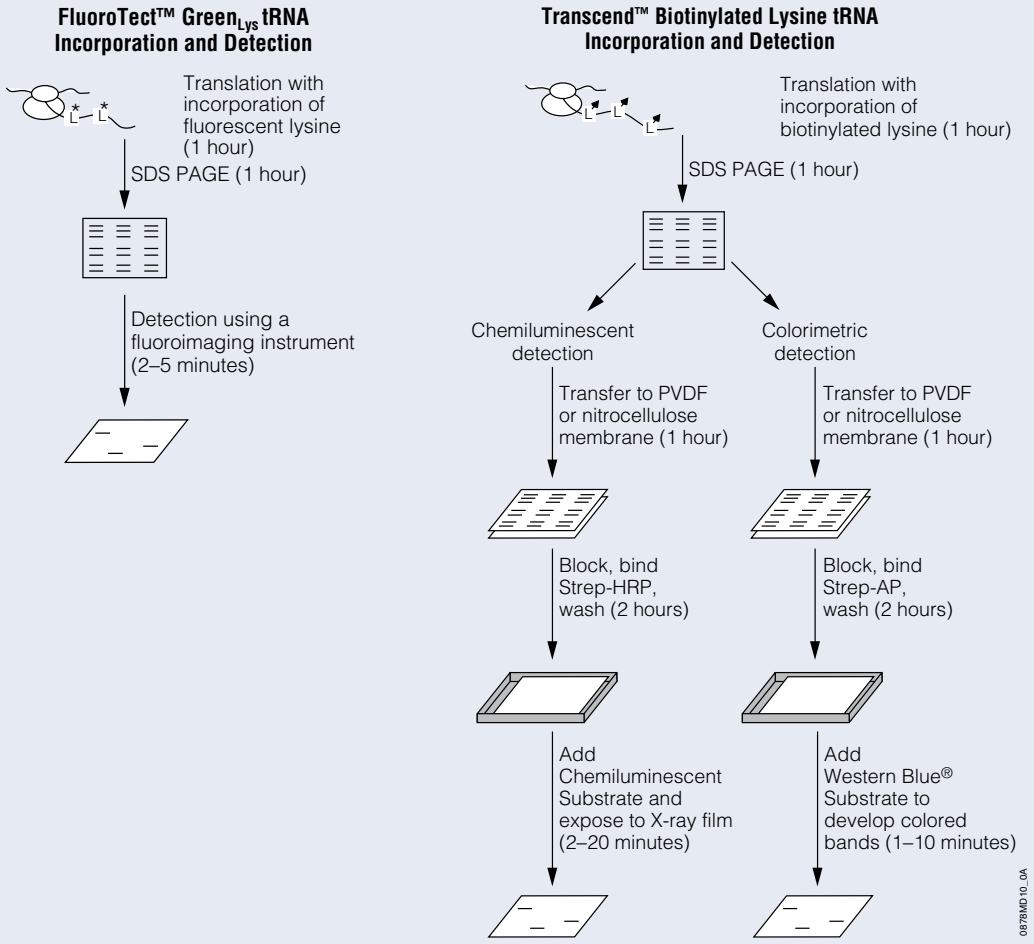


Figure 3.9. Detection protocols using FluoroTect™ Green$_{Lys}$ tRNA and Transcend™ tRNA.

FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System

Labeling and detection of in vitro synthesized proteins.

Description

The FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System allows fluorescent labeling and detection of proteins synthesized in vitro. The system is based on a lysine-charged tRNA, which is labeled at the ε position of the lysine with the fluorophore BODIPY®-FL. Fluorescent lysine residues will be incorporated into synthesized proteins during in vitro translation reactions, eliminating the need for radioactivity.

Principle

Detection of the labeled proteins is accomplished in 2–5 minutes directly "in-gel" by use of a fluorescence gel scanner. This eliminates any requirements for protein gel manipulation, such as fixing/drying or any safety, regulatory or waste disposal issues associated with the use of radioactively-labeled amino acids. The convenience of "in-gel" detection also avoids the time-consuming electroblotting and detection steps of conventional non-isotopic systems.

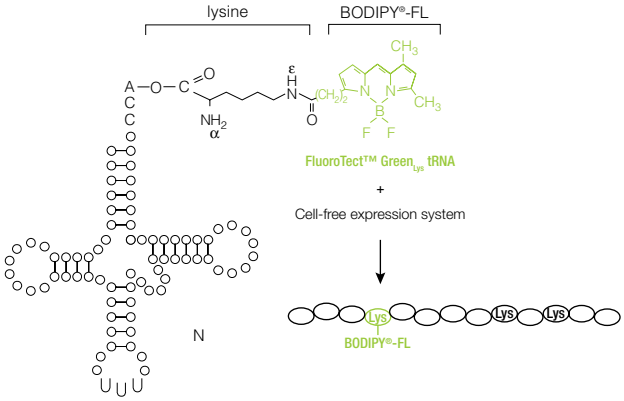


Figure 3.10. Schematic diagram of the incorporation of FluoroTect™ Green$_{Lys}$-labeled lysine into nascent protein.

Features and Benefits

- **Fast:** Data can be obtained in minutes. No requirement to transfer, fix or dry gels.
- **Nonradioactive:** No safety, regulatory or waste disposal issues associated with radioactivity.
- **Flexible:** The modified charged tRNA can be used with: Rabbit Reticulocyte Lysate, TNT® Coupled Transcription/Translation System, Wheat Germ Extract and *E. coli* S30 Extract.

Ordering Information

FluoroTect™ Green$_{Lys}$ in vitro Translation Labeling System (Cat.# L5001)

Transcend™ Nonradioactive Translation Detection Systems

Labeling and detection of in vitro synthesized proteins.

Description

The Transcend™ Nonradioactive Translation Detection Systems allow nonradioactive detection of proteins synthesized in vitro. Using these systems, biotinylated lysine residues are incorporated into nascent proteins during translation, eliminating the need for labeling with [^{35}S]methionine or other radioactive amino acids

Principle

This biotinylated lysine is added to the translation reaction as a precharged ε-labeled biotinylated lysine-tRNA complex (Transcend™ tRNA) rather than a free amino acid. After SDS-PAGE and blotting, the biotinylated proteins can be visualized by binding either Streptavidin-Alkaline Phosphatase (Streptavidin-AP) or Streptavidin-Horseradish Peroxidase (Streptavidin-HRP), followed either by colorimetric or chemiluminescent detection (see Chapter 8). Typically, these methods can detect 0.5–5ng of protein within 3–4 hours after gel electrophoresis. This sensitivity is equivalent to that achieved with [^{35}S]methionine incorporation and autoradiographic detection 6–12 hours after gel electrophoresis.

Features and Benefits

- **Sensitive:** The biotin tag allows detection of 0.5–5ng of translated protein.
- **Safe:** No radioisotope handling, storage or disposal is required.
- **Flexible:** Results can be visualized by using colorimetric or chemiluminescent detection.

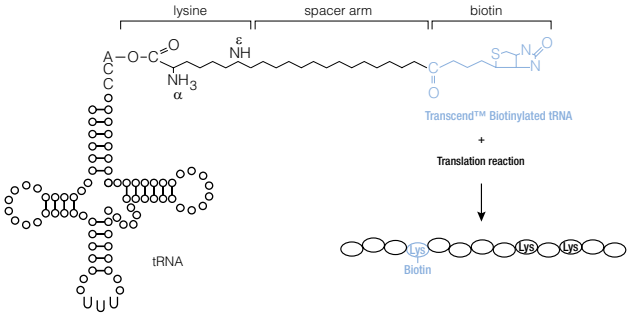


Figure 3.11. Schematic diagram of the incorporation of Transcend™ labeled lysine into nascent protein.

Ordering Information

Transcend™ Colorimetric Translation Detection System (Cat.# L5070)

Transcend™ Chemiluminescent Translation Detection System (Cat.# L5080)

3.4 Membrane Vesicles for Signal Peptide Cleavage and Core Glycosylation

OVERVIEW

Microsomal vesicles are used to study cotranslational and initial post-translational processing of proteins. Processing events such as signal peptide cleavage, membrane insertion, translocation and core glycosylation can be examined by the translation of the appropriate mRNA in vitro in the presence of these microsomal membranes.

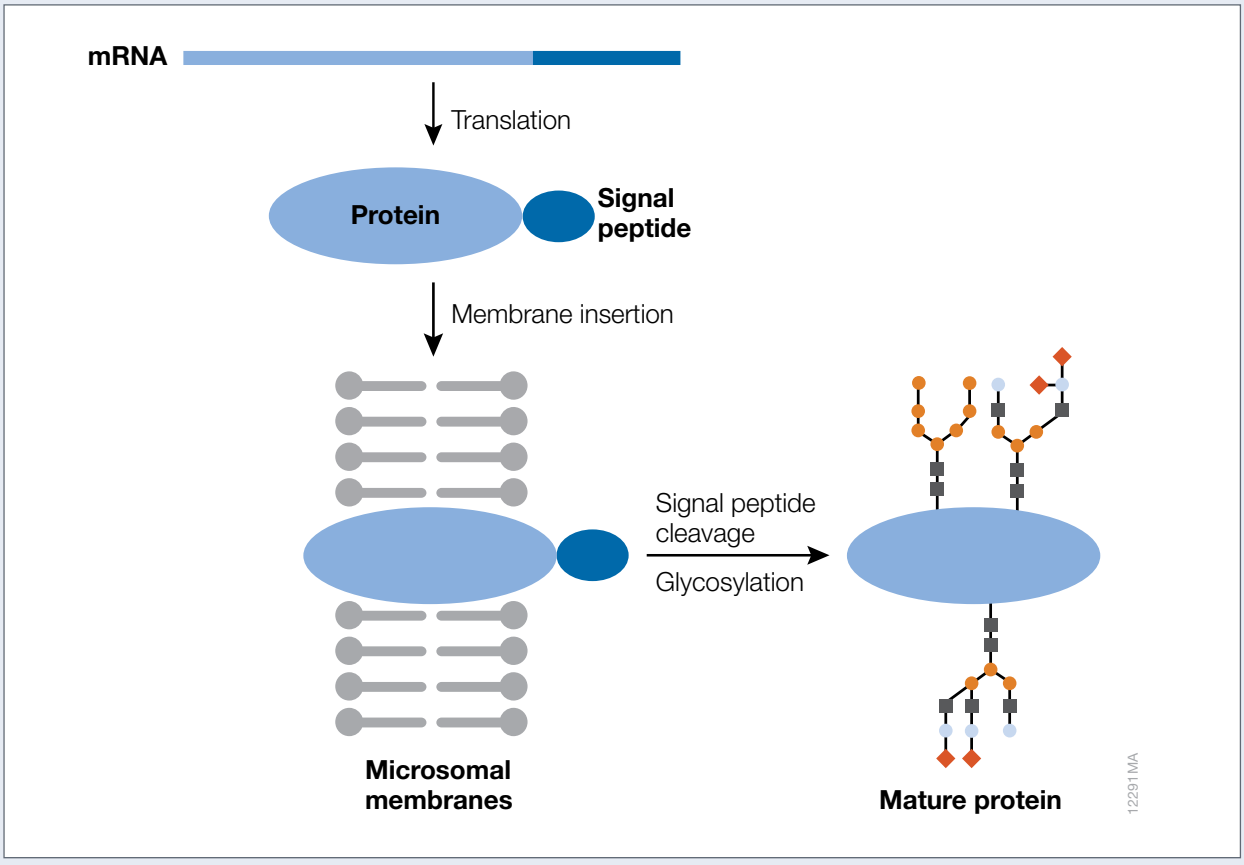


Figure 3.12. Schematic of signal peptide cleavage and introducing core glycosylation by use of canine microsomal membranes in combination with rabbit reticulocyte lysate cell-free protein expression system.

Canine Pancreatic Microsomal Membranes

Examination of signal peptide cleavage, membrane insertion, translocation and core glycosylation of in vitro expressed proteins.

Description

Canine Pancreatic Microsomal Membranes are used to study cotranslational and initial posttranslational processing of proteins in combination with in vitro expressed protein using Rabbit Reticulocyte Systems (RRLs). Processing events such as signal peptide cleavage, membrane insertion, translocation and core glycosylation can be examined by the translation of the appropriate mRNA in vitro in the presence of these microsomal membranes. In addition, processing and glycosylation events may be studied by transcription/translation of the appropriate DNA in TnT® RRL Systems.

Principle

Processing and glycosylation events can be studied with Rabbit Reticulocyte Lysate Cell-free expression systems. To assure consistent performance with minimal translational inhibition and background, microsomes have been isolated free of contaminating membrane fractions and stripped of endogenous membrane-bound ribosomes and mRNA. Membrane preparations are assayed for both signal peptidase and core glycosylation activities using two different control mRNAs. The two control mRNAs supplied with this system are the precursor for β-lactamase (or ampicillin resistance gene product) from *E. coli* and the precursor for α-mating factor (or α-factor gene product) from *S. cerevisiae*.

Features and Benefits

- **Minimal Translational Inhibition, Minimal Background:** Microsomes are stripped of endogenous membrane-bound ribosomes and mRNA.
- **Compatible:** Can be used with TnT® RRL Systems, Rabbit Reticulocyte Lysate and Flexi® Lysate.
- **Reliable Results:** Control mRNAs are supplied.

Ordering Information

Canine Pancreatic Microsomal Membranes (Cat.# Y4041)

Chapter 3 References

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Cell-toxic Proteins, Membrane Protein, Viral Proteins, Kinases

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Protein Labeling

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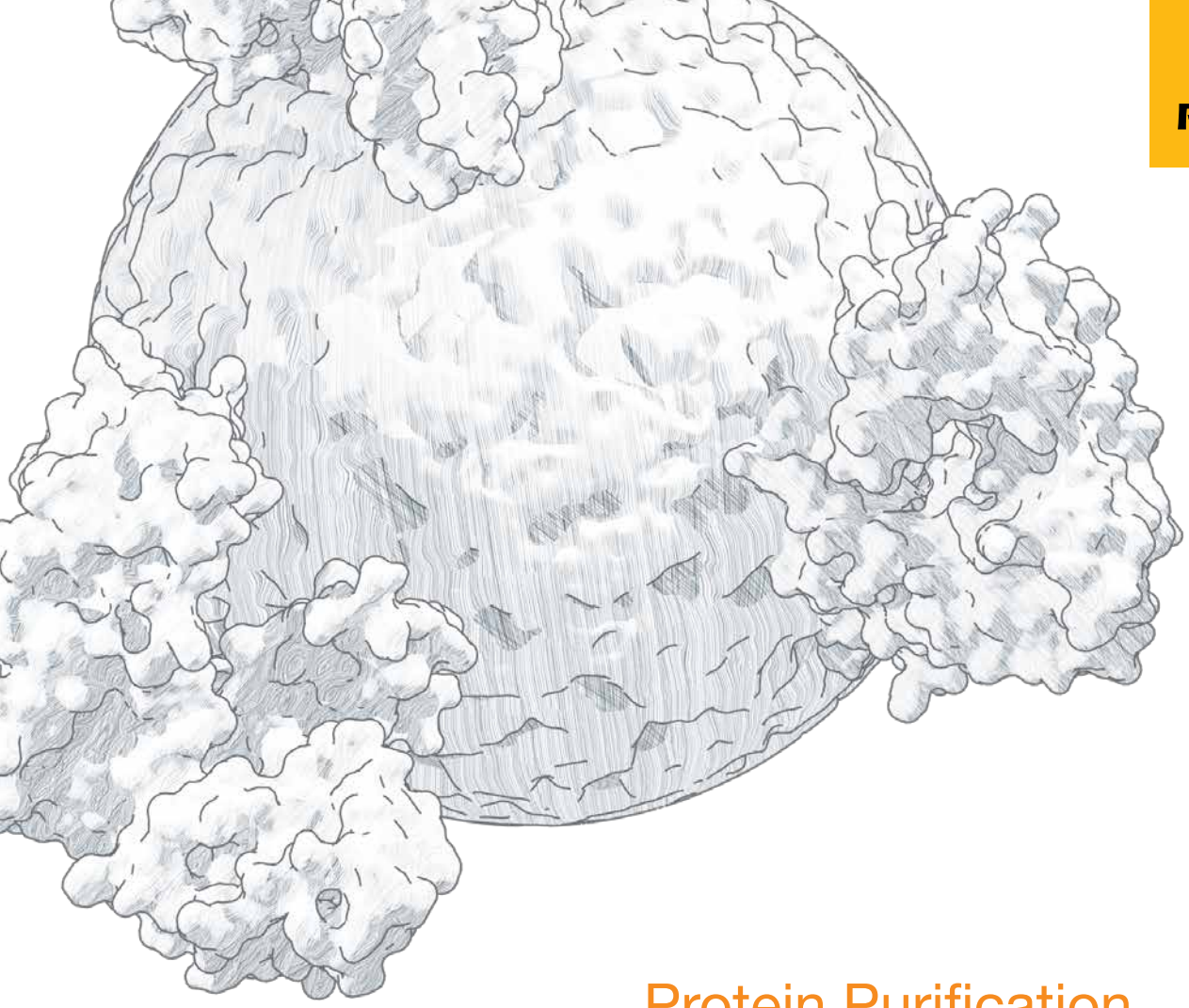
4 Protein Purification



Protein Purification

Protein purification is fundamental to the study of protein function and involves a series of processes to express, enrich and purify a protein of interest from a complex mixture such as cell lysate. The fastest and most powerful method for this purpose is affinity purification. During the purification of recombinant proteins it is convenient to use affinity tags such as HaloTag, GST- or His-tag. By introducing a cleavage-site between the protein fusion tag and the protein sequence, the tag can be enzymatically removed after the purification procedure.

In this chapter different affinity tags will be introduced as well as the corresponding resins and magnetic supports that can be used for low- to high-throughput protein purification.

4.1 Affinity-based Protein Purification

OVERVIEW

Affinity purification is a powerful method to enrich and purify a recombinant protein from a cell lysate. For this purpose an affinity tag is placed either on the N- or C-terminus of the protein of interest. There are numerous affinity tags that can be used for protein purification; the most common tags are listed in **Table 4.1**. Affinity tags vary greatly based on size, strength-of-binding and effect on protein solubility and stability.

The first step to purification of a recombinant protein is the preparation of the cell lysate or supernatant. Cell lysis can be accomplished using a variety of methods, including nonenzymatic methods (e.g., sonication or French press) or the use of hydrolytic enzymes, such as lysozyme (for *E. coli*) or detergent reagents such as FastBreak™ Cell Lysis Reagent. For secreted proteins, minimal supernatant preparation is required, followed by selective binding, washing and elution of the purified protein. After purification the protein may be cleaved with a protease to remove the affinity tag.

Table 4.1. Comparison of Properties of Commonly Used Affinity Tags for Protein Purification.

Tag	Size (kDa)	Affinity Strength	Solubility Enhancement
His	~1	++	–
Strep	~1	+++	–
FLAG HA	~1	+++	–
GST	~26	+	++
MBP	~42	+	+++
HaloTag	~34	++++	+++
SUMO	~12	+	+++

Types of Affinity Tags

Polyhistidine (His) Tags

The most commonly used tag to purify and detect recombinant expressed proteins is the polyhistidine tag. Protein purification using His tags relies on the affinity of histidine residues for immobilized chelated metal such as nickel, which allows selective protein purification. The metal is immobilized and is covalently attached to a solid support such as agarose beads.

His tags offer several advantages for protein purification. The small size of the His tag makes it less immunogenic than larger tags, although it does not enhance the solubility of the fused proteins. The His tag can be placed on either the N- or C-terminus of the protein of interest. And finally, the interaction of the polyhistidine tag with the metal support material does not depend on the tertiary structure of the His tag, making it possible to purify proteins under denaturing conditions such as 8M urea and 6M guanidine hydrochloride. Post binding and washing, the protein is eluted with a high concentration of imidazole, (greater than 100mM) or with other elution methods, including low pH, and EDTA.

Chapter 4.1 Overview (continued)

Biotin Tags

The power of the streptavidin-biotin interaction is used in a number of applications, including the detection of various biomolecules. Bacterial streptavidin and its analog chicken avidin have a very high binding affinity for biotin (vitamin H). In fact, this is one of the strongest non-covalent interactions known in biology. The strength of the binding is a major drawback for its use as a purification technique, since the elution conditions would have to be so harsh that they would destroy the purified protein.

Nevertheless, there are several ways to circumvent this strong binding and harness the power of the streptavidin-biotin interaction for the purification of recombinant proteins. One strategy is to fuse the protein of interest (POI) to a peptide sequence that is biotinylated in vivo. The biotinylation peptide sequence is fused to a protease cleavage site allowing a gentle release of the protein of interest by protease digestion. Other strategies are based on biotin-mimicking peptides such as Strep Tag, which binds to native and modified versions of streptavidin and avidin, enabling successful protein elution under mild conditions (e.g., competitive elution with D-desthiobiotin).

Glutathione-S-Transferase (GST)

The use of the affinity tag glutathione-S-transferase (GST) is based on the strong affinity of GST for immobilized glutathione-covered matrices. Glutathione-S-transferases are a family of multifunctional cytosolic proteins that are present endogenously in eukaryotic organisms but normally not found in bacteria. As such, GST-based purification is not recommended for eukaryotic systems (e.g., insect, mammalian). The 26kDa GST affinity tag enhances the solubility of many eukaryotic proteins expressed in bacteria. After capture and washing, the tagged protein is eluted with soluble glutathione.

HaloTag® Protein Tag

Protein fusion tags are frequently used to aid in expression of suitable levels of soluble protein as well as for purification. The HaloTag® fusion protein is engineered to enhance expression and solubility of recombinant proteins in *E. coli*, as well as to provide superb recovery of even low-expressing proteins, such as in mammalian expression systems. HaloTag® technology is based on the formation of a covalent bond between a protein fusion tag (HaloTag®) and synthetic chemical ligands. By interchanging different synthetic ligands researchers ultimately control the function and properties of the HaloTag® fusion protein. The HaloTag® system is designed to provide broad experimental flexibility and superb performance in both cell-based and biochemical assays such as protein purification, without switching fusion tags, or recloning. Available HaloTag® ligands include magnetic and nonmagnetic beads, glass slides, cell-permeable and impermeable fluorescent ligands, biotin, and reactive ligands to enable researchers to build their own custom HaloTag® ligands and surfaces. **(Figure 4.1)**.
For protein purification HaloTag® Technology is compatible with many protein expression systems and can be applied to proteins expressed in *E. coli*, mammalian cells and cell-free systems. HaloTag® protein is a good affinity tag for purification and also a good solubility tag. The lack of an endogenous equivalent of the HaloTag® protein in mammalian and most prokaryotic cells minimizes the chances of nonspecific interactions. The combination of covalent capture with rapid binding kinetics overcomes the equilibrium-based limitations associated with traditional affinity tags, and enables efficient capture even at low expression levels.

Chapter 4.1 Overview (continued)

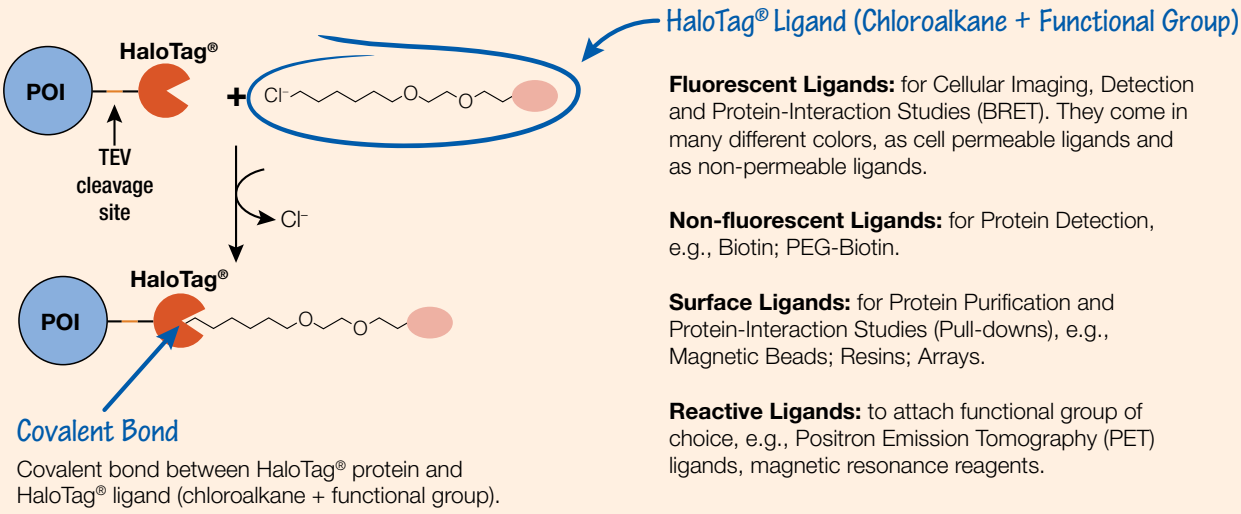


Figure 4.1. Schematic of HaloTag® technology consisting of the HaloTag® protein fused to a protein of interest (POI) and a selection of synthetic HaloTag® ligands carrying different functional groups. HaloTag® ligands specifically and covalently bind to the HaloTag® protein fusions.

HaloTag® Protein Purification System *(E. coli)*

Purification of HaloTag® fusion proteins from *E. coli* lysates.

Description

The HaloTag® Protein Purification System allows covalent, efficient and specific capture of proteins expressed in *E. coli* as HaloTag® fusion proteins. When a HaloTag® protein is fused to a target protein it functions as a robust solubility tag in addition to an affinity purification tag (note that solubility enhancement is achieved when a HaloTag® is fused to the N-terminus of the protein).The HaloTag® Protein Purification System allows stringent washing because of the covalent attachment of the HaloTag® fusion protein to the resin. ProTEV Protease cleaves the protein of interest from the HaloTag® protein, which is bound to the HaloLink™ Resin.

Principle

A protein of interest (POI) fused to a HaloTag® protein will covalently bind to the HaloLink™ Resin. The covalent nature of the linkage enables stringent and lengthy wash conditions without leaching the POI off the resin. After washing, the POI is eluted by proteolytic cleavage with ProTEV Protease. The ProTEV Protease contains a HQ-tag similiar to His-tag, allowing protease removal with the included HisLink™ resin.

Features and Benefits

- **Higher Solubility and Activity:** Compared to His-tag, GST and MBP affinity tags.
- **High Purity:** Covalent capture allows extensive and/or stringent washes without loss of bound protein, resulting in very low (<0.1%) nonspecific binding and a highly pure protein.
- **High Protein Recovery:** Rapid covalent capture; recovery is highly efficient > 75%.
- **High Yield:** >7mg of HaloTag® fusion protein per ml of HaloLink™ Resin.
- **Tag-Free Protein of Interest:** Due to proteolytic release coupled with protease removal.

Additional Information

Depending on the orientation of fusion constructs, 6 amino acids for N-terminal fusion constructs or 13 amino acids for C-terminal fusion constructs will remain on the POI after ProTEV cleavage.

References

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Ordering Information

HaloTag® Protein Purification System (Cat.# G6280)

HaloTag® Mammalian Protein Purification and Detection Systems

Quick purification of HaloTag® fusion proteins from mammalian cell culture lysates and supernatant.

Description

The HaloTag® Mammalian Protein Purification System is an optimized kit for rapid purification of HaloTag® fusion proteins from mammalian cell culture lysates and cell culture supernatants. An optimized TEV protease recognition site within the interconnecting polypeptide separates the HaloTag® protein and the fusion partner. The kit contains HaloLink™ Resin, HaloTEV Protease, 50X Protease Inhibitor Cocktail as well as Spin Columns. The covalent binding of HaloTag® fusion proteins, coupled with the low nonspecific binding of the HaloLink™ Resin, provides superior purity and recovery of recombinant proteins from cultured mammalian cells, even at low expression levels. The HaloTag® Mammalian Protein Detection and Purification System also contains a fluorescent ligand (TMRDirect™ Ligand) for the easy detection of HaloTag® fusion proteins by in-gel imaging, flow cytometry or microscopy applications. The simple-to-use fluorescent detection of HaloTag® fusion proteins facilitates rapid optimization of expression and purification conditions.

Principle

The protein of interest (POI), when fused to the HaloTag® protein, will covalently bind to the HaloLink™ Resin **(Figure 4.2)**. The covalent nature of the linkage enables stringent and lengthy wash conditions without concern of leaching the protein of interest off the resin. Post wash the POI is eluted by proteolytic cleavage with HaloTEV Protease. Since the HaloTEV Protease is fused to HaloTag® protein, the cleavage step and protease capture can be performed in a single step that separates the POI from the HaloTag fusion tag and the HaloTEV Protease.

Features and Benefits

- **High Purity:** Covalent capture allows extensive and/or stringent washes without loss of bound protein, resulting in very low (<0.1%) nonspecific binding and a highly pure protein.
- **High Protein Recovery:** Rapid covalent capture. Recovery is highly efficient, commonly >75%.
- **High Yield:** >7mg HaloTag® fusion protein per ml of HaloLink™ Resin.
- **Easily Scalable:** From 1ml up to 1L of mammalian cell culture.
- **Easy Detection:** Fluorescent HaloTag® ligands facilitate monitoring and optimization of the protein expression and purification procedure.

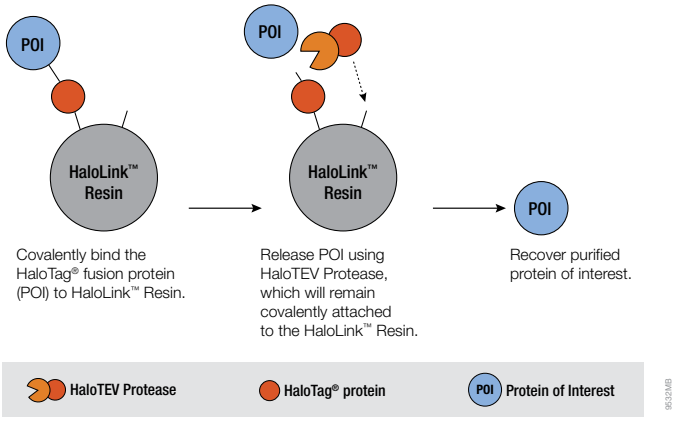

Figure 4.2. Schematic of the purification steps used with the HaloTag® Mammalian Protein Purification System.

HaloTag® Mammalian Protein Purification and Detection Systems (continued)

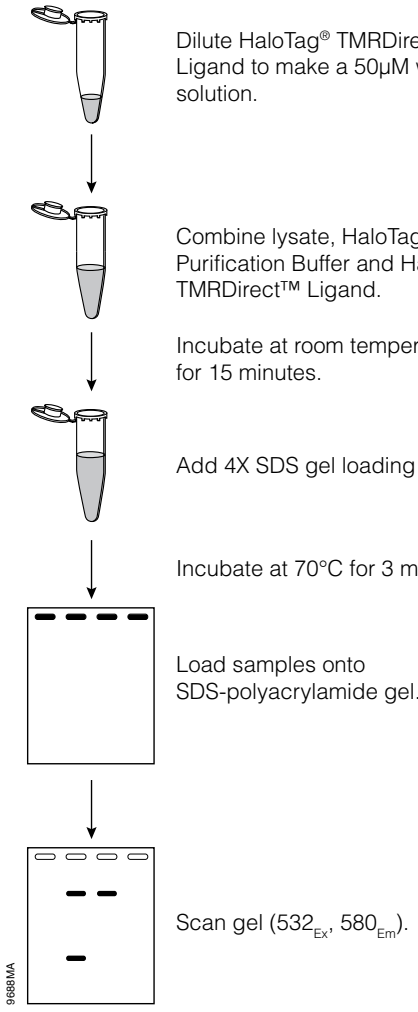

Figure 4.3 Schematic diagram of the fluorescent labeling of HaloTag® fusion protein with the HaloTag® TMRDirect™ Ligand.

Additional Information

Depending on the orientation of fusion constructs, 6 amino acids for N-terminal fusion constructs or 13 amino acids for C-terminal fusion constructs will remain on the POI after HaloTEV cleavage.

Fluorescent labeling of HaloTag® fusion proteins with the HaloTag® TMRDirect™ Ligand provides a rapid and convenient method to optimize protein expression and to monitor purification efficiency. HaloTag® protein fusions are briefly incubated with HaloTag® TMRDirect™ Ligand prior to SDS-PAGE **(Figure 4.3)**. After electrophoresis the proteins can be imaged without additional processing using a either a fluorescence gel scanner, such as GE Typhoon, or BIORAD ChemiDoc™ MP system.

References

Mammalian Cells

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Ordering Information

HaloTag® Mammalian Protein Purification System (Cat.# G6790)

HaloTag® Mammalian Protein Detection and Purification System (Cat.# G6795)

HisLink™ Protein Purification Resin

Purification of polyhistidine (His)-tagged proteins from bacterial and mammalian cells.

Description and Principle

HisLink™ Protein Purification Resin is a macroporous silica resin modified to contain a high level of tetradentate chelated nickel (>20mmol Ni/ml settled resin). The resin is designed to efficiently capture and purify overexpressed polyhistidine-tagged proteins. The HisLink™ Resin also may be used for general applications requiring an immobilized metal affinity chromatography (IMAC) matrix. The resin performs well in either column, batch or vacuum-based methods with a binding capacity of >15mg/ml of resin. The HisLink™ Protein Purification Resin is useful in all general immobilized metal affinity chromatography (IMAC) applications matrix as well as in low- to medium-pressure liquid chromatography systems. In batch format, HisLink™ Resin settles easily and may be separated from the lysate without filtration or centrifugation, simply by decanting. This enables rapid processing of larger quantities of lysate and the ability to purify protein without clearing the lysate of insoluble cellular debris.

Features and Benefits

- **Optimized Yields:** Binding capacity >15mg/ml.
- **Save Time:** Purify polyhistidine- or HQ-tagged proteins from cleared or crude cell lysates.
- **Flexible:** Use standard gravity column chromatography or automated applications such as FPLC.

Ordering Information

HisLink™ Protein Purification Resin
(Cat.# V8823, V8821)

References

Mochida, S. *et al.* (2010) A recombinant catalytic domain of matriptase induces detachment and apoptosis of small-intestinal epithelial IEC-6 cells cultured on laminin-coated surface. *J. Biochem.* **148**(6), 721-32.

Wei, L. *et al.* (2010) LPA19, a Psb27 homolog in Arabidopsis thaliana, facilitates D1 protein precursor processing during PSII biogenesis. *J. Biol. Chem.* **285**(28), 21391-8.

Herold, S. *et al.* (2009) Sab, a novel autotransporter of locus of enterocyte effacement-negative shiga-toxigenic Escherichia coli O113:H21, contributes to adherence and biofilm formation. *Infect. Immun.* **77**(8), 3234-43.

HisLink™ Spin Protein Purification System

Purification of polyhistidine (His)-tagged proteins in small volumes from bacterial cells.

Description

The HisLink™ Spin Protein Purification System provides a simple and fast system for purifying overexpressed His-tagged proteins from a 700µl sample of *E. coli* cell culture, using either a centrifuge- or vacuum-based method. The system contains cell lysis buffer, HisLink™ Resin, DNase I, Buffers, Collection Tubes and Spin Columns.

Principle

Protein can be purified directly from culture medium containing bacterial cells expressing a polyhistidine-tagged protein. The bacterial cells are lysed using FastBreak™ Cell Lysis Reagent, followed immediately by addition of HisLink™ Protein Purification Resin to the culture. Addition of these reagents results in simultaneous bacterial cell lysis and binding of the polyhistidine-tagged proteins. The samples then are transferred to a Spin Column where unbound protein is removed while the affinity resin is washed, and the target protein is recovered by elution. This system requires the use of a tabletop centrifuge or vacuum manifold. A schematic diagram of protein purification using the HisLink™ Spin System is shown in **Figure 4.4**.

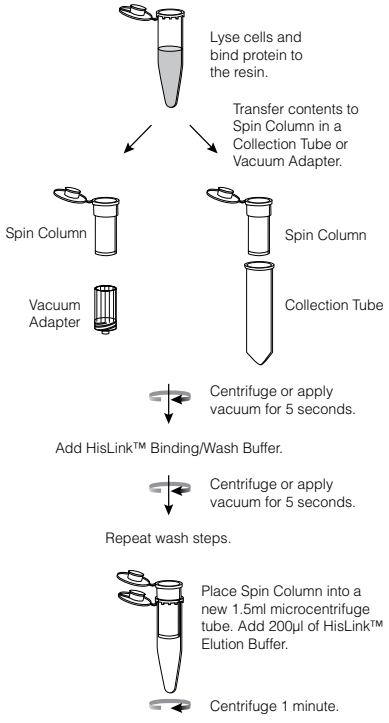


Figure 4.4. Schematic of polyhistidine-tagged protein purification using the HisLink™ Spin Protein Purification System.

Features and Benefits

- **Simple:** No cell culture preparation steps (no preclearing) required.
- **Quick:** No lengthy lysozyme incubations required to lyse cells.
- **Efficient:** Binding capacity of 1mg of polyhistidine-tagged protein per spin column.

Reference

Engel, L. *et al.* (2006) HisLink™ Spin Protein Purification System: Maximum Versatility in a Small Package. *Promega Notes* **93**, 2-4.

Ordering Information

HisLink™ Spin Protein Purification System
(Cat.# V1320)

HisLink™ 96 Protein Purification System

Purification of polyhistidine (His)-tagged proteins from bacterial and mammalian cells using a vacuum-based format.

Description

The HisLink™ 96 Protein Purification System provides a simple, and quick method of high-throughput purification of polyhistidine- or HQ-tagged overexpressed proteins from *E. coli* using a vacuum-based method. The system is designed to purify expressed polyhistidine-tagged proteins directly from deep-well 96-well culture plates. The HisLink™ System is amenable to manual or automated methods, such as the Beckman Coulter Biomek® 2000 or FX for high-throughput applications. The System contains cell lysis buffer, HisLink™ Resin, DNase I, Buffers and HisLink™ 96 Filtration & Collection Plates.

Principle

In preparation for protein purification, bacterial cells expressing a polyhistidine-tagged protein are lysed in culture using the provided FastBreak™ Cell Lysis Reagent. The HisLink™ Resin is added to the lysate and mixed; the polyhistidine-tagged proteins bind within a few minutes. Transfer the samples to a Filtration Plate, wash the resin to remove contaminants, and recover the target protein by elution **(Figure 4.5)**.

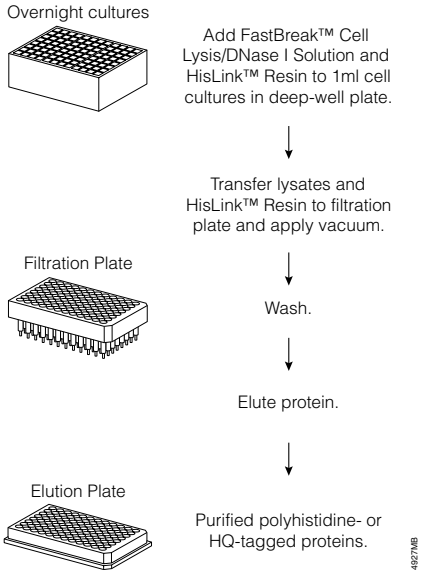


Figure 4.5. Flow diagram of polyhistidine-tagged protein purification using the HisLink™ 96 Protein Purification System.

Features and Benefits

- **Simple:** No centrifugation required—lysis buffer is added directly to cells in culture medium.
- **Quick:** No long lysozyme incubations are required for cell lysis.
- **Versatile:** Perform purification manually or on an automated platform.
- **Efficient:** Binding capacity of 1mg of polyhistidine-tagged protein per well.

Additional Information

Note: This system requires the use of the Vac-Man® 96 Vacuum Manifold or compatible vacuum manifold.

Reference

Engel, L. *et al.* (2005) HisLink™ 96 Protein Purification System: Fast Purification of Polyhistidine-Tagged Proteins. *Promega Notes* **90**, 15–18.

Ordering Information

HisLink™ 96 Protein Purification System
(Cat.# V3680, V3681)

SoftLink™ Soft Release Avidin Resin

Reversible binding and purification of biotinylated proteins under mild nondenaturing conditions, purification of biotinylated antibodies and other proteins as well as in vivo biotinylated fusion proteins from PinPoint™ Vectors.

Description

SoftLink™ Avidin Resin can be used for the isolation and purification of biotinylated molecules. SoftLink™ Resin is a rigid, methacrylate polymeric gel filtration matrix, functionalized with a covalently bound, monomeric avidin that does not leach under elution conditions. The monomeric avidin enables purification of biotinylated proteins under mild elution conditions due to a lower K_d than tetrameric biotin (10^{-7}M vs. 10^{-15}M). Monomeric avidin allows specific capture and gentle elution that preserves the integrity of sensitive proteins.

Features and Benefits

- **Sensitive:** Binds 20–40nmol of biotinylated protein per 1ml of settled resin. Up to 4mg of biotinylated protein has been purified per ml resin.
- **Gentle:** Elution of biotinylated proteins under mild nondenaturing conditions (5mM biotin).
- **Reusable:** Regenerates at least 10 times without loss of binding capacity.
- **Flexible:** Purification by batch or column method, in both mild and stringent conditions such as pH 2-13, ionic strength up to 2M NaCl, denaturing conditions (6M guanidine, 1% SDS).

Ordering Information

SoftLink™ Avidin Resin
(Cat.# V2011, V2012)

References

Hoke, D.E. *et al.* (2008) LipL32 is an extracellular matrix-interacting protein of Leptospira spp. and Pseudoalteromonas tunicata. *Infect. Immun.* **76**(5), 2063–9.

Field, S. *et al.* (2008)The 'zinc knuckle' motif of Early B cell Factor is required for transcriptional activation of B cell-specific genes. *Mol. Immunol.* **45**(14), 3786–96.

Zamft, B. *et al.* (2012) Nascent RNA structure modulates the transcriptional dynamics of RNA polymerases. *Proc. Natl. Acad. Sci. USA* **109**(23), 8948–53.

PinPoint™ Xa Protein Purification System

Production and purification of fusion proteins that are biotinylated in *E. coli*.

Description and Principle

The PinPoint™ Xa Protein Purification System is designed for the production and purification of fusion proteins that are biotinylated at the N-terminus in vivo. The DNA coding for the protein of interest is cloned into a PinPoint™ Vector downstream of a sequence encoding a 14kDa peptide that becomes biotinylated in vivo. Biotinylated fusion proteins are produced in *E. coli* (JM109 and HB101 *E. coli* bacterial strains, or other strains without the birA mutation) and are affinity-purified using the SoftLink™ Soft Release Avidin Resin. The PinPoint™ Vectors feature the encoded endoproteinase Factor Xa, for proteolytic removal of the biotinylated peptide post-purification.

The system contains 3 cloning vectors in multiple combinations of sense reading frames, an avidin-conjugated resin, Streptavidin-Alkaline Phosphatase, a purification column and biotin. The PinPoint™ Xa Control Vector contains the chloramphenicol acetyl-transferase (CAT) gene and is provided as a means of monitoring protein expression, purification and processing conditions. The system generally yields 1–5mg of protein per liter of *E. coli* culture.

Features and Benefits

- **In vivo Biotinylation Tag:** Allows purification of fusion proteins labeled with biotin.
- **Easy-to-Use:** Purification of biotinylated proteins with the SoftLink™ Resin can be performed by column or batch purification.
- **Flexible:** PinPoint™ Vectors are supplied for all reading frames.
- **Gentle Release Conditions:** SoftLink™ Resin allows release of the fusion protein under nondenaturing conditions.

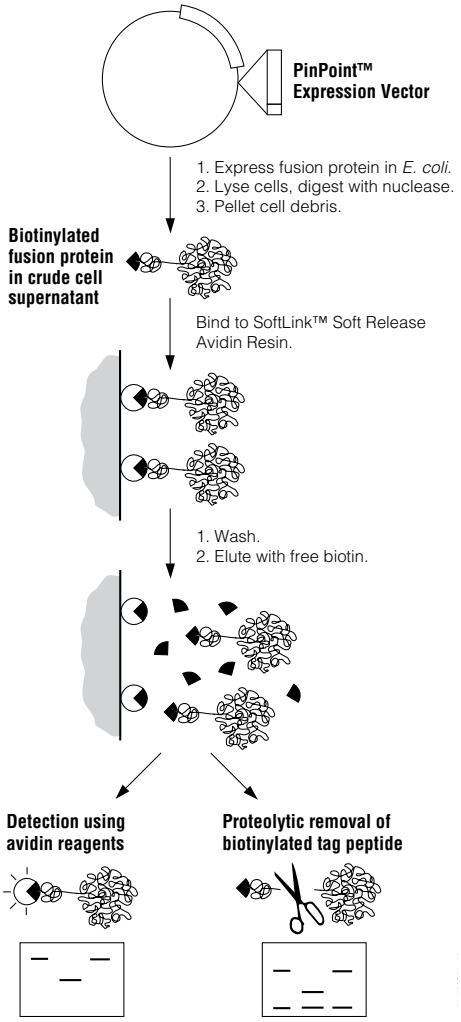


Figure 4.6. Schematic diagram of recombinant protein expression and purification using the PinPoint™ Xa Protein Purification System.

Ordering Information

PinPoint™ Xa Protein Purification System (Cat.# V2020)

4.2 Magnetic Affinity-based Purification and Pull-down Strategies

OVERVIEW

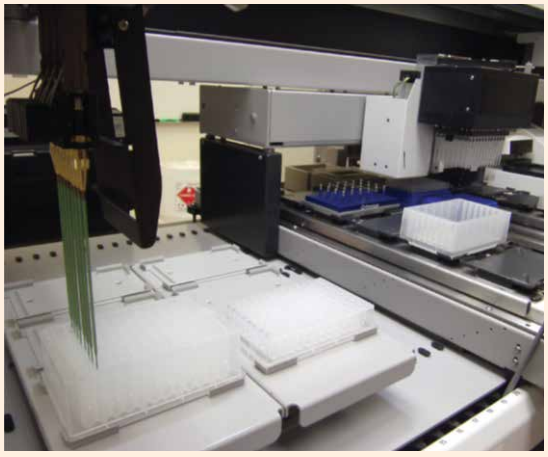
The use of magnetic beads for protein purification/pull-down provides a rapid and efficient method to extract and capture recombinant proteins from cell lysates and cell culture supernatant. Virtually all affinity chemistries discussed previously can be transferred on magnetic beads. There are many advantages of a magnetic approach:

1. Easily process very small sample volumes. Proteins from volumes as low as 20µl can be processed with good efficiency and minimal target protein losses.
2. Simple and cost effective approach that does not require extensive plumbing, chromatography instrumentation or centrifugation.
3. Protein samples can be eluted into a minimal volume resulting in higher concentration of purified protein. This can be used for protein concentration purposes.
4. Faster washing and elution steps because no centrifugation steps are required.
5. More readily adapted for high-throughput applications on automated liquid-handling robots **(Figure 4.7).**
6. Magnetic approach excels at parallel sample processing, where multiple samples need to be processed as fast as possible.

All previously discussed affinity chemistries (His, HaloTag® GST) are available for protein capture in a magnetic bead format and are amenable to both protein purification and protein pull-down applications.

A.

B.



10543TB

Figure 4.7. Magnetized beads in an Eppendorf tube during protein purification from small (µl) sample volumes **(Panel A)**. Tecan automated liquid handler processing magnetic beads during high-throughput purification **(Panel B)**.

MagneGST™ Protein Purification System

Manual or automated magnetic purification or pull-down of glutathione-S-transferase (GST) fusion proteins from crude or cleared *E. coli* lysates.

Description

The MagneGST™ Protein Purification System includes immobilized glutathione paramagnetic particles, buffers and cell lysis reagents to isolate GST-tagged protein directly from a crude or cleared lysate. GST-tagged proteins can be purified from 1ml to 50ml of culture. Samples also can be processed using a robotic platform. MagneGST™ particles are supplied as a 25% slurry and have a binding capacity of 5–10mg of GST protein per 1ml of settled resin.

Principle

Bacterial cells containing a GST-fusion protein are lysed using the provided MagneGST™ Cell Lysis Reagent or with an alternative lysis method, then the MagneGST™ particles are added directly to the crude lysate. GST-fusion proteins bind to the MagneGST™ particles. Unbound proteins are washed away, and the GST-fusion target protein is recovered by elution with 50mM glutathione **(Figure 4.8)**.

For capture and verification of interacting proteins (pull-down), the GST-tagged bait protein is expressed in *E. coli* and the potential prey proteins are expressed in cell-free system. The bait is bound on the MagneGST™ particles and is then used to capture different prey proteins from cell-free expression reactions **(Figure 4.8).**

Features and Benefits

- **Simple:** One-step purification of multiple samples with easy handling. No lysate clearing needed.
- **Scalable Protocol:** Use 1–50ml of cell culture. Obtain 5–10mg of GST protein per 1ml of settled beads.
- **Efficient:** Achieve high yields with little or no nonspecific background.

References

Meloni, A. *et al.* (2010) DAXX is a new AIRE-interacting protein. *J. Biol. Chem.* **285**(17), 13012–21.

Maier R.H, *et al.* (2010) Epitope mapping of antibodies using a cell array-based polypeptide library. *J. Biomol. Screen.* **15**(4), 418–26.

Morimoto, H. *et al.* (2008) Procollagen C-proteinase enhancer-1 (PCPE-1) interacts with beta2-microglobulin (beta2-m) and may help initiate beta2-m amyloid fibril formation in connective tissues. *Matrix Biol.* **27**(3), 211–9.

Ordering Information

MagneGST™ Protein Purification System (Cat.# V8600, V8603)

MagneGST™ Protein Purification System (continued)

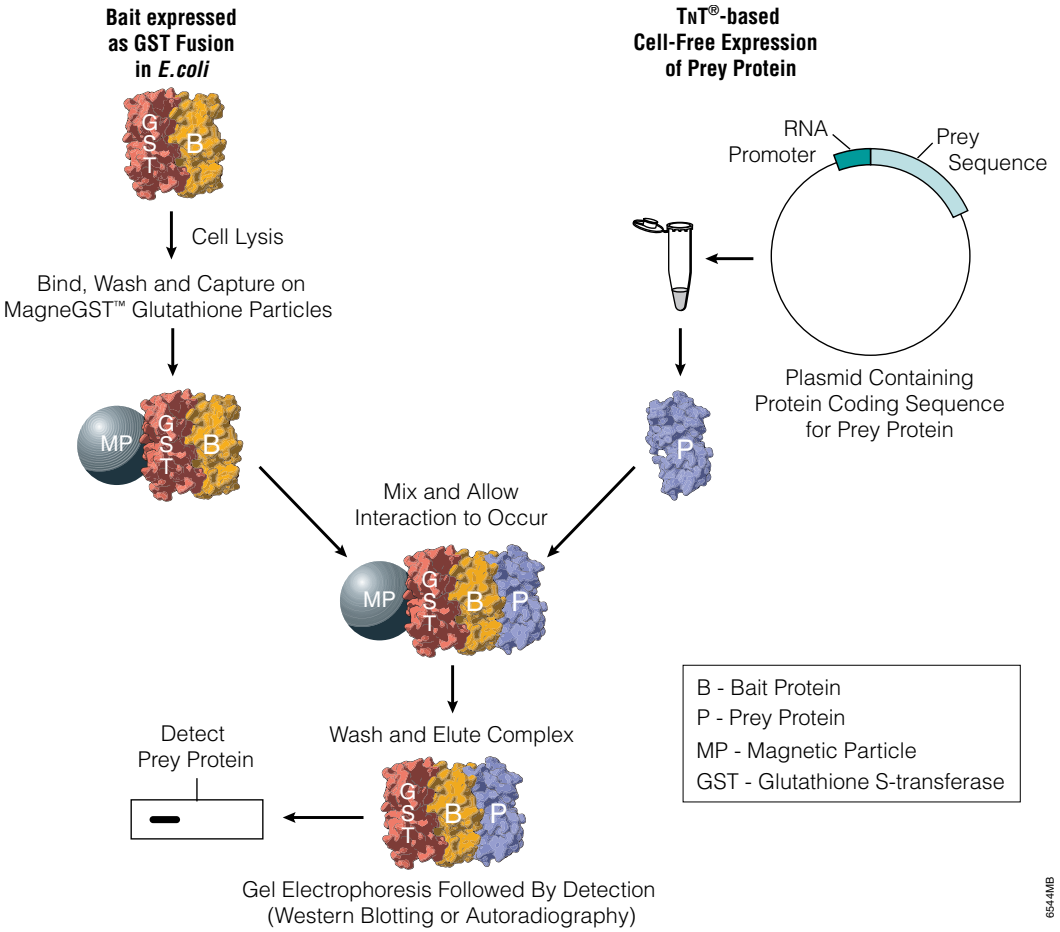


Figure 4.8. Schematic diagram of MagneGST™ Protein Purification in combination with protein pull-down.

Magne™ HaloTag® Beads

Manual or automated high-throughput protein purification and pull-down of HaloTag® fusion proteins from *E. coli* and mammalian cell cultures, at input volumes from 20µl to 50ml.

Description and Principle

The Magne™ HaloTag® Beads provide a convenient method to covalently capture HaloTag® fusion proteins with magnetic particles for protein pull-downs and purification. HaloTag® fusion proteins may be expressed in cell-based and cell-free systems. Cell lysates are mixed with Magne™ HaloTag® Beads and HaloTag® fusion proteins bound covalently to the beads. These magnetic beads offer a high-binding capacity (≥20mg/ml) for purifying HaloTag® fusion proteins with low nonspecific protein binding. After washing, the protein of interest can be released from the beads by TEV Protease cleavage (either HaloTEV or ProTEV) at the optimized TEV recognition site. Proteolytic release yields the protein of interest, while the HaloTag® protein and HaloTEV Protease remain covalently attached to the beads **(Figure 4.9)**.

Features and Benefits

- **Simple:** No centrifugation or vacuum is required once the cells are lysed.
- **High Recovery:** Binding capacity ≥20mg of purified HaloTag® fusion protein per ml of settled particles.

References

Verger A. *et.al.* (2013) The Mediator complex subunit MED25 is targeted by the N-terminal transactivation domain of the PEA3 group members. *Nucl. Acids Res.* **41**(9), 4847–59.

Yoshida S. *et al.* (2013) Androgen receptor promotes sex-independent angiogenesis in response to ischemia and is required for activation of vascular endothelial growth factor receptor signaling. *Circulation* **2**(128), 60–71.

Nagaki K. *et al.* (2012) Isolation of centromeric-tandem repetitive DNA sequences by chromatin affinity purification using a HaloTag7-fused centromere-specific histone H3 in tobacco. *Plant Cell Rep.* **31**(4), 771–9.

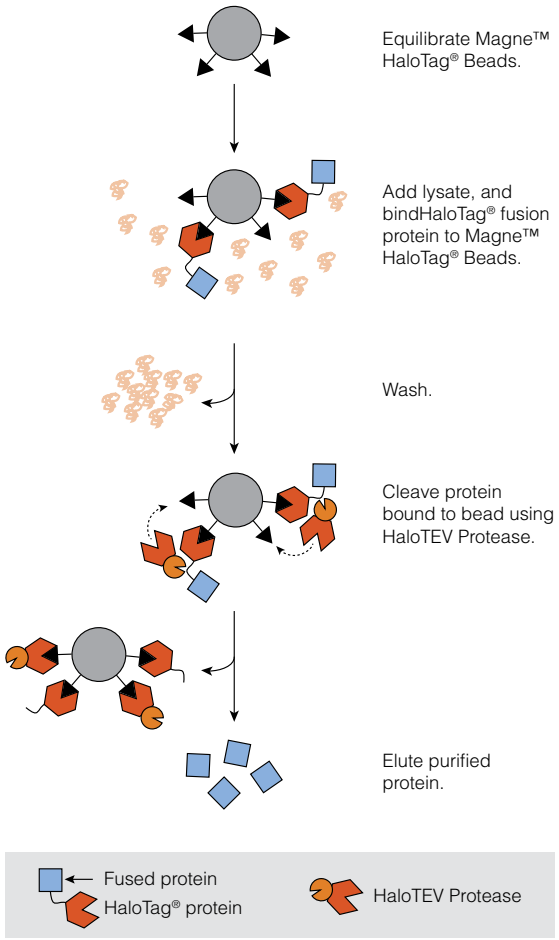


Figure 4.9 Schematic diagram of the HaloTag® fusion purification protocol using Magne™ HaloTag® Beads.

Ordering Information

Magne™ HaloTag® Beads
(Cat.# G7281, G7282)

MagneHis™ Protein Purification System

Manual or automated high-throughput protein purification and pull-down of polyhistidine-tagged proteins, from crude *E. coli* cell lysates, insect or mammalian cell lysates or cell supernatant.

Description and Principle

The MagneHis™ Protein Purification System provides a quick and simple method for the purification of polyhistidine-tagged, overexpressed proteins. Paramagnetic precharged nickel particles (MagneHis™ Ni-Particles) are used to isolate His-tagged fusion proteins directly from a crude cell lysate. Sample volumes from 20µl to 50ml can be easily processed manually. Samples can also be processed using a robotic platform such as the Beckman Coulter Biomek® 2000 or FX or Tecan Genesis® RSP. This Purification System contains magnetic beads and buffers as well as FastBreak™ Cell Lysis Reagent. His tagged proteins can be expressed either intracellularly or secreted into cell media. Bacterial, insect or mammalian cells can be lysed with FastBreak™ Cell Lysis Reagent or with other lysis methods. MagneHis™ Ni-Particles are then added to the lysate and bind to His tag containing proteins. Unbound proteins are washed away, and the target protein is recovered by elution with imidazole **(Figure 4.10)**. Purification can be performed under native and denaturing conditions.

Features and Benefits

- **Fast:** No long incubations with lysozyme are required for cell lysis.
- **Simple:** No centrifugation or vacuum after cell lysis.
- **Efficient:** Binding capacity of up to 1mg of polyhistidine-tagged protein per 1ml of MagneHis™ Ni-particles.
- **Versatile:** Purify His-tagged proteins in manual format or at high-throughput with an automated liquid handler.

References

Guo, H. *et al.* (2010) Osteopontin and protein kinase C regulate PDLIM2 activation and STAT1 ubiquitination in LPS-treated murine macrophages. *J Biol Chem.* **285**(48), 37787–96.

Yun CS, *et al.* (2010) Pmr, a histone-like protein H1 (H-NS) family protein encoded by the IncP-7 plasmid pCAR1, is a key global regulator that alters host function. *J Bacteriol.* **192**(18), 4720–31.

Koschubs, T. *et al.* (2010) Preparation and topology of the Mediator middle module. *Nucleic Acids Res.* **38**(10), 3186–95.

Ordering Information

MagneHis™ Protein Purification System
(Cat.# V8500, V8550)

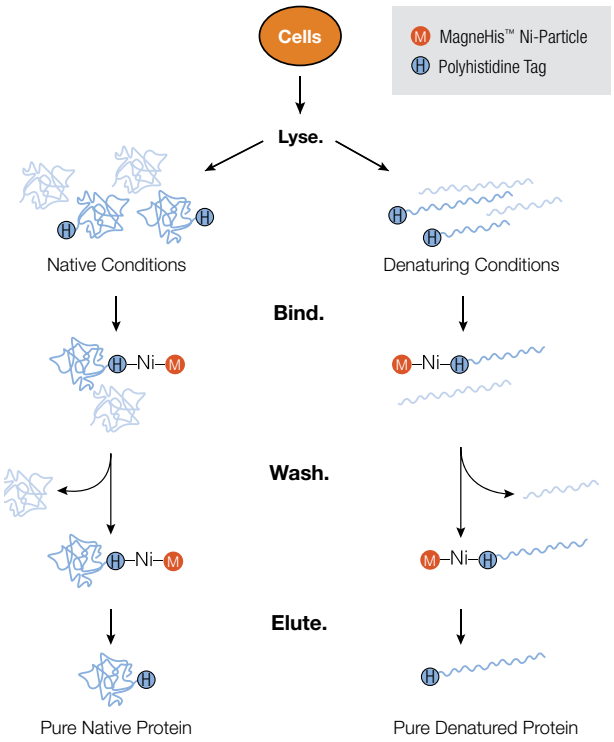


Figure 4.10. Schematic of the MagneHis™ Protein Purification System protocol.

Maxwell® 16 Polyhistidine Protein Purification Kit

Automated purification of polyhistidine-tagged proteins from bacterial cultures and mammalian and insect proteins.

Description and Principle

The Maxwell® 16 Polyhistidine Protein Purification Kit consists of cartridges prefilled with MagneHis™ Ni-Particles and buffers that are used with the Maxwell® 16 Instrument to provide an easy method for efficient, automated purification of polyhistidine-tagged protein from various cells **(Table 4.2)**. The Maxwell® Instrument is supplied with a preprogrammed purification procedure and reagent cartridges specifically designed to maximize simplicity and convenience. The instrument can process up to 16 samples in approximately 40 minutes.

Features and Benefits

- **Save Hands-On Time:** Prefilled cartridges eliminate reagent preparation, multiple pipetting steps, centrifugation and additional sample manipulation.
- **Consistent Results:** Across all samples.

Table 4.2. Sample Types and Maximum Processing Capacity per Cartridge.

Sample Type	Processing Capacity
Bacterial Culture	Up to 20 $O.D._{600}$
Mammalian Cell Culture Cells	Up to 5 x 10^6 cells
Insect Cell Culture Cells	Up to 5 x 10^6 cells
Mammalian or Insect Cell Culture Medium	1ml

Figure 4.11. The Maxwell® 16 Instrument uses paramagnetic-particle technology to extract DNA, RNA or protein from up to 16 samples in less than 45 minutes. The paramagnetic particles and reagents are supplied in prefilled cartridges for faster separations with less hands-on time.

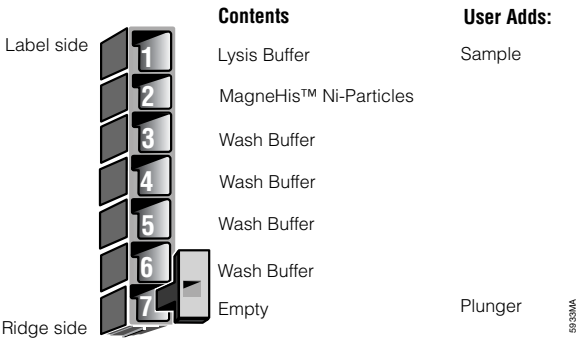


Figure 4.12. Maxwell 16® Polyhistidine Protein Purification Sample Cartridge

Ordering Information

Maxwell® 16 Polyhistidine Protein Purification Kit (Cat.# AS1060)

Magnetic Separation Devices

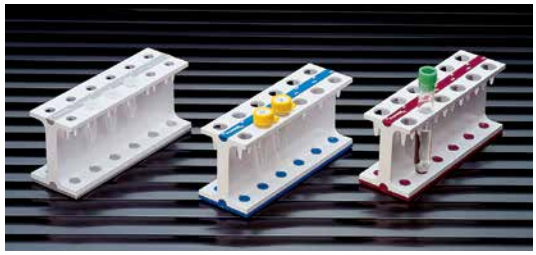
Manual or automated protein purification using magnetic particles.

Description

Promega offers a wide range of magnetic devices for separations from 0.5ml microcentrifuge tubes to 15ml or 50ml conical tubes, to 96- and 384-well standard and deep-well plates. The magnetic separation device for plates is useful for both manual and automated liquid-handling.

MagneSphere® Technology Magnetic Separation Stands

MagneSphere® Technology Magnetic Separation Stand (two-position). Up to two sample volumes (50µl –1.0ml). Left to Right: Cat.# Z5331, Z5332, Z5333.



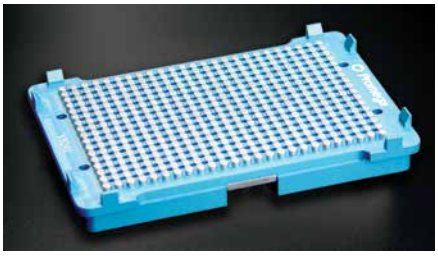
MagneSphere® Technology Magnetic Separation Stand (twelve-position). Up to two sample volumes (50µl–1.0ml). Left to Right: Cat.# Z5341, Z5342, Z5343.

PolyATtract® System 1000 Magnetic Separation Stand. One Sample volume (1-50ml). Cat.# Z5410.

MagnaBot® Magnetic Separation Devices

MagnaBot® 96 Magnetic Separation Device for 96-well standard- or deep-well-plates (20µl– 1.0ml). Cat.# V8151.

MagnaBot® II Magnetic Separation Device for 96-well PCR plate. Cat.# V8351.



MagnaBot® 384 Magnetic Separation Device. Cat.# V8241.

5 Antibody Purification and Labeling

Antibody Purification and Labeling

Antibodies are an indispensable tool for bioresearch, diagnosis and therapy, thus there is a great demand for antibody purification strategies. These strategies include antibody purification from various sources, such as serum (polyclonal antibodies), ascites (monoclonal antibodies) and cell culture supernatant of a hybridoma cell line (monoclonal antibodies). Polyclonal antibodies are a mixture of different antibodies produced by a diverse population of B cells. They may be of different isotypes, have different antigen specificities or recognize a different antigen epitope.

In contrast, moncolonal antibodies are produced by specific B cells that are clonally related; these antibodies are the same isotype, and have identical specificity for the antigen epitope. Depending on the starting material used, various antibody purification strategies exist, including classical chromatography (e.g., ion exchange), affinity-purification (e.g., on immobilized protein A and protein G beads) and antigen-specific affinity purification.

This chapter introduces the use of magnetic protein A and G beads for antibody purification as well as for on-bead antibody labeling.

5.1 Antibody Purification

OVERVIEW

The choice of the antibody purification method depends on the starting material, the intended application, and the manufacturing scale. However, most antibody purification strategies include the use of immobilized Protein A or Protein G **(Table 5.1)**. These bacterial proteins have different affinities for various antibody species and isotypes **(Table 5.2)**. Immobilized Protein A and Protein G can be used to purify monoclonal antibodies from ascites fluid or cell culture supernatant. However, in the case of polyclonal antibodies (serum samples) the use of Protein A or Protein G will enrich mainly the IgG fraction **(Figure 5.1)**. IgG is the main antibody isotype found in blood. For target-specific antibody isolation from serum, antigen-specific affinity-purification is required.

Table 5.1. Features of Native Ig-binding Proteins: Protein A and Protein G.

	Protein A	Protein G
Species	*Staphylococcus aureus*	*Streptococcus* spp. (group C and G)
Native Size	40-60 kDa	40-65 kDa
Ig-Binding Target	Heavy chain constant region (F_c) of IgG (CH2-CH3 region)	Heavy chain constant region (F_c) of IgG (CH2-CH3 region)

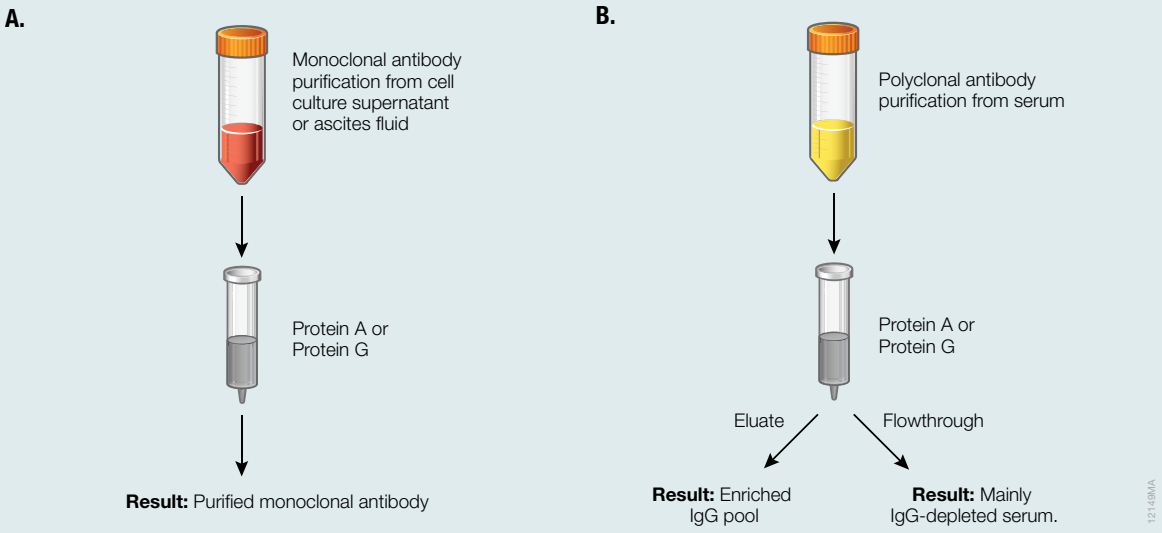


Figure 5.1. Different workflows for antibody purification using Protein A and Protein G. Monoclonal antibodies can be purified using Protein A or Protein G **(Panel A)**. The IgG fraction of polyclonal antibodies from serum can be enriched using Protein A or Protein G. Furthermore, Protein A or Protein G can be used to deplete the IgG fraction from serum. Note: For target-specific antibody purification from serum antigen-specific affinity-purification is required **(Panel B)**.

Chapter 5.1 Overview (continued)

Table 5.2. Relative Recovery of Different Antibody Species and Isotypes by Magne™ Protein A Beads and Protein G Beads.

Species	Isotype	Magne™ Protein A Beads (µg)	Magne™ Protein G Beads (µg)
Bovine	IgG	36.2	33
Goat	IgG	26.2	26
Human	IgA	4.8	0.4
	IgG1	35.2	39.4
	IgG2	34.4	32.6
	IgG3	0	37.6
	IgG4	28.8	38
	IgM	8.2	0
Mouse	IgG1	18.6	30.4
	IgG2a	33.2	31.6
	IgG2b	29.6	31.4
	IgG3	16	9.2
Porcine	IgG	35.2	27.6
Rat	IgG1	29.4	34.2
	IgG2a	0	33
	IgG2b	0	31.2
Sheep	IgG	26.6	25.6

Note: Starting material for the recovery experiment was comprised of 50µg of purified antibody diluted in 1ml of buffer and captured with 50µl of bead slurry (20%). Experiment performed in triplicate (CV ≤10%).

Magne™ Protein A Beads and Magne™ Protein G Beads

Manual or automated antibody purification from different sample types; antibody enrichment or antibody depletion from serum samples; antibody labeling with small molecules or fluorophores.

Description

Magne™ Protein A Beads and Magne™ Protein G Beads are magnetic affinity beads with high specificity and high capacity for binding antibodies from cell culture supernatant, ascites fluid and serum samples. Antibody purification can be performed easily from a single sample or multiple samples in parallel, or in a high-throughput automated fashion. The magnetic beads allow for superior purification and recovery of concentrated antibodies from small input volumes (20µl) by decreasing losses normally associated with handling of small volumes and nonmagnetic resins.

Principle

Recombinant Protein A from *Staphylococcus* and recombinant Protein G from *Streptococcus* are covalently attached in an oriented fashion to magnetic beads. Biological samples are added, and antibodies are captured by the beads. Using magnetic devices, beads are attached and unbound material is washed away. Finally, antibodies are eluted using lower pH buffer and the solution is neutralized **(Figure 5.2)**.

Features and Benefits

- **High Binding Capacity:** Binding capacities of 25mg of antibody per ml of settled beads are observed, depending on antibody species and isotype.
- **Simple:** Easy to handle beads with fast magnetic response.
- **High Purity:** Due to low nonspecific binding.
- **Flexible:** Handles sample volumes from 20µl–50ml.

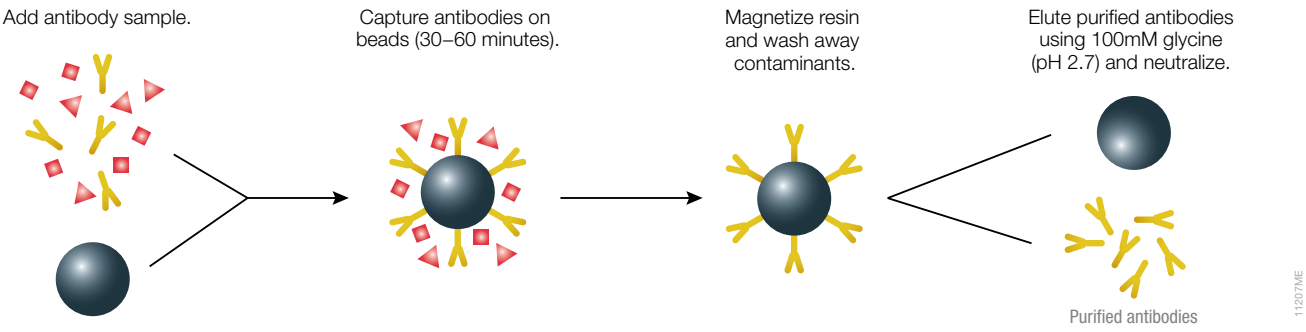
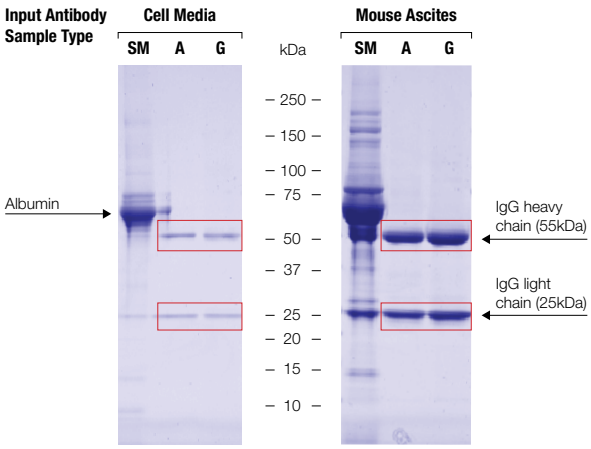


Figure 5.2. Antibody purification using Magne™ Protein A Beads or Magne™ Protein G Beads.

Magne™ Protein A Beads and Magne™ Protein G Beads (continued)



SM = Starting Material
A = Antibody purified with Magne™ Protein A Beads
G = Antibody purified with Magne™ Protein G Beads

Figure 5.3. Antibody purified from various sample types using Magne™ Protein A and Magne™ Protein G Beads. Antibody was purified from 50µl of cell culture media (mouse IgG1), mouse ascites (IgG2a) using 50µl of Magne™ Protein A Beads (A) and Magne™ Protein G Beads (G). Samples were analyzed by adding 1µl of starting material (SM) or 5µl of purified sample to SDS buffer, and heating at 80°C for ten minutes before loading onto a 4–20% Tris-glycine gel. The gel was stained with SimplyBlue® Safe Stain.

Additional Information:

- Magnetic stands and spacer for the use with this product are listed in Chapter 4.
- High-throughput antibody purification platforms such as the ReliaPrep™ LV 32 HSM Instrument (Cat.# A1715) or a robotic liquid handler platform such as the Beckman Coulter Biomek® FX can be used.
- Custom solutions for using the beads with a Maxwell® Instrument are possible upon request.
- Note: Magne™ Protein A Beads and Magne™ Protein G Beads are not recommended for use in immunoprecipitation (IP) or Co-IP applications.

References

Godat, B. and Nath, N. (2012) High-Capacity and High-Purity Antibody Purification Using Magnetic Beads. [Promega Corporation web site. November 2012. Accessed 1-20-2014. http://www.promega.com/resources/pubhub/high-capacity-and-high-purity-antibody-purification/

Ordering Information

Magne™ Protein A Beads
(Cat.# G8781, G8782, G8783)

Magne™ Protein G Beads
(Cat.#. G7471, G7472, G7473)

5.2 Antibody Labeling

OVERVIEW

Antibodies can be labeled with a diverse number of small molecules including biotin, fluorophores (e.g., FITC, Alexa Dyes, Cy3) and other large proteins (e.g., horseradish peroxidase). These antibody conjugates are useful reagents in bioresearch mainly for analyte/antigen detection in ELISA, fluorescent microscopy and for antibody-based assays. A specific class of antibody conjugates, Antibody Drug Conjugates (ADCs), is comprised of antibodies labeled with cytotoxic compounds intended for human therapy to target and kill specific cells (e.g., solid tumors). An ADC binds to target cells via a specific antibody-cell surface antigen interaction, the antibody and drug conjugate are internalized into endosomes. In the endosomes the linker between the cytotoxic drug and antibody are cleaved causing the release of the cytotoxic molecule and subsequent killing of the target cell. There are three aspects of ADC production that require optimization: the targeting antibody, the labile linker and the cytotoxic agent.

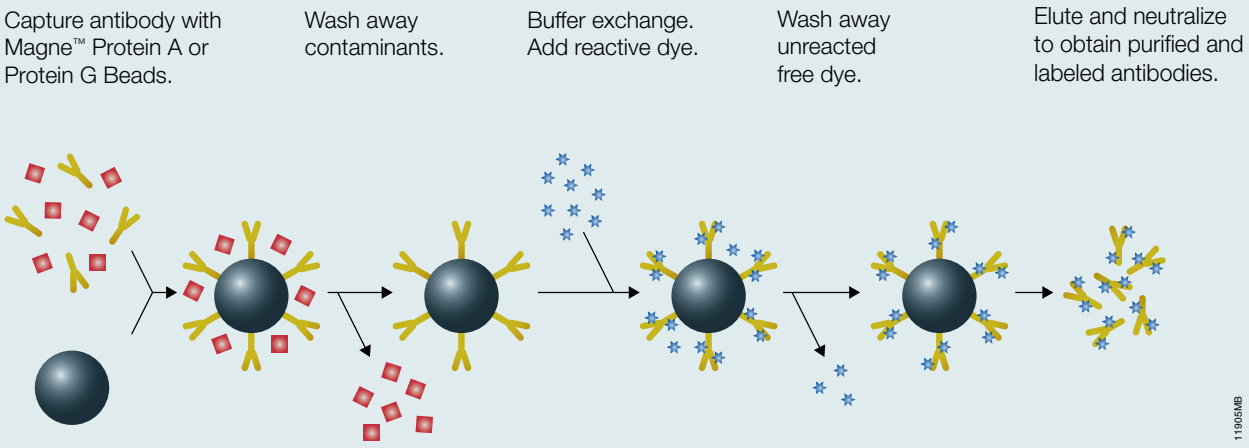


Figure 5.4. Schematic diagram of on-bead antibody conjugation/labeling.

Chapter 5.2 Overview (continued)

Conjugation and Labeling Strategies

Antibodies are labeled with small molecules using two common chemistries: 1) a small molecule that reacts with primary amines on the lysines amino acids; and 2) reducing interchain disulfide bonds in antibodies with a reducing agent (DTT or TCEP) followed by reaction with thiol-reactive small molecules.

Antibody labeling is typically performed in solution and consists of several steps. For example, labeling antibody with a thiol-reactive small molecule starts with a purified antibody preparation that is buffer exchanged or dialyzed into an appropriate buffer for reduction of interchain disulfide bonds using reducing agents. After reduction, the antibody solution is buffer exchanged to remove the reducing agent. Antibody is then reacted with a small molecule followed by another dialysis step to remove any nonreacted small molecule. The method is tedious and time-consuming because of the several dialysis steps involved. In addition, the method has a very limited throughput because of the need for purified antibody.

On-Bead Antibody Labeling

To simplify the workflow for labeling antibody and to increase throughput, we have developed an on-bead antibody conjugation method using high-capacity magnetic Protein G and Protein A beads **(Figure 5.4)**. The method involves binding antibody to Protein G or Protein A beads. Instead of dialysis, simple wash steps are performed to remove unreacted small molecules. In addition, antibody can be directly captured from cell media, serum or ascites fluid without the need for pre-purification. Magnetic beads allow 1–96 samples to be labeled in-parallel (for sample volumes of 100µl–1ml), significantly improving the throughput.

Advantages of On-Bead vs. In-Solution Labeling

- Avoid pre-purification, dialysis and concentration steps required for in-solution labeling.
- Resulting antibody is labeled and highly concentrated.
- Conjugate/Label from 20µl to 50ml sample size.
- Automatable for 1–96 samples.
- Eluted, labeled antibody is compatible with downstream applications such as cell internalization and Antibody-dependent Cell-mediated Cytotoxicity (ADCC assay).

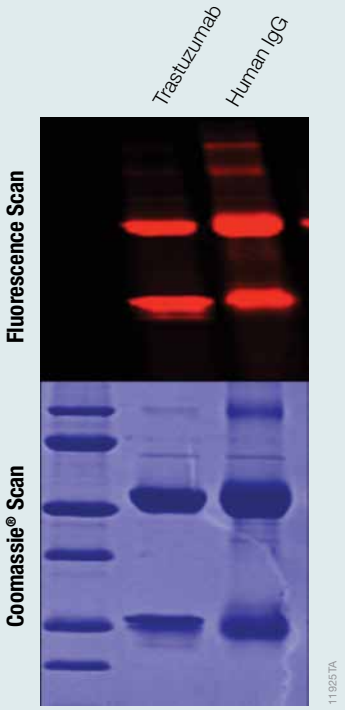
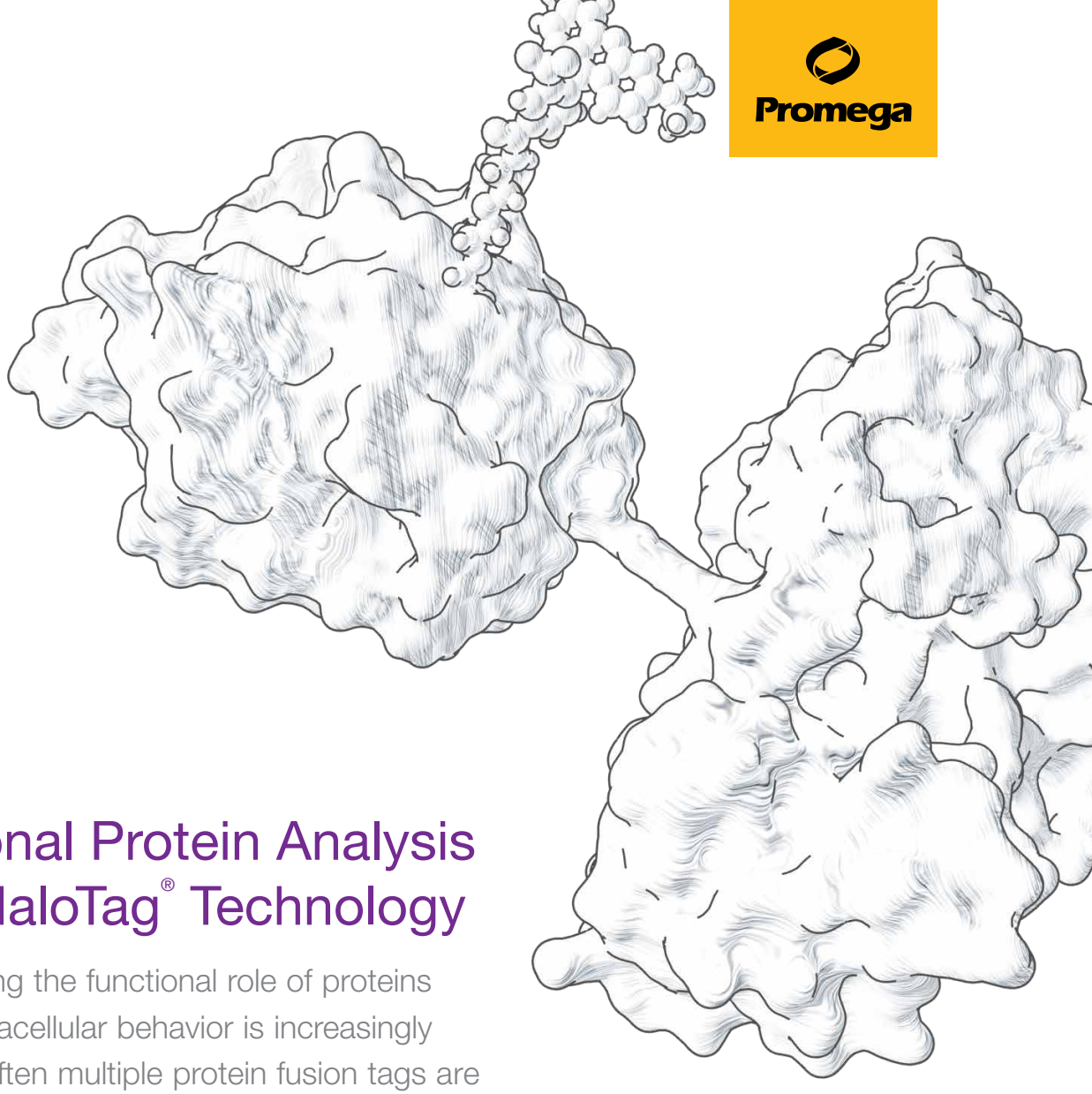


Figure 5.5. Gel images showing purification and labeling of anti-Her2 antibody (Trastuzumab) and control human IgG with Alexa Fluor® 647.

6 Functional Protein Analysis using HaloTag® Technology



Functional Protein Analysis using HaloTag® Technology

Understanding the functional role of proteins and their intracellular behavior is increasingly important. Often multiple protein fusion tags are required to fully characterize a specific protein of interest, such as use of one or more fusion tags for imaging and protein capture/purification applications. Recloning the same protein-coding DNA sequence of interest with multiple tags can be slow and cumbersome, requiring revalidation of the new construct in functional assays prior to use. What is needed is a single recombinant protein tag that provides application flexibility and superb performance for protein expression and localization, protein purification, protein interaction discovery, screening and further functional analyses. The HaloTag® Technology and the HaloTag® fusion proteins address this research need.

Chapter 6 Overview (continued)

HaloTag® technology is based on an engineered catalytically inactive bacterial hydrolase of 34kDa that reacts, under physiological conditions, with chloroalkane ligands (HaloTag® ligands) to form a highly specific and irreversible covalent bond. By changing the ligands it is possible to control the function and application of the tagged protein of interest.

The HaloTag® system is designed to provide broad experimental flexibility in both cell-based and biochemical assays without switching fusion tags or recloning. HaloTag® ligands coupled to magnetic and nonmagnetic beads and glass slides are available. Cell-permeable and impermeable fluorescent ligands and biotin ligands are also available as well as reactive ligands to enable the creation of custom HaloTag®-linked constructs **(Figure 6.1)**.

Features and Benefits

- **Multiple Applications:** Use a single protein fusion tag for all your needs.
- **Minimal Background:** HaloTag® protein has no homology to other mammalian cell proteins.
- **Irreversible Covalent Attachment:** Covalent binding to HaloTag® ligands and surfaces allows stringent washing, resulting in low background.

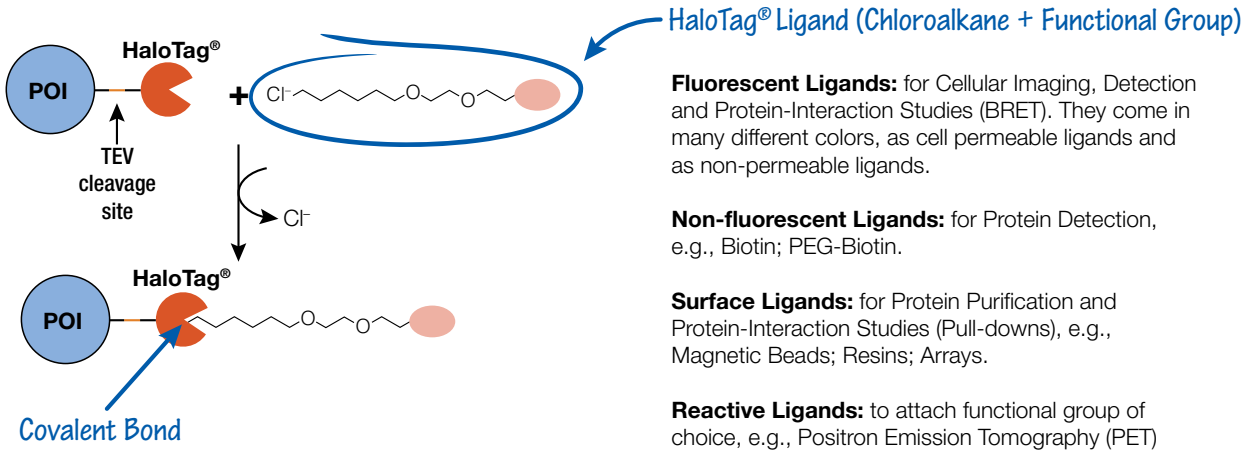


Figure 6.1. Schematic of HaloTag® technology consisting of the HaloTag® protein fused to a protein of interest (POI) and a selection of synthetic HaloTag® ligands carrying different functional groups. HaloTag® ligands specifically and covalently bind to the HaloTag® protein fusions.

Chapter 6 Overview (continued)

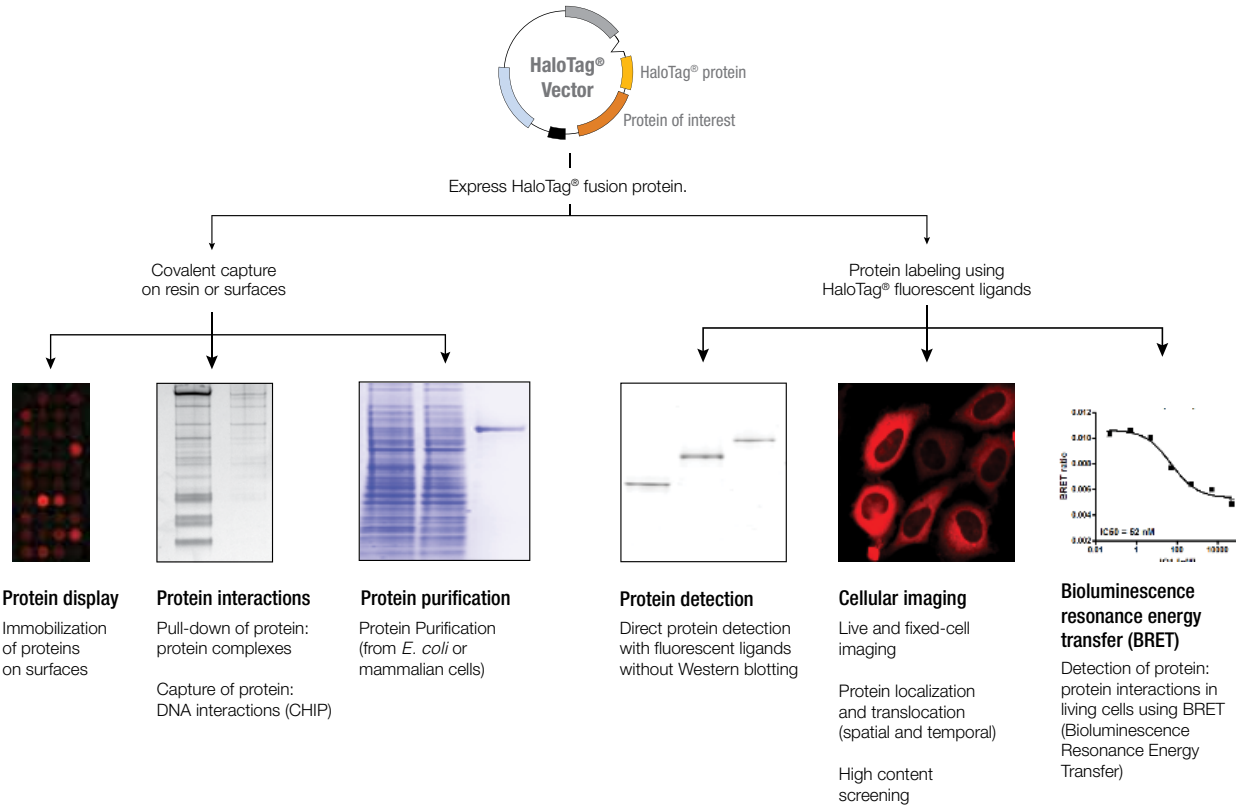


Figure 6.2. One genetic HaloTag® construct can be used for multiple applications.

References

Urh, M. *et al.* (2012) HaloTag, a Platform Technology for Protein Analysis. *Curr Chem Genomics.* **6**,72-8.

Los, G.V. *et al.* (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol.* **3**(6), 373-82.

Fluorescent HaloTag® Ligands for Cellular Imaging

Cellular Imaging: Live and fixed cells; protein localization and trafficking; protein turnover and pulse-chase analysis; FACS analysis.

Description

Fluorescent HaloTag® ligands **(see Figure 6.3)** are available for the detection of HaloTag® protein fusions in live cells for direct imaging or for labeling in fixed cells. HaloTag® imaging can be multiplexed with fluorescent proteins (e.g., GFP) in live cells and fluorescent antibody staining in fixed cells and tissues. HaloTag® ligands are based on small organic dyes; they retain their fluorescent properties after fixation, enabling multiplexing of immunocytochemistry experiments.

Principle

Proteins of interest fused to HaloTag® protein can be labeled with various fluorescent ligands in live or fixed cells using a simple labeling protocol **(Figure 6.4).** HaloTag® ligands are noncytotoxic and allow permanent labeling due to covalent attachment to the HaloTag® protein. There are different types of fluorescent ligands for intracellular protein labeling (cell-permeant) as well as for cell surface protein labeling (cell-impermeant). Rapid ligands can label as quickly as 5–15 minutes after addition to cells expressing the HaloTag® protein. The direct label ligands can be added at the time of cell plating or transfection and will label the tagged protein of interest (POI) as it is expressed (without the need to wash away the unbound ligand). In addition to direct labeling, the dyes can be used in standard labeling protocols that include wash steps. Lastly, permeant and impermeant ligands of different colors can be used in tandem to track protein translocation to and from the plasma membrane **(Figure 6.5)**. The range of differently colored HaloTag® ligands allows easy changes in POI color and easy integration with other fluorophores like GFP or other cellular labeling dyes.

Features and Benefits

- **Localization:** Robust protein imaging in live or fixed cells (e.g., 4% PFA-fixation).
- **Trafficking and Turnover:** Directly observe spatially- or temporally-separated protein populations with one or two colors in live cells. Label first with cell-impermeable and then with cell-permeable ligands.
- **Cell Sorting:** Simple non-antibody based cell labeling with multiple fluorophores.
- **Flexible:** Variety of HaloTag® ligands in different colors allows flexible combination with other labeling dyes (e.g., DNA staining dyes) as well as with immunofluorescence reagents.

Ligand	Excitation Maximum	Emission Maximum	Intended Use of Ligands	Ordering Information
HaloTag® Coumarin	362nm	450nm	Intracellular labeling	G8581, G8582
HaloTag® Alexa Fluor® 488	499nm	518nm	Cell-surface labeling	G1001, G1002
HaloTag® Oregon Green®	492nm	520nm	Intracellular labeling	G2801, G2802
HaloTag® DiAcFAM	492nm	521nm	Intracellular labeling	G8272, G8273
HaloTag® R110 Direct™ ("No Wash" ligands)	498nm	528nm	Intracellular labeling	G3221
HaloTag® TMR Direct™ ("No Wash" ligands)	552nm	578nm	Intracellular labeling	G2991
HaloTag® Alexa Fluor® 660	654nm	690nm	Cell-surface labeling	G8471, G8472

Figure 6.3. Maximum excitation and emission spectra for the HaloTag® Ligands.

Fluorescent HaloTag® Ligands for Cellular Imaging (continued)

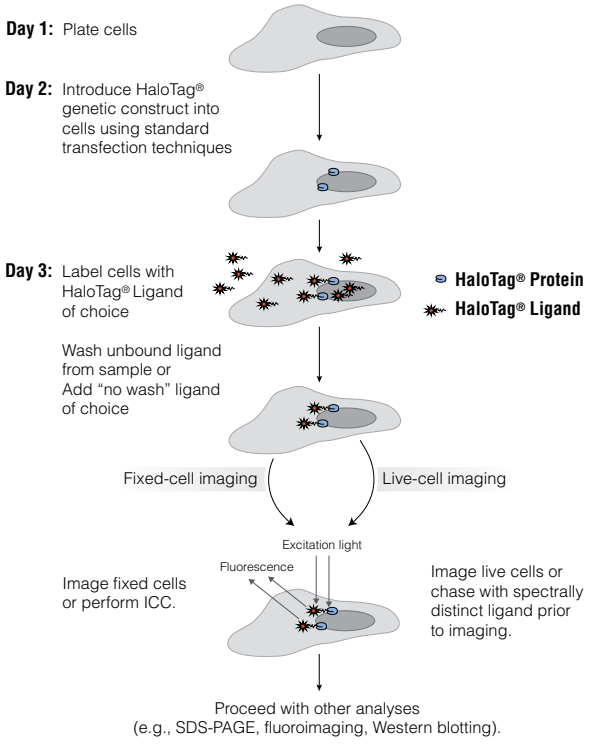


Figure 6.4. Overview of protocol for live-cell and fixed-cell imaging using HaloTag® Technology. No washing steps are required with the "No Wash" DIRECT® HaloTag® ligands.

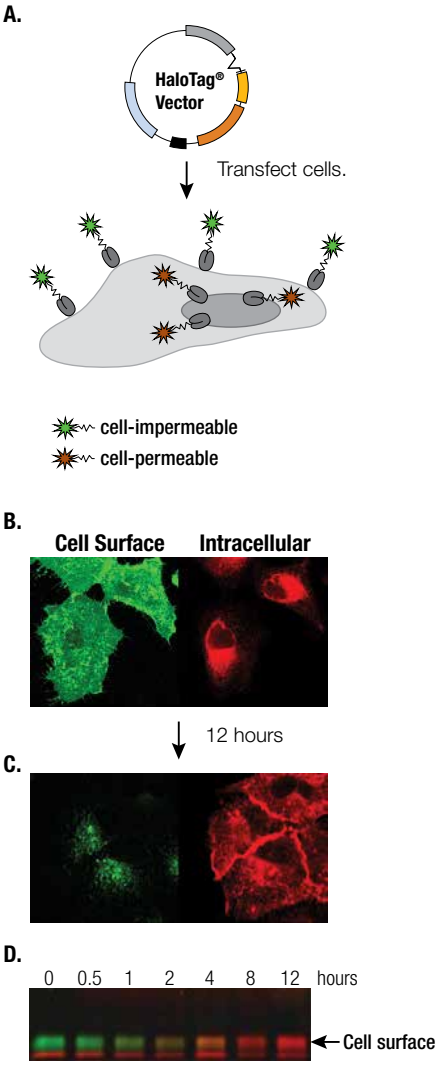


Figure 6.5. Membrane trafficking of ß-integrin fragment fused to HaloTag® (N-terminal fusion). **(A)** Cells stably expressing ß1Int-HaloTag were labeled first with the cell-impermeable ligand HaloTag® Alexa Fluor® 488 and then with the cell-permeable HaloTag® TMR ligand. **(B)** Images were taken with a confocal microscope immediately after labeling to show cell-surface and intracellular localization of ß1Int-HaloTag. **(C)** Cells were re-imaged 12 hours after labeling to show receptor internalization and trafficking to the plasma membrane. **(D)** Fluoroscan image of an SDS gel: Cell lysates taken at 0, 0.5, 1, 2, 4, 8 and 12 hours after labeling were run on SDS-PAGE. Fluoroscan shows bidirectional trafficking of ß1Int-HaloTag as reflected in the upper band (cell surface ß1Int-HaloTag) and lower band (intracellular ß1Int-HaloTag). Image copyright BMC Cell Biology. Usage license can be found at: http://www.biomedcentral.com/authors/license. Image has been modified from original published version.

References

Yamaguchi, K. *et al.* (2009) Pulse-chase experiment for the analysis of protein stability in cultured mammalian cells by covalent fluorescent labeling of fusion proteins. In: *Reverse Chemical Genetics, Methods in Molecular Biology* **577**, H. Koga ed. Humana Press.

Huybrechts, S.J. *et al.* (2009) Peroxisome dynamics in cultured mammalian cells. *Traffic* **10**(22),1722-33.

Svendsen, S. *et al.* (2008) Spatial separation and bidirectional trafficking of proteins using a multi-functional reporter. *BMC Cell Biol.* **9**(17).

NanoBRET™ Technology: Live–Cell Protein: Protein Interaction Assay

Study protein:protein interactions in living cells, even at physiological expression levels, using Bioluminescence Resonance Energy Transfer (BRET) real-time measurements.

Description

NanoBRET™ Technology uses the very bright NanoLuc® Luciferase as a BRET donor and the HaloTag® Protein as an acceptor **(Figure 6.6)**. NanoBRET™ Technology has many advantages compared to other BRET technologies including higher signal-to-background ratios that enable BRET measurements in a broad dynamic assay window. NanoLuc® Luciferase is a small protein (19 kDa) with a very high light output (100X brighter than *Renilla* Luciferase). In combination with the HaloTag® protein and the optimized red-shifted NanoBRET™ Ligand, this newly developed technology allows BRET measurements at physiological expression levels and also may be suitable for BRET measurements in difficult-to-transfect cells and primary cells.

Principle

NanoBRET™ technology measures the interaction between the energy donor fusion Protein X-NanoLuc® Luciferase and the energy acceptor fusion, Protein Y-HaloTag® protein. As it can be difficult to predict the optimal orientation and placement of either the energy donor or acceptor, we recommend that you evaluate all possible combinations of N- and C-terminal protein fusions to NanoLuc® and HaloTag® proteins on both protein interaction partners (X, Y), since both the orientation and the composition of the fusion protein can affect protein expression and/or activity. To select the best BRET pairs, eight different fusion constructs are used (four NanoLuc® protein fusions and four HaloTag® protein fusions). See **Table 6.1**.

To obtain the optimal NanoBRET™ signal, we recommend minimizing the amount of unbound donor protein (the NanoLuc® fusion). For this reason, transfection dilutions are recommended, where the NanoLuc® fusion is decreased relative to the HaloTag® fusion. NanoBRET™ technology measurements require instruments capable of filtered luminescent readout. In addition, we highly recommend the use of filters that can read the donor at approximately 460nm, and for the acceptor, a 610nm long pass filter over a bandpass filter for optimal results.

Features and Benefits

- **High Signal-to-Background Values:** Excellent signal-to-background and high dynamic range due to large spectral separation of donor emission and acceptor emission. Very low bleeding of donor emission into acceptor emission **(Figure 6.7)**.
- **Bright Donor Signal:** Strong signal from donor enables use of weak promoters for physiological expression levels; lower cell number; difficult-to-transfect cells.
- **Simple Background Calculation:** A simple background calculation is possible by performing the assay in the absence of the NanoBRET™ fluorescent ligand. This feature is not possible in other BRET systems that use intrinsically fluorescent proteins.
- **Easy Cloning of Different Fusion Pairs:** NanoLuc® and HaloTag® protein sequences are available in Flexi® Vectors, which allow easy shuttling of ORFs from one vector to the other (see Chapter 1).
- **Ready to use ORF Clones Available:** Choose from >9,000 human HaloTag® ORF clones (see Chapter 1).

NanoBRET™ - Live Cell Protein:Protein Interaction Assay (continued)

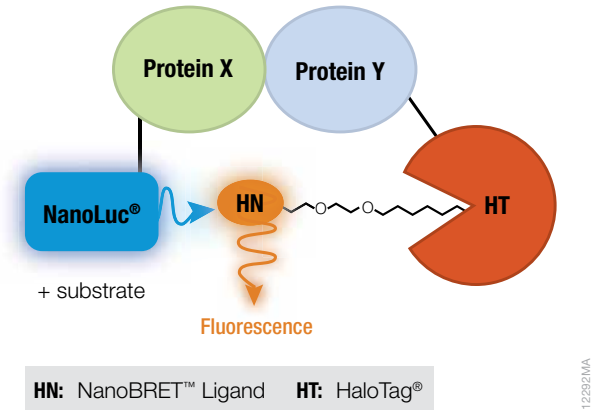


Figure 6.6. NanoBRET™ Technology. Interaction partners Protein X and Protein Y are fused to NanoLuc® luciferase and a HaloTag® ligand or vice versa. Upon interaction of Protein X with Protein Y (<10nm) the energy produced by NanoLuc® Luciferase is transferred to the fluorescent HaloTag® Ligand (NanoBRET™ Ligand). Using filter-based plate readers, the luminescent and fluorescent signals are recorded to calculate the BRET ratio.

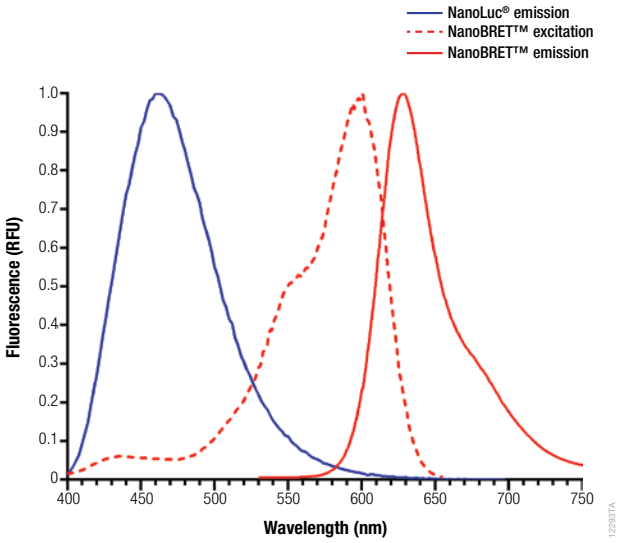


Figure 6.7. Emission spectra of NanoLuc® Luciferase and NanoBRET™ Ligand. Large spectral separation of donor emission (blue line; Em_{Max} 460nm) and acceptor emission (red line; Em_{Max} 629nm). The dotted red line represents the excitation of NanoBRET™ Ligand (Ex_{Max} 600nm).

Table 6.1. HaloTag® and NanoLuc® Fusion Construct Combinations.

Test Pair	N-Terminal HaloTag® Fusion Protein	C-Terminal HaloTag® Fusion Protein	N-Terminal NanoLuc® Fusion Protein	C-Terminal NanoLuc® Fusion Protein
1	Protein X		Protein Y	
2	Protein X			Protein Y
3		Protein X	Protein Y	
4		Protein X		Protein Y
5	Protein Y		Protein X	
6	Protein Y			Protein X
7		Protein Y	Protein X	
8		Protein Y		Protein X

Additional Information

Please contact Promega for additional information.

References

Deplus, R. *et al.* (2013) TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. *EMBO J.* **32**(5),645-55.

HaloTag® Mammalian Pull-Down System

Capture and analysis of interacting proteins.

Description

HaloTag® Mammalian Pull-Down System is used to isolate and identify intracellular protein complexes from mammalian cells. HaloTag® fusion proteins form a highly specific covalent bond with the HaloLink™ Resin, allowing rapid and efficient capture of protein complexes even at very low, endogenous levels of bait protein expression. These benefits, coupled with the low nonspecific binding of the HaloLink™ Resin, improve the rate of successful complex capture and identification of physiologically relevant protein interactions in mammalian cells. The HaloTag® Mammalian Pull-Down and Labeling System also includes the HaloTag® TMRDirect™ Ligand, which allows optimization of protein expression levels, to study cellular localization, trafficking and protein turnover using the same HaloTag® genetic construct.

Principle

The basic HaloTag® pull-down experimental scheme is depicted in **Figure 6.8**. HaloTag® fusion proteins can be expressed in mammalian cells either transiently or stably, and used as bait to capture interacting proteins or protein complexes. After cell lysis, the HaloTag® fusion protein, bound to its interacting protein partners, is captured on the HaloLink™ Resin. The captured complexes are gently washed and eluted either using SDS elution buffer (or other denaturing conditions such as 8M urea), or cleaved from the resin using TEV protease*. The recovered complexes are suitable for analysis by a variety of methods including, Western blotting and mass spectrometry.

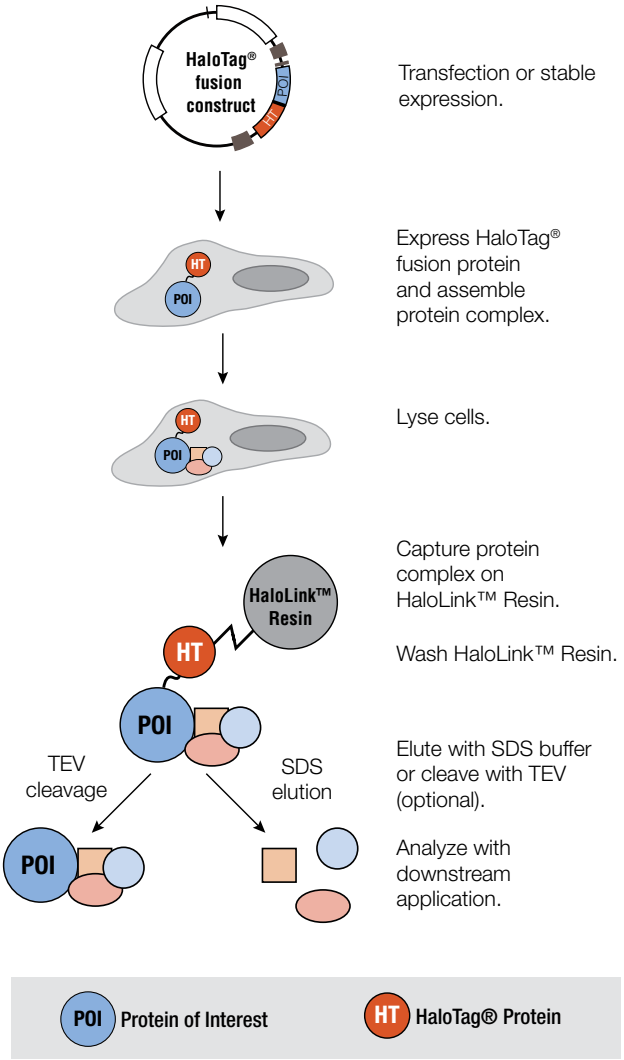


Figure 6.8. Schematic of the HaloTag® Mammalian Pull-Down System protocol.

HaloTag® Mammalian Pull-Down Kit (continued)

Features and Benefits

- **Improved Capture:** Rapid and covalent attachment of HaloTag® protein to its resin enhances capture of protein partners, including transient interactions.
- **Compatibility:** Compatible with all downstream methods of analysis, including mass spectrometry.
- **Ability to Fluorescently Label HaloTag® Fusion Protein:** Optimize protein expression levels and determine cellular protein localization.

Ordering Information

HaloTag® Mammalian Pull-Down System (Cat.# G6504)

HaloTag® Mammalian Pull-Down and Labeling System (Cat.# G6500)

HaloTag® TMRDirect™ Ligand (Cat.# G2991)

Additional Information

*TEV protease is also available separately.

References

Galbraith, M.D. *et al.* (2013) HIF1A Employs CDK8-Mediator to Stimulate RNAPII Elongation in Response to Hypoxia. *Cell* **153**(6), 1327–39.

Kalashnikova, A. *et al.* (2013) Linker histone H1.0 interacts with an extensive network of proteins found in the nucleolus. *Nucl. Acids Res.* **41**(7) 4026-35.

HaloCHIP™ System

Capture of Protein:DNA interactions.

Description

The HaloCHIP™ System is used for the covalent capture of intracellular protein:DNA complexes without the use of antibodies. This kit offers an efficient and robust alternative to the standard chromatin immunoprecipitation (ChIP) method and contains HaloLink™ Resin, Mammalian Lysis Buffers, Wash Buffers and HaloCHIP™ Blocking ligands.

Principle

Proteins of interest (e.g., transcription factors) are expressed in cells as HaloTag® fusion proteins, crosslinked to DNA with formaldehyde, and captured covalently on HaloLink™ Resin. Covalent capture allows the use of extensive and stringent wash conditions that are not possible when antibodies and other noncovalent tags are used for pull-down. The ability to use stringent wash conditions results in reduced background and an increased signal-to-background ratio of detected DNA fragments. After covalent capture, stringent washing removes nonspecifically bound nuclear proteins and DNA; heating reverses the crosslinking between the DNA and the HaloTag®-bound transcription factor, releasing the DNA for subsequent analysis (either RT-PCR or sequencing). See **Figure 6.9**.

Features and Benefits

- **No Requirement for Antibody:** No need for ChIP-qualified antibodies.
- **Obtain Results Faster:** Obtain data in 24–48 hours with fewer steps, minimizing potential experimental errors and reducing artifacts.
- **Improved Signal-to-Background Ratios:** Enables detection of small changes in protein binding patterns using a minimal number of cells.

References

Nagaki, K. *et al.* (2012) Isolation of centromeric-tandem repetitive DNA sequences by chromatin affinity purification using a HaloTag7-fused centromere-specific histone H3 in tobacco. *Plant Cell Rep*. **31**(4), 771–9.

Felder, T. *et al.* (2011) Characterization of novel proliferator-activated receptor gamma coactivator-1alpha (PCG-1alpha) in human liver. *J. Biol. Chem*. **286**, 42923-36.

Ordering Information

HaloCHIP™ System (Cat.# G9410)

HaloCHIP™ System (continued)

Covalent Capture of Chromatin Complexes Using HaloTag® Technology

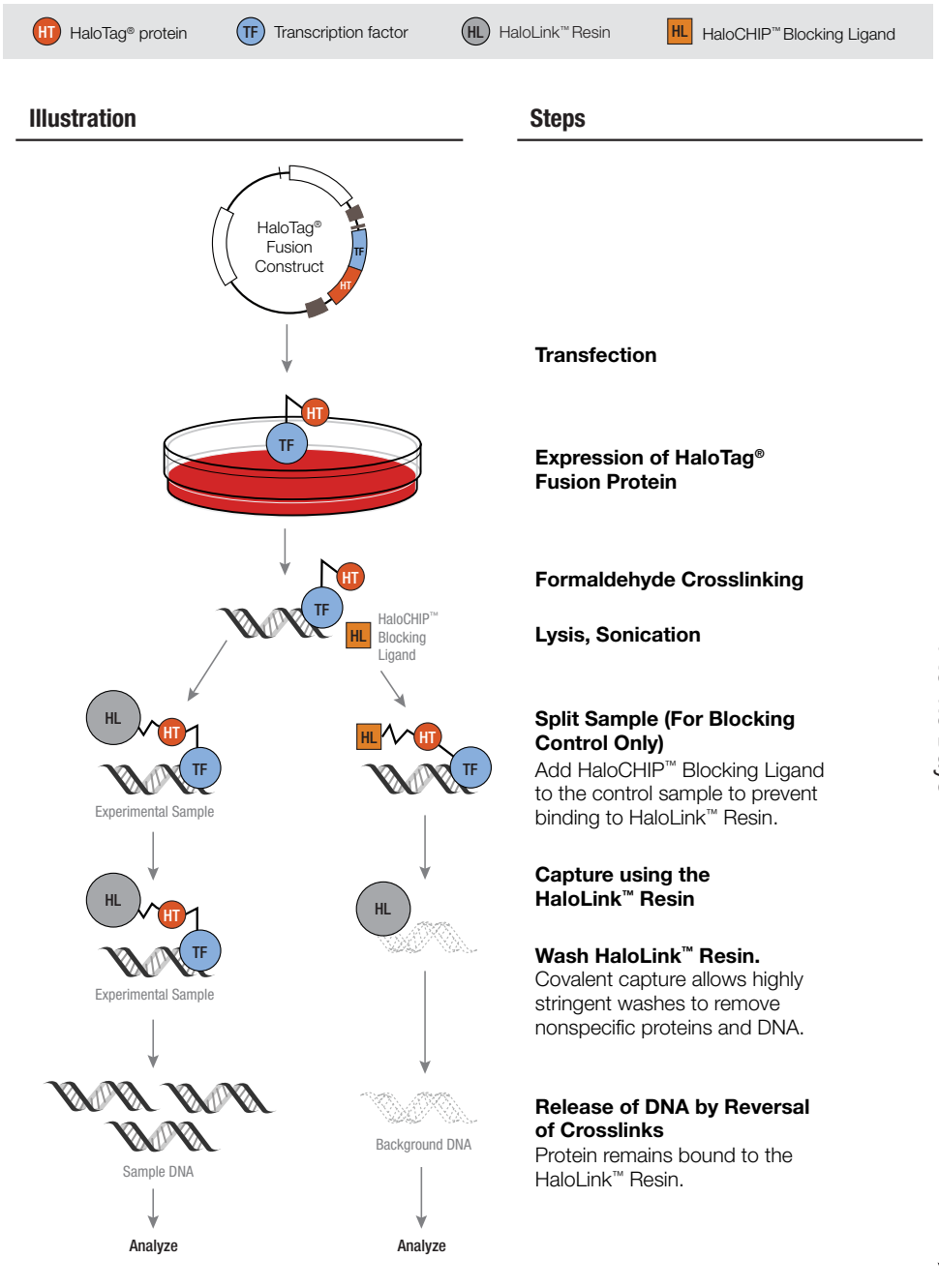


Figure 6.9. Schematic diagram of the HaloCHIP™ System protocol.

HaloLink™ Protein Array System

Create your individual HaloTag® protein array to study protein:protein, protein:drug, protein:nucleic acid interactions; arrays for antibody screening and enzymatic functional analysis.

Description

The HaloLink™ Array Six Slide System provides an easy way to create custom protein arrays. HaloTag® fusion proteins are covalently attached to hydrogel-coated glass slides (optimized for low nonspecific binding) and can be used for many different binding and interaction studies.

Principle

To produce custom HaloTag® protein arrays, protein-coding sequences are cloned into appropriate HaloTag® Flexi® Vectors (see Chapter 1 for Flexi® Cloning System). HaloTag® fusion proteins are expressed either in cell-free (purchased separately) or cell-based expression systems **(Figure 6.10)**. The HaloLink™ Array Gasket is applied to the HaloLink™ Slide, creating 50 leak-free wells. HaloTag® protein fusions are applied and captured on the HaloLink™ Slide, creating a custom array.

Features and Benefits

- **Irreversible Binding of Captured Protein:** HaloTag® fusion proteins bind to the HaloLink™ Slide via a covalent bond.
- **No Protein Pre-Purification Step:** The protein of interest is immobilized directly from crude cell-free or cell-based expression system lysates.
- **Reduced Nonspecific Binding:** Less nonspecific binding issues due to unique hydrogel coating of the HaloLink™ Slides.
- **Extensive, and Stringent Washing Allowed:** Covalent binding of HaloTag® fusion proteins to the HaloLink™ Slide, allows extensive washing, possibly resulting in lower background.

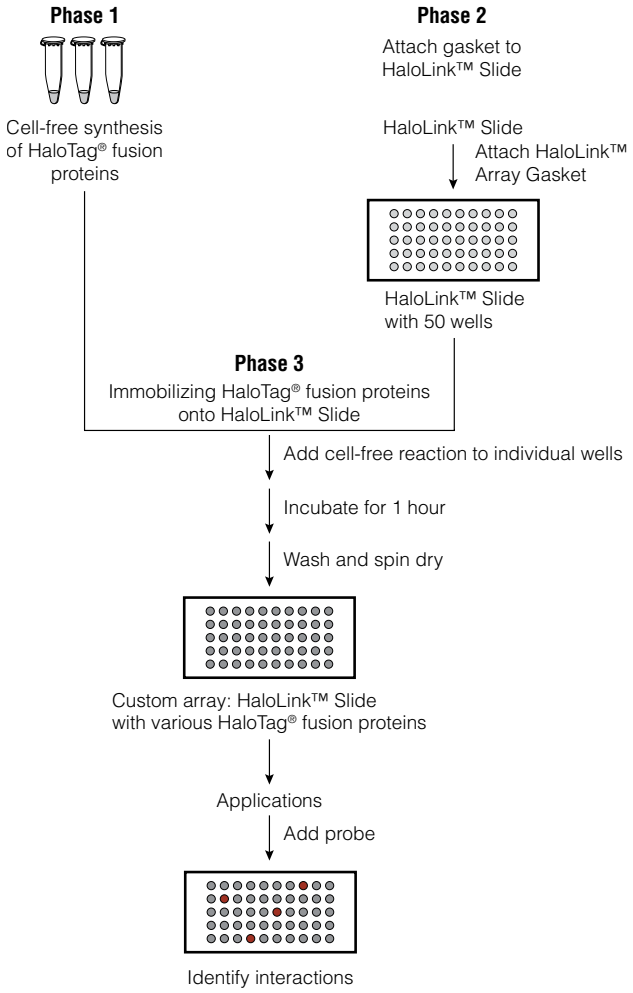


Figure 6.10. HaloLink™ Protein Array System overview.

HaloLink™ Protein Array System (continued)

References

Hurst, R. *et al.* (2009) Protein-protein interaction studies on protein arrays: Effect of detection strategies on signal-to-background ratios. *Analytical Biochem*. **392**, 45–53.

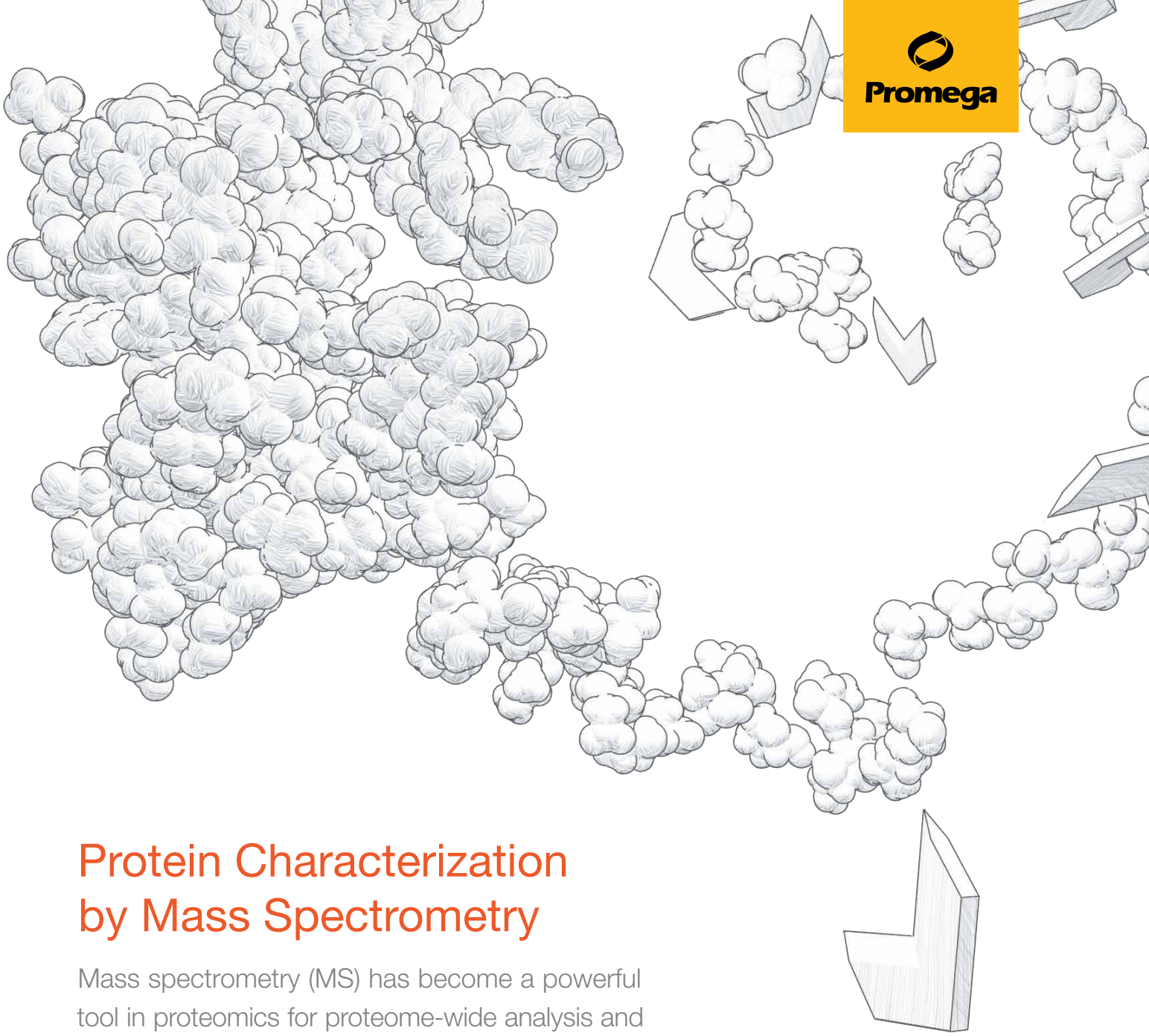
Hoppe, S. *et al.* (2012) Microarray-based method for screening of immunogenic proteins from bacteria. *J. Nanobiotechnol*. **10**,12.

Wang, J. *et al.* (2013) A versatile protein microarray platform enabling antibody profiling against denatured proteins. *Proteomics Clin. App*. **7**, 378–8

Promega Products

HaloLink™ Array Six Slide System
(Cat.# G6190)

7 Protein Characterization by Mass Spectrometry



Protein Characterization by Mass Spectrometry

Mass spectrometry (MS) has become a powerful tool in proteomics for proteome-wide analysis and characterization of proteins from a variety of organisms and cell types. Recent advances in mass spectrometry provide tools for protein identification, protein characterization, relative and absolute quantitation, and the study of post-translational modifications and protein:protein interactions. Proteins are generally digested with proteases to generate peptides for MS analysis followed by sequencing (tandem MS). Promega provides high-quality proteases and other reagents that are critical to sample preparation for mass spectrometry.

7.1 Trypsin: Mass Spectrometry Sample Preparation

OVERVIEW

Trypsin is the most widely used protease in mass spectrometry sample preparation. It is a highly-specific serine protease, which cleaves at the carboxylic side of lysine and arginine residues. Protein digestion with trypsin generates peptides of optimal sizes for mass spec analysis. Tryptic peptides have a strong C-terminal charge, and therefore they can be efficiently ionized.

Trypsin is used for in-gel or in-solution digestion of proteins. Post digestion, the resulting peptides are introduced into the mass spectrometer and identified by *peptide mass fingerprinting or tandem mass spectrometry* (MS/MS). This approach is called "bottom-up" proteomics and uses identification at the peptide level to detect and characterize proteins.

The stringent specificity of trypsin is essential for characterizing proteins using mass spectrometry. Promega's high quality trypsin is derived from porcine trypsin and modified to give the highest proteolytic activity and cleavage specificity **(Figure 7.1)**.

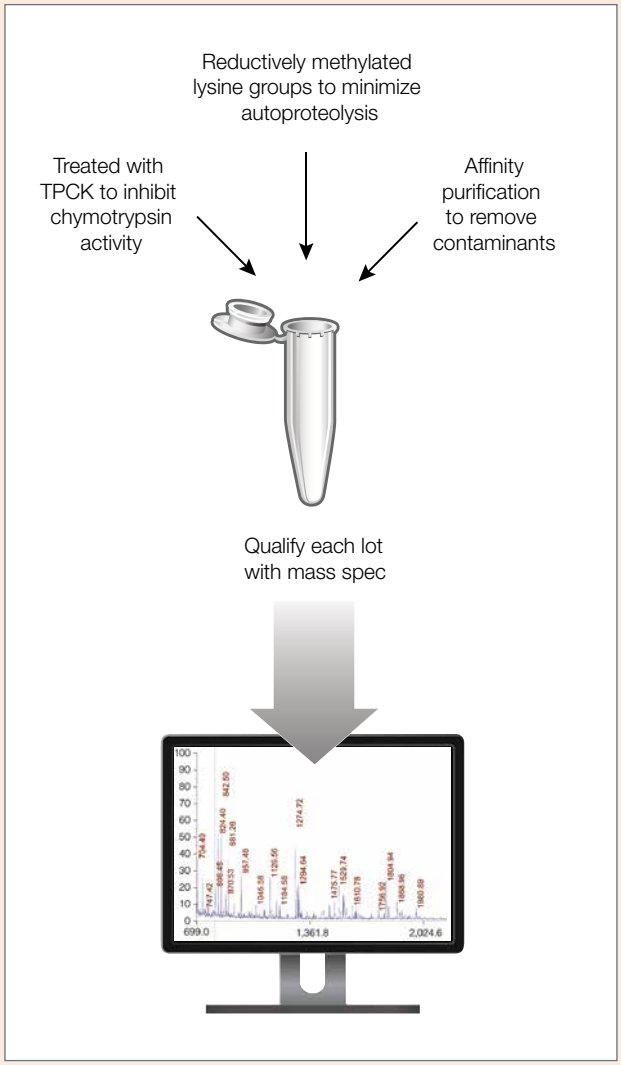


Figure 7.1. The high quality of Promega trypsin is achieved by chemical modification, affinity purification and strict lot-release criteria. Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280) is mass spectrometry-qualified.

Trypsin Gold, Mass Spectrometry Grade

Digestion of proteins into peptides via in-gel or in-solution techniques, followed by mass spec. Typical applications include protein identification, protein quantitation and post-translation modifications via liquid chromatography-mass spectrometry/ mass spectrometry analysis.

Description

Trypsin Gold, Mass Spectrometry Grade, is manufactured to provide maximum specificity. Lysine residues in porcine trypsin are modified by reductive methylation, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion. The specificity of the purified trypsin is further improved by TPCK treatment, which inactivates chymotrypsin. Additionally, the high performance of Trypsin Gold is assured by selecting affinity fractions with the highest proteolytic activity.

Principle

For in-gel digestion, protein samples are run on SDS-PAGE. Protein bands are stained and the bands of interest are excised. A buffer containing resuspended trypsin is added and the reaction is incubated overnight at 37°C. The resulting peptides are then extracted from the gel and analyzed by mass spec.

For in-solution digestion, protein samples are denatured, reduced and alkylated. Trypsin is added and the solution is incubated for 3-12 hours at 37°C. The resulting peptides are desalted and analyzed by mass spectrometry.

Features and Benefits

- **Application Qualified:** Each lot is qualified by mass spectrometry.
- **Pure:** Trypsin Gold is prepared by TPCK treatment followed by affinity purification.
- **Good Value:** Stable for up to 5 freeze-thaw cycles, thus minimizing reagent waste.
- **Referenced in Thousands of Papers:** Reliable and customer proven.

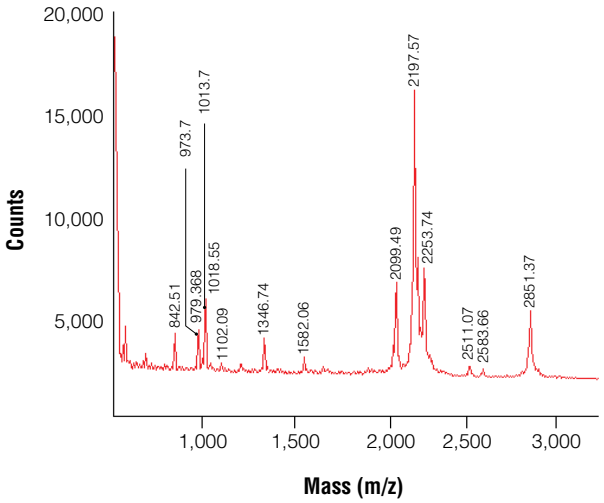


Figure 7.2. Spectrogram of bovine carbonic anhydrase II digested by Trypsin Gold, Mass Spectrometry Grade. A 500ng sample of carbonic anhydrase II was separated by gel electrophoresis and digested with 500ng of Trypsin Gold, Mass Spectrometry Grade, overnight at 37°C.*

Ordering Information

Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280)

Trypsin/Lys-C Mix, Mass Spectrometry Grade

Enhanced in-solution protein digestion. Applications include: protein identification, protein quantitation and characterization of post-translation modifications via LC-MS/MS analysis.

Description

Trypsin/Lys-C Mix, Mass Spectrometry Grade, is a mixture of Trypsin Gold, Mass Spectrometry Grade, and rLys-C, Mass Spec Grade. The Trypsin/Lys-C Mix is designed to improve digestion of proteins or protein mixtures in solution. Replacing trypsin with Trypsin/Lys-C Mix has multiple benefits for protein analysis, including more accurate mass spectrometry-based protein quantitation and improved analytical reproducibility. Trypsin/Lys-C Mix also provides greater tolerance to trypsin-inhibiting agents, assuring efficient digestion of proteins for which purification is limited or not feasible.

Principle

Typical trypsin reactions do not digest proteins to completion, missing 10–30% of cleavage sites **(Figure 7.3)**. Incomplete digestion affects protein identification, reproducibility of mass spectrometry analysis and accuracy of protein quantitation. The number of missed cleavage sites may be even higher if the protein is not properly purified or contains protease-inhibiting contaminants. Using the conventional trypsin digestion protocol (i.e., overnight incubation under nondenaturing conditions), Trypsin/Lys-C Mix improves protein digestion by eliminating the majority of missed lysine cleavages. The mixture is stable since trypsin is modified and therefore resistant to digestion by Lys-C.

References

Saveliev, S. *et al.* (2013) Trypsin/Lys-C protease mix for enhanced protein mass spectrometry analysis. *Nature Methods* 10, Published online 30 November 2013. http://www.nature.com/app_notes/nmeth/2013/131211/pdf/nmeth.f.371.pdf

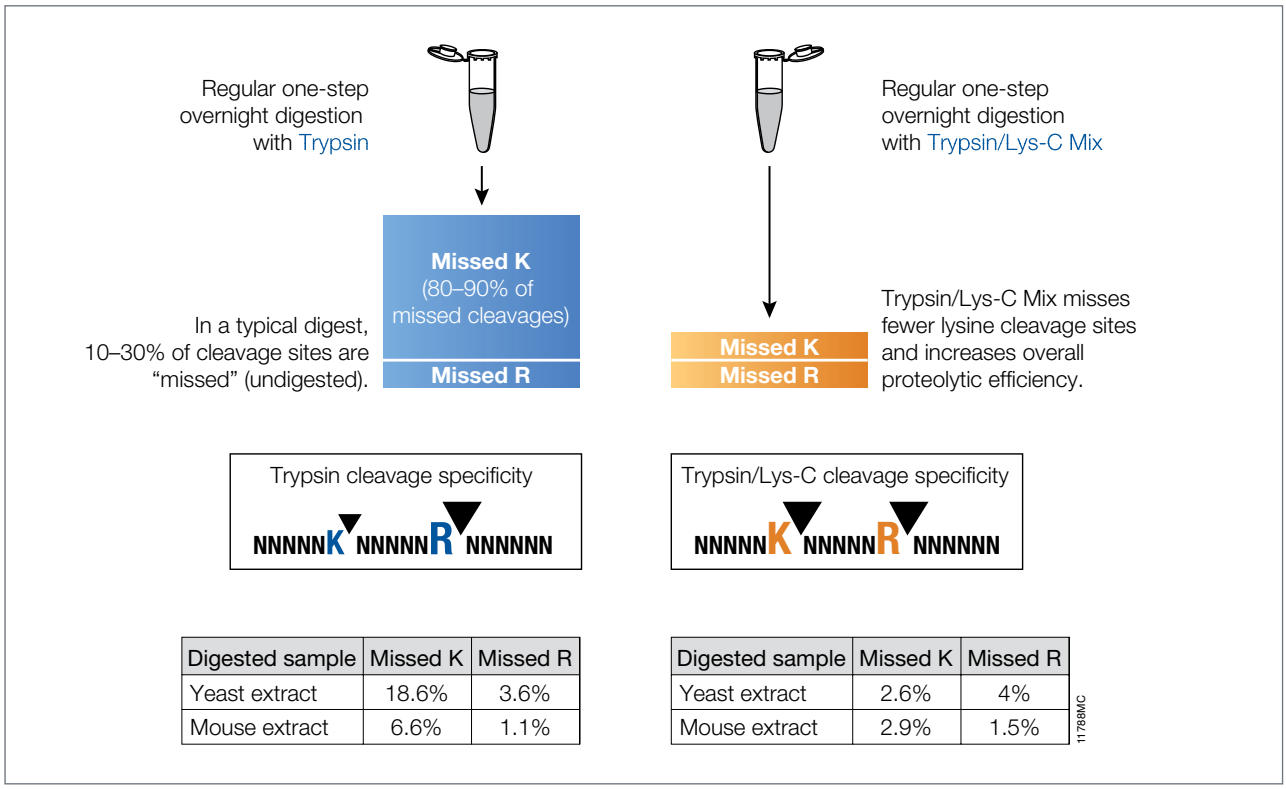
Features and Benefits

- **Simple to Use:** Use standard overnight digestion with non-denaturing conditions.
- **Enhanced Proteolysis:** Increase peptide recovery by enhancing cleavage efficiency at lysine residues and eliminating the majority of missed cleavages **(Figures 7.3 and 7.4)**.
- **Tolerant to Trypsin-Inhibiting Contaminants:** Generate mass spectrometry data from low-quality sample material.

Ordering Information

Trypsin/Lys-C Mix, Mass Spec Grade
(Cat.# V5071, V5072, V5073)

Trypsin/Lys-C Mix, Mass Spectrometry Grade (continued)



Digested sample	Missed K	Missed R
Yeast extract	18.6%	3.6%
Mouse extract	6.6%	1.1%

Digested sample	Missed K	Missed R
Yeast extract	2.6%	4%
Mouse extract	2.9%	1.5%

Figure 7.3. Side-by-side comparison of cleavage sites missed by trypsin or the Trypsin/Lys-C Mix using a standard digestion protocol.

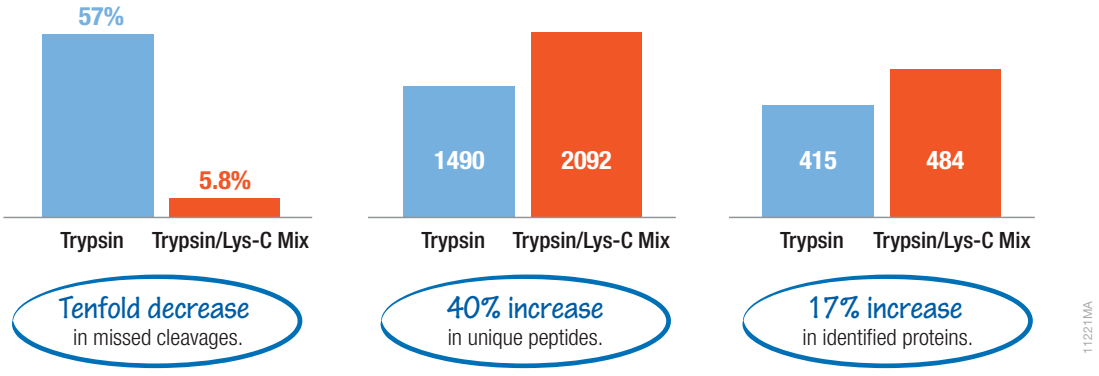


Figure 7.4. Improved mass spec analysis of a protein extract containing residual amounts of methanol and chloroform, which inhibit trypsin but have no effect on Trypsin/Lys-C Mix performance.

Sequencing Grade Modified Trypsin

Digestion of proteins into peptides via in-gel or in-solution techniques, followed by mass spec analysis. Typical applications include protein identification, protein quantitation and analysis of post-translation modifications via LC-MS/MS analysis.

Description

Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The stringent specificity of trypsin is essential for protein identification. Native trypsin is subject to autoproteolysis, generating pseudo-trypsin, which exhibits a broadened specificity including a chymotrypsin-like activity. Such autoproteolysis products present in a trypsin preparation could result in additional peptide fragments that may interfere with database analysis of the mass of fragments detected by mass spectrometry. Sequencing Grade Modified Trypsin is chemically modified to prevent auto-proteolysis and assure high proteolytic activity over the course of digestion reaction.

Principle

For in-gel digestion, protein samples are run on SDS-PAGE. Protein bands are stained and bands of interest are excised. A buffer containing resuspended trypsin is added and incubated overnight at 37°C. The resulting peptides are then extracted from the gel and analyzed by mass spec.

For in-solution digestion, protein samples are denatured, reduced and alkylated. Trypsin is then added and the solution is incubated for 3-12 hours at 37°C. The resulting peptides are desalted and analyzed by mass spec.

Features and Benefits

- **Maximum Specificity and Purity:** Trypsin is prepared by TPCK treatment followed by affinity purification.
- **High Stability:** Due to reductive methylation of lysine residues.
- **Reliable and Customer-Proven:** Referenced in more than 2,000 publications.
- **Additional Value:** Stability is ensured for up to 5 freeze-thaw cycles, minimizing leftover reagents.
- **Variety of Packaging:** We offer flexibility in packaging depending on experimental design and scope.

Ordering Information

Sequencing Grade Modified Trypsin (Cat.# V5111, V5117)

Sequencing Grade Modified Trypsin, Frozen (Cat.# V5113)

References

Trask. S. *et al.* (2013) Mutations in the rotavirus spike protein VP4 reduce trypsin sensitivity but not viral spread. *J. Gen, Vir.* **94**, 1296-300.

Trautner. C. *et al.* (2013) The sll1951 gene encodes the surface layer protein of Synechocystis sp. strain PCC 6803. *J. Bact.* **195**(23), 5370-80.

Lord, M. *et al.* (2013) Sulfation of the bikunin chondroitin sulfate chain determines heavy chain·hyaluronan complex formation. *J. Biol. Chem.* **288**(22), 22930-41.

Gordon,S. *et al.* (2013) Multi-dimensional co-separation analysis reveals protein-protein interactions defining plasma lipoprotein subspecies. *Mol. Cell, Prot.* **12**(11), 3123-34.

Jiang,W. *et al.* (2013) Mass spectrometry method to identify aging pathways of Sp- and Rp-tabun adducts on human butyrylcholinesterase based on the acid labile P-N bond. *Toxicol. Sci.* **132**(2), 390-98.

Burkhart J. *et al.* (2012) Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J. Proteomics* **75**(4), 1454-1462.

Immobilized Trypsin

Protein digestion of simple or complex protein mixtures over a wide range of concentrations.

Description

Trypsin in solution is the most widely used protease for digestion of proteins. However, there are a few limitations in using free trypsin. The limitations include:

1) The digestion requires a long time (three hours to overnight).
2) High concentrations of trypsin cannot be used because of the generation of dominant trypsin autolytic fragments in the digested samples.

To overcome these limitations we offer Immobilized Trypsin. It provides a fast and convenient method for protein digestion.

Principle

Immobilized Trypsin reduces digestion time and allows easy removal of trypsin from the digestion reaction **(Figure 7.5)**. Immobilized Trypsin provides the ability to digest 20–500µl of protein in solution simply by adjusting the amount of resin used in the reaction. This flexibility facilitates digestion while decreasing potential trypsin interference in downstream sample analysis. The percentage of digestion and sequence coverage is comparable to overnight digestion with free trypsin. Immobilized Trypsin has been used to study protein expression profiling in serum, microwave-assisted digestion of proteins, phoshopeptide analysis, analysis of membrane proteins and for $^{18}O/^{16}O$ labeling of peptides.

Features and Benefits

- **Fast:** Digestion can be accomplished in as little as 30 minutes.
- **Scalable:** Easily adjustable protocol to accommodate various protein concentrations.
- **Easy Setup:** Minimal centrifugation steps.

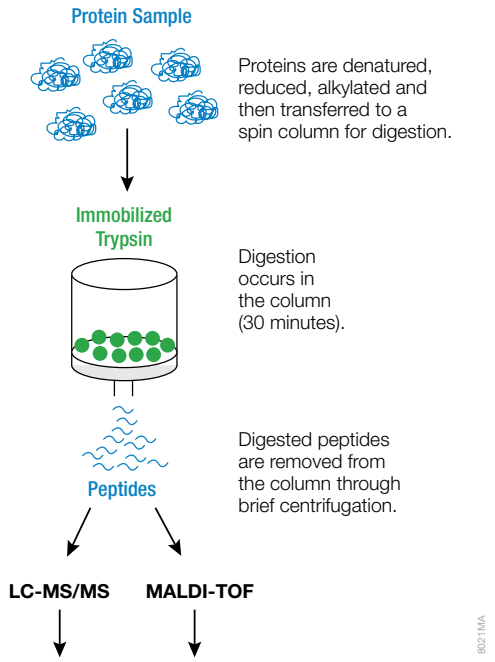


Figure 7.5. Overview of the Immobilized Trypsin digestion protocol.

Ordering Information

Immobilized Trypsin (Cat.# V9012, V9013)

7.2 Alternative Proteases for Mass Spectrometry

OVERVIEW

Trypsin is the most widely used endoprotease. It cleaves proteins in a highly specific manner and generates peptides 7–20 amino acids in length, with a strong C-terminal charge, making trypsin ideal for mass spectrometry analysis.

However, there are certain proteins and protein mixtures where trypsin digestion alone is not efficient enough. Examples include digestion of membrane proteins and analysis of histone post-translational modifications (PTMs). Furthermore, certain tryptic peptides are too small or too large for mass spec analysis. In these situations alternative proteases provide a viable solution, either alone or in combination with trypsin.

Table 7.1. Alternative Proteases and Corresponding Cleavage Sites.

Protease	Cleavage Site	Optimal pH	Example of Use
Lys-C Specific Protease	NNNN**K**⌄NNN (**K** is lysine)	7.0–9.0	Digests membrane and other proteolytically resistant proteins; generates larger peptides than trypsin–advantage for certain mass spec methods (for example, ETD).
Arg-C Specific Protease	NNNN**R**⌄NNN (**R** is arginine) Arg-C can, at a lesser degree, cleave at lysine also.	7.6–7.9	Facilitates analysis of histone posttranslational modifications; used in proteome-wide analysis.
Glu-C Specific Protease	NNNN**E**⌄NNN (**E** is glutamate) Glu-C can, at a lesser degree, cleave at aspartate residue also.	4.0–9.0	Used as alternative to trypsin if trypsin produces too short or too long peptides or if tryptic cleavage sites are not accessible.
Asp-N Specific Protease	NNNN⌄**D**NNN (**D** is aspartate)	4.0–9.0	Similiar to Glu-C.
Chymotrypsin Low Specific Protease	NNNN(**F**/**Y**/**W**)⌄NNN (**F**,**Y** and **W**) are aromatic residues phenylalanine, tyrosine and tryptophan)	7.0–9.0	Digests hydrophobic proteins (for example, membrane proteins).
Pepsin Nonspecific Protease	Nonspecific Protease (advantage: most active at low pH)	1.0–3.0	Used in structural protein studies and antibody analysis; digests proteolytically resistant, tightly folded proteins.
Thermolysin Nonspecific Protease	Nonspecific Protease (advantage: remains active at high temperature)	5.0–8.5	Digests proteolytically resistant, tightly folded proteins; used in structural protein studies.
Elastase Nonspecific Protease	Nonspecific Protease	9.0	Used to increase protein coverage.

Chapter 7.2 Overview (continued)

Digestion with Alternative Proteases Increase Protein Coverage

Digestion with an alternative protease, individually or in combination with trypsin, creates a unique peptide map that may include sequences not seen with trypsin digestion alone. Overlaying peptides obtained with alternative proteases and trypsin, increases protein coverage and overall confidence in protein identification **(Figure 7.6)**.

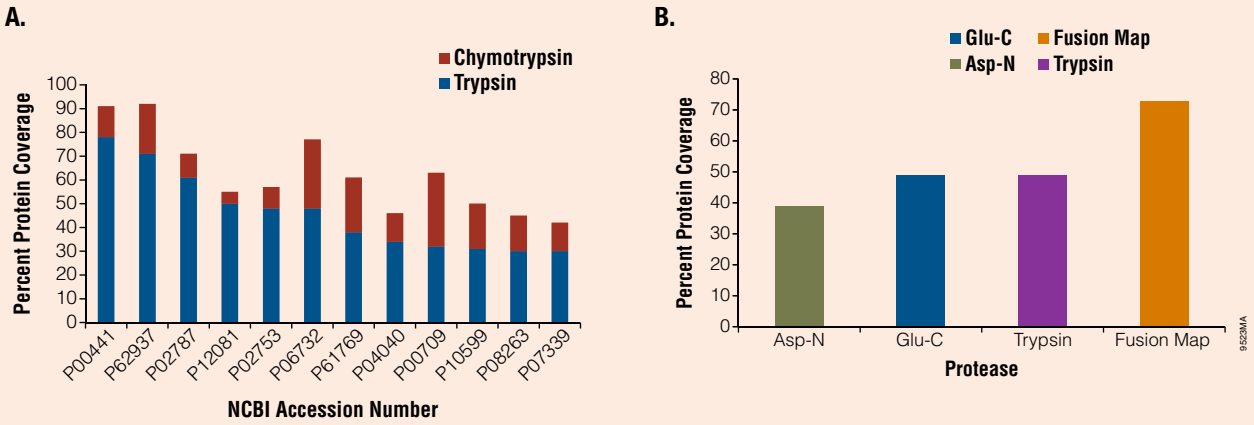


Figure 7.6. Increased protein coverage using **(Panel A)** both trypsin and chymotrypin; **(Panel B)** multiple proteases in separate digestion reactions. Note that the fusion map is the combination of the results obtained by digestion with each protease alone.

Alternative Proteases Improve Analysis of Post-Translational Modifications (PTMs)

In certain cases, trypsin is not suitable for PTM analysis. For example, histone PTMs are complex and some, such as acetylation and methylation, prevent trypsin digestion. As shown in **Figure 7.7**, trypsin digestion of histone H4 identified several PTMs; however, certain PTMs were missing. By digesting histone H4 with Arg-C, additional PTMs were identified, including mono- and dimethylated, and acetylated lysine and arginine residues.

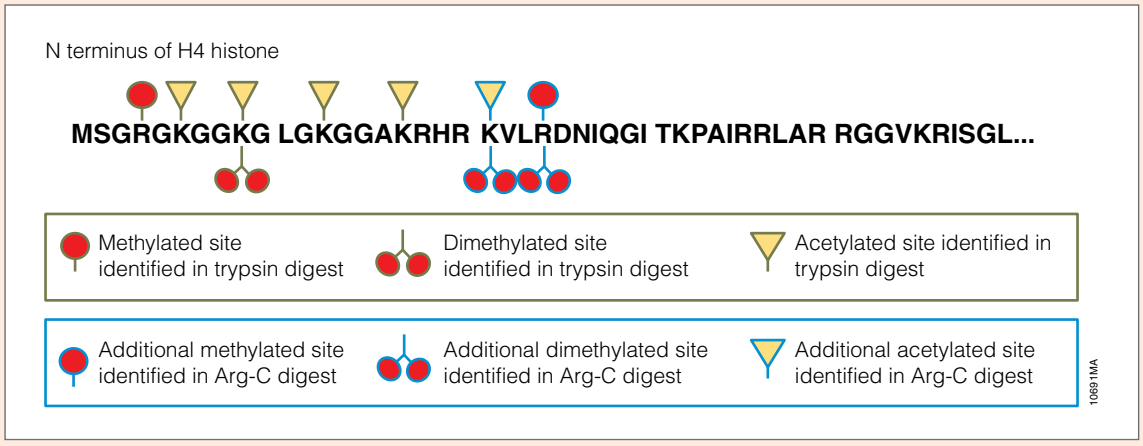


Figure 7.7. Histone H4 post-translational modifications identified in trypsin and Arg-C digests.

Alternative Proteases

Improve protein sequence coverage, enhance digestion of tightly-folded proteins, improve the identification of post-translation modifications (PTMs).

Specific Proteases

Arg-C (Clostripain), Sequencing Grade is an endopeptidase that cleaves at the C-terminus of arginine residues, including the sites next to proline. Arg-C activity is optimal in the pH range of 7.6–7.9.

Asp-N, Sequencing Grade is an endoproteinase that hydrolyzes peptide bonds on the N-terminal side of aspartic acid residues. Asp-N activity is optimal at pH 4.0–9.0.

Glu-C, Sequencing Grade is a serine protease that specifically cleaves at the C-terminus of glutamic acid residues. In ammonium bicarbonate and ammonium acetate the enzyme specificity is higher at the glutamic residues. In phosphate buffers cleavage occurs at the aspartic and glutamic residues. Glu-C activity is optimal at pH 4.0–9.0.

Endoproteinase Lys-C, Sequencing Grade is a serine protease isolated from *Lysobacter enzymogenes* as a highly purified protease, that hydrolyzes specifically at the C-terminus of Lys residues. Lys-C activity is optimal at pH 7.0–9.0.

rLys-C, Mass Spec Grade is a recombinant Lys-C expressed in *E. coli*. The sequence origin of rLys-C is Protease IV from *Pseudomonas aeruginosa*. Similarly to a native Lys-C, rLys-C cleaves at the C-terminus of lysine residues with exceptional specificity. rLys-C retains proteolytic activity under protein denaturing conditions such as 8M Urea, used to improve digestion of proteolytically resistant proteins. rLys-C activity is optimal at pH 8.0–9.0.

Low-specific Proteases

Chymotrypsin, Sequencing Grade is a highly purified serine endopeptidase derived from bovine pancreas that preferentially hydrolyzes at the carboxyl side of aromatic amino acids: tyrosine, phenylalanine and tryptophan. Chymotrypsin activity is optimal at pH 7.0–9.0.

Nonspecific Proteases

Elastase is a serine protease that preferentially cleaves at the C-terminus of alanine, valine, serine, glycine, leucine or isoleucine. Elastase activity is optimal at pH 9.0.

Pepsin preferentially cleaves at the C-terminus of phenylalanine, leucine, tyrosine and tryptophan. Pepsin activity is optimal at pH 1.0–3.0.

Thermolysin is a thermostable metalloproteinase. Thermolysin preferentially cleaves at the N-terminus of the hydrophobic residues leucine, phenylalanine, valine, isoleucine, alanine and methionine. The optimal digestion temperature range is 65–85°C. Thermolysin activity is optimal at pH 5.0–8.5.

Poster

Enhancing Trypsin Digestion with Lys-C and Arg-C Proteases. Promega Scientific Poster: **www.promega.com/resources/scientific_posters/posters/enhancing-trypsin-digestion-with-lysc-and-argc-proteases-scientific-poster/**

Alternative Proteases (continued)

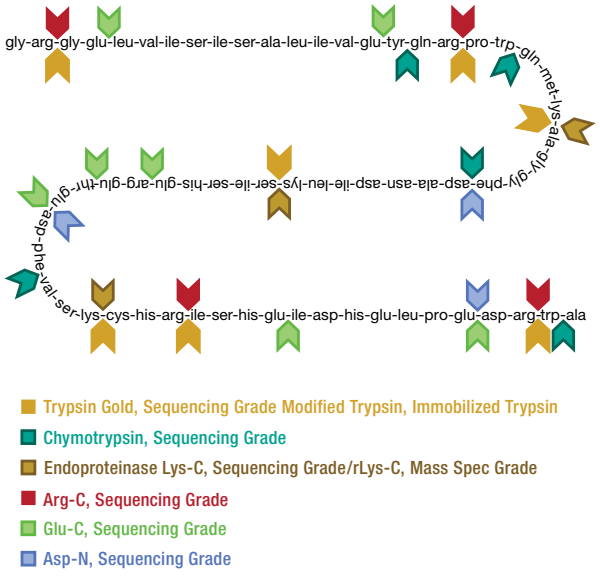


Figure 7.8. Cleavage sites of proteases frequently used in mass spectrometry sample preparation.

Ordering Information

Specific Proteases:

Arg-C (Clostripain), Sequencing Grade (Cat.# V1881)

Asp-N, Sequencing Grade (Cat.# V1621)

Glu-C, Sequencing Grade (S. aureus V8; Cat.# V1651)

Endoproteinase Lys-C, Sequencing Grade (Cat.# V1071)

rLys-C, Mass Spec Grade (Cat.# V1671)

Low-specific Proteases:

Chymotrypsin, Sequencing Grade (Cat.# V1061, V1062)

Nonspecific Proteases:

Elastase (Cat.# V1891)

Pepsin (Cat.# V1959)

Thermolysin (Cat.# V4001)

7.3 Glycosidases

OVERVIEW

Glycosylation is the most abundant post-translational modification (PTM) in eukaryotic proteins. Glycosylation structures on proteins have diverse functions and can serve as disease biomarkers, such as for the progression of cancer. Therefore, tools that facilitate glycoprotein characterization are extremely important.

Endoglycosidases and exoglycosidases are tools for structural and functional analysis of glycoproteins. Endoglycosidases like PNGase F **(see Figure 7.9)** and Endo H are routinely used to remove carbohydrates from N-glycosylated proteins. For the analysis of O-glycosylated proteins, a mixture of endoglycosidases and exoglycosidases is recommended.

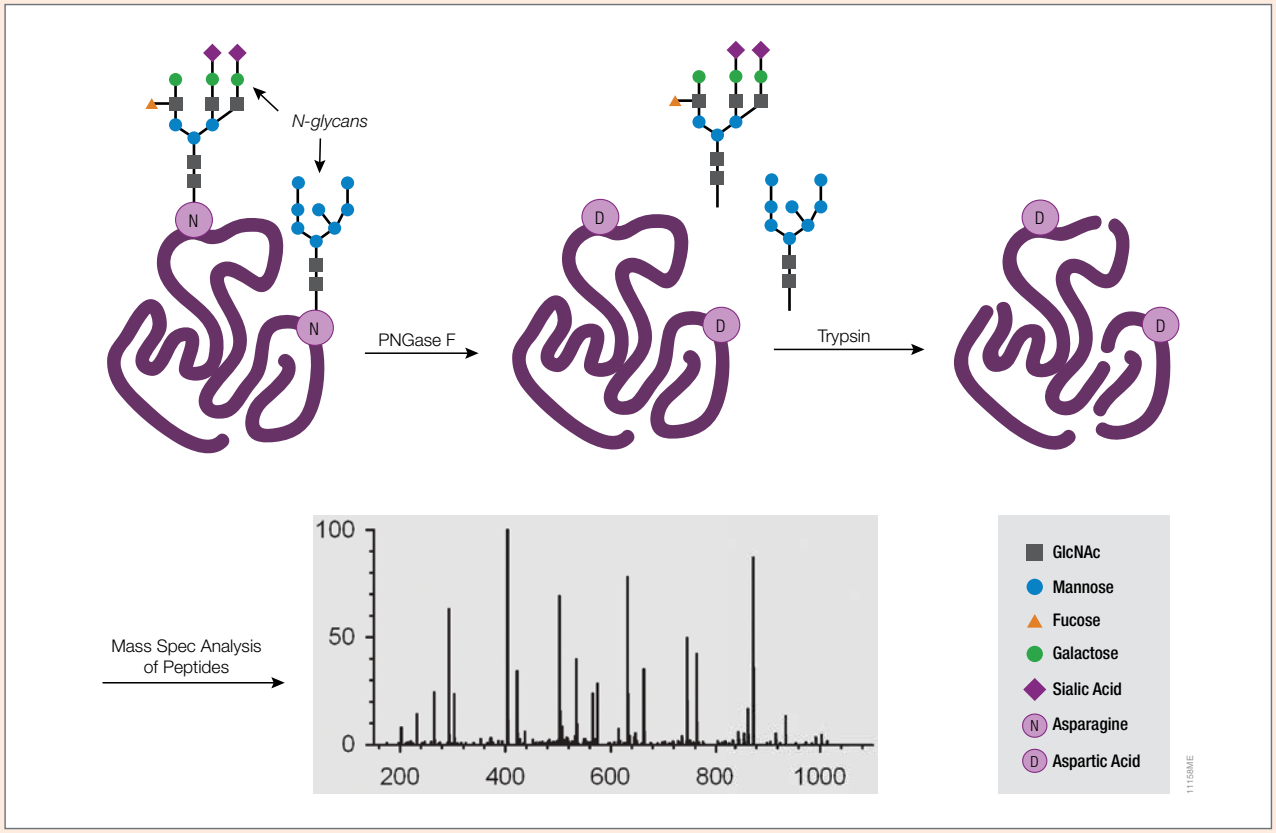


Figure 7.9. Identification of N-glycosylated sites in proteins. Asn-linked glycans can be cleaved enzymatically by PNGase F, yielding intact oligosaccharides and a protein in which Asn residues at the site of N-glycosylation are converted to Asp.

Glycosidases

Identification of glycosylation sites by LC-MS/MS; characterization of glycoproteins and glycan structures; monitor protein trafficking (endoplasmic reticulum, golgi); correlation of structural and functional data.

Description

PNGase F is a recombinant glycosidase cloned from *Elizabethkingia miricola* and overexpressed in *E. coli.* PNGase F has a molecular weight of 36kDa. PNGase F catalyzes the cleavage of N-linked oligosaccharides between the innermost N-Acetylglucosamine (GlcNAc) and asparagine residues (Asn) of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins **(Figure 7.10)**. Due to the enzymatic reaction, intact oligosaccharides are released and Asn residues are converted to aspartic acids (Asp) at the former sites of N-glycosylation. The conversion (deamination) of Asn to Asp leads to a monoisotopic mass shift of 0.9840 Da that is used in tandem mass spectrometry (MS/MS) for the identification of N-glycosylated sites in proteins. PNGase F will not remove oligosaccharides containing α-(1,3)-linked core fucose commonly found on plant glycoproteins.

Endo H is a recombinant glycosidase cloned from *Streptomyces plicatus* and overexpressed in *E. coli.* Endo H cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. In contrast to PNGase F, Endo H does not cleave N-glycosylated proteins of the complex type. The enzymatic activity of EndoH leaves one GlcNac residues at the N-glycosylated protein site leading to a shift in molecular weight (M.W.). This M.W. shift is used in mass spectrometry for the identification of N-glycosylated sites.

Protein Deglycosylation Mix is a mixture of five protein deglycosidases (PNGase F, O-Glycosidase, Neuraminidase, β1-4 Galactosidase, β-N- Acetylglucosaminidase) capable of removing glycans from both O-linked and N-linked glycosylation sites. Fetuin is provided as a deglycosylation substrate control.

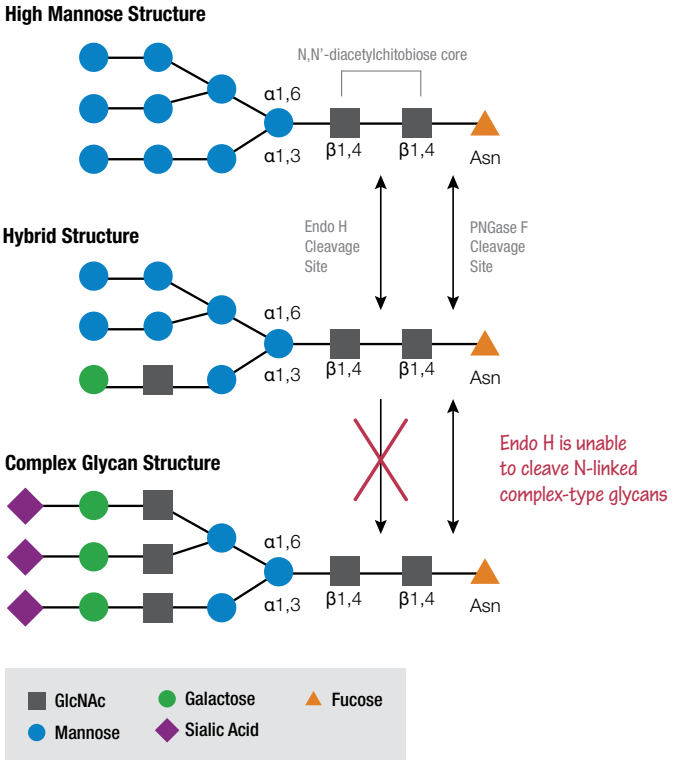


Figure 7.10. Cleavage specificity of PNGase F and Endo H on N-glycans.

Glycosidases (continued)

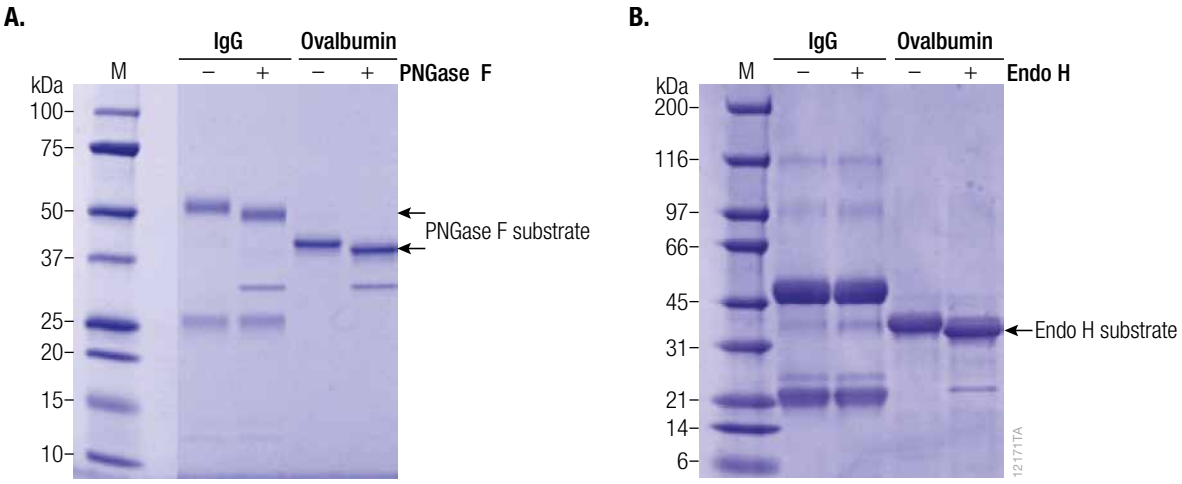


Figure 7.11. Detection of N-glycosylated proteins in SDS-PAGE. After glycan cleavage, the respective proteins show a higher mobility in SDS-PAGE. The application of both PNGase F and Endo H is informative for the presence of complex glycan structures, as shown here for Immunoglobulin G (IgG).

Ordering Information

PNGase F (Cat.# **V4831**)

Endo H (Cat.# **V4871, V4875**)

Protein Deglycosylation Mix (Cat.# **V4931**)

Fetuin (Cat.# **V4961**)

7.4 ProteaseMAX™ Surfactant

OVERVIEW

Detergents are routinely used for protein extraction, solubilization and/or denaturation. However, detergents are known to interfere with mass spectrometry analysis. ProteaseMAX™ Surfactant is a mass spec-compatible detergent that can be used for improved protein extraction from cells and tissues, for protein solubilization/denaturation as well as for improved in-gel and in-solution protein digestion. Unlike other detergents/denaturants such as SDS and Urea, ProteaseMAX™ Surfactant is compatible with MS analysis. It degrades over the course of digestion reaction. Samples can then be easily analyzed with mass spectrometry.

A comparison of different detergents in cell-lysis buffers (Urea, SDC, ProteaseMAX™ Surfactant) revealed that ProteaseMAX™ Surfactant is optimal for efficient cell lysis/protein extraction for mass spectrometry sample preparation. The results of Pirmoradian *et al.* suggest that the addition of ProteaseMAX™ Surfactant to the cell-lysis buffer/extraction buffer provides the highest number of peptide/protein identifications **(Table 7.2)**.

References

Pirmoradian, M. *et al.* (2013) Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol. Cell. Prot.* **12**, 3330–8.

Table 7.2. Comparison of Cell-lysis/Protein Extraction Methods

Cell-lysis/ Extraction Buffer	Number of Peptides	Number of Proteins
Urea	17,024 ± 148	3,326 ± 20
SDC*	22,171 ± 403	3,698 ± 18
ProteaseMAX™ Surfactant	29,884 ± 228	4,465 ± 100
ProteaseMAX™ Surfactant and Cell Debris**	33,098 ± 283	4,655 ± 51

*SDC: Sodium desoxycholate.

** Cell debris was included in the trypsin digestion step.

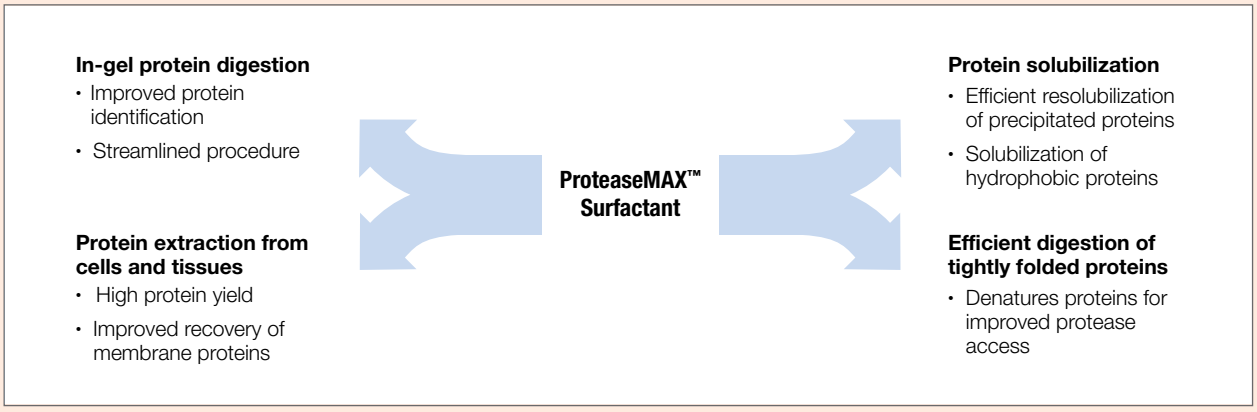


Figure 7.12. Multiple applications of ProteaseMAX™ Surfactant for improved protein mass spectrometry sample preparation.

ProteaseMAX™ Surfactant, Trypsin Enhancer

Enhanced in-gel and in-solution protein digestions; protein solubilization/ denaturation; protein extraction from cells and tissues.

Description

ProteaseMAX™ Surfactant, Trypsin Enhancer is designed to improve in-gel and in-solution protein digestions. It ensures fast and efficient protein digestion with proteases such as Trypsin, Chymotrypsin and Lys-C. ProteaseMAX™ Surfactant degrades over the course of a digestion reaction **(Figure 7.13)**, resulting in products that are compatible with mass spectrometry (MS) and liquid chromatography (LC). No long-term negative effect of the residual surfactant on the ion optics and capillary of mass spectrometry instruments has been observed. ProteaseMAX™ Surfactant can be used with existing in-gel or in-solution digestion protocols.

Principle

In-gel protein digestion is the major application for ProteaseMAX™ Surfactant. Benefits for in-gel digestion include increased peptide recovery from gels, minimized peptide absorption to plasticware and a streamlined digestion procedure. Trypsin and ProteaseMAX™ Surfactant (final concentration 0.01%) are added to a gel slice containing the protein band of interest. The digestion is incubated at 50°C for 1 hour. ProteaseMAX™ Surfactant also improves recovery of longer peptides that are retained in the gel under a standard extraction protocol.

ProteaseMAX™ Surfactant solubilizes proteins, including difficult proteins (i.e., membrane proteins) and enhances in-solution protein digestion by providing a denaturing environment. For cytoplasmic proteins, add ProteaseMAX™ Surfactant to a final concentration of 0.03%; for membrane proteins add to a final concentration 0.05%.

Features and Benefits

- **No Interference with Mass Spec Analysis:** Avoid the negative effects of common solubilizing agents (such as SDS).
- **Improved Peptide Recovery from Gels:** Protein sequence coverage and identification increased.
- **Enhanced Protein Solubilization :** Avoid high temperature.
- **Enhanced proteolysis rate:** Better access to cleavage sites.
- **Degrades Over Course of Digestion Reaction:** No need for post-digestion inactivation.

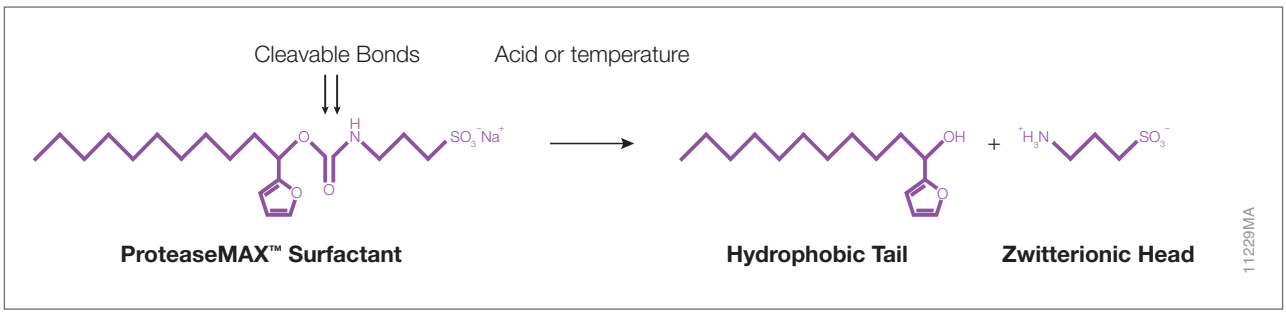


Figure 7.13. Chemical Structure of ProteaseMAX™ Surfactant and its decomposition pathway.

ProteaseMAX™ Surfactant, Trypsin Enhancer (continued)

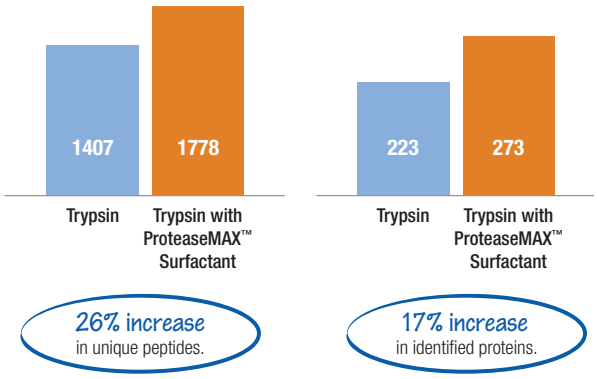


Figure 7.14. Example of improved peptide/protein identifications when using ProteaseMAX™ Surfactant for in-gel digestion of a complex protein sample.

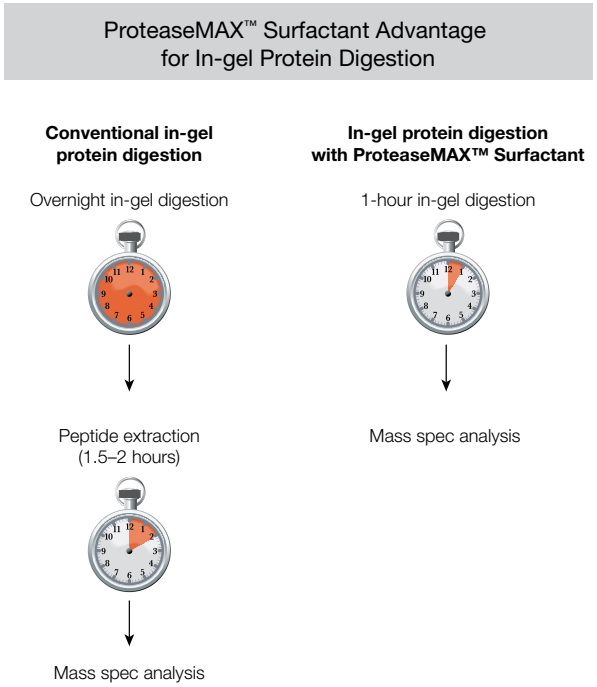


Figure 7.15. Comparison of ProteaseMAX ™ Surfactant-assisted versus standard in-gel digestion procedure ProteaseMAX™ Surfactant offers time and labor savings.

References

Saveliev, S. (2013) Mass spectrometry compatible surfactant for optimized in-gel protein digestion. *Anal. Chem.* **85**(2) 907-14.

Pirmoradian, M. *et al.* (2013) Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol. Cell Prot.* **12**(11), 3330-38.

Kalashnikova, A. *et al.* (2013) Linker histone H1.0 interacts with an extensive network of proteins found in the nucleolus. *Nucl. Acids. Res.* **41**(7), 4026-35.

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Ordering Information

ProteaseMAX™ Surfactant, Trypsin Enhancer (Cat.# V2071, V2072)

7.5 Protein Extracts for LC/MS Instrument Performance Monitoring

OVERVIEW

Monitoring Liquid Chromatography/Mass Spectrometry (LC/MS) instrument performance is required to assure efficient MS-analysis. The complexity of biological samples puts high demands on LC/MS analytical capabilites. Adequate monitoring of instrument performance for proteomic studies requires complex protein reference materials. Whole-cell protein extracts provide the needed complexity. We offer mass speccompatible whole-cell reference protein extracts from yeast and human cells, in a convenient, pre-digested lyophilized form. The yeast reference extract offers the convenience of a relatively small and well-annotated proteome, whereas the human reference extract provides a complex proteome with large dynamic range. Material reproducibility (i.e., lot-to-lot consistency in protein composition and abundance) is assured by tight control over cell culture conditions and manufacturing processes. Consistency is monitored by various protein and peptide qualitative and quantitative methods including amino acid analysis and LC/MS analysis.

Validated Proteome Mass Spectrometry Reference Material

- Full compatibility with LC and MS
- Minimal nonspecific protein fragmentation and low levels of nonbiological PTMs, including deamidation, oxidation and carbamylation
- Minimal level of missed tryptic cleavages
- Lot-to-lot protein compositional reproducibility (monitored by LC/MS)
- Lot-to-lot protein quantitative reproducibility (monitored by spectral counting)

Table 7.3. Representative MS Analysis of MS Compatible Human and Yeast Protein Extracts.

Extract	Human	Yeast
Identified proteins	2120	1637
Total spectra	26440	21264
Spectrum match	65%	52%
Unique peptides	16375	12079
Deamidation spectra	4.9%	6.5%
Oxidation spectra	0.6%	1.3%
Carbamylated spectra	0.2%	0.6%
Nonspecific cleavages	1%	2%
Missed Tryptic cleavages	4.9%	4.8%

The extracts were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive (1µg injection).

MS Compatible Human Protein Extract, Digest

Ready-to-use predigested human extracts for Liquid Chromatography/Mass Spectrometry (LC/MS) instrument performance monitoring and method development.

Description

MS Compatible Human Protein Extract, Digest is a lyophilized tryptic peptide mixture from K562 human cells (myelogenous leukemia cell line) designed for monitoring of LC/MS instrument performance. The digest is also suitable for MS method development such as isobaric mass tag labeling. Stringent quality control over the manufacturing process assures reproducibility of this reference material. MS Compatible Human Protein Extract, Digest is provided in ready-to-use format eliminating the need for sample preparation prior to MS analysis.

Preparation

MS Compatible Human Protein Extract, Digest, is prepared by digestion of K562 whole-cell protein extract with the Trypsin/Lys-C Mix. The digestion procedure is optimized to assure the most efficient proteolysis with less than 8% tryptic missed cleavages. The digest is SPE (C_{18}) cleaned-up to remove nonpeptide material and lyophilized. The digest can be reconstituted in formic acid or TFA and used for instrument performance monitoring. In general, 1µg of digest per injection is recommended.

Features and Benefits

- **Ready-to-Use:** No clean-up or digestion required.
- **High Material Complexity:** Complex proteome with a large dynamic range.
- **Lot-to-Lot Consistency:** Stringent QC over the production process.
- **Validated Reference Material:** Tested with all the major LC/MS platforms.

Ordering Information

http://www.promega.com/extracts

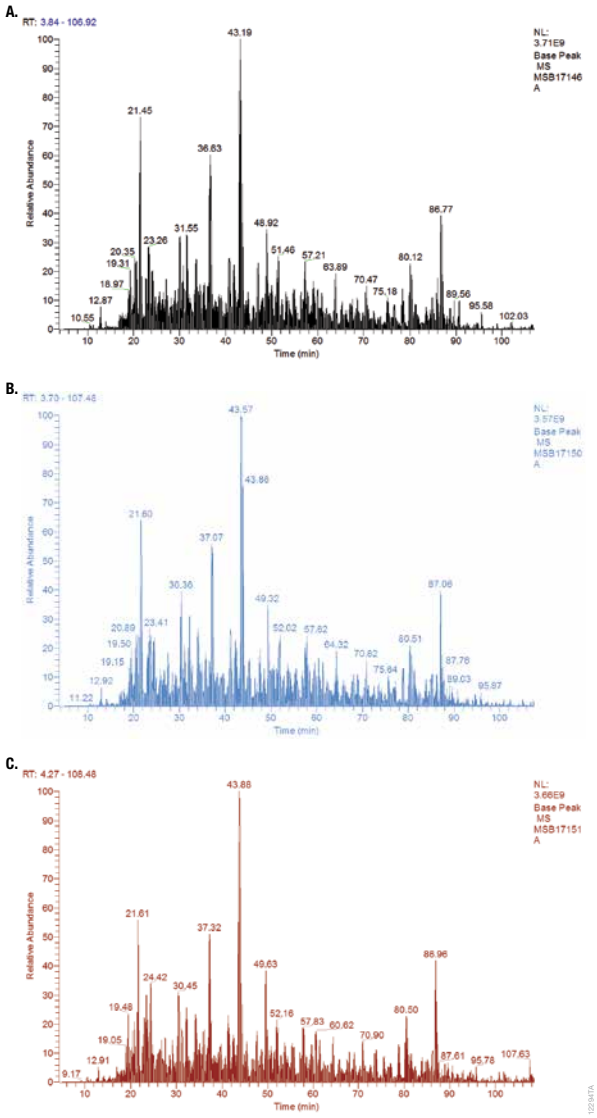


Figure 7.16. Lot-to-lot reproducibility. Base peak chromatograms of three different human protein extract batches analyzed by LC/MS. Each run used 1µg of human pre-digested protein extract injected into the instrument (Waters NanoAquity HPLC system interfaced to a ThermoFisher Q Exactive). Peptides were resolved with 2h gradient.

Promega

Protein Extracts for LC/MS Instrument Performance Monitoring

MS Compatible Yeast Protein Extract, Digest

Ready-to-use predigested yeast extracts for Liquid Chromatography/Mass Spectrometry LC/MS instrument performance monitoring and method development.

Description

MS Compatible Yeast Protein Extract, Digest from *Saccharomyces cerevisiae* is a lyophilized tryptic peptide mixture designed for monitoring LC/MS instrument performance. The digest is also suitable for MS method development such as isobaric mass tag labeling. Stringent quality control over the manufacturing process assures reproducibility of this reference material.

Preparation

MS Compatible Yeast Protein Extract, Digest, is prepared by digestion of yeast whole-cell protein extract with the Trypsin/Lys-C Mix. The digestion procedure is optimized to assure the most efficient proteolysis with less than 8% tryptic missed cleavages. The digest is SPE (C_{18}) cleaned-up to remove nonpeptide material and lyophilized. The digest can be reconstituted in formic acid or TFA and used for instrument performance monitoring. In general, 1µg of digest per injection is recommended.

Features and Benefits

- **Ready-to-Use:** No clean-up or digestion required.
- **Well-Annotated Proteome:** The small yeast proteome is a convenient reference material.
- **Lot-to-Lot Consistency:** Stringent QC throughout the production process.
- **Validated Reference Material:** Tested with all the major LC/MS platforms.

Ordering Information

http://www.promega.com/extracts

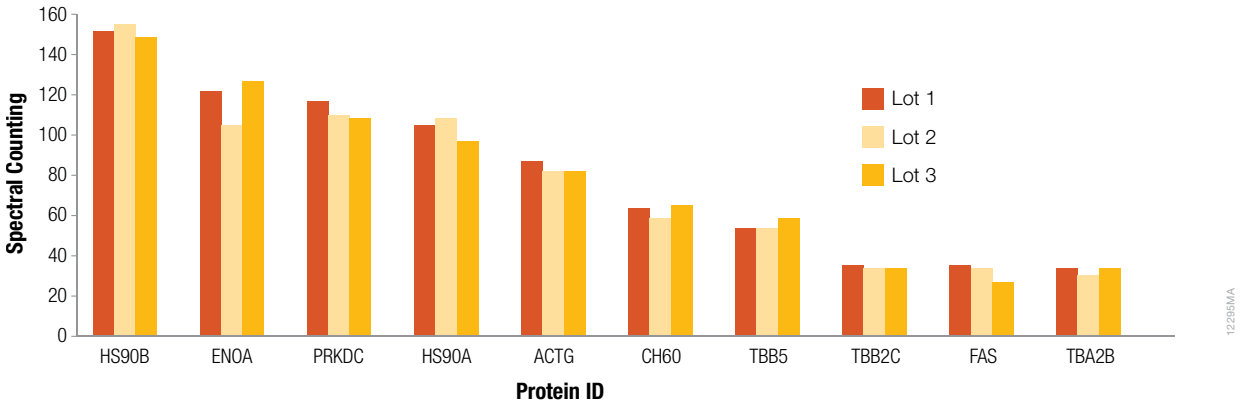


Figure 7.17. Lot-to-lot quantitative reproducibility of yeast extract. Relative protein quantitative levels were determined by spectral counting. The data showed high protein quantitative reproducibility between the different lots.

7.6 Proteases for Mass Spectrometry Sample Preparation

ADDITIONAL INFORMATION

Table 7.4. Characteristics and Reaction Conditions for Trypsin Proteases.

	Trypsin Gold, Sequencing Grade Modified Trypsin, Sequencing Grade Modified Trypsin, Frozen	Immobilized Trypsin	Trypsin/Lys-C Mix
Cat.#	V5280; V5111; V5117; V5113	V9012; V9013	V5071; V5072; V5073
Source and Size	Porcine Pancreas (23.8kDa)	Porcine Pancreas (23.8kDa)	Mix of Tryspin-Gold & rLys-C
Cleavage Sites	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. Asp and Glu at C-terminal side of Lys and Arg inhibit cleavage.	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. Asp and Glu at C-terminal side of Lys and Arg inhibit cleavage.	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. In contrast to trypsin, Trypsin/Lys-C efficiently tolerates presence of Glu and Asp at C-terminal side of Lys and Arg.
Protease Protein Ratio (w/w)	1:20 to 1:100	see Technical Manual	1:25 to 1:50
pH Range for Digestion	pH 7–9	pH 5–9	pH 8
Reaction Conditions	50–100mM Tris-HCl (pH 8) or 50–100mM NH_4HCO_3(pH 7.8). Digest overnight hours at 37°C.	50mM NH_4HCO_3 (pH 7.8). Digest for 30 minutes at room temperature.	50–100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH7.8). Digest overnight at 37°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3
In-Gel Digestion Compatibility	Yes	No	Yes
ProteaseMAX™ Compatibility	Yes	Yes	Not tested
Notes	Most widely used protease in mass spectrometry. All Promega trypsin is treated with TPCK to inactivate chymotrypsin contamination and chemically modified (methylated) to minimize autoproteolysis. All Promega trypsin is resistant to mild denaturing conditions (1–2M urea and 0.1% SDS). They retain 48% activity in 2M Guanidine Chloride.	Used if rapid digestion is required. Urea ≤ 4M; guanidine HCl ≤ 3M; methanol < 60%; DTT ≤ 50mM; TCEP ≤ 5mM; pH 5–9; Iodoacetamide ≤ 300mM.	Trypsin/Lys-C mix is the most robust trypsin preparation. It is designed to improve general protein digestion. Trypsin/Lys-C mix uses the same regular overnight digestion at 37°C under non- or mildly denaturing conditions as trypsin alone.

Table 7.5. Characteristics and Reaction Conditions for Specific Alternative Proteases.

	rLys-C	Endoproteinase Lys-C	Arg-C	Asp-N	Glu-C
Cat.#	V1671	V1071	V1881	V1621	V1651
Source and Size	*Pseudomonas aeruginosa.* Expressed in *E.coli* (27.7kDa)	*Lysobacter enzymogenes* (30kDa)	*Clostridium histolyticum* (Subunits: 45kDa and 12kDa)	*Pseudomonas fragi* (24.5kDa)	*Staphylococcus aureus V8* (27kDa)
Cleavage Sites	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp and Glu at C -terminal side of Lys inhibit cleavage.	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp and Glu at C -terminal side of Lys inhibit cleavage.	C-terminal of Arg. Also cleaves at Lys albeit at lower efficiency.	N-terminal of Asp.	C-terminal of Glu. Low level cleavages might occur at Asp residues too albeit at 100–300 fold lower efficiency.
Protease Protein Ratio (w/w)	1:20 to 1:50	1:20 to 1:100	1:20 to 1:350	1:20 to 1:200	1:20 to 1:200
pH Range for Digestion	pH 8–9	pH 7–9	pH 7.6–7.9	pH 4–9	pH 4–9
Reaction Conditions	50-100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8). Digestion 2–18 hours at 37°C.	50-100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8) Digestion 2–18 hours at 37°C.	50mM Tris-HCl (pH 7.6-7.9), 5mM $CaCl_2$, 2mM EDTA, >2mM DTT. Digestion 2–18 hours at 37°C.	50mM Tris-HCl (pH 8).Digestion 2–18 hours at 37°C.	100mM NH_4HCO_3 (pH 7.8), 50-100 mM HCL (pH8). Digestion 2–18 hours at 37°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	NH_4HCO_3, Ammonium acetate
In-Gel Digestion Compatibility	Yes	Yes	Yes	Yes	Yes
ProteaseMAX™ Compatibility	Yes	Yes	Yes	Yes	Yes
Notes	Inexpensive alternative to a native Lys-C protease. Similarly to a native protease, rLys-C tolerates high denaturing conditions such as 8M urea. Used to digest tightly folded proteolytically resistant proteins. Also used as a trypsin alternative if larger peptides are preferable for the analysis. If urea is used in protein sample preparation, avoid high temperature. High temperature induces protein carbamylation in the presence of urea.	Tolerates high denaturing conditions such as 8M urea. Used to digest tightly folded proteolytically resistant proteins. Also used as a trypsin alternative if larger peptides are preferable for analysis. If urea is used in protein sample preparation, avoid high temperature. High temperature induces protein carbamylation in the presence of urea.	Used in analysis of histone modifications. Requires DTT, cysteine or other reducing agent and $CaCl_2$ for activity.	Can be used as a trypsin alternative to achieve better distribution of cleavage sites.100% activity retained in the presence of urea (up to 3.5 M), guanidine HCL (1M), SDS (up to 0,028%), ProteaseMax™ Surfactant (up to 0,026%), acetonitrile (up to 60%), EDTA (up to 2 mM); DTT or ß-mercaptoethanol	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. Glu-C activity and cleavage specificity is affected by buffer conditions. In ammonium biocarbonate and other non-phosphate buffers, Glu-C cleaves at C-term of Glu. Glu-C cleaves at C-term of Glu and Asp in phosphate buffer.

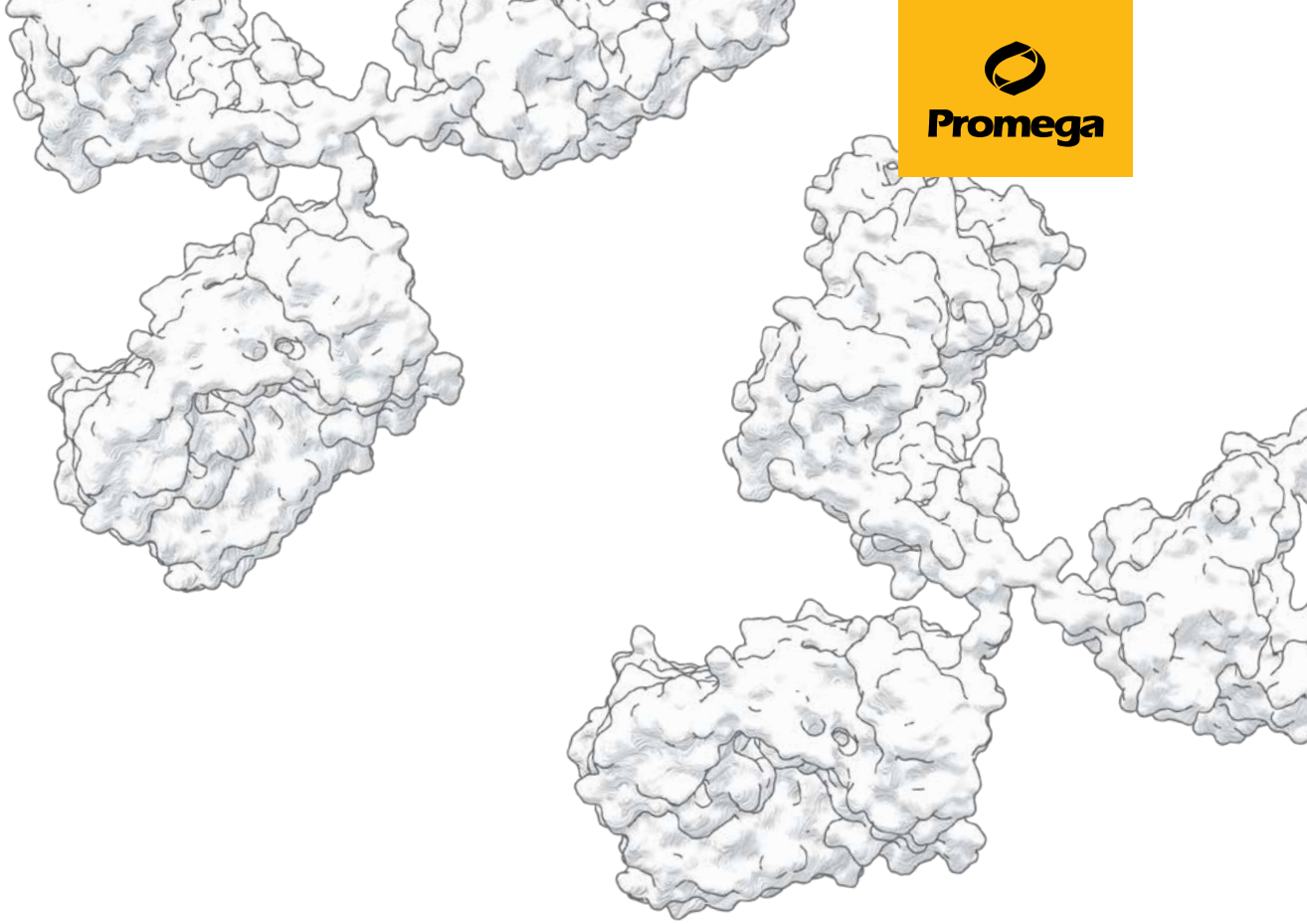
Table 7.6. Characteristics and Reaction Conditions for Low Specific Alternative Proteases.

	Chymotrypsin
Cat.#	V1061; V1062
Source and Size	Bovine pancreas (25kDa)
Cleavage Sites	Preferentially C-terminal of Trp, Tyr and Phe but it also cleaves at other residues albeit at a lower efficiency.
Protease Protein Ratio (w/w)	1:20 to 1:200
pH Range for Digestion	pH 7–9
Reaction Conditions	100mM Tris HCl (pH 8), 10mM $CaCl_2$. Digestion 2–18 hours at 25°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3
In-Gel Digestion Compatibility	Yes
ProteaseMax™ Compatibility	Yes
Notes	Often used to digest hydrophobic proteins including membrane proteins. 80% activity retained in the presence of urea (up to 1M) or 1M guanidine HCl (up to 1M). No reduction in activity in the presence of ProteaseMAX™ Surfactant (up to 0,025%).

Table 7.7. Characteristics and Reaction Conditions for Nonspecific Proteases.

	Elastase	Pepsin	Thermolysin
Cat.#	V1891	V1959	V4001
Source and Size	Porcine pancreas (25.9kDa)	Porcine stomach (34.6kDa)	*Bacillus thermoproteolyticus rokko* (36.2kDa)
Cleavage Sites	Preferentially C-terminal of Ala, Val, Ser, Gly, Leu and Ile.	Preferentially C-terminal of Phe, Leu, Tyr and Trp	Preferentially N-terminal of Leu, Phe, Val, Ile and Met.
Protease Protein Ratio (w/w)	1:20 to 1:100	1:20 to 1:100	1:20 to 1:50
pH Range for Digestion	pH 9	pH 1–3	pH 5.0–8.5
Reaction Conditions	50-100mM Tris-HCl (pH 8.5-9.5), digestion 2–18 hours at 37°C.	Adjust protein solution to pH 1-3 with 1N HCl prior to digestion. Digestion 1–18 hours at 37°C.	50mM Tris-HCl (pH 8), 0.5mM $CaCl_2$. Digestion 0.5-6 hours at 70–95°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	(—)	Tris-HCl
In-Gel Digestion Compatibility	Yes	Yes	Not tested
ProteaseMAX™ Compatibility	Yes	Yes	Yes
Notes	Used as a trypsin alternative to increase protein coverage.	Used in structural protein studies (HDX exchange based) and antibody analysis; used to digest proteolyti-cally resistant, tightly folded proteins.	Used to digest proteolytically resistant, tightly folded proteins and in structural studies.

8 Protein Detection Tools for Western Blotting & ELISA



Protein Detection Tools for Western Blotting and ELISA

Western blot and ELISA are widely used analytical techniques for the specific detection of proteins in samples such as cells, tissues and other extracts. For both techniques protein-specific antibodies (primary antibodies) are required. Upon binding of a primary antibody to its target, a conjugated secondary antibody directed against a species-specific heavy chain portion of the primary antibody is added: for example, an anti-mouse secondary antibody binds to a primary antibody generated in mouse. Secondary antibodies, which are most frequently conjugated to reporter enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), will bind to one primary antibody. Depending on the substrates used, the conjugated enzymes catalyze a colorimetric, fluorescent or chemiluminescent reaction enabling sensitive detection with different instruments and scanners.

Chapter 8 Overview (continued)

Table 8.1. Overview of Substrates for Western Blotting and ELISA.

Readout	Secondary Antibody Conjugates	
	Alkaline Phosphatase	Horseradish Peroxidase
Luminescent	-	ECL Western Blotting Substrate[W] (Cat.# W1001, W1015)
Fluorescent	AttoPhos® AP Fluorescent Substrate[E] (Cat.# S1000)	-
Colorimetric	ProtoBlot® AP Systems (BCIP/NBT)[W] (Cat.# W3940, W3950, W3960) Western Blue® Stabilized Substrate[W] (Cat.# S3841)	TMB Stabilized Substrate[W] (Cat.# W4121) TMB One Solution[E] (Cat.# G7431)

[E] ELISA
[W] Western Blotting

Table 8.2. Overview of Promega Conjugated Secondary Antibodies.

Cat.#	Conjugated Secondary Antibodies	Size	Recommended Dilution	Storage	Applications*
Alkaline Phosphatase conjugates (AP)					
S3721	Goat Anti-Mouse IgG (H+L), AP Conjugate	100µl	1:7,500	+4°C	W, D, E
S3731	Goat Anti-Rabbit IgG (Fc), AP Conjugate	100µl	1:7,500	+4°C	W, D, E
S3821	Goat Anti-Human IgG (H+L), AP Conjugate	100µl	1:7,500	+4°C	W, D, E
S3831	Goat Anti-Rat IgG (H+L), AP Conjugate	100µl	1:2,500	+4°C	W, D, E
Horseradish Peroxidase conjugates (HRP)					
W4021	Goat Anti-Mouse IgG (H+L), HRP Conjugate	300µl	1:2,500	+4°C	W, D, E
W4011	Goat Anti-Rabbit IgG (H+L), HRP Conjugate	300µl	1:2,500	-20°C/+4°C	W, D, E
W4031	Goat Anti-Human IgG (H+L), HRP Conjugate	300µl	1:2,500	+4°C	W, D, E
G1351	Rabbit Anti-Chicken IgY, HRP Conjugate	300µl	1:1,000	–20/+4°C	W, D, E
Anti-ACTIVE® qualified antibodies					
V1151	Donkey Anti-Goat IgG, AP	60µl	1:5,000-10,000	-20°C	W
V7951	Donkey Anti-Rabbit IgG (H+L) HRP	60µl	1:5,000-10,000	-20°C/+4°C	W
V8051	Donkey Anti-Goat IgG, HRP	60µl	1:5,000-10,000	-20°C	W

*W: Western Blotting; D: Dot Blotting; E: ELISA

Conjugated Secondary Antibodies

Detection of primary antibodies in Western blotting, enzyme-linked immunosorbent assay (ELISA) and dot blotting.

Description

High-quality, polyclonal secondary antibodies are raised in goat, rabbit or donkey. These polyclonal antibodies are immunoaffinity-purified using corresponding immobilized antigens. They are conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes.

The Anti-ACTIVE® qualified secondary antibodies are specifically tested for the use with Promega Anti-ACTIVE® primary antibodies, which are tools to measure activation of three members of the Mitogen-Activated Protein Kinase (MAPK) superfamily. The primary antibodies are specific for dually-phosphorylated active forms of MAPK, p38 and JNK. They exhibit minimal cross-reactivity to goat, mouse and sheep IgG, bovine serum albumin (BSA) and proteins in mammalian cell extracts. These secondary antibody conjugates provide low backgrounds and highly specific signals.

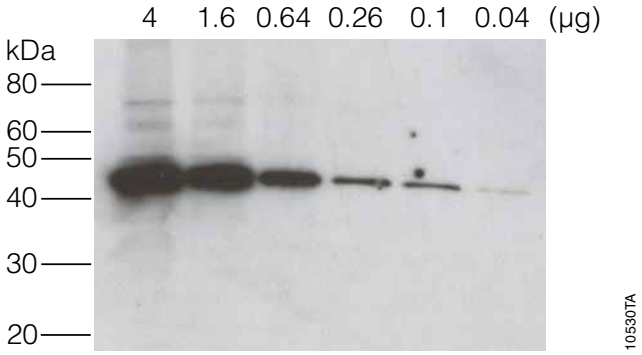


Figure 8.1. Western blot (immunoblot) for β-actin in cytoplasmic lysate from HEK293T cells. The blot was performed on a serial dilution of lysate; each lane contains an indicated amount of total protein (µg). Primary antibody (monoclonal) was used at 1:5,000; Anti-Mouse IgG, HRP Conjugate secondary antibody (Cat.# W4021) was used at 1:2,500; blot imaged with ECL Western Blotting Substrate (Cat.# W1015) and 1-minute exposure.

Features and Benefits

- **Approved:** Use with confidence, as supported by numerous publications.
- **Ready-to-Use Formulation:** No need to reconstitute the antibody.
- **Flexible Dispensing:** We can readily accommodate large-scale custom orders.

References

Chang, H.-Y. *et al.* (2011) Domain analysis of protein P30 in *Mycoplasma pneumoniae* cytadherence and gliding motility. *J. Bacteriol.* **193**(7), 1726–33.

Prenner, G. *et al.* (2011) Is LEAFY a useful marker gene for the flower-inflorescence boundary in the *Euphorbia cyathium*? *J. Exp. Bot.* **62**(1), 345–50.

Hu, D. *et al.* (2012) Novel insight into KLF4 proteolytic regulation in estrogen receptor signaling and breast carcinogenesis. *J. Biol. Chem*. **287**(17), 13584–97.

AttoPhos® AP Fluorescent Substrate System

Fluorescent development of ELISA.

Description

The AttoPhos® AP Fluorescent Substrate System provides a highly sensitive fluorescent alkaline phosphatase (AP) substrate. The system includes AttoPhos® Substrate, AttoPhos® Buffer and Calibration Solution. AttoPhos® Substrate is cleaved by alkaline phosphatase to produce inorganic phosphate (Pi) and the alcohol 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole (BBT).

This enzyme-catalyzed conversion of the phosphate form of AttoPhos® Substrate to BBT is accompanied by an enhancement in fluorescence properties. Relative to AttoPhos® Substrate, the BBT anion has highly increased quantum efficiency and fluorescence excitation, also the emission spectra are shifted into the visible region. Relative to other fluorometric reporters, the BBT anion has an unusually large Stokes' shift of 120nm, resulting in a higher signal-to-noise ratio and higher overall detection sensitivity. The excitation of the fluorescence is at 435nm, emission at 555nm.

Features and Benefits

- **Sensitivity:** Low fluorescence signal until enzymatically activated, detection of AP to 0.1 attomole.
- **Low Background:** Low fluorescence from interfering biological molecules.
- **Linearity:** Linear kinetics over five orders of magnitude of AP concentration.

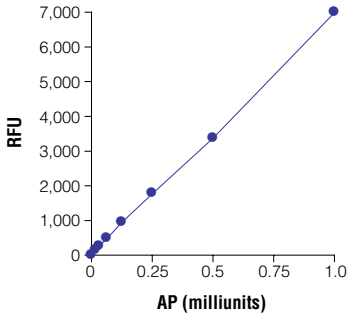


Figure 8.2. Fluorescence signal observed for a serial dilution of calf intestinal alkaline phosphatase (CIAP) treated with 1mM AttoPhos® Substrate in a multiwell plate. The graph represents the increasing fluorescence per unit of AP at 15 minutes post addition of AttoPhos® Substrate.

References

Michaud, A. *et al.* (2013) Absence of cell surface expression of human ACE leads to perinatal death. *Hum. Mol. Genet.* Nov. 14 [Epub ahead of print]

Meinel, S. *et al.* (2013) Mineralocorticoid receptor interaction with SP1 generates a new response element for pathophysiologically relevant gene expression. *Nucl. Acids Res.* **41**(17), 8045–60.

Chen, Y. *et al.* (2013) Common tolerance mechanisms, but distinct cross-reactivities associated with gp41 and lipids, limit production of HIV-1 broad neutralizing antibodies 2FS and 4E10. *J. Immunol.* **191**(3), 1260–75.

Ordering Information

AttoPhos® AP Fluorescent Substrate System
(Cat.# S1000)

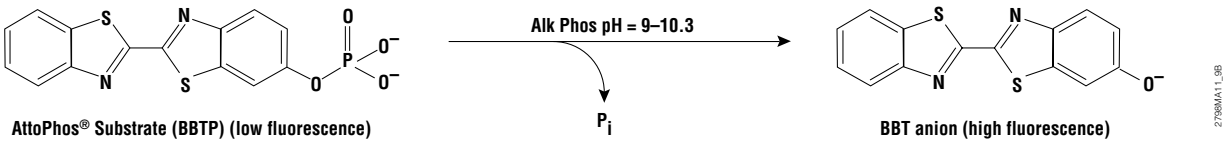


Figure 8.3. The reaction of AttoPhos® Substrate with AP to produce highly fluorescent BBT and inorganic phosphate (Pi).

TMB One Solution

Colorimetric Development of ELISA.

Description

TMB One Solution is a safe, convenient ready-to-use working solution for the detection of HRP-conjugated antibodies in an ELISA format. HRP-conjugated antibodies react with the chromogenic substrate 3,3´,5,5´-tetramethylbenzidine (TMB) yielding a blue-colored solution. After reaching the desired color intensity, the reaction is stopped by addition of an acidic solution, which leads to a change in color from blue to yellow. Plates are analyzed on an ELISA reader at 450nm.

Features and Benefits

- **Convenient:** Single solution provided ready-to-use; add, incubate, stop and read. This homogeneous reagent reduces assay variation.
- **Stable:** Stable for 12 months at 4°C, providing extended shelf life; the assay end product is stable for at least one hour after stopping the assay.
- **Safe:** Provided in a slightly acidic, nonhazardous proprietary buffer without aprotic solvents; non-caustic to plastics used in automated systems.
- **Sensitive:** Low background provides higher assay sensitivity.

Additional Information

The TMB One Solution has been developed to work with Promega E_{max}® ELISAs for BDNF, GDNF, and TGFβ1. It can be used for any ELISA using HRP conjugated secondary antibodies.

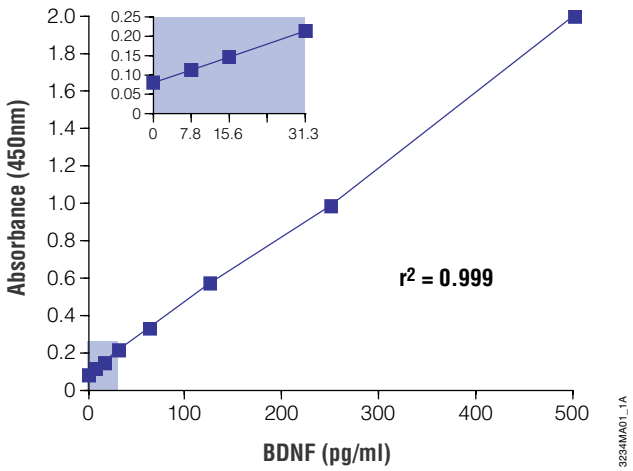


Figure 8.4. Representative BDNF standard curve obtained using the ELISA "BDNF E_{max}® ImmunoAssay System" (Cat.# G7610). The inset is an enlargement of the 0–31.3pg/ml portion of the graph.

References

Smith, A.D. *et al.* (2013) Selenium status alters the immune response and expulsion of adult *Heligosomoides bakeri* worms in mice. *Infect. Immun.* **81**, 2546–53.

Yamazaki, T. *et al.* (2012) The ddY mouse: a model of postprandial hypertriglyceridemia in response to dietary fat. *J. Lipid Res*. **53**, 2024–37.

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Ordering Information

TMB One Solution (Cat.# G7431)

Western Blue® Stabilized Substrate for Alkaline Phosphatase (AP)

Substrate for Alkaline Phosphatase for Western blots and Dot blots.

Description

Western Blue® Stabilized Substrate for Alkaline Phosphatase is a stable, ready-to-use substrate for Western blots and immunoscreening. It is a mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) in a proprietary stabilizing buffer. Western Blue® Substrate should be used directly and without dilution. This liquid substrate deposits a permanent dark purple stain on membrane at sites with an alkaline phosphatase-conjugated antibody. Western Blue® Substrate is as sensitive as other reagents based on the BCIP/NBT formulation.

Features and Benefits

- **Convenient:** Ready-to-use formulation does not require dilution or reagent mixing.
- **Sensitive:** Substrate is as sensitive as other commercially available BCIP/NBT formulations and reagents.
- **Stable:** Stable for one year at room temperature.

References

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To, W.S. and Midwood, K.S. (2011) Identification of novel and distinct binding sites within Tenascin-C for soluble and fibrillar fibronectin. *J. Biol. Chem*. **286**(17), 14881–91.

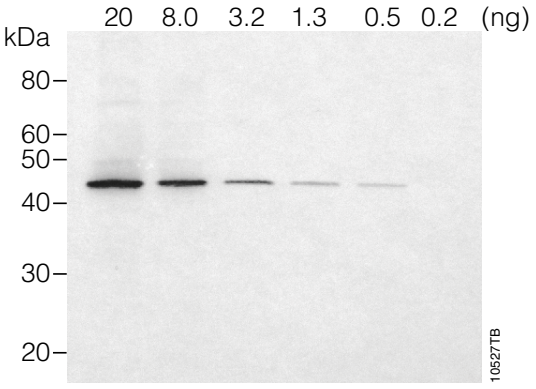


Figure 8.5. Western blot (immunoblot) for β-actin in cytoplasmic lysate from HEK293T cells. The blot was performed on a serial dilution of lysate; each lane contains an indicated amount of β-actin (ng). Primary antibody (monoclonal) used at 1:5,000; Anti-Mouse IgG, AP Conjugate secondary antibody (Cat.# S3721) used at 1:2,500; blot imaged with Western® Blue Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841).

Ordering Information

Western Blue® Stabilized Substrate for Alkaline Phosphatase (Cat.# S3841)

TMB Stabilized Substrate for Horseradish Peroxidase (HRP)

Substrate for Western blots and Dot blots.

Description

TMB Stabilized Substrate for horseradish peroxidase is a stable, ready-to-use TMB (3,3´, 5,5´-tetramethyl-benzidine) color development substrate for localization of horseradish peroxidase-conjugated antibodies on Dot blots and Western blots. It is easier to use than 4-chloro-1-naphthol (CN), which must be prepared immediately before use. TMB Stabilized Substrate comes premixed and fully diluted in a proprietary buffer containing less than 0.5% organic solvent.

Features and Benefits

- **Convenient:** Premixed, ready-to-use; in proprietary buffer containing less than 0.5% organic solvents.
- **Stable:** Stable at room temperature for 12 months.
- **Sensitive:** At least 3X more sensitive than 4-chloro-1-naphthol (CN).
- **Long-Lasting Color:** Color is much more stable than CN and photographs more easily.

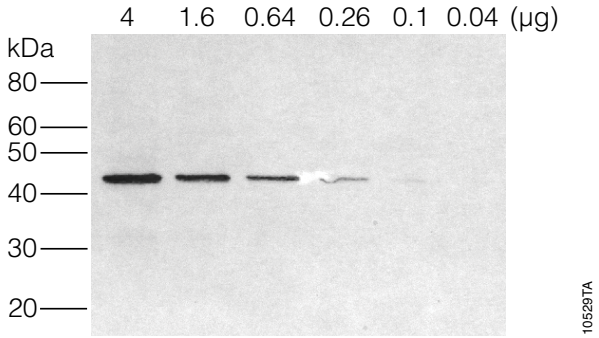


Figure 8.6. Western blot (immunoblot) for β-actin in cytoplasmic lysate from HEK293T cells. The blot was performed on a serial dilution of lysate; each lane contains an indicated amount of total protein (µg). Primary antibody (monoclonal) used at 1:5,000; Anti-Mouse IgG, HRP Conjugate secondary antibody (Cat.# W4021) used at 1:2,500; blot imaged with TMB Stabilized Substrate for horseradish peroxidase (Cat.# W4121).

Ordering Information

TMB Stabilized Substrate for HRP
(Cat.# W4121)

ECL Western Blotting Substrate

Substrate for the detection of HRP-conjugated antibodies for Western blots and Dot blots.

Description

ECL Western Blotting Substrate is a nonradioactive, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) conjugates on immunoblots. The ECL Western Blotting Substrate detects and visualizes the presence of picogram (pg) amounts of antigen through the use of photographic or other suitable chemiluminescent imaging methods.

Features and Benefits

- **Save Time:** No optimization required; you can switch from other entry-level ECL substrates.
- **Save Money:** Use Promega's Entry Level ECL.

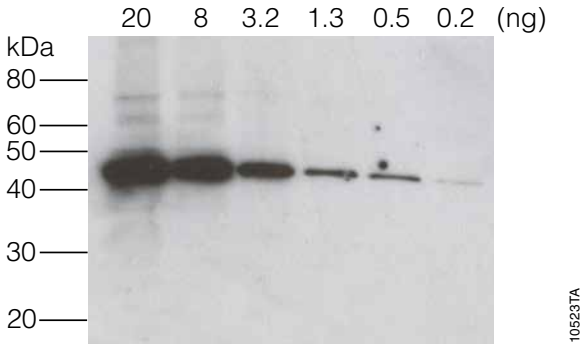


Figure 8.7. Western blot (immunoblot) for β-actin in cytoplasmic lysate from HEK293T cells. The blot was performed on a serial dilution of lysate; each lane contains an indicated amount of β-actin (ng) as quantitated by ELISA.

Ordering Information

ECL Western Blotting Substrate
(Cat.# **W1001, W1015**)

ProtoBlot® II AP Systems with Stabilized Substrate and Western Express® Fast Blotting

Substrate for the detection of alkaline phosphatase (AP) conjugated antibodies in Western blots.

Description and Principle

The ProtoBlot® II AP Systems with Stabilized Substrate are designed for the rapid and sensitive detection of proteins or other macromolecular antigens immobilized on nitrocellulose or PVDF membranes. Proteins can be transferred from gels after electrophoresis (Western blots) or bound directly from solution ("dot" blots).

The Western Express® Fast Blotting Protocol is included with the system and can reduce dramatically the time required to perform immunodetection.

Features and Benefits

- **Fast:** Easy-to-use Western Express® Protocol allows the detection of dot blots in 30–45 minutes and the detection of Western blots in 1–2 hours.
- **Convenient:** The system contains Western Blue® Stabilized Substrate for AP, which is a ready-to-use solution of BCIP/NBT. No reagent preparation is required for the substrate.

Ordering Information

ProtoBlot® II AP System with Stabilized Substrate, Human (Cat.# W3940)

ProtoBlot® II AP System with Stabilized Substrate, Mouse (Cat.# W3950)

ProtoBlot® II AP System with Stabilized Substrate, Rabbit (Cat.# W3960)

Broad Range Protein Molecular Weight Markers

SDS-PAGE protein size marker.

Description

The Broad Range Protein Molecular Weight Markers consist of nine clearly identifiable bands at convenient molecular weights. The protein sizes are 10, 15, 25, 35, 50, 75, 100, 150 and 225kDa. Each protein is present at a concentration of 0.1µg/µl, except for the 50kDa protein, which is present at 0.3µg/µl and serves as a reference indicator, having triple the band intensity of the other proteins.

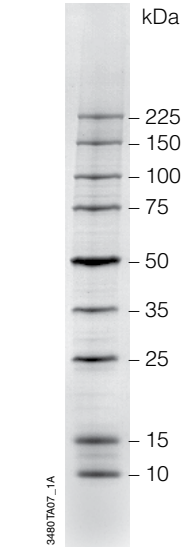
These markers are intended for use as a size standard when performing SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) for estimation of the molecular weight of the protein of interest. Note that they are not stained and will need to be visualized using common in-gel staining reagents such as Coomassie®, Silver, or other staining methods

Features and Benefits

- **Reference Band:** Band at 50kDa is 3X intensity for use as a reference.
- **Convenient:** Nine bands at evenly spaced intervals.
- **Fast:** Ready to load.

Additional Information

Sufficient for 100 lanes at 5µl per lane.



4–20% Tris-Glycine SDS-PAGE gel

Figure 8.8. Broad Range Protein Molecular Weight Markers; 4-20% Tris-Glycine SDS-PAGE.

Ordering Information

Broad Range Protein Molecular Weight Markers (Cat.# V8491)

Protease Inhibitor Cocktail

Inhibition of endogenous proteases during protein purification from mammalian or insect cell cultures.

Description

Protease Inhibitor Cocktail is used to prevent protein degradation after lysing cells. The product is a mixture of six different protease inhibitors with different target protease specificities. The inhibitor cocktail is EDTA-free and provided as a powder, ready for reconstitution in 1ml of either 100% ethanol or DMSO to obtain a 50X working solution.

Features and Benefits

- **Broad Specificity:** Inhibitor cocktail is effective against a diverse number of proteases.
- **Excellent Potency:** Reagent provides the best-in-class level of protease inhibition.
- **Highly Compatible:** Works with a wide array of protein fusion tags (e.g., Flag® tag, His tag, GST tag) and capture technologies. It is ideally suited for HaloTag® Technology-based approaches.

References

Galbraith, M.D. *et al.* (2013)HIF1A Employs CDK8-Mediator to Stimulate RNAPII Elongation in Response to Hypoxia. *Cell* **153**(6), 1327–1339.

Deplu, R. *et al.* (2013) TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. *EMBO J.* **32**(5), 645–55.

Additional Information

Table 8.3. Compounds included in the Protease Inhibitor Cocktail.

Inhibitor	Mode of Action	Target
Benzamidine HCl	Reversible	Trypsin like/Serine Proteases
Leupeptin	Reversible	Serine/Cysteine Proteases
Pepstatin A	Reversible	Aspartic Acid Proteases
1,10 Phenanthroline	Chelator	Metalloproteases
PMSF	Reversible	Serin Proteases
Bestatin	Reversible	Amino Peptidases

Ordering Information

Protease Inhibitor Cocktail (Cat.# G6521)

Expression Vectors

Table 9.1. Overview of Protein Expression Vectors.

Flexi® Vector Name[1]	Cat. #	Drug Selection[2]	Expression Application	Promoter for Expression			Peptide Fusion Tag	
				E. coli	Mammalian Cells	Cell-Free Transcription/ Translation	N-terminus	C-terminus
pF1A T7 Flexi® Vector pF1K T7 Flexi® Vector	C8441 C8451	Ampicillin Kanamycin	Inducible expression of native protein	T7		T7		
pFN2A (GST) Flexi® Vector pFN2K (GST) Flexi® Vector	C8461 C8471	Ampicillin Kanamycin	Soluble expression and purification via an N-terminal GST tag	T7		T7	GST	
pF3A WG (BYDV) Flexi® Vector pF3K WG (BYDV) Flexi® Vector	L5671 L5681	Ampicillin Kanamycin	In vitro wheat germ expression of native protein			T7, SP6		
pF25A ICE T7 Flexi® Vector pF25K ICE T7 Flexi® Vector	L1061 L1081	Ampicillin Kanamycin	In vitro insect cell free expression of native protein			T7		
pFN6A (HQ) Flexi® Vector pFN6K (HQ) Flexi® Vector	C8511 C8521	Ampicillin Kanamycin	Inducible expression and protein purification via a metal affinity resin	T7		T7	HQHQHQ	
pFC7A (HQ) Flexi® Vector pFC7K (HQ) Flexi® Vector	C8531 C8541	Ampicillin Kanamycin	Inducible expression and protein purification via a metal affinity resin	T7		T7		HQHQHQ
pF4A CMV Flexi® Vector pF4K CMV Flexi® Vector	C8481 C8491	Ampicillin Kanamycin	Constitutive high expression of native protein		CMV	T7		
pF5A CMV-neo Flexi® Vector pF5K CMV-neo Flexi® Vector	C9401 C9411	Ampicillin/ Neomycin Kanamycin/ Neomycin[M]	Constitutive high expression of native protein with selection for stable transfectants		CMV	T7		

[1] The "pF" indicates the vector is a Flexi® Vector suitable for Flexi® cloning (see chapter 1 for details).The letter after "pF" indicates the position of any expression tags ("N" for an N-terminal expression tag and "C" for a C-terminal expression tag). The letters "A" and "K" designate the bacterial drug selection for the vector ("A"= ampicillin and "K"= kanamycin).

[2] The "M" indicates the vector provides resistance to the indicated drug in mammalian cells.

Table 9.1. Overview of Protein Expression Vectors (continued).

Flexi® Vector Name[1]	Cat. #	Drug Selection[2]	Expression Application	Promoter for Expression			Peptide Fusion Tag	
				E. coli	Mammalian Cells	Cell-Free Transcription/ Translation	N-terminus	C-terminus
pF9A CMV hRluc-neo Flexi® Vector	C9361	Ampcillin NeomycinM	Constitutive high expression of native protein with selection and reporter screening for stable transfectants		CMV	T7		
pFN10A (ACT) Flexi® Vector	C9331	Ampicillin/ Neomycin	Mammalian in vivo protein:protein interaction		CMV	T7	HSV VP16 activation domain	
pN11A (BIND) Flexi® Vector	C9341	Ampicillin	Mammalian in vivo protein:protein interaction		CMV	T7	GAL4 DNA-binding domain	
pF12A RM Flexi® Vector pF12K RM Flexi® Vector	C9431 C9441	Ampicillin Kanamycin	Regulated mammalian protein expression		12XOP-Mini CMV			
pFN18A HaloTag® T7 Flexi® Vector pFN18K HaloTag® T7 Flexi® Vector	G2751 G2681	Ampicillin Kanamycin	Protein interaction analysis and protein purification	T7		T7	HaloTag®	
pFN19A HaloTag® T7 SP6 Flexi® Vector pFN19K HaloTag® T7 SP6 Flexi® Vector	G1891 G1841	Ampicillin Kanamycin	Protein interaction analysis	T7		T7, SP6	HaloTag®	
pFC20A HaloTag® T7 SP6 Flexi® Vector pFC20K HaloTag® T7 SP6 Flexi® Vector	G1681 G1691	Ampicillin Kanamycin	Protein interaction analysis	T7		T7, SP6		HaloTag®
pFC14A HaloTag® CMV Flexi® Vector pFC14K HaloTag® CMV Flexi® Vector	G9651 G9661	Ampicillin Kanamycin	Cell imaging and protein interaction analysis; high constitutive expression		CMV	T7		HaloTag®

[1] The "pF" indicates the vector is a Flexi® Vector suitable for Flexi® cloning (see chapter 1 for details).The letter after "pF" indicates the position of any expression tags ("N" for an N-terminal expression tag and "C" for a C-terminal expression tag). The letters "A" and "K" designate the bacterial drug selection for the vector ("A"= ampicillin and "K"= kanamycin).

[2] The "M" indicates the vector provides resistance to the indicated drug in mammalian cells.

Table 9.1. Overview of Protein Expression Vectors (continued).

Flexi® Vector Name[1]	Cat. #	Drug Selection[2]	Expression Application	Promoter for Expression			Peptide Fusion Tag	
				E. coli	Mammalian Cells	Cell-Free Transcription/ Translation	N-terminus	C-terminus
pFC15A HaloTag® CMVd1 Flexi® Vector	G1611	Ampicillin	Cell imaging and protein interaction analysis; medium constitutive expression	T7	CMVd1	T7, SP6		HaloTag®
pFC15K HaloTag® CMVd1 Flexi® Vector	G1601	Kanamycin						
pFC16A HaloTag® CMVd2 Flexi® Vector	G1591	Ampicillin	Cell imaging and protein interaction analysis; low constitutive expression	T7	CMVd2	T7, SP6		HaloTag®
pFC16K HaloTag® CMVd2 Flexi® Vector	G1571	Kanamycin						
pFC17A HaloTag® CMVd3 Flexi® Vector	G1551	Ampicillin	Cell imaging and protein interaction analysis; very low constitutive expression	T7	CMVd3	T7, SP6		HaloTag®
pFC17K HaloTag® CMVd3 Flexi® Vector	G1321	Kanamycin						
pFN21A HaloTag® CMV Flexi® Vector	G2821	Ampicillin	Cell imaging and protein interaction analysis; high constitutive expression		CMV	T7	HaloTag®	
pFN21K HaloTag® CMV Flexi® Vector	G2831	Kanamycin						
pFN22A HaloTag® CMVd1 Flexi® Vector	G2841	Ampicillin	Cell imaging and protein interaction analysis; medium constitutive expression	T7	CMVd1	T7, SP6	HaloTag®	
pFN22K HaloTag® CMVd1 Flexi® Vector	G2851	Kanamycin						
pFN23A HaloTag® CMVd2 Flexi® Vector	G2861	Ampicillin	Cell imaging and protein interaction analysis; low constitutive expression	T7	CMVd2	T7, SP6	HaloTag®	
pFN23K HaloTag® CMVd2 Flexi® Vector	G2871	Kanamycin						

[1] The "pF" indicates the vector is a Flexi® Vector suitable for Flexi® cloning (see chapter 1 for details).The letter after "pF" indicates the position of any expression tags ("N" for an N-terminal expression tag and "C" for a C-terminal expression tag). The letters "A" and "K" designate the bacterial drug selection for the vector ("A"= ampicillin and "K"= kanamycin).

[2] The "M" indicates the vector provides resistance to the indicated drug in mammalian cells.

Table 9.1. Overview of Protein Expression Vectors (continued).

Flexi® Vector Name[1]	Cat. #	Drug Selection[2]	Expression Application	Promoter for Expression			Peptide Fusion Tag	
				E. coli	Mammalian Cells	Cell-Free Transcription/ Translation	N-terminus	C-terminus
pFC30A His_6HaloTag® T7 Flexi® Vector	G8321	Ampicillin	Inducible expression and protein purification via metal affinity resin or HaloTag®	T7		T7		His_6 HaloTag®
pFC30K His_6HaloTag® T7 Flexi® Vector	G8381	Kanamycin						
pFC27A HaloTag® CMV-neo Flexi® Vector	G8421	Ampicillin/ Neomycin[M]	Cell imaging and protein interaction analysis; high constitutive expression with selection for stable transfectants		CMV	T7		HaloTag®
pFC27K HaloTag® CMV-neo Flexi® Vector	G8431	Kanamycin/ Neomycin[M]						
pFC30A His6HaloTag® T7 Flexi® Vector	G8321	Ampicillin	Inducible expression and protein purification via metal affinity resin or HaloTag®	T7		T7		His_6 HaloTag®
pFC30K His6HaloTag® T7 Flexi® Vector	G8381	Kanamycin						
pFC27A HaloTag® CMV-neo Flexi® Vector	G8421	Ampicillin/ Neomycin[M]	Cell imaging and protein interaction analysis; high constitutive expression with selection for stable transfectants		CMV	T7		HaloTag®
pFC27K HaloTag® CMV-neo Flexi® Vector	G8431	Kanamycin/ Neomycin[M]						
pFN31A *Nluc* CMV-Hygro Flexi® Vector	N1311	Ampicillin/ Hygromycin[M]	NanoLuc® fusion proteins; NanoBRET™		CMV		NanoLuc®	
pFN31K *Nluc* CMV-neo Flexi® Vector	N1321	Kanamycin/ Neomycin[M]						
pFC32A *Nluc* CMV-Hygro Flexi® Vector	N1331	Ampicillin/ Hygromycin[M]	NanoLuc® fusion proteins; NanoBRET™		CMV			NanoLuc®
pFC32K *Nluc* CMV-neo Flexi® Vector	N1341	Kanamycin/ Neomycin[M]						

[1] The "pF" indicates the vector is a Flexi® Vector suitable for Flexi® cloning (see chapter 1 for details).The letter after "pF" indicates the position of any expression tags ("N" for an N-terminal expression tag and "C" for a C-terminal expression tag). The letters "A" and "K" designate the bacterial drug selection for the vector ("A"= ampicillin and "K"= kanamycin).

[2] The "M" indicates the vector provides resistance to the indicated drug in mammalian cells.

Standard Multiple Cloning Site (MCS) Vectors

Table 9.2. Overview of Protein Expression Vectors.

Vector Name	Cat. #	Drug Selection*	Expression Application	Promoter for Expression			Peptide Fusion Tag	
				E. coli	Mammalian Cells	Cell-Free Transcription/ Translation	N-terminus	C-terminus
pH6HTN His_6HaloTag® T7 Vector	G7971	Ampicillin	Inducible expression and protein purification via metal affinity resin or HaloTag®	T7		T7	His_6 HaloTag®	
pH6HTC His_6HaloTag® T7 Vector	G8031	Ampicillin	Inducible expression and protein purification via metal affinity resin or HaloTag®	T7		T7		His_6 HaloTag®
pHTC HaloTag® CMV-neo Vector	G7711	Ampicillin/ Neomycin^M	Cell imaging and protein interaction analysis; high constitutive expression with selection for stable transfectants		CMV	T7		HaloTag®
pHTN HaloTag® CMV-neo Vector	G7721	Ampicillin	Cell imaging and protein interaction analysis; high constitutive expression with selection for stable transfectants		CMV	T7	HaloTag®	
pNLF1-N [CMV/Hygro]	N1351	Ampicillin/ Neomycin^M	NanoLuc® fusion proteins; NanoBRET™		CMV		NanoLuc®	
pNLF1-C [CMV/Hygro]	N1361	Ampicillin/ Neomycin^M	NanoLuc® fusion proteins; NanoBRET™		CMV			NanoLuc®
pNLF1-secN [CMV/Hygro]	N1371	Ampicillin/ Neomycin^M	Secretory NanoLuc® fusion proteins; NanoBRET™		CMV		NanoLuc®	

*The "M" indicates the vector provides resistance to the indicated drug in mammalian cells.

For all vectors visit our website: **http://www.promega.de/resources/vector-sequences/all-vectors/**

Protein Conversion

Table 9.3. Protein Conversions.

Protein Molar Conversion	
100pmol of 100kDa protein	10µg
100pmol of 50kDa protein	5µg
100pmol of 10kDa protein	1µg
100pmol of 1kDa protein	100ng

Protein/DNA Conversions	
1kb of DNA	333 Amino Acids of Coding Capacity 37kDa protein
270bp DNA	10kDa protein
810bp DNA	30kDa protein
1.35kb DNA	50kDa protein
2.7kb DNA	100kDa protein
Average MW of an Amino Acid	110 daltons

Amino Acids

Table 9.4. Amino Acid Abbreviations and Molecular Weights.

Amino Acid	Three-Letter Abbreviation	One-Letter Symbol	Molecular Weight
Alanine	Ala	A	89Da
Arginine	Arg	R	174Da
Asparagine	Asn	N	132Da
Aspartic acid	Asp	D	133Da
Asparagine or Aspartic acid	Asx	B	–
Cysteine	Cys	C	121Da
Glutamine	Gln	Q	146Da
Glutamic Acid	Glu	E	147Da
Glutamine or Glutamic acid	Glx	Z	–
Glycine	Gly	G	75Da
Histidine	His	H	155Da
Isoleucine	Ile	I	131Da
Leucine	Leu	L	131Da
Lysine	Lys	K	146Da
Methionine	Met	M	149Da
Phenylalanine	Phe	F	165Da
Proline	Pro	P	115Da
Serine	Ser	S	105Da
Threonine	Thr	T	119Da
Tryptophan	Trp	W	204Da
Tyrosine	Tyr	Y	181Da
Valine	Val	V	117Da

The average molecular weight of an amino acid is 110Da.

Genetic Code

Table 9.5. Genetic Code.

1st Position	2nd Position U	2nd Position C	2nd Position A	2nd Position G	3rd Position
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	**UAA** Stop	**UGA** Stop	A
	UUG Leu	UCG Ser	**UAG** Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Termination codons are in bold.
AUG start codon is in bold italics.

Gel Percentages

Table 9.6. Recommended Acrylamide Gel Percentages for Resolution of Proteins on Polyacrylamide Gels.

Recommended % Acrylamide	Protein Size Range
8	40–200kDa
10	–100kDa
12	10–40kDa

Chapter 1: Cloning System and Protein Expression Vectors	Size	Cat.#
Flexi® Cloning System		
Flexi® System, Entry/Transfer	5 entry & 20 transfer reactions	C8640
Flexi® System, Transfer	100 transfer reactions	C8820
Carboxy Flexi® System, Transfer	50 transfer reactions	C9320
HaloTag® Cloning Starter System	1 each	G6050
HaloTag® Flexi® Vectors—CMV Deletion Series Sample Pack	9x2µg	G3780
10X Flexi® Enzyme Blend (SgfI & PmeI)	25µg	R1851
	100µg	R1852
Carboxy Flexi® Enzyme Blend (SgfI & EcoICRI)	50µg	R1901
Kazusa Human ORF Library *www.promega.com/findmygene		
GeneX- Native human ORF in pF1K	100ng–10ng/µl	FHCxxxx*
GeneX - HaloTag® human ORF in pFN21A	100ng–10ng/µl	FHCxxxx*
Mammalian Expression Vectors (see Table 9.1; Table 9.2)		
Regulated Mammalian Expression System	1 system	C9470
Coumermycin A1	5mg	C9451
Novobiocin Sodium Salt	1g	C9461
pReg neo Vector	20µg	C9421
pF12A RM Flexi® Vector	20µg	C9431
pF12K RM Flexi® Vector	20µg	C9441
pTARGET™ Mammalian Expression Vector System	20 reactions	A1410
pGEM®-T Vector System I	20 reactions	A3600
pGEM®-T Vector System II	20 reactions	A3610
pGEM®-T Easy Vector System I	20 reactions	A1360
pGEM®-T Easy Vector System II	20 reactions	A1380
Competent Bacteria for Cloning		
Single-Use JM109 Competent Cells, >10^8cfu/µg	1ml - (20 × 50µl)	L2005
JM109 Competent Cells, >10^8cfu/µg	1ml - (5 × 200µl)	L2001
JM109 Competent Cells, >10^7cfu/µg	1ml - (5 × 200µl)	L1001
Single-Use HB101 Competent Cells, >10^8cfu/µg	1ml - (20 × 50µl)	L2015
HB101 Competent Cells, >10^8cfu/µg	1ml - (5 × 200µl)	L2011
Single-Use Pro 5-alpha Competent Cells, >10^9cfu/µg	1ml - (20 × 50µl)	L1221
IPTG, Dioxane-Free	1g	V3955
	5g	V3951
	50g	V3953
X-Gal	100mg/2ml	V3941
Chapter 2: Bacterial Strains for Protein Expression	**Size**	**Cat.#**
Single Step (KRX) Competent Cells for Protein Expression		
Single Step (KRX) Competent Cells	20 x 50µl	L3002
L-Rhamnose Monohydrate	10g	L5701
	50g	L5702
BL21 Competent Cells for Protein Expression		
Single-Use BL21(DE3)pLysS Competent Cell	1ml (20 × 50µl)	L1195
BL21(DE3)pLysS Competent Cells, >106cfu/µg	1ml (5x 200µl)	L1191
Chapter 3: Cell-Free Protein Expression Systems	**Size**	**Cat.#**
mRNA-based Translation Systems		
Rabbit Reticulocyte Lysate System, Nuclease Treated	30 reactions	L4960

Chapter 3: Cell-Free Protein Expression Systems	Size	Cat.#
Flexi® Rabbit Reticulocyte Lysate System	30 reactions	L4540
Wheat Germ Extract	5 x 200µl	L4380
Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination System	24 reactions	L4330
DNA-based Transcription and Translation Systems: Rabbit Reticulocyte Lysate Systems		
TnT® SP6 Coupled Reticulocyte Lysate System	8 reactions (Trial Size)	L4601
	40 reactions	L4600
TnT® T7 Coupled Reticulocyte Lysate System	8 reactions (Trial Size)	L4611
	40 reactions	L4610
TnT® T3 Coupled Reticulocyte Lysate System	40 reactions	L4950
TnT® T7/T3 Coupled Reticulocyte Lysate System	40 reactions	L5010
TnT® T7/SP6 Coupled Reticulocyte Lysate System	40 reactions	L5020
TnT® T7 Quick Coupled Transcription/Translation System	5 reactions (Trial Size)	L1171
	40 reactions	L1170
TnT® SP6 Quick Coupled Transcription/Translation System	5 reactions (Trial Size)	L2081
	40 reactions	L2080
TnT® T7 Quick for PCR DNA	40 reactions	L5540
DNA-based Transcription and Translation Systems: Wheat Germ Extracts		
TnT® SP6 Coupled Wheat Germ Extract System	40 reactions	L4130
TnT® T7 Coupled Wheat Germ Extract System	40 reactions	L4140
TnT® T7/SP6 Coupled Wheat Germ Extract System	40 reactions	L5030
TnT® SP6 High-Yield Wheat Germ Protein Expression System	10 reactions	L3261
	40 reactions	L3260
DNA-based Transcription and Translation Systems: Insect Cell Extract		
TnT® T7 Insect Cell Extract Protein Expression System	10 reactions	L1101
	40 reactions	L1102
pF25A ICE T7 Flexi® Vector	20µg	L1061
pF25K ICE T7 Flexi® Vector	20µg	L1081
DNA-based Transcription and Translation Systems: *E. coli* S30 Extract Systems		
E. coli S30 Extract System for Linear Templates	30 reactions	L1030
E. coli S30 Extract System for Circular DNA	30 reactions	L1020
E. coli T7 S30 Extract System for Circular DNA	30 reactions	L1130
S30 T7 High-Yield Protein Expression System	8 reactions	L1115
	24 reactions	L1110
Luciferase Control RNA	20µg - 1mg/ml	L4561
Magnesium Acetate	100 µl - 25mM	L4581
Potassium Chloride	200µl - 2.5M	L4591
Amino Acid Mixture, Complete	175µl - 1mM	L4461
Amino Acid Mixture Minus Cysteine	175µl - 1mM	L4471
Amino Acid Mixture Minus Methionine and Cysteine	175µl - 1mM	L5511
Amino Acid Mixture Minus Leucine	175µl - 1mM	L9951
Amino Acid Mixture Minus Methionine	175µl - 1mM	L9961
Cell-Free Protein Labeling Reagents		
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System	40 reactions	L5001
Transcend™ Colorimetric Translation Detection System	30 reactions	L5070
Transcend™ Chemiluminescent Translation Detection System	30 reactions	L5080

Chapter 3: Cell-Free Protein Expression Systems	Size	Cat.#
Cell-Free Protein Labeling Reagents		
Transcend™ tRNA	30µl	L5061
Membrane Vesicles for Signal Peptide Cleavage and Core Glycosylation		
Canine Pancreatic Microsomal Membranes	50µl	Y4041
Chapter 4: Protein Purification	**Size**	**Cat.#**
Affinity-based Protein Purification: HaloTag® Fusion Proteins		
HaloTag® Protein Purification System (*E.coli*)	1 each	G6280
HaloTag® Protein Purification System Sample Pack	1 each	G6270
HaloTag® Mammalian Protein Detection and Purification System Sample Pack (*E.coli*)	1 each	G6799
HaloTag® Mammalian Protein Purification System	1 each	G6790
HaloTag® Mammalian Protein Detection and Purification System	1 each	G6795
HaloTEV Protease	1,000u	G6601
	4,000u	G6602
HaloTag® TMRDirect™ Ligand	30µl	G2991
HaloLink™ Resin	1.25ml	G1912
	2.5ml	G1913
	10ml	G1914
	25ml	G1915
Protease Inhibitor Cocktail, 50X	1ml	G6521
Mammalian Lysis Buffer	40ml	G9381
Affinity-based Protein Purification: His-tagged Proteins		
HisLink™ Protein Purification Resin	5ml	V8823
	50ml	V8821
HisLink™ Spin Protein Purification System	25 reactions	V1320
HisLink™ 96 Purififfcation System	1 x 96 blank	V3680
	5 x 96 blank	V3681
Affinity-based Protein Purification: Biotinylated Proteins		
SoftLink™ Soft Release Avidin Resin	1 mL	V2011
	5 mL	V2012
Single-Use Pro 5-alpha Competent Cells, >10^9cfu/µg	1ml - (20 × 50µl)	L1221
PinPoint™ Xa Protein Purification System (Production & Purification)	1 system	V2020
Magnetic Affinity-based Purification and Pull-Down Strategies: Biotinylated Proteins		
MagneGST™ Protein Purification System	40 reactions	V8600
	200 reactions	V8603
Magnetic Affinity-based Purification and Pull-Down Strategies: HaloTag® Fusion Proteins		
Magne™ HaloTag® Beads, 20% Slurry	1 mL	G7281
	5 mL	G7282
Magnetic Affinity-based Purification and Pull-Down Strategies: His-tagged Proteins		
MagneHis™ Protein Purification System	65 reactions	V8500
	325 reactions	V8550
Maxwell® 16 Polyhistidine Protein Purification Kit	48 preps	AS1060
Magnetic Stands and Spacers		
MagneSphere® Technology Magnetic Separation Stand (two-position)	0.5ml	Z5331
	1.5ml	Z5332
	12 x 75mm	Z5333

Chapter 4: Protein Purification	Size	Cat.#
MagneSphere® Technology Magnetic Separation Stand (twelve-position)	0.5ml	Z5341
	1.5ml	Z5342
	12 x 75mm	Z5343
PolyATtract® System 1000 Magnetic Separation Stand	1 each	Z5410
MagnaBot® 96 Magnetic Separation Device	1 each	V8151
MagnaBot® II Magnetic Separation Device	1 each	V8351
MagnaBot® Flat Top Magnetic Separation Device	1 each	V6041
Plate Clamp 96	1 each	V8251
Plate Stand	1 each	V8261
Deep Well MagnaBot® 96 Magnetic Separation Device	1 each	V3031
Heat Transfer Block	1 each	Z3271
Heat Block Insert	1 each	Z3651
Chapter 5: Antibody Purification and Labeling	**Size**	**Cat.#**
Antibody Purification		
Magne™ Protein G Beads, 20% Slurry	1ml	G7471
	5ml (5 x 1ml)	G7472
	50ml	G7473
Magne™ Protein A Beads, 20% Slurry	1ml	G8781
	5ml (5 x 1ml)	G8782
	50ml	G8782
Chapter 6: Functional Protein Analysis using HaloTag® Technology	**Size**	**Cat.#**
Cellular Imaging: HaloTag® Ligands		
HaloTag® Alexa Fluor® 488 Ligand	30µl	G1001
	15µl	G1002
HaloTag® Oregon Green® Ligand	30µl	G2801
	15µl	G2802
HaloTag® TMRDirect™ Ligand	30µl	G2991
HaloTag® R110Direct™ Ligand	30µl	G3221
HaloTag® TMR Ligand	30µl	G8251
	15µl	G8252
HaloTag® diAcFAM Ligand	30µl	G8272
	15µl	G8273
HaloTag® Biotin Ligand	30µl	G8281
	15µl	G8282
HaloTag® Alexa Fluor® 660 Ligand	30µl	G8471
	15µl	G8472
HaloTag® Coumarin Ligand	30µl	G8581
	15µl	G8582
HaloTag® PEG-Biotin Ligand	30µl	G8591
	15µl	G8592
HaloTag® Iodoacetamide (O2) Ligand	5mg	P1681
HaloTag® Succinimidyl Ester (O2) Ligand	5mg	P1691
HaloTag® Amine (O2) Ligand	5mg	P6711
HaloTag® Amine (O4) Ligand	5mg	P6741
HaloTag® Succinimidyl Ester (O4) Ligand	5mg	P6751
HaloTag® Thiol (O4) Ligand	5mg	P6761

Chapter 6: Functional Protein Analysis using HaloTag® Technology	Size	Cat.#
HaloTag® Iodoacetamide (O4) Ligand	5mg	P6771
Protein Interaction Analysis: HaloTag® Mammalian Pulldown Kits		
HaloTag® Mammalian Pull-Down and Labeling System	24 reactions	G6500
HaloTag® Mammalian Pull-Down System	24 reactions	G6504
HaloTag® Complete Pull-Down System	1 each	G6509
Protein Interaction Analysis: HaloCHIP™ System		
HaloCHIP™ System	20 reactions	G9410
Protein Interaction Analysis: HaloTag® Arrays		
HaloLink™ Array Six Slide System	6 slides	G6190
Additional Reagents		
Protease Inhibitor Cocktail, 50X	1ml	G6521
HaloTag® Control Vector	20µg	G6591
Anti-HaloTag® Monoclonal Antibody	200µg	G9211
Anti-HaloTag® pAb	200µg	G9281
Mammalian Lysis Buffer	40ml	G9381
HaloTag® Standard Protein	30µg	G4491
Chapter 7: Protein Characterization by Mass Spectrometry	**Size**	**Cat.#**
Trypsin		
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
Sequencing Grade Modified Trypsin	100µg (5 x 20µg)	V5111
	100µg	V5117
Sequencing Grade Modified Trypsin, Frozen	100µg (5 x 20µg)	V5113
Trypsin/Lys-C Mix, Mass Spec Grade	20µg	V5071
	100µg	V5072
	100µg (5x 20 µg)	V5073
Immobilized Trypsin	2 ml	V9012
	2x2 ml	V9013
Alternative Proteases		
rLys-C, Mass Spec Grade	15µg	V1671
Endoproteinase Lys-C, Sequencing Grade	5µg	V1071
Arg-C, Sequencing Grade	10µg	V1881
Asp-N, Sequencing Grade	2µg	V1621
Glu-C, Sequencing Grade	50µg (5 x 10µg)	V1651
Low-Specific Proteases		
Chymotrypsin, Sequencing Grade	25µg	V1061
	100µg (4 x 25µg)	V1062
Nonspecific Proteases		
Elastase	5mg	V1891
Pepsin	250mg	V1959
Thermolysin	25mg	V4001
Glycosidases		
PNGase F	500 units (10u/µl)	V4831
Endo H	10,000 units (500u/µl)	V4871
	50,000 units (500u/µl)	V4875
Protein Deglycosylation Mix	20 reactions	V4931

Chapter 7: Protein Characterization by Mass Spectrometry	Size	Cat.#
Fetuin (control protein)	500µg (10mg/ml)	V4961
ProteaseMAX™ Surfactant		
ProteaseMAX™ Surfactant, Trypsin Enhancer	1mg	V2071
	5 x 1mg	V2072
Chapter 8: Protein Detection Tools for Western Blotting & ELISA	**Size**	**Cat.#**
Conjugated Secondary Antibodies: AP		
Anti-Mouse IgG (H+L), AP Conjugate	100µl	S3721
Anti-Rabbit IgG (Fc), AP Conjugate	100µl	S3731
Anti-Human IgG (H+L), AP Conjugate	100µl	S3821
Anti-Rat IgG (H+L), AP Conjugate	100µl	S3831
Conjugated Secondary Antibodies: HRP		
Anti-Rabbit IgG (H+L), HRP Conjugate	300µl	W4011
Anti-Mouse IgG (H+L), HRP Conjugate	300µl	W4021
Anti-Human IgG (H+L), HRP Conjugate	300µl	W4031
Anti-Chicken IgY, HRP Conjugate	300µl	G1351
Anti-ACTIVE® Qualified Antibodies		
Donkey Anti-Goat IgG, AP	60µl	V1151
Donkey Anti-Rabbit IgG (H+L) HRP	60µl	V7951
Donkey Anti-Goat IgG, HRP	60µl	V8051
Substrates for ELISA		
AttoPhos® AP Fluorescent Substrate System	3 × 36mg	S1000
	1 × 36mg (Trial Size)	S1001
AttoPhos® Substrate	36mg	S1011
	100mg	S1012
	1g	S1013
AttoPhos® Buffer	60ml	S1021
	240ml	S1022
TMB One Solution	100ml	G7431
Substrates for Western Blotting		
BCIP/NBT Color Development Substrate	1.25/2.5ml	S3771
Western Blue® Stabilized Substrate for Alkaline Phosphatase	100ml	S3841
ECL Western Blotting Substrate	250ml	W1001
	500ml	W1015
TMB Stabilized Substrate for Horseradish Peroxidase	200ml	W4121
Tween® 20	2.5ml	W3831
Blot-Qualified BSA	10g	W3841
ProtoBlot® II AP System with Stabilized Substrate, Human	1 each	W3940
ProtoBlot® II AP System with Stabilized Substrate, Mouse	1 each	W3950
ProtoBlot® II AP System with Stabilized Substrate, Rabbit	1 each	W3960
Additional Reagents		
Protease Inhibitor Cocktail, 50x	1ml	G6521
Broad Range Protein Molecular Weight Markers	100 lanes	V8491

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