

PROTEIN EXPRESSION & ANALYSIS

IMPACT™ Kit

Instruction Manual

NEB #E6901S
Store at -20°C

 NEW ENGLAND
BioLabs® Inc.
enabling technologies in the life sciences



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Kit Components:

Vector DNA – pTXB1, pTYB21	10 µg of each (50 µl)
Control Vector – pMXB10	10 µg (50 µl)
<i>E. coli</i> ER2566 (T7 Express)	0.2 ml cells (not competent)
Anti-Chitin Binding Domain Serum (rabbit)	50 µl
Chitin Beads (store at 4°C)	20 ml
1, 4-Dithiothreitol (DTT), 1 M	5 ml
3X Sample Buffer	1.5 ml

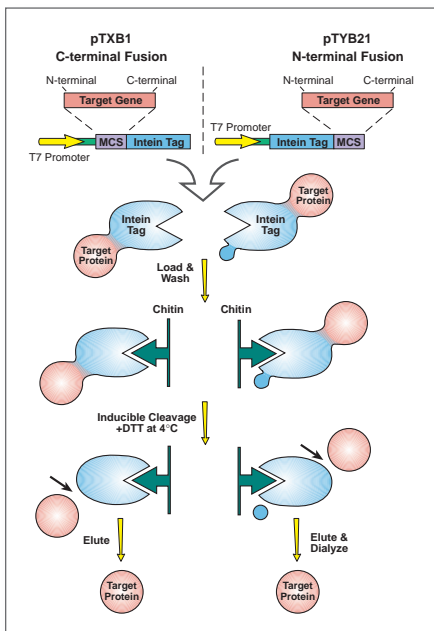
Introduction:

The IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system is a novel protein purification system which utilizes the inducible self-cleavage activity of protein splicing elements (termed inteins) to separate the target protein from the affinity tag (1). It distinguishes itself from all other purification systems by its ability to purify, in a single chromatographic step, a native recombinant protein without the use of a protease. Each intein tag contains a chitin binding domain (CBD) for the affinity purification of the fusion protein on a chitin resin (2-4). Induction of on-column cleavage, using thiol reagents such as dithiothreitol (DTT), releases the target protein from the intein tag (Figures 1,2).

The vectors included in this kit allow for the fusion of the target protein at its C-terminus (pTXB1) (3,5) or at its N-terminus (pTYB21) (4,6) to the intein tag.

In addition, with the use of pTXB1, native recombinant proteins that possess a reactive C-terminal thioester can be isolated for applications, including protein semisynthesis and site-specific labeling [3,7, Intein Mediated Protein Ligation (IPL, Appendix II)].

Figure 1: Schematic illustration of the IMPACT System.



Advantages of the IMPACT System:

Single column purification – without the use of a protease to remove the affinity tag

Ability to produce a native target protein without vector derived amino acids

Fusion to either the C-terminus or the N-terminus of the target protein

Isolation of proteins with or without an N-terminal methionine residue

Ligation and labeling of recombinant proteins

T7 promoter-driven system to achieve high levels of expression and tight transcriptional control in *E. coli*.

Description of System Components:

Vectors

pTXB1 (NEB #N6707) contains a mini-intein from the *Mycobacterium xenopi gyrA* gene (Mxe GyrA intein; 198 amino acid residues) that has been modified to undergo thiol-induced cleavage at its N-terminus (3,5). The vector allows for the purification of a target protein without any extra amino acids by cloning into the NdeI and SapI sites. The target protein is fused at its C-terminus to a self-cleavable intein tag (~28 kDa) that contains the chitin binding domain (CBD, 6 kDa) allowing for affinity purification of the fusion precursor on a chitin column.

The pTYB21 (NEB #N6701) vector utilizes an intein from the *Saccharomyces cerevisiae* VMA1 gene (Sce VMA1 intein; 454 amino acids)(4,6). The target protein is fused at its N-terminus to a self-cleavable VMA1 intein-CBD tag (56 kDa); the tag allows for the affinity purification of the fusion precursor on a chitin column. The vector is designed to allow for purification of a target protein without any extra amino acids, or without an N-terminal methionine residue, by cloning its 5' end into the SapI site. The SapI site should be used as the 3' cloning site in pTXB1 or the 5' cloning site in pTYB21. If another restriction site is used an unfavorable amino acid for cleavage will be encoded at the cleavage site, adjacent to the intein. In addition, many restriction sites within the polylinker of pTYB21 are shared with pTYB22, pMAL-5 and pKLAC2 vectors. If an insert is subcloned using these sites, it can be subcloned into any of these vectors either in parallel or as a subsequent experiment. This simplifies an examination of expression in the IMPACT System (*E. coli*), pMAL Protein Fusion and Purification System (*E. coli*) (NEB #E8200) and *K. lactis* Protein Expression Kit (*Kluyveromyces lactis*) (NEB #E1000). Please see "Construction of the Fusion Plasmid" for details.

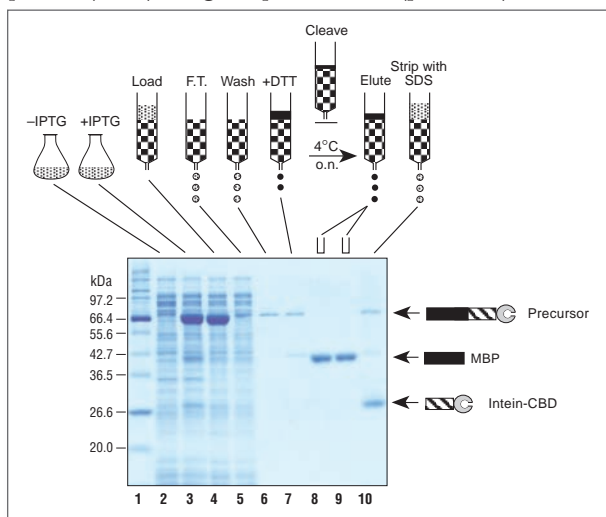
The control vector, pMXB10 (NEB #N6903), derived from pTXB1, carries the control target protein, maltose binding protein (MBP), already inserted upstream of the Mxe GyrA intein-CBD. Induction yields the MBP-Mxe GyrA intein-CBD fusion (~71 kDa) which, when cleaved, results in the elution of MBP (43 kDa). The polylinker regions flanking the coding region for MBP can conveniently be used to clone a gene of interest. However, after intein cleavage the target protein will contain additional amino acids at its C-terminus, including (LEY), which has had a high rate of successful cleavage (see Appendix 1 for cloning into pMXB10).

The IMPACT vectors utilize a T7 promoter to provide stringent control of expression of the fusion gene in *E. coli* (8). The IMPACT vectors carry the Amp^r gene marker (the *bla* gene), which conveys ampicillin resistance to the host strain; one vector sold separately, pKYB1 (NEB #N6706), is kanamycin resistant.

Other vectors and applications

Additional IMPACT vectors with different multiple cloning sites or inteins (pTYB series, pTWIN series, pKYB1, pTXB3) are also available for the fusion of the cleavable intein tag to either the C-terminus or the N-terminus of a target protein (for a complete list see www.neb.com). The flexibility in fusion protein construction is designed to increase the probability of successful expression and purification of a target protein, as well as to conduct sophisticated protein engineering projects such as protein ligation and cyclization [pTWIN1 (NEB #N6951) or pTWIN2 (NEB #N6952)]. If you do not wish to use a thiol reagent for cleavage the Intein1 (*Ssp* DnaB intein) in the pTWIN vectors may be used; cleavage is induced by temperature (room temperature) and/or pH shift (from pH 8.5 to 6).

Figure 2: Expression and Purification of *E. coli* maltose-binding protein (MBP) using the pTXB vector (pMXB10).



Lane 1: Protein Marker, Broad Range (NEB #P7702, 15 µl).

Lane 2: Crude extract from uninduced cells (15 µl).

Lane 3: Crude extract from cell induced at 15°C for 16 hours (15 µl).

Lane 4: Load or clarified lysate (4 µl).

Lane 5: Flow through from chitin column (4 µl).

Lane 6: Wash (4 µl).

Lane 7: DTT flush to distribute it evenly throughout the column (4 µl).

Lanes 8-9: Elution of MBP after stopping flow and inducing a cleavage reaction at 4°C for 16 hours.

Lane 10: Chitin beads aliquot after elution (6 µl).

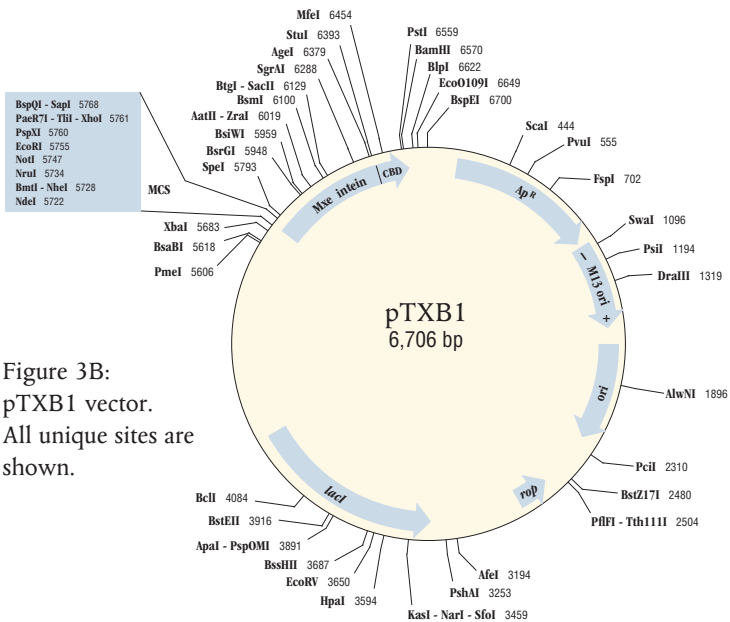
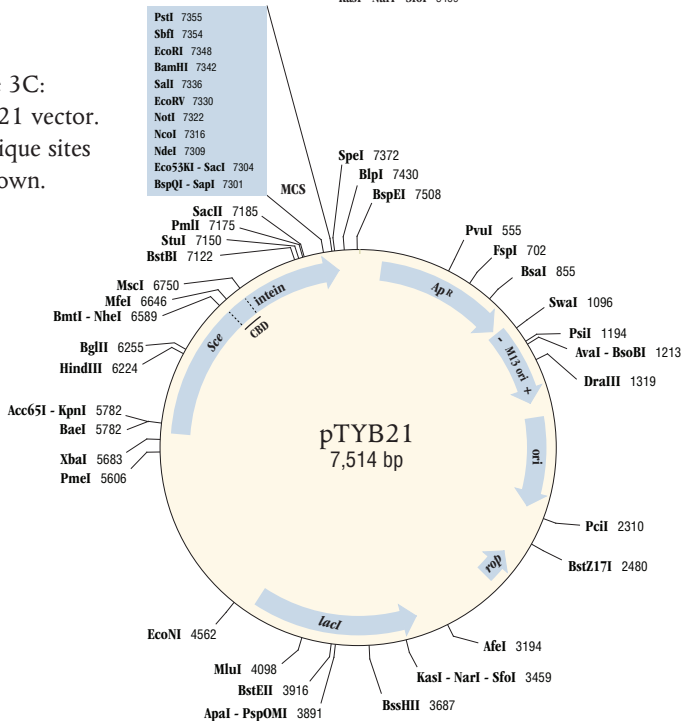


Figure 3B:
pTXB1 vector.
All unique sites are shown.

Figure 3C:
pTYB21 vector.
All unique sites are shown.



E. coli strain ER2566

ER2566 is provided as a host strain for the expression of a target gene cloned into the IMPACT vectors. ER2566 carries a chromosomal copy of the T7 RNA polymerase gene inserted into the *lacZ* gene, and thus is under the control of the *lac* promoter. The strain is deficient in both *lon* and *ompT* proteases.

ER2566 is supplied as 50% glycerol stock; **these cells are not competent.**

Recommended long term storage (>30 days) is at -70°C. In addition, T7 Express Competent *E. coli* from NEB (NEB #C2566), and other T7 expression strains and derivatives can also be used, including BL21(DE3), etc.

ER2566 Genotype: *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10--Tet^s)2 [dcm] R(zgb-210::Tn10--Tet^s) endA1 Δ(mcrC-mrr)114::IS10.*

Chitin Beads (NEB #S6651)

A chitin affinity matrix is used to isolate the fusion precursor that contains the target protein, intein and a chitin binding domain (CBD). 20 ml of chitin beads (NEB #S6651) are supplied as a 40 ml slurry in 20% ethanol. The binding capacity for the intein tag fused to the target protein, maltose binding protein (MBP), is 2 mg of eluted MBP protein per ml of chitin beads. **Chitin beads should be stored at 4°C.**

Anti-Chitin Binding Domain Serum (anti-CBD)

(NEB #S6654)

Rabbit serum raised against a peptide derived from the *Bacillus circulans* chitin binding domain is provided for Western blot analysis (1:5000). Anti-CBD Monoclonal Antibody (NEB #E8034) is also available separately. Store at -20°C.

1, 4-Dithiothreitol (DTT)

A 1.0 M DTT solution is provided in the IMPACT Kit. Since DTT is not particularly stable after dilution, the cleavage buffer should be freshly prepared before use. DTT stock solutions should be aliquoted to minimize freeze/thaw cycles. Store at -20°C.

3X SDS Sample Buffer

(Blue Loading Buffer Pack, NEB #B7703)

187.5 mM Tris-HCl (pH 6.8 @ 25°C), 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) bromophenol blue (store at room temperature). DTT should be added to the 3X SDS Sample Buffer, to a final concentration of 40 mM (see note in Table 1A). Store at -20°C.

Construction of the Fusion Plasmid:

Cloning considerations

The identity of the amino acid residues adjacent to the intein has been shown to affect the cleavage reaction and should be taken into consideration (see Tables 1A and 1B). These tables are only a guide, since the folding of the entire fusion protein, not just a single amino acid, affects cleavage.

It is difficult to accurately predict the expression of a target protein-intein fusion, thus we recommend cloning the insert into different vectors and conducting small scale purifications. In our experience we have found pTXB1 to be the most consistent and reliable expression vector.

If a target protein without vector-derived amino acids is required, the pTXB1 or the pTYB21 vector can be used.

If a C-terminal fusion is required, where the C-terminus of the target protein is fused to the intein, then the pTXB1 vector should be used. If a N-terminal fusion is required the pTYB21 vector should be used.

In the presence of thiols such as DTT, β -mercaptoethanol or cysteine, the intein undergoes specific self-cleavage which releases the target protein from the chitin-bound intein tag resulting in a single-column purification of the target protein. Furthermore, when pTXB1 is utilized, the use of thiol reagents, such as 2-mercaptoethanesulfonic acid (MESNA), releases a protein with a reactive thioester at the C-terminus of the target protein for use in intein mediated protein ligation (IPL).

Cloning into pTXB1

The mini-intein (*Mxe* GyrA intein) in pTXB1 has been used to purify a wide range of target protein fusions. Cloning into the *Nde*I and the *Sap*I sites results in the fusion of the intein to the C-terminus of the target protein, without any extra amino acids on the target protein after cleavage of the intein tag. **The *Sap*I site must be used to clone the 3' end of an insert.** The *Sap*I site can also be used for the inclusion of extra amino acid residues favorable for controllable cleavage (by engineering the extra codons into the primers; Table 2).

If the insert sequence contains an internal *Sap*I site the following options exist:

1. Use the pMXB10 vector (See Appendix I).
2. If the insert does not contain a *Spe*I site, then the *Spe*I site present near the N-terminus of the *Mxe* GyrA intein can be used by designing a primer that contains the intein sequence; this can result in a fusion without any vector-derived residues following cleavage.
3. PCR with a proofreading polymerase to generate a blunt-end product for ligation with pTXB1 that has been digested with *Sap*I and filled in. The Quick Blunting Kit (NEB #E1201) can be used to generate a vector with blunt ends.

Table 1A

C-TERMINAL RESIDUE OF THE TARGET PROTEIN	% CLEAVAGE AFTER 16 HOURS*		% CLEAVAGE AFTER 40 HOURS*	
	4°C	23°C	4°C	23°C
Tyr Phe Gln Asn Thr Lys Ala His Leu ¹ Met	65-80	80-95	75-90	85-95
Ile Arg Glu Trp Cys	30-55	60-85	50-70	70-95
Val	30	70	60	90
Gly	10	40	20	60
Asp ²	10	20	20	30
Ser Pro	5-15	5-15	5-15	5-20

Effect of the C-terminal residue of a target protein on DTT-induced cleavage with pTXB1. The C-terminal amino acid of the target protein, paramyosin, was mutated immediately upstream of the intein cleavage site. Cleavage was induced with 40 mM DTT in 30 mM Tris, pH 8.5, 0.5M NaCl. Percent cleavage was determined by Coomassie stained SDS-PAGE analysis of chitin beads before and after DTT cleavage.

Note: Boiling in SDS Sample Buffer containing DTT can cause partial or complete cleavage, resulting in an overestimation of in vivo cleavage. If substantial in vivo cleavage is observed, the cell extract should be evaluated in a SDS Sample Buffer containing no DTT.

¹ Leu showed ~50% in vivo cleavage when induced at 15°C; at 37°C in vivo cleavage was less than 5%.

² Asp showed ~50% in vivo cleavage when expression was induced at 15°C and 37°C.

Table 1B

N-TERMINAL RESIDUE OF THE TARGET PROTEIN	% CLEAVAGE AFTER 16 HOURS*		% CLEAVAGE AFTER 40 HOURS*	
	4°C	23°C	4°C	23°C
Met Ala Gln	40-60	> 95	60-90	> 95
Gly Leu Asn Trp Phe Tyr	10-40	75-95	40-60	> 90
Val Ile Asp Glu Lys Arg His	< 10	50-80	10-20	70-95
Pro	< 10	< 10	< 10	< 10
Thr Ser Cys	< 10 not determined not determined	80 not determined not determined	20 not determined not determined	> 90 not determined not determined

Effect of the N-terminal residue of a target protein on DTT-induced cleavage with pTYB21 or pTYB11. The N-terminal amino acid of the target protein, T4 DNA ligase, was mutated immediately downstream of the intein cleavage site. Cleavage was induced with 40 mM DTT in 30 mM Hepes, pH 8.0, 0.5M NaCl. Percent cleavage was determined by Coomassie stained SDS-PAGE analysis of chitin beads before and after DTT cleavage.

Cloning into pTYB21

Use of the Sapl site results in the fusion of the target gene adjacent to the intein tag so that the target protein can be purified without any extra non-native residues. The Sapl site can be used for the inclusion of extra amino acid residues favorable for cleavage (by engineering the extra codons into the primers; Table 2). If the NdeI site is used four extra amino acids (Gly-Arg-Ala-His) will be added to the N-terminus of the protein. A stop codon should be included in the reverse primer when constructing a N-terminal fusion.

When pTYB21 is used, a small peptide (15 amino acid residues, 1.6 kDa) is also cleaved from the intein tag and co-eluted with the target protein. It cannot be detected on a regular SDS-PAGE and can be dialyzed out.

Cloning into pMXB10

For cloning into pMXB10 NdeI, Sapl or NcoI can be used as the 5' cloning site. The 3' cloning site can be SacI, HindIII, NotI, EcoRI or XhoI (See Appendix I).

Note: BspQI (NEB #R0712) is an isoschizomer of Sapl (NEB #R0569) and can be used instead of Sapl.

Primer Design:

Appropriate restriction sites, absent in the target gene, are incorporated in the forward and reverse primers when a target gene is generated by PCR. The choice of the restriction site in the primers determines whether any, or which, extra amino acid residues will be attached to the terminus of the target protein after the cleavage of the intein tag.

Table 2 illustrates some examples of designing forward and reverse primers for pTXB1 and pTYB21. For cloning into pTXB1 one should clone a target gene between the NdeI (forward primer) and the Sapl (reverse primer) sites in pTXB1. For the pTYB21 vector the Sapl site can be used to clone the 5' end of the target gene (PstI as the 3' cloning site for pTYB21 is shown as an example for a reverse primer in the table below).

When constructing a N-terminal fusion (pTYB21) a stop codon should be encoded in the reverse primer. The reverse primer for the C-terminal fusion (pTXB1) should not include a stop codon.

We recommend writing out your primers and cloning strategy in order to check Sapl (or BspQI) digestion, the reading frames etc. For more information on cloning with Sapl, please refer to our web site: http://www.neb.com/nebecomm/tech_reference/protein_expression/IMPACTFaq.asp

In general, more than 15 bp of target gene sequence is required for PCR (represented by "NNNNNN..."). In Table 2 the restriction site is underlined. The "GGTGGT" sequence at the 5' end of the primer is a random sequence of 6 bp to ensure efficient DNA cleavage by the restriction enzyme.

Table 2: Primer design for pTXB1 and pTYB21.

RESTRICTION SITE	SEQUENCE (RESTRICTION SITE UNDERLINED)	CLONING VECTOR
NdeI	5' - GGT GGT <u>CAT ATG</u> NNN NNN... -3' (forward primer)	pTXB1
SapI ¹	5' - GGT GGT <u>TGC TCT TCC</u> GCA NNN NNN...-3' (reverse primer)	pTXB1
SapI ²	5' - GGT GGT <u>TGC TCT TCC</u> AAC NNN NNN... -3' (forward primer)	pTYB21
PstI ³	5' - GGT GGT <u>CTG CAG</u> TCA NNN NNN... -3' (reverse primer)	pTYB21

¹ SapI digestion creates a 3-nt overhang (GCA) for ligation with the SapI-digested pTXB1 vector (containing a TGC overhang), resulting in an in-frame fusion to the N-terminus of an intein. The SapI site can be used to add one or more extra amino acid residue(s) to the target protein by including an appropriate sequence (e.g. add ACC in the reverse primer corresponding to a GGT codon for a glycine residue). The SapI site is not regenerated after cloning.

² SapI digestion creates a 3-nt overhang (AAC) compatible with the SapI digested pTYB21 (containing a GTT overhang). The SapI site is not regenerated after cloning.

³ A stop codon should be included in the reverse primer when constructing a N-terminal fusion.

Cloning a PCR Fragment:

The following brief protocol describes the cloning of an amplified target gene fragment using restriction enzymes that create non-compatible sticky ends for direct cloning into the IMPACT vectors. Users can follow standard techniques to clone the PCR amplified gene. For blunt-end or single site cloning, the vector may need to be treated with a phosphatase (Antarctic Phosphatase, NEB #M0289 or Calf Intestinal Phosphatase, NEB #M0290). If necessary, the PCR product can be sub-cloned into a PCR cloning vector before cloning into an IMPACT vector.

1. Restriction Digestion and Ligation: The amplified gene fragment is electrophoresed on an agarose gel and the gene fragment is purified. The purified fragment is double-digested with the appropriate restriction enzymes; the vector (0.5–1 µg) is also digested with the same enzymes.

Note: SapI tends to settle in the tube so mix it with a pipette prior to removing it from the vial.

Following a 1 to 2 hour digestion, both reaction mixtures are loaded onto a 1% low melting TAE agarose gel. Each of the gel slices (100–200 µl, the smaller the volume the better) containing the digested gene fragment and IMPACT vector are melted at 65°C for 10 minutes and treated with β-Agarase I (NEB #M0392; 1 µl per 50 µl) for one hour at 42°C. Alternatively, a spin column can be used to isolate the DNA. Ligation is conducted using the Quick Ligation Kit (NEB #M2200) for five minutes at room temperature by mixing the vector and the insert in an appropriate ratio (about 1:3). A control ligation reaction containing digested vector alone should be included.

2. Transformation: To reduce the background from the vector's self-ligation, the ligation sample can be digested, prior to transformation, with a restriction enzyme whose recognition site is deleted from the polylinker during cloning and is also absent from the insert. This linearizes any remaining parental vector. Fusion constructs should initially be established in a non-restricting (r_m^- or r_m^+), non-expression *E. coli* strain [i.e. NEB 10-beta (NEB #C3019) or NEB Turbo (NEB #C2984), not ER2566 or T7 Express], especially when cloning a potentially toxic gene.

For more information on transformation and competent cell selection please refer to our web site: http://www.neb.com/nebecomm/tech_reference/competent/default.asp

3. Screening for the Presence of Inserts:
 - A. Analysis of plasmid DNA: Restriction digests with the same restriction enzymes that were used for cloning the target gene fragment can be used to screen for correct clones, **except when SapI is used because the SapI site is lost after ligation**. It is also possible to use other restriction sites present in the plasmid.

Colony PCR or colony hybridization can be used to screen a large number of transformants for the presence of the target gene.

- B. Analysis of protein expression: Transform the correct plasmid construct into competent ER2566 or T7 Express. Inoculate 5–10 freshly grown colonies, each in 4 ml LB+Amp media. Grow the culture at 37°C until it reaches an OD₆₀₀ of ~0.5 or slightly turbid. Transfer 2 ml to a sterile tube as an uninduced control.

Induce protein expression with 0.4 mM IPTG at 37°C for 2–3 hours or at 15°C overnight. Mix 40 µl culture with 20 µl 3X SDS-PAGE Sample Buffer. Boil for 5 minutes and load 15 µl of both uninduced and induced samples on SDS-PAGE and stain with Coomassie blue.

Immunodetection with Anti-Chitin Binding Domain Serum can be used to detect the intein-CBD fusion proteins in total cell lysates. Immunodetection is not necessary if an induced band can be easily visualized by Coomassie blue staining of a SDS-PAGE.

- C. Sequencing: Clones should be further confirmed by DNA sequencing before proceeding to the cell culture and protein expression steps. See section 3.9.5 of IMPACT FAQs; www.neb.com – Technical Reference – Protein Expression and Purification Technologies – FAQs for IMPACT Kit.
- D. Storage: The plasmid encoding the correct fusion protein should be stored at –20°C or a glycerol stock should be made of the cells containing the expression plasmid and stored at –80°C.

Fusion Protein Expression:

The expression of the fusion protein may be affected by a variety of factors such as the (a) *E. coli* strain, (b) cell growth conditions (e.g. temperature, aeration, cell density, IPTG concentration, etc.), (c) toxicity of the target protein, (d) codon usage and (e) structure and stability of mRNA. *E. coli* ER2566 cells are supplied in the kit (not competent) as a host for fusion protein expression from an IMPACT vector. High efficiency competent cells of this strain, T7 Express (NEB #C2566) are available separately. For expression of toxic proteins T7 Express ^h Competent *E. coli* (High Efficiency) (NEB #C3016), T7 Express *lysY*/^h Competent *E. coli* (High Efficiency) (NEB #C3013) and T7 Express *lysY* Competent *E. coli* (High Efficiency) (NEB #C3010) are also available. Expression of a toxic protein may require lowering the culture temperature.

Induction of protein expression at 12–15°C can often help the folding and the solubility of the fusion protein and increase the cleavage efficiency of the intein.

For all protein samples remove a 40 µl aliquot and mix with 20 µl 3X SDS Sample Buffer (Figure 2). The samples are then boiled for five minutes before loading onto a gel. If necessary, the samples can be stored at –20°C for a few days.

The following protocol is provided as a general guideline (see Figure 4, page 19).

Cell Culture

1. Inoculate 1 liter of LB medium, containing 100 µg/ml ampicillin, with a freshly grown colony. Using cells stored at 4°C or an overnight culture may lower the protein yield.
2. Incubate the culture in an orbital shaker at 37°C until the OD₆₀₀ reaches 0.5.

Induction of Protein Expression

3. IPTG is added to a final concentration of 0.4 mM for induction of protein expression. Before the addition of IPTG, an aliquot of cell culture should be removed and incubated separately as an uninduced control (sample 1, uninduced). Initially induction at 37°C for 2–4 hours can be tested for expression and solubility.
4. Remove a sample (40 µl) and mix with 20 µl 3X SDS Sample Buffer for the total cell extract or induced protein sample (sample 2, crude cell extract or induced). The cells from the IPTG-induced culture are spun down at 5000 x g for 15 minutes at 4°C and the supernatant is discarded. The cell pellet can be stored at –80°C.
5. The cell pellet from a one-liter culture is resuspended in 100 ml of the appropriate ice-cold Column Buffer (See Media and Solutions, page 26).

The inclusion of nonionic detergents in the cell lysis buffer can reduce nonspecific protein binding to the chitin resin during the affinity column step. Oxidation-sensitive proteins can be stabilized during purification by using the reducing agents TCEP [tris-(2-carboxyethyl)phosphine] or TCCP [tris-(2-cyanoethyl)phosphine] (0.1 mM) in the cell lysis buffer. Egg white

lysozyme is not recommended for cell lysis because it is known to bind and degrade the chitin beads. Cell lysis reagents, such as B-PER (Thermo Scientific, Rockford, IL), can be used.

6. Lyse the cells by sonication on ice.

Sonicate in short pulses, keep the cell culture cold during sonication and do not allow the build up of bubbles/foam. The release of protein can be monitored by using Bradford Reagent (BioRad, Hercules, CA) or OD₂₈₀. Continue sonication until the level of the released protein level reaches a maximum.

7. Centrifuge the cells at 15,000 x g for 30 minutes at 4°C; the supernatant is the clarified extract to be loaded on the chitin column. Remove 40 µl from the supernatant for the clarified protein sample (sample 3, clarified extract). Save the pellet at -80°C for future analysis.

Optimization of Expression

If the target fusion protein is present as an induced band in the crude cell extract but absent from the clarified cell extract, this may indicate a solubility problem. Also if *in vivo* cleavage is detected (the induced sample contains the intein-CBD and target protein) different induction conditions should be tested. Usually induction at lower temperatures and/or with lower IPTG concentrations results in increased solubility and improved folding and subsequent thiol induced cleavage. In order to optimize expression conditions we recommend splitting a one liter culture into several samples (100–200 ml each) and testing for optimal expression conditions. The optimal incubation temperature and time for induction will vary depending on the target protein. We recommend varying induction temperature and time to optimize expression (37°C for 2–4 hours, 30°C for 4–6 hours, 22–25°C for 6–16 hours and 12–15°C overnight using 0.4 mM IPTG). One sample with no IPTG should be incubated as a control for uninduced cells. Varying IPTG concentrations (up to 1 mM) can also be tested. Lowering the IPTG concentration (0.01–0.1 mM) may also reduce the fusion protein expression in inclusion bodies. For low temperature induction (e.g. 12–15°C) the culture can be incubated at 37°C until the OD₆₀₀ reaches 0.6–0.7.

Fusion protein expression can be examined by SDS-PAGE, followed by Coomassie staining or western blot analysis, or by an activity assay. Analyze samples from both the total cell extract (soluble and insoluble proteins) and clarified extract (soluble). If the fusion protein is not detected by Coomassie staining, a Western blot with the Anti-Chitin Binding Domain Serum may be performed.

If the cell pellet needs to be tested, dissolve the pellet from a 100–200 ml culture in 10–20 ml column buffer and mix 40 µl of the pellet suspension with 20 µl of the 3X SDS Sample Buffer. The pellet can also be dissolved in a buffer containing urea. This sample can be saved for analysis (by loading 5–10 µl on a SDS-PAGE) if the fusion protein is not detected in the clarified (soluble) extract.

Affinity Purification and On-column Cleavage:

This should be performed with cold solutions at 4°C, unless otherwise stated.

Preparation of Chitin Column

The chitin column should be washed with 10 column volumes of the Column Buffer prior to the loading of the crude cell extract. The chitin-binding domain (CBD) present in the intein-tag, allows for the affinity purification of the fusion protein using chitin beads. Generally, a column packed with 10 ml of chitin beads (10 ml bed volume or 20 ml chitin beads slurry) should be used for a one liter culture (adjust the amount of beads according to expression level).

Loading the Clarified Cell Extract

Load the clarified extract onto the chitin column at a flow rate no faster than 0.5–1 ml/min. Take a sample of the flow through (sample 4) and compare it to the clarified cell extract sample to indicate the binding efficiency of the fusion precursor to the chitin column. If some of the fusion precursor is present in the flow through you may need to increase the amount of resin or load more slowly.

Washing the Chitin Column

At least 20 bed volumes of the Column Buffer should be used to wash the column (sample 5). Due to the high affinity of the CBD for the chitin beads, a higher flow rate (e.g., 2 ml/min) and stringent wash conditions can be used to reduce nonspecific binding of other *E. coli* proteins [high salt concentration (0.5–1 M NaCl) and/or nonionic detergents].

Induction of On-column Cleavage

To release the target protein, on-column cleavage is induced by a thiol reagent. Induction of the on-column cleavage is conducted by quickly flushing the column with 3 bed volumes of the Cleavage Buffer, containing 50 mM DTT, to evenly distribute thiols throughout the column (sample 7). After the quick flush, stop the column flow and leave at 4–23°C for 16–40 hours (see Tables 1A and 1B). Before adding the thiol reagent, check cleavage efficiency by removing 100 µl of resin and mixing with 50 µl 3X SDS Sample Buffer. After boiling for 5 minutes, spin the resin down. The supernatant (3–10 µl) is directly used for SDS-PAGE analyses (sample 6).

If intein mediated protein ligation (IPL) or expressed protein ligation (EPL) is to be conducted, typically 2-mercaptoethanesulfonic acid (MESNA) is used as the thiol reagent to induce cleavage (Appendix II).

Several factors affect the cleavage efficiency and thus the final yield: (i) amino acid residue(s) at the cleavage site; (ii) temperature of the cleavage reaction; (iii) duration of the cleavage reaction; (iv) pH of the Cleavage Buffer.

Since cleavage is dependent on the protein structure, a single amino acid residue at the cleavage site is not the only determinant for efficient cleavage,

and only serves as a guideline. In most cases (see Tables 1A & 1B), incubation of the column at 16–23°C for 16 hours (overnight) results in more than 50% cleavage of the fusion precursor. When the C-terminal fusion vector (pTXB1) is used, the on-column cleavage reaction can be conducted at 4°C overnight. When the N-terminal fusion vector (pTYB21) is used, higher temperatures (16–23°C) and longer cleavage reaction times (40 hours) are normally required. The data in Tables 1A & 1B provide a guideline for selecting an appropriate temperature and duration for the cleavage reaction. The cleavage efficiency can be determined by a SDS-PAGE by analyzing samples of the chitin resin after thiol cleavage (sample 9).

If most of the precursor is not cleaved, longer incubation time and higher temperature for the cleavage reaction are recommended.

Elution of the Target Protein

Following on-column cleavage the target protein is eluted from the column using the Column Buffer. The intein-CBD tag remains bound to the resin. Fraction sizes of about one third of the column bed volume typically result in the elution of the target protein within the first few fractions (sample 8).

The protein concentration in each fraction can be determined by the Bradford Assay and the eluted fractions should be analyzed by SDS-PAGE. To check cleavage efficiency, remove 100 µl of resin and mix with 50 µl 3X SDS Sample Buffer. Boil for 5 minutes and spin the resin down. Analyze the supernatant (3–10 µl, sample 9) by SDS-PAGE. If a large amount of the precursor still remains uncleaved, continue incubation of the column for an additional 12–24 hours before conducting a second elution.

When pTYB21 is used, a small peptide (1.6 kDa) is also cleaved from the intein tag and co-eluted with the target protein. Due to its small molecular weight, the cleaved peptide cannot be detected on a regular SDS-PAGE gel and can be removed by dialysis.

Stripping the Chitin Column

Uncleaved fusion precursor protein and the intein-tag remain bound to the chitin resin during elution and can be stripped from the resin by 1% SDS or 0.3 N NaOH in column buffer. The elution should be conducted at room temperature to prevent the precipitation of the SDS. Since protein concentrations cannot be determined by the Bradford dye binding assay when SDS is present, the samples should be analyzed by SDS-PAGE.

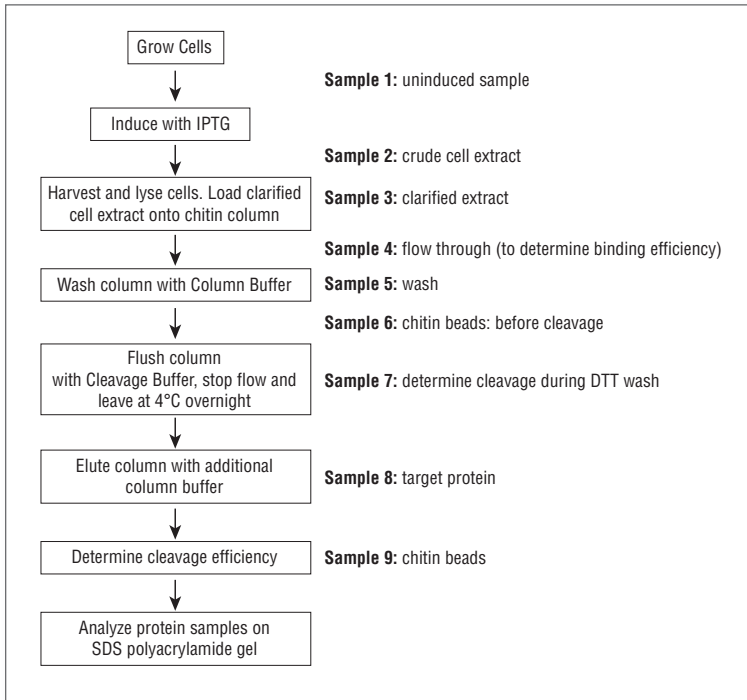
Regeneration of the Chitin Resin

The chitin resin can be regenerated 4–5 times using the following protocol. Wash with 3 bed volumes of 0.3 M NaOH (stripping solution). Allow the resin to soak for 30 minutes and then wash with an additional 7 bed volumes of Stripping Solution. Rinse with 20 bed volumes of water followed by 5 bed volumes of Column Buffer. The resin can be stored at 4°C. For long term storage 0.02% sodium azide should be added to the Column Buffer.

Simplified Expression and Purification Protocol:

1. Transformation: Transform the plasmid bearing the target gene into competent T7 Express or competent cells prepared from ER2566.
2. Cell Culture: Inoculate a freshly grown colony in LB medium containing 100 µg/ml ampicillin and grow the cells at 37°C. When the OD₆₀₀ reaches 0.5, induce protein expression by adding IPTG to a final concentration of 0.4 mM, and incubate at 30–37°C.
3. Column Preparation: Equilibrate a chitin column (20 ml slurry for 1 liter culture) with 10 column volumes of Column Buffer [20 Tris-HCl (pH 8.5), 500 mM NaCl].
4. Cell Harvest: Centrifuge cell culture at 5,000 x g for 15 minutes at 4°C. Discard supernatant. Resuspend cell pellet in column buffer.
5. Loading: Break cells by sonication in Column Buffer, and centrifuge at 15,000 g for 30 minutes at 4°C. Slowly load the clarified lysate onto the chitin column (0.5–1.0 ml/minute).
6. Washing: Wash the column with at least 20 bed volumes of Column Buffer to thoroughly remove the unbound proteins (up to 2.0 ml/minute).
7. Adding Thiols: Quickly wash the column with 3 column volumes of Cleavage Buffer [Column buffer containing 50 mM DTT (for purification) or 50 mM MESNA (for IPL)].
8. On-column Cleavage: Stop the flow and incubate the column at 4°C–23°C for 16–40 hours. The temperature and duration of the cleavage reaction are dependent on the on-column cleavage efficiency which can be checked by analyzing samples of chitin resin before and after cleavage.
9. Elution: Elute the target protein with Column Buffer by continuing the column flow.
10. Dialysis: Dialyze the target protein in to an appropriate storage buffer; this will also remove the excess thiol reagent used in the Cleavage Buffer and the co-eluted small peptide (when using pTYB21).
11. Cleavage: To examine cleavage efficiency remove 100 µl of chitin resin and mix with 50 µl of 3X SDS Sample Buffer. After boiling for 5 minutes, analyze the supernatant on a Coomassie stained SDS-PAGE gel to determine the cleavage efficiency.
12. Regeneration of Chitin Resin: Wash the column with 3 bed volumes of the 0.3 M NaOH (Stripping Solution). Allow the resin to soak for 30 minutes and wash the resin with an additional 7 bed volumes of 0.3 M NaOH. Wash with 20 bed volumes of water, followed by 5 bed volumes of column buffer.

Figure 4: Flow chart for Protein Expression and Purification using the IMPACT System. Sample collection for analysis by SDS-PAGE is indicated.



Media and Solutions:

The following are suggested media for cell culture, cell lysis and protein purification. They can be modified according to the specific properties of the target protein.

LB broth (per liter)

10 g tryptone

5 g yeast extract

10 g NaCl

Adjust pH to 7.0 with NaOH

Cell Lysis Buffer

20 mM Na-HEPES (or Tris-HCl), pH 8.5

500 mM NaCl (or 50–1,000 mM NaCl)

1 mM EDTA (optional)

0.1% Triton X-100 (optional)

Nonionic detergents (0.1–0.5% Triton X-100 or 0.1–0.2% Tween 20) and protease inhibitors [e.g., PMSF (20 μ M)] can also be included. For a target protein sensitive to oxidation, 1 mM of TCEP [tris-(2-carboxyethyl)phosphine] or TCCP [tris-(2-cyanoethyl)phosphine] may be used.

Column Buffer

20 mM Na-HEPES (or Tris-HCl), pH 8.5

500 mM NaCl (or 50–1,000 mM NaCl)

1 mM EDTA (optional)

Cleavage Buffer

20 mM Na-HEPES (or Tris-HCl), pH 8.5

500 mM NaCl (or 50–1,000 mM NaCl)

50 mM DTT or β -mercaptoethanol or cysteine*

1 mM EDTA (optional)

* (use 2-mercaptoethanesulfonic acid and HEPES buffer for IPL see Appendix II, page 27)

Stripping Solution

0.3 M NaOH

Frequently Asked Questions (FAQs):

Cloning

Which vector should I use?

We routinely use pTXB1 with success. The mini-intein of bacterial origin usually allows for higher level of expression; however, we have not conducted a systematic scientific comparison between pTXB1 and the other IMPACT vectors.

pTXB1 allows for the fusion of the C-terminus of a target protein to the intein tag whereas with the pTYB21 vector, the N-terminus of the target protein is fused to the intein tag. It is conceivable that different target proteins, due to certain structural constraints, may prefer either a C-terminal or a N-terminal fusion to allow proper folding of the fusion precursor and for a high level of protein expression.

Check the "Guide to IMPACT Vectors and Applications" in our catalog and on our web site (Section 1.35 of the IMPACT FAQs on the web site). Since inteins exhibit different preferences for the amino acid at the cleavage site, the user should follow guidelines for choosing a vector based on the information given in Tables 1A and 1B. These tables are only a guide, since the folding of the entire fusion protein, not just a single amino acid, affects cleavage. Since each fusion protein varies in expression level and cleavage we often clone the target protein into more than one IMPACT vector.

The choice of vector will also depend on the restriction map of the gene; the presence of an internal NdeI or SapI site will affect the selection of the cloning vector.

If the purified target protein is also intended for intein mediated protein ligation (IPL), C-terminal labeling or peptide ligation, pTXB1 should be used.

If you wish to generate a target protein with a N-terminus other than methionine, you may use pTYB21, pTYB21 or the Intein 1 (Ssp DnaB intein) in the pTWIN1 (NEB #N6951) or pTWIN2 (NEB #N6952) vector. A N-terminal cysteine can be generated for IPL using Intein 1 (Ssp DnaB intein) in the pTWIN vectors.

How do I design primers with the SapI site?

SapI (or its isoschizomer, BspQI) is a Type II enzyme; it cuts outside its recognition sequence to give a staggered cut. To design the primer please refer to Table 2, **write out the cloning procedure** and it will be apparent how compatible ends between the vector and insert are generated. Further information is given in the IMPACT FAQ section on our web site.

Expression

What if I observe *in vivo* cleavage, where a band corresponding to intein-CBD but not the fusion protein, is detected in the crude cell extract?

First, to accurately assess the extent of *in vivo* cleavage, the samples should be prepared in SDS Sample Buffer without DTT or β -mercaptoethanol, since boiling in DTT-containing Sample Buffer may cause cleavage of the fusion protein (see note in Table 1A). If proteolysis is evident, try different hosts or include protease inhibitors. *In vivo* cleavage may be reduced by varying induction temperature. If necessary, perform a Western blot with anti-target protein serum to differentiate between proteolysis and intein-mediated cleavage. Sometimes changing one or several residues between the target protein and the intein tag may reduce intein-mediated *in vivo* cleavage. However, the purified target protein will contain extra residues after cleavage.

What if the fusion protein is insoluble?

If you have problems with solubility one of the first things to try is varying the induction temperature (15°C or lower; some customers have used 8–12°C induction temperatures) and/or the concentration of IPTG (0.01 mM–0.4 mM).

A fresh colony from an overnight plate, maybe necessary for optimal expression.

Resuspend the cell pellet in at least 100 ml Column Buffer/L culture.

During sonication, it is crucial to handle the sample cautiously. If the protein solution gets warm or the solution foams there is a possibility that the proteins are being denatured. Use an ice-chilled water bath to keep the cell suspension cool.

If you wish to use a detergent in your Column and Cleavage Buffers, we recommend 0.1–0.5% Triton X-100 or 0.1–0.2% Tween-20. Too much detergent will impair binding to the chitin column.

If the fusion is still insoluble you can use urea to resuspend the protein. If urea is used, several factors should be considered:

1. The binding efficiency of the intein-tag to the chitin resin is lower at 4 M urea or higher.
2. The intein-mediated cleavage reaction should be carried out in 0–2 M urea.

The following refolding protocol has been successfully applied to the recovery of insoluble proteins

1. Resuspend the cell pellet from 1 L of *E. coli* culture in 100 ml Cell Lysis Buffer.
2. Break cells by sonication.
3. Spin down cell debris containing the inclusion bodies at 15,000 g at 4°C for 30 minutes.
4. Pour out supernatant and resuspend pellet in 100 ml Breaking Buffer.

5. Stir solution for 1 hour at 4°C.
6. Spin remaining cell debris down at 15,000 g and 4°C for 30 minutes.
7. Load supernatant into dialysis bag and dialyze against Renaturation Buffer A, B, C, D and 2 times E. Each step is against 1 L of a renaturation buffer and should take at least 3 hours at 4°C. During dialysis the buffer should be continuously stirred.
8. Centrifuge the dialyzed solution containing the renatured protein at 15,000 g and 4°C for 30 minutes to remove any remaining impurities or incorrectly folded protein which is again aggregated.
9. Use a standard protocol for chitin chromatography and cleavage reaction. Elute the protein product and analyze both the eluate and chitin beads for cleavage efficiency and protein solubility.

Solutions

Cell Lysis Buffer:	20 mM Tris-HCl, pH 8.5 and 0.5 M NaCl
Breaking Buffer:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 7M Guanidine-HCl
Renaturation Buffer A:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 8 M urea.
Renaturation Buffer B:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 6 M urea
Renaturation Buffer C:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 4 M urea
Renaturation Buffer D:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 2 M urea, 0.1 mM oxidized glutathione, 1 mM reduced glutathione
Renaturation Buffer E:	20 mM Tris-HCl, pH 8.5, 0.5 M NaCl, 0.1 mM oxidized glutathione, 1 mM reduced glutathione

Cleavage

What should I do if the fusion precursor is the major product on the chitin resin after target protein elution?

This means that the thiol-induced on-column cleavage is not efficient – invariably leading to a low yield of the target protein. The following options can be tried to increase the cleavage efficiency: (i) increase the duration of the on-column cleavage (ii) increase the temperature from 4°C to room temperature (iii) increase the pH of the Cleavage Buffer to 8.5–9.0 (iv) change the residue(s) adjacent to the intein cleavage site.

What does it mean if the target protein is not eluted after on-column cleavage but is present on the chitin beads after target protein elution?

If both the target protein and the intein tag are present on the chitin beads after elution, it suggests that the target protein becomes insoluble after induced on-column cleavage. Increase the salt concentration (0.5–2 M NaCl) or add a nonionic detergent to the Cleavage Buffer to improve the solubility of the target protein. A number of nonionic detergents examined (0.1–0.5% Triton X-100 or 0.1–0.2% Tween 20) had little effect on binding or cleavage and may improve solubility. If urea is used to elute, some intein tag may co-elute with the target protein. In this case, it may be necessary to repurify and refold the target protein.

If my target protein is sensitive to DTT, are there alternative means to induce the on-column cleavage?

If the activity of the target protein is affected by high concentrations of DTT or β -mercaptoethanol, lower concentrations of DTT or β -mercaptoethanol (5–10 mM) may be used for on-column cleavage. However, longer incubation time or higher temperatures (up to room temperature) may be required for efficient cleavage. Alternatively, 50 mM of freshly prepared hydroxylamine (for pTXB1 at pH 6) or cysteine solution (at pH 8–9) can be used to induce cleavage at 4–25°C. Be aware that when hydroxylamine or cysteine is used with C-terminal IMPACT vectors (pTXB1), they form a stable covalent bond with the C-terminus of the target protein. One should determine whether a C-terminal hydroxamate or cysteine affects the activity of the target protein. When cysteine is used for cleavage with pTYB21, the cysteine is not attached to the target protein.

If you do not wish to use a thiol reagent for cleavage, the Intein1 (Ssp DnaB intein) in the pTWIN vectors may be used; cleavage is induced by temperature (room temperature) and/or pH (from pH 8.5 to 6).

How do I remove DTT after cleavage?

After elution of the target protein, free DTT can be removed from the sample by dialyzing at least twice against an appropriate buffer (at pH 8–9).

For more FREQUENTLY ASKED QUESTIONS (FAQs) please refer to the web site – www.neb.com – Technical Reference – Protein Expression and Purification Technologies – FAQs for IMPACT Kit.

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IMPACT User Publications:

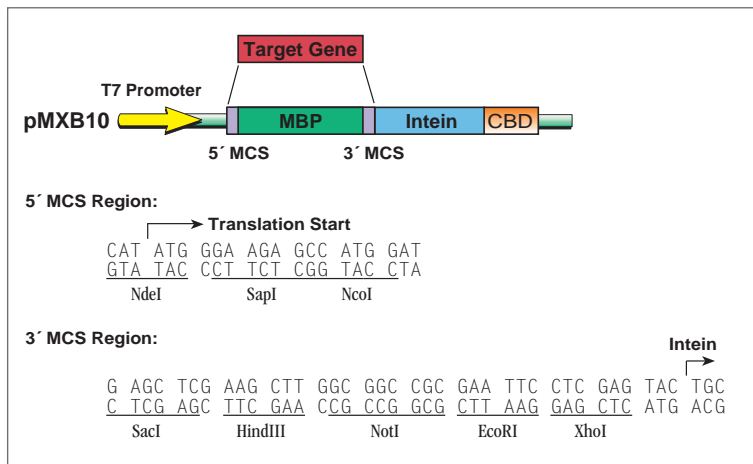
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For more references from IMPACT Users please go to the Section 1.11 in the IMPACT FAQs.

Appendix I: Cloning into pMXB10

The control vector pMXB10 encodes the fusion protein maltose binding protein (MBP) – Mxe GyrA intein – Chitin Binding Domain (CBD). The polylinker regions flanking the coding region for MBP can conveniently be used to clone a gene of interest (Figure 5). The 5' end of the target gene can be cloned into the NdeI, SapI or NcoI site. If the SapI or NcoI sites are used the primer should be designed so that the gene is in frame with the translational start in the NdeI site. For cloning the 3' end of the gene the SacI, HindIII, NotI, EcoRI or XhoI site can be used; make sure that the target protein is in frame with the intein. However, after intein cleavage the target protein will contain additional amino acids at its C-terminus, including (LEY), which has been shown to have a high rate of successful cleavage. For the 3' site you may also use the SpeI site, present near the N-terminus of the Mxe GyrA intein, by designing a primer that contains the intein sequence; this can result in a fusion without any vector-derived residues following cleavage.

Figure 5: pMXB10 vector and multiple cloning site.



Appendix II: Intein-mediated Protein Ligation (IPL) and Protein Labeling

The IPL reaction, also referred to as expressed protein ligation, allows the ligation of a bacterially expressed protein or a synthetic peptide with an N-terminal cysteine residue to a protein with a C-terminal thioester through a native peptide bond (Figure 6; 3,7). In addition to protein purification, pTXB, and other C-terminal fusion vectors (pTYB and pTWIN series), can be used to generate a protein with a C-terminal thioester for IPL. Typically 2-mercaptoethanesulfonic acid (MESNA) is used as the thiol reagent to induce intein-mediated cleavage; this produces a C-terminal thioester on the target protein. The C-terminus of the target protein can then be covalently labeled or ligated to a synthetic peptide with an N-terminal cysteine.

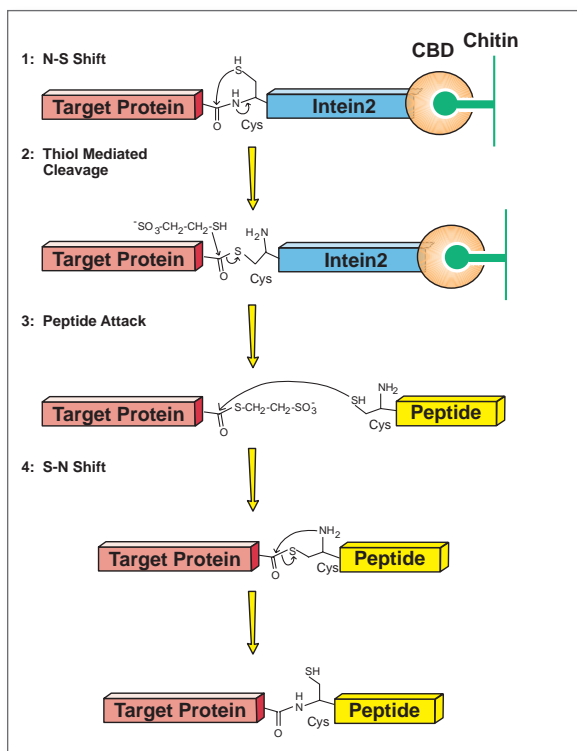
The following protocol illustrates a typical ligation or labeling experiment:

1. One of the components should have a final concentration of at least 0.5–1 mM. For the ligation of a peptide to a protein we use 0.01 mM protein with 0.5–1 mM peptide.
2. Combine the components in the presence of 0.1 M Tris, pH 8.5 and 10 mM MESNA and incubate overnight at 4°C. Alternatively, the reaction can be incubated at 25°C for 1–4 hours.
3. The ligation may be visualized by a 10% or 12% SDS-PAGE as a shift in mobility of the ligated protein, or can easily be detected by Western blot using an antibody specific for the peptide. Though a purified peptide is not required, it can usually yield a higher ligation efficiency (75%–90%). Add 20 μ l of 3X SDS Sample Buffer (with DTT) to 40 μ l of the protein sample. Boil for 5 minutes and analyze by SDS-PAGE, with unligated protein as a control.

To label a protein with biotin or a fluorescent moiety, a peptide with an N-terminal cysteine and a biotinylated or fluorescinated residue can be used. After ligation the protein samples can be analyzed by SDS-PAGE and/or detected by Western blot.

For cleavage, HEPES buffer should be used (9). For long term storage of MESNA-tagged proteins, dialyze the protein into 5 mM Bis Tris, pH 6.5, 250 mM NaCl and store at –80°C.

Figure 6: Mechanism of intein-mediated protein ligation (IPL).



Appendix III: Research Use Assurance Statement

The buyer and user have a non-exclusive sub-license to use this system or any component thereof for **RESEARCH PURPOSES ONLY**, based upon agreement to the following assurances. US Patent 5,496,714 and 5,834,247.

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PRODUCT	NEB #	SIZE
IMPACT™ Kit	E6901S	
KIT COMPONENTS SOLD SEPARATELY		
pTXB1 Vector	N6707S	10 µg
pTYB21 Vector	N6709S	10 µg
pMXB10 Control Plasmid	N6903S	10 µg
Chitin Beads	S6651S/L	20/100 ml
Anti-Chitin Binding Domain Serum	S6654S	0.05 ml
Blue Loading Buffer Pack	B7703S	8 ml
COMPANION PRODUCTS		
Anti-CBD Monoclonal Antibody	E8034S	0.05 ml
T7 Express Competent <i>E. coli</i> (High Efficiency)	C2566H	20 tubes
NEB 10-beta Competent <i>E. coli</i> (High Efficiency)	C3019H	20 tubes
NEB Turbo Competent <i>E. coli</i> (High Efficiency)	C2984H	20 tubes
pTYB1 Vector	N6701S	10 µg
pTYB2 Vector	N6702S	10 µg
pTYB3 Vector	N6703S	10 µg
pTYB4 Vector	N6704S	10 µg
pTXB3 Vector	N6708S	10 µg
pTYB11 Vector	N6901S	10 µg
pTYB12 Vector	N6902S	10 µg
pKYB1 Vector	N6706S	10 µg
pTWIN1 Vector	N6951S	10 µg
pTWIN2 Vector	N6952S	10 µg
pTWIN-MBP1 Vector	N6953S	10 µg
pTYB22 Vector	N6710S	10 µg
Quick Ligation Kit	M2200S/L	30/150 reactions
Quick Blunting Kit	E1201S/L	20/100 reactions

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USA

New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

Canada

New England Biolabs, Ltd.
Telephone: (905) 837-2234
Toll Free: 1-800-387-1095
Fax: (905) 837-2994
Fax Toll Free: 1-800-563-3789
e-mail: info@ca.neb.com

China, People's Republic

New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com

France

New England Biolabs France
Free Call: 0800/100 632
Free Fax: 0800/100 610
e-mail: info@fr.neb.com

Germany

New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info@de.neb.com

Japan

New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax +81 (0)3 5669 6192
e-mail: info@neb-japan.com

United Kingdom

New England Biolabs (UK), Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info@uk.neb.com

