Novagen®

Perfectly Blunt® Cloning Kits

Table of Contents

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About the Kits

Introductory pETBlue™-1 Perfectly Blunt® Cloning Kit	70633-3
pETBlue-1 Perfectly Blunt Cloning Kit	70634-3, 70634-4
pETBlue-1 Blunt Vector	70620-3, 70620-4
Introductory pETBlue-2 Perfectly Blunt Cloning Kit	70635-3
pETBlue-2 Perfectly Blunt Cloning Kit	70636-3, 70636-4
pETBlue-2 Blunt Vector	70621-3, 70621-4
Introductory pSTBlue-1 Perfectly Blunt Cloning Kit	70184-3
pSTBlue-1 Perfectly Blunt Cloning Kit	70191-3, 70191-4
pSTBlue-1 Blunt Vector	70188-3, 70188-4
Introductory pT7Blue Perfectly Blunt Cloning Kit	70183-3
pT7Blue Perfectly Blunt Cloning Kits	70189-3, 70189-4
pT7Blue Blunt Vector	70174-3, 70174-4
pT7Blue-2 Blunt Vector	70186-3, 70186-4
Introductory pT7Blue-3 Perfectly Blunt Cloning Kit	70075-3
pT7Blue-3 Perfectly Blunt Cloning Kit	70182-3, 70182-4
pT7Blue-3 Blunt Vector	70187-3, 70187-4

Description

The Perfectly Blunt Cloning Kits are designed for simplified cloning of DNA generated by PCR using any type of DNA polymerase. This approach enables the use of high-fidelity proofreading enzymes for amplification, thus decreasing the probability of generating mutations in the target sequence. In addition, under many conditions blunt cloning is more efficient than T-cloning, most likely due to the observation that the efficiency of single dA addition by *Taq* DNA polymerase varies significantly depending on the sequence context of the DNA ends, and even the number of PCR cycles performed (1–5).

With the Perfectly Blunt Cloning protocol, you can go from PCR product to plating transformations less than one hour with minimal hands-on time (see flow chart at right). The finished PCR product is converted to a blunt, phosphorylated form in a 15-minute reaction using premixed reagents. Following a 5 minute heat inactivation step, the treated insert is combined with the ready-to-use vector and ligated in an optimized 15 minute reaction. An exclusive 8-minute transformation procedure using highly efficient NovaBlue Singles™ Competent Cells generates recombinant colonies that are easily visualized by blue/white screening. The Perfectly Blunt® method is not limited to cloning PCR products; these kits are also suitable for cloning restriction fragments, cDNA, or sheared DNA with the same protocols in this User Protocol.

In addition to the components required for cloning, the pETBlue™ Perfectly Blunt Cloning Kits also include Tuner™(DE3)pLacI Competent Cells. This strains carries a chromosomal copy of the T7 RNA polymerase gene and is designed for IPTG-inducible expression of target genes under the control of the T7*lac* promoter in pETBlue vectors. The NovaBlue host is used for initial cloning and verification of constructs in the pETBlue Perfectly Blunt Vectors, and then the recombinant plasmids are transformed into the Tuner(DE3)pLacI strain for expression in *E. coli*. Several other (DE3)pLacI strains are available separately (see User Protocol TB009). Please refer to User Protocol TB249 (pETBlue System Manual) for expression of target genes in *E. coli* using the pETBlue vectors.

*Note: Highest cloning efficiencies are obtained with the Perfectly Blunt Cloning Kits when cloning fragments less than 3 kbp in length. In general, fragments over 3 kbp will not be cloned with high efficiency using this method. For large inserts, we recommend using the Novagen **A**ccep**T**or™ Vector Kits or LIC Vector Kits.*

Components and Storage

Each of the Perfectly Blunt Vectors is available in 3 different formats: Introductory Kits (10 reaction) and 20 or 40 reaction kits with NovaBlue Singles™ Competent Cells. Blunt Vectors also come in 20 and 40 reaction kits without the ligase or transformation components. Components are listed in the table below.

Component	Storage Conditions	Introductory Kits	Perfectly Blunt Cloning Kits		Perfectly Blunt Vectors	
		10 rxn	20 rxn	40 rxn	20 rxn	40 rxn
Blunt Vector, 50 ng/µl	–20°C	0.5 µg	2 × 0.5 µg	4 × 0.5 µg	2 × 0.5 µg	4 × 0.5 µg
Positive Control Insert, 4.5 ng/µl	–20°C	10 µl	10 µl	10 µl	10 µl	10 µl
End Conversion Mix	–70°C	1 × 100 µl	1 × 100 µl	2 × 100 µl	100 µl	2 × 100 µl
T4 DNA Ligase (Weiss units)	–20°C	1 × 100 U	1 × 100 U	2 × 100 U		
Nuclease-free Water	–20°C	1.5 ml	1.5 ml	1.5 ml		
NovaBlue Singles™ Competent Cells	–70°C	11 × 50 µl	22 × 50 µl	44 × 50 µl		
SOC Medium	–70°C	2 × 2 ml 3 × 2 ml*	4 × 2 ml 6 × 2 ml *	7 × 2 ml 9 × 2 ml *		
Test Plasmid for Transformation, 0.2 ng/µl (Amp^R)	–20°C	10 µl	10 µl	10 µl		
Tuner™(DE3)pLacI Competent Cells*	–70°C	0.2 ml	2 × 0.2 ml	4 × 0.2 ml		

* Included only in pETBlue™-1 and pETBlue-2 Perfectly Blunt Cloning Kits.

Perfectly Blunt® Vectors

Six different plasmids are available as Perfectly Blunt Vectors, and vary in their features and applications. The vector features are summarized below.

Perfectly Blunt Vector	Applications	Vector Advantages
pSTBlue-1	Archiving, Subcloning, Sequencing, *In vitro* transcription	Dual opposed SP6/T7 promoters Amp or Kan selection Dual *Eco*R I sites flank insert
pT7Blue-3	Archiving, Subcloning, Sequencing, *In vitro* transcription	T7 promoter Amp or Kan selection Dual *Eco*R I sites flank insert
pT7Blue	Archiving, Subcloning, Sequencing, *In vitro* transcription	T7 promoter *Nde* I/*Bam*H I sites flank insert
pT7Blue-2	Protein expression: *In vitro* transcription/translation, Sequencing	T7-driven *in vitro* protein synthesis N-terminal S•Tag™ sequence Optimal Kozak translation initiation *Xenopus* globin 5' UTR
pETBlue™-1	Protein expression: T7*lac*-driven, tightly controlled, high level expression in *E. coli*	No fusion tags Insert provides ATG start codon
pETBlue-2	Protein expression: T7*lac*-driven, tightly controlled, high level expression in *E. coli*	Optional C-terminal HSV•Tag® + His•Tag® sequences Vector provides ATG start codon

Insert design for expression from pT7Blue-2, pETBlue™-1 and pETBlue-2 Blunt Vectors

pT7Blue-2 Blunt Vector

The blunt cloning site in pT7Blue-2 (*Sma* I) is located such that an appropriately cloned insert will specify the third amino acid following the enterokinase cleavage site (AspAspAspAspLys↓). To achieve the correct reading frame in the pT7Blue-2 Blunt Vector, the 5' end of the insert (and the sense PCR primer) should begin with the third base of a Pro codon.

```
enterokinase site                          lacZ reading frame
AspAspAspAspLysSerPro     insert       GlyPheSer
GATGACGACGACAAGAGCCCNXXX.........XXXXGGGCTTCTCC
CTACTGCTGCTGTTCTCGGGNXXX.........XXXXCCCGAAGAGG
```

Sense primer: `5'-NXXX...,` where N = any base (completes Pro codon; G is recommended), and XXX is the initial codon of the insert.

Note: Although the pT7Blue-2 vector is designed primarily for in vitro *transcription/translation from the T7 promoter, inserts cloned in this manner may restore the reading frame of* lacZ *beyond the cloning site and would then express the target gene from the* lac *promoter in* E. coli*, and, if they lack a stop codon, will also produce a blue colony phenotype on IPTG/X-gal plates. Because target gene expression is induced from the* lac *promoter in the presence of IPTG, omitting IPTG from the plates may enhance cloning of some genes whose products are toxic to* E. coli*. This is not the case with the pETBlue vectors, in which the* lacZ *sequences are expressed by a different promoter (*tet*) in the opposite orientation relative to the T7 promoter.*

pETBlue-1 Vector

The blunt cloning site in pETBlue-1 (*Eco*R V) is located such that an appropriately cloned insert will specify the N-terminus of the expressed protein. Amplification with sense primers beginning with the ATG Met codon at their 5' end will ensure optimal spacing between the RBS and translation initiation sites for efficient protein synthesis in *E. coli*. There are no restrictions on the design of primers that specify the carboxyl terminus of the target sequence.

Sense primer: `5'-ATGXXX....`
Antisense primer: No restrictions

pETBlue-2 Vector

The blunt cloning site (*Eco*R V) is located such that an appropriately cloned insert will specify the fourth amino acid following Met-Ala-Ile/Met at the N-terminus of the expressed protein. To achieve the correct reading frame in the pETBlue-2 Blunt Vector, the 5' end of the insert (and the sense PCR primer) should begin with the third base of the Ile/Met codon. To express a target protein fused with C-terminal HSV•Tag® and His•Tag® peptides, the antisense primer should begin with two bases in any combination except TA or CA, and specify an antisense codon beginning with the third base.

```
        MetAlaIle....insert... Ser
Vector: ATGGCGATNXXX..........ATCC
        TACCGCTA.........YYYNNTAGG
```

Sense primer: `5'-NXXX......,` where XXX is the first codon of the target insert.
If N = G, Met codon is generated instead of Ile.
Antisense primer: `5'-NNYYY.....,` where YYY is the last codon of the target insert (antisense)
If NN = CA or TA, a stop codon is generated in the sense strand.

Preparation of the Insert

The Perfectly Blunt® Cloning Kits enable cloning of DNA with any type of end, because all types of ends are converted to blunt-, phosphorylated-ends during the End Conversion step. Typical applications include cloning of PCR products, restriction fragments, and cDNA synthesis products. Whereas the following sections are oriented to preparing PCR products for cloning, the procedures described below can be used for preparation of restriction fragments and cDNA. For restriction fragments we recommend gel purification with SpinPrep™ Gel DNA Kit, and for cDNA we recommend precipitation with Pellet Paint® Co-Precipitant to remove small DNA (< 50 bp) and unincorporated dNTPs.

Direct cloning without purification

The need for PCR product purification is determined by the quality of the amplified material (except when the procedure is used to subclone an insert from an Amp^R plasmid into an Amp^R Perfectly Blunt Vector; see notes below). If the PCR is very clean (i.e., the gel shows a clear, distinct band of the desired size with no extraneous bands), a small sample (≤ 2 µl) of the reaction can be added to the end-conversion reaction after extracting the PCR reaction with chloroform. The chloroform extraction inactivates the *Taq* DNA polymerase and thereby avoids the possible regeneration of heterogeneity on the PCR product termini due to *Taq* DNA polymerase activity. After removal of the oil overlay (if relevant), 1 volume chloroform:isoamyl alcohol (24:1) is added to the PCR reaction, the mixture is vortexed vigorously for 1 minute, centrifuged at 12,000 × g for 1 minute, and then up to 2 µl of the aqueous phase is added to the end conversion reaction. Precipitation is not necessary unless the PCR product is at a low concentration.

Note: Adding more than 2 µl of PCR reaction mix to the end-conversion reaction will significantly decrease cloning efficiency. Up to 5-fold higher cloning efficiencies may be attained by purifying the PCR product by gel or Pellet Paint Co-Precipitant methods (see below).

Note: When using PCR to subclone into a pT7Blue, pT7Blue-2, pETBlue™-1 or pETBlue-2 Blunt Vector from ampicillin-resistant plasmid templates, it is necessary to gel-purify the fragment of interest to remove the original plasmid, which will transform very efficiently (This is not necessary with pT7Blue-3 or pSTBlue-1 when plated on kan because they have kanamycin resistance). As little as 10 pg of contaminating supercoiled plasmid can typically result in several hundred white colonies when using NovaBlue competent cells and the following protocols.

Purification of PCR products

Pellet Paint® Co-Precipitant procedures are presented below. Gel purification with SpinPrep™ Gel DNA Kit is found in User Protocol TB274. Other methods of partial purification, such as spin columns, may be substituted.

PCR and cDNA clean-up with Pellet Paint Co-Precipitant

This rapid method removes dNTPs and DNA less than 50 bp in size. It will not remove ampicillin-resistant plasmids used as template in PCR reactions.

1. Thaw Pellet Paint Co-Precipitant and 3 M Sodium Acetate. Invert Pellet Paint to mix; do not vortex.
2. Add 2 µl Pellet Paint and 0.1 vol Sodium Acetate to the nucleic acid sample and mix.
3. Add 2 volumes of ethanol or 1 vol of isopropanol and vortex briefly.
4. Incubate at room temperature for 2 min.
5. Centrifuge at 14,000–16,000 × g for 5 min.
6. Remove supernatant using a pipet tip. Wash pellet with 70% ethanol and 100% ethanol. Dry pellet.
7. Resuspend in a small volume (e.g., 10 µl) of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
8. Determine DNA concentration according to the following protocol.

The absorbance contribution of Pellet Paint® Co-Precipitant in a given sample can be easily calculated. Because the absorbance ratio at 260 nm and 555 nm (A_{260}/A_{555}) is constant for each lot of Pellet Paint, you can calculate the Pellet Paint contribution to the total absorbance at 260 nm by taking an additional reading of your precipitated, resuspended

sample at 555 nm. This method can be used for DNA and RNA solutions as dilute as 0.5 µg/ml and is compatible with either water or TE buffer as the solvent.

a. Read the A_{260} of a dilution of the resuspended sample (e.g., 3 µl in 300 µl water) vs. a water blank.

b. Read the A_{555} of the same diluted sample.

c. Calculate the Pellet Paint contribution to the A_{260} of the sample:
Pellet Paint contribution to A_{260} = sample A_{555} × (Pellet Paint A_{260}/A_{555} ratio)
(the Pellet Paint A_{260}/A_{555} ratio is on the tube label)

d. Calculate the corrected A_{260} of the sample:
Corrected A_{260} = A_{260} reading from (a) – Pellet Paint contribution to A_{260} (c)

The DNA concentration is then calculated as usual by multiplying the Corrected A_{260} × dilution factor × 50 µg/ml.

Note: Novagen provides the absorbance ratio with each lot of Pellet Paint, which is printed on the tube label. However, because of the variability in readings between different spectrophotometers, the highest degree of accuracy is achieved by measuring the Pellet Paint A_{260}/A_{555} ratio with the same instrument and solvents that you use for your nucleic acid determinations.

End conversion

Note: No more than 2 µl of the chloroform-extracted PCR reaction should be added to the end conversion reaction. If the insert to vector molar ratio of 1:1 to 2.5:1 cannot be obtained by adding this volume of PCR reaction, then precipitate the PCR product and resuspend in a smaller volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). See the Pellet Paint protocol above for quick precipitation.

For a standard reaction when cloning into pT7Blue-3, pSTBlue-1, pETBlue™-1 or pETBlue-2, use 0.05 pmol amplified product, restriction fragment or cDNA (0.05 pmol = 16.5 ng of a 500 bp fragment) in the end-conversion reaction. When cloning into pT7Blue or pT7Blue-2, use 0.067 pmol amplified product (22 ng of a 500 bp fragment).

1. Assemble the following components in a 0.5 ml tube (add the End Conversion Mix last):

0.5–2.0 µl	PCR product (or 2 µl Blunt Vector Control Insert; *see note below)
X µl	Nuclease-free Water to a total of 10 µl
5.0 µl	End Conversion Mix
10 µl	total volume

**Note: Set up a positive control to test the efficiency of the vector: substitute 2 µl (9 ng = 0.067 pmol) of the Blunt Vector Control Insert provided with the kit in place of the amplified product in the above end conversion reaction. The Blunt Vector Control Insert is a 212 bp PCR product amplified with Taq DNA polymerase.*

   Mix gently by stirring with a pipet tip. Also prepare a negative control, omitting the PCR product or control insert.

2. Incubate the reaction at 22°C for 15 min.
3. Inactivate the reaction by heating at 75°C for 5 min. Note that complete inactivation of the kinase in the End Conversion Mix is required to avoid high vector background.
4. Cool the reaction briefly on ice (2 min). Please note that the reaction must be chilled prior to proceeding to the ligation step to avoid inactivating the ligase.
5. Briefly centrifuge the cooled reaction to collect the condensate and proceed to the ligation reaction.

Ligation

For a standard reaction, 1 µl (50 ng) Blunt Vector and 1 µl (4 U) T4 DNA Ligase are added directly to the end-conversion reaction, which brings the total volume to 12 µl. The 50 ng vector used corresponds to the following molar amounts of the different Blunt Vectors: 0.027 pmol for pT7Blue; 0.025 pmol for pT7Blue-2; 0.020 pmol for pT7Blue-3 and pSTBlue-1, 0.022 pmol for pETBlue-1, 0.021 pmol for pETBlue-2.

1. To the cooled end conversion reaction, add 1 µl Blunt Vector, and then 1 µl T4 DNA Ligase. Mix gently by stirring with the pipet tip used to add the ligase.
2. Incubate at 22°C for 15 min.

The molar ratio of insert to vector is 2.5:1 under these conditions. This ratio has been shown to produce maximum efficiency for ligation to the Blunt Vector Positive Control 212 bp insert. We recommend using insert to vector molar ratios from 1:1 to 2.5:1. Using higher molar ratios is possible but the frequency of obtaining insert multimers will increase.

Ligate the positive and negative controls prepared in the end conversion step in exactly the same manner as the insert-containing sample.

Transformation

Transformation of NovaBlue Singles™ Competent Cells

For transformation, 1 µl of the ligation reaction usually yields sufficient numbers of colonies for screening. Up to 5 µl of the ligation reaction containing high quality reagents can be added to Singles Competent Cells without reducing transformation efficiency.

Note: Upon receipt from Novagen, verify that the competent cells are still frozen and that dry ice is still present in the shipping container. Immediately place the competent cells at –70°C or below. For optimal results, do not allow the cells to thaw at any time prior to use. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible. To mix cells, flick the tube 1–3 times. NEVER vortex competent cells.

1. Remove the appropriate number of tubes of NovaBlue Singles Competent Cells from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently finger-flick the tube 1–2 times to evenly resuspend the cells. The cells are then ready for the addition of the ligation reaction.
3. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
4. Add 1 µl of a ligation reaction or purified plasmid DNA directly to the cells. Stir gently to mix and return the tube to the ice; making sure that the tube is immersed in ice except for the cap. Repeat for additional samples.
5. Place tubes on ice for 5 min.
6. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.

   Note: This "heat shock" step is most easily accomplished if the tubes are in a rack that leaves the lower halves of the tubes exposed. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 30 s, and then replace the rack on ice.
7. Place on ice for 2 min.
8. Add 250 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
9. a. If selecting for the expression of β-lactamase (Amp^R), no "outgrowth" step (shaking incubation) is required, although slightly higher cloning efficiencies may be obtained with 30–60 min outgrowth. Plate immediately as described in step 10.

   b. If using pSTBlue-1 or pT7Blue-3 Blunt Vectors, it is also possible to select for the expression of aminoglycoside 3'-phosphotransferase (Kan^R). For this purpose an outgrowth step is required. Shake at 200–250 rpm at 37°C for 30 min prior to plating. See notes below and then go to step 10.

*Note: **When selecting for the ampicillin resistance marker**, the antibiotic carbenicillin is recommended over ampicillin. Carbenicillin is less sensitive to the drop in the pH of the growth media that typically accompanies bacterial growth.*

*Note: **When selecting for the kanamycin resistance marker** (possible only with pSTBlue-1 and pT7Blue-3 vectors), substitute 30 µg/ml kanamycin for carbenicillin in Step 10.*

*Note: **When using the Test Plasmid**, plate no more than 5 µl of the final transformation mix in a pool of SOC on an LB agar plate containing 50 µg/ml carbenicillin or ampicillin (because the Test Plasmid carries the ampicillin resistance gene, bla).*

*Note: **For blue/white screening of recombinants**, also include IPTG and X-gal in the LB agar in the following steps. These can be pre-spread on the plates and allowed to soak in for about 30 minutes prior to plating. Use 35 µl of 50 mg/ml X-gal in dimethyl formamide and 20 µl 100 mM IPTG (in water) per 82 mm plate. Alternatively, X-gal and IPTG can be added to the LB agar at a final concentration of 70 µg/ml and 80 µM, respectively, just prior to pouring the plates.*

10. Plate 5–50 µl transformation mixture directly on LB agar media containing 50 µg/ml carbenicillin, 12.5 µg/ml tetracycline, 70 µg/ml X-gal and 80 µM IPTG. Tetracycline ensures that the selectable F'-containing lacZΔM15 is maintained and thus eliminates the background of non-recombinant white colonies that have lost the F'. If plating less than 50 µl, apply transformation mixture to a 50 µl cushion of SOC before spreading.

    Important: The appropriate amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. For recombinants in NovaBlue, expect 10^5–10^7 transformants/µg plasmid, depending on the particular insert and the ligation efficiency.

11. Using a sterile bent glass rod or specialized spreader, spread until the sample is evenly distributed on the plate. If the plates are fairly dry, the sample and cushion will quickly absorb into the plate. Once the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. Do not continue to spread until the sample and cushion have completely absorbed into the plate.

    Note: ColiRollers™ Plating Beads (Cat. No. 71013-3) are sterile glass beads that eliminate the use of the spreader and alcohol flame while evenly distributing cells without damaging the cells.

12. Set the plates on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

    Note: If performing blue/white screening, blue color development can be enhanced by placing the plates in a 4°C refrigerator for a few hours after the colonies have reached the desired size.

Transformation of Tuner™(DE3)pLacI Competent Cell swith pETBlue™ recombinants

Note: Always use NovaBlue Single™ Competent Cells and the above transformation procedure for the initial cloning in pETBlue vectors. NovaBlue enables blue/white screening, high quality plasmid preps for mapping and sequencing, and contains no source of T7 RNA polymerase, which is ideal for the establishment of recombinant plasmids under non-expression conditions.

For IPTG-inducible expression of target genes in *E. coli* using pETBlue vectors, it is necessary to transform recombinants that have been established in NovaBlue into the Tuner(DE3)pLacI host (included in the pETBlue Kits), or other pLacI host strains (available separately, see User Protocol TB009 for a listing). Unlike other T7*lac* promoter-containing pET vectors, the pETBlue vectors do not contain a copy of the *lacI* gene, and thus they require an additional source of *lac* repressor to suppress basal expression of target genes in the absence of inducer. The pLacI plasmid carried by these hosts is compatible with pETBlue recombinants and serves as the source for additional *lac* repressor. After the target plasmid is established and verified in NovaBlue, 1 µl of a 1:50 dilution of a miniprep (approximately 1 ng plasmid) can be easily transformed into (DE3)pLacI Competent Cells with the following protocol.

Tuner(DE3)pLacI Competent Cells are provided in 0.2-ml aliquots. The standard transformation reaction calls for 20 µl cells, so each tube contains enough cells for 10 transformations.

Note: Do not allow the cells to thaw at any time prior to use. Handle only the very top of the tube and the tube cap to prevent the cells from warming. Keep the cells on ice whenever possible.

Note: To mix cells, flick the tube 1–3 times. NEVER vortex competent cells.

Note: Cells can be refrozen at –70°C and reused; however, transformation efficiencies may decline several-fold with each freeze-thaw cycle. To avoid multiple freeze-thaw cycles of the standard cells, dispense the cells into aliquots after the initial thaw and store them at –70°C or below. To dispense aliquots of cells from the 0.2-ml stock, remove the stock tube quickly from the ice and flick 1–2 times to mix prior to opening the tube. Remove a 20 µl aliquot from the middle of the cells, and replace the tube immediately on ice. Place the aliquot immediately into the bottom of a pre-chilled 1.5 ml tube, mix by pipetting once up and down, and then immediately close the tube and replace on ice. After all of the aliquots are taken, return any unused tubes to the freezer before proceeding with the transformation.

Procedure: Transformation of Tuner™(DE3)pLacI Competent Cells

1. Remove the appropriate number of competent cell tubes from the freezer (include one extra sample for the Test Plasmid positive control, if desired). Immediately place the tubes on ice, so that all but the cap is surrounded by ice. Allow the cells to thaw on ice for 2–5 min.
2. Visually examine the cells to see that they have thawed and gently finger-flick the tube 1–2 times to evenly resuspend the cells. The cells are then ready for removal of an aliquot.
3. Place the required number of 1.5 ml snap-cap polypropylene tubes on ice to pre-chill. Pipet 20 µl aliquots of cells into the pre-chilled tubes.
4. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl pETBlue recombinant plasmid (~1 ng/µl) directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is immersed in ice except for the cap. Repeat for additional samples.
6. Incubate the tubes on ice for 5 min.
7. Heat the tubes for exactly 30 s in a 42°C water bath; do not shake.
8. Place the tubes on ice for 2 min.

9. Add 80 µl room temperature SOC medium to each tube. Keep the tubes on ice until all have received SOC.
10. Incubate at 37°C while shaking at 250 rpm for 60 min prior to plating on selective medium.
11. Plate 5–50 µl cells directly on LB agar media containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol, and 1% glucose. The addition of glucose to LB agar media and subsequent liquid media reduces basal expression.

    Note: Chloramphenicol (34 µg/ml) must be included in the plates used for pLacI hosts.

12. Set the plates on the bench for several min to allow excess liquid to be absorbed, and then invert and incubate overnight at 37°C.

    Note: Procedure for protein expression from pETBlue™ recombinants are described in the pETBlue System Manual, User Protocol TB249.

Screening

Blue/white phenotype

The Perfectly Blunt® Vectors provide for blue/white screening of recombinants. The plasmids encode a functional *lacZ* α-peptide that complements the *lacZ* ω-fragment expressed by the host strain (*lacZ*ΔM15 on F' in NovaBlue). The resulting active β-galactosidase can cleave the chromogenic substrate X-gal to yield a blue colony phenotype. Inserts are cloned within the α-peptide open reading frame (ORF). Inserts disrupt this ORF, thereby preventing the production of functional α-peptide, which results in the white colony phenotype when plated on X-gal/IPTG indicator plates.

The ligation with the insert should produce 20–50 fold whiter colonies than the negative control (no insert). In addition to dark blue and white phenotypes, a light blue phenotype can be observed with relatively high frequency in vector + insert ligations. We have found that more than 90% of these light blue colonies generated from vector + insert ligations contain inserts of the expected size. Also, note that the white colonies may develop a light blue center or *bullseye* appearance when grown to large sizes or during prolonged storage at 4°C. Presumably a small amount of functional α-peptide is produced in these recombinants by means of ribosomal frameshifting, second site translational initiation, or as an α-peptide fusion protein. The very small numbers of light blue or white colonies that arise from self-ligated vector appear to be the result of religating vector ends that may have been damaged during the vector preparation. The background due to self-ligated vector can be determined by performing control ligations in the absence and presence of the Positive Control Insert.

Note: Cloning into the blunt vector typically destroys the EcoR V site (or the Sma *I site in the case of pT7Blue-2); therefore, EcoR V is not a useful enzyme for restriction mapping of recombinant plasmids.*

Rapid screening by colony PCR

Prior to growing colonies for plasmid isolation, the presence of the appropriate insert and its orientation can be determined using direct colony PCR. This additional step may be particularly helpful if a "dirty" (many extraneous bands), unpurified PCR product was cloned. To determine insert orientation and size, 5 pmol (1 µl) of one of the vector- specific primers (see the following table) can be used with 5 pmol of an insert-specific primer. Alternatively, just the vector-specific primers can be used if insert orientation information is not desired.

Verification of the recombinants generated from the Positive Control Insert reaction can also be accomplished by performing PCR with the vector primers. The expected sizes of PCR products for each vector in both the presence and absence of the 212 bp Positive Control Insert are listed in the following table.

		Expected PCR Product	
Vector	**Vector Primers (5' + 3')**	**No Insert**	**+ 212 bp Insert**
pT7Blue	T7 promoter + U-19mer	139 bp	351 bp
	R-20mer* + U-19mer	166 bp	378 bp
pT7Blue-2	T7 promoter + U-19-mer	355 bp	566 bp
	R-20mer* + U-19mer	382 bp	593 bp
pT7Blue-3	T7 promoter + U-19-mer	198 bp	410 bp
	R-20mer* + U-19mer	225 bp	437 bp
pSTBlue-1	T7 promoter + U-19-mer	229 bp	441 bp
	R-20mer* + U-19mer	256 bp	468 bp
pETBlue™-1	pETBlueUP + pETBlueDOWN	155 bp	367 bp
	pETBlueT7UP* + pETBlueDOWN	177 bp	389 bp
pETBlue-2	pETBlueUP+ pETBlueDOWN	332 bp	544 bp
	pETBlueT7UP* + pETBlueDOWN	354 bp	566 bp

* recommended as the 5' primer for STP3® and EcoPro™ transcription/translation analysis (see following section). The R-20mer and pETBlueT7UP primers are not available from EMD Chemicals, Inc.. The sequence of these primers can be found on our website, www.merck4biosciences.com.

Colony PCR

1. Pick a colony from an agar plate using a 200 µl pipet tip or sterile toothpick. Choose colonies that are at least 1 mm in diameter and try to collect as many cells as possible. If a copy of the colony is desired, touch the pipet tip to a fresh plate, before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 0.5 ml tube containing 50 µl sterile water. Vortex to disperse the cells.
3. Place the tubes in boiling water or a heat block at 99°C for 5 min to lyse the cells and denature DNases.
4. Centrifuge at 12,000 × g for 1 min to remove cell debris.
5. Transfer 10 µl of the supernatant to a fresh 0.5 ml tube for PCR. Place on ice until use.
6. Make a master reaction mix on ice using the following amounts per reaction. To account for pipetting losses, it is convenient to multiply the amounts by X.5, where X is the number of reactions.

   Per reaction:

31.75 µl	PCR-grade Water
1 µl	dNTP Mix (10 mM each dATP, dCTP, dGTP, dTTP)
1 µl	5' primer, 5 pmol/µl
1 µl	3' primer, 5 pmol/µl
5 µl	10X Nova*Taq*™ Buffer (10X = 100 mM Tris-HCl pH 8.8 at 25°C, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100)
0.25 µl	Nova*Taq* DNA Polymerase (1.25 U)
40 µl	total volume

   Note: If using NovaTaq Buffer without $MgCl_2$, compensate by adding mM MgCl2 to a final concentration of 1.5–2.5 mM and decreasing the volume of water added to compensate.

7. Mix gently and, if necessary centrifuge briefly. Add 40 µl of the master mix to each sample, mix gently, overlay 2 drops of mineral oil if appropriate, cap the tubes, and place the samples in a thermal cycler.

   Note: As an optional step, a hot start procedure can be used in which the cell lysate samples are warmed to 80°C before the addition of the master mix. Alternatively, use NovaTaq Hot Start DNA Polymerase. For greatest accuracy, specificity, and yield of long complex target use KOD HiFi, KOD Hot Start, and KOD XL DNA Polymerase, respectively.

8. Process in the thermal cycler for 35 cycles as follows:

   Denature 1 min at 94°C
   Anneal 1 min at the proper annealing temperature (usually 55°C for vector primers)
   Extend 2 min at 72°C
   Repeat for 35 cycles
   Final extension for 5 min at 72°C

9. To remove the oil overlay and inactivate the polymerase, add 100 µl chloroform, mix 30 s, and centrifuge for 1 min. The top aqueous phase (which may appear cloudy) contains the DNA products. Transfer the aqueous phase to a fresh tube. If desired, remove a 5–10 µl sample for gel analysis. Store the remainder at –20°C.
10. If an appropriate PCR primer combination was used, 2 µl of the PCR products can be added directly to a Single Tube Protein® System 3, T7 reaction for protein synthesis.
11. Prior to use in EcoPro™ T7 reactions, PCR products should be precipitated to remove salts. To precipitate a 50 µl PCR samples, add 5.2 µl 3 M sodium acetate, and 115 µl 95% ethanol. Vortex briefly and spin at 14,000 × g for 5 min. Wash the pellet briefly with 70% ethanol, followed by 100% ethanol. Dry the pellet to remove residual ethanol and resuspend in 50 µl deionized water. The addition of Pellet Paint® Co-Precipitant to the DNA facilitates recovery in the precipitation step without affecting performance in the EcoPro™ reaction. Use 2–4 µl in the EcoPro T7 reaction.

Analysis of recombinants by *in vitro* transcription/translation

If the target insert encodes an open reading frame, PCR can be used to generate templates for *in vitro* transcription/translation analysis of the expressed polypeptide. This approach can be used to rapidly identify and/or verify recombinants that will produce the desired product when put into an *in vivo* expression system. Depending on the construct, this method can be used with any of the Perfectly Blunt® Vectors; however, it is particularly useful with the pT7Blue-2 and pETBlue™ vectors, which are designed specifically for protein expression.

By using a 3' insert-specific primer and a primer that anneals upstream of and in the same orientation as the T7 promoter, a fragment containing the T7 promoter and target insert can be amplified. The PCR product can serve as a suitable template in a Single Tube Protein® System 3 (STP3®, T7) or EcoPro™ T7 System for *in vitro* transcription/translation analysis of the target protein. STP3 is a linked reaction in which transcription by a bacteriophage RNA polymerase is directly followed by translation in an optimized rabbit reticulocyte lysate (for details refer to User Protocol TB206). The EcoPro System employs a proprietary fractionated E. *coli* extract to perform coupled transcription and translation in one step (see User Protocol TB278). Note that use of the T7 promoter primer itself will not allow efficient transcription by T7 RNA polymerase because it does not provide sufficient spacer sequence upstream from the promoter. The table on page 12 lists the 5' primers appropriate for use with STP3 and EcoPro analysis. The 3' primers listed can also be used if the insert orientation is known.

In addition to plasmids and colonies, vector-insert ligation reactions can also be used as templates to generate PCR products suitable for transcription/translation. When cloning using a non-directional strategy, it is recommended to use an insert-specific 3' primer to ensure amplification of only the desired orientation of the insert relative to the T7 promoter.

For amplification, the same protocol as colony PCR can be used; templates can be single colonies, 1–10 ng purified plasmid DNA, or 1 µl of a ligation reaction. Assuming appropriate amplification yields use 1–2 µl or 2–4 µl of the completed reaction as the template for STP3, T7 or EcoPro T7 respectively.

Note: Superior in vitro *transcription and translation with the STP3 System can be achieved by using the pT7Blue-2 vector. This vector encodes the β-globin 5' UTR translation enhancer upstream of an optimal Kozak translation initiation site.*

DNA Isolation and Sequencing

After positive clones are identified, high-copy Perfectly Blunt® pUC-based plasmids can be isolated for transformation into expression hosts, restriction mapping, and sequence analysis. NovaBlue is *recA endA* deficient and therefore is recommended for highest quality plasmid preparations. Plasmid DNA from candidate recombinants may also be evaluated using *in vitro* transcription/translation analysis. It is important that the template be RNase-free for *in vitro* transcription and translation. Purify Plasmid DNA isolated using protocols from scientific literature. The plasmid DNA must be RNase-free, and thus may require an additional phenol:CIAA extraction to eliminate RNases.

A satisfactory procedure is to add TE to 100 µl, and then extract successively with 1 vol TE-buffered phenol, 1 vol phenol:CIAA (1:1; CIAA is chloroform:isoamyl alcohol, 24:1), and 1 vol CIAA. Transfer the final aqueous phase to a fresh tube and add 0.1 vol 3M Na acetate and 2 vol 100% ethanol. Mix and place at –20°C for 30 min, spin 5 min at 14,000 × g, remove the supernatant, and rinse the pellet with 70% ethanol. Dry and resuspend the DNA in 30 µl TE. If desired, 2 µl Pellet Paint® or Pellet Paint NF Co-precipitant can be added with the TE buffer before extraction to facilitate recovery of the DNA (the –20°C incubation can be eliminated if using Pellet Paint Co-Precipitant).

Sequencing

Detailed protocols for sequencing with double stranded and single stranded templates are available from many manufacturers of sequencing kits. Primers for sequencing are indicated on the vector maps available at www.merck4biosciences.com.

It is possible to prepare single stranded DNA template from PCR products with the Strandase™ Kit (Cat. No. 69202-3). Also, because the Perfectly Blunt vectors contain an f1 origin of replication, it is possible to prepare single stranded DNA by infection with a single stranded DNA helper phage. The required helper phage (strain R408 or M13KO7) and protocols for infection and DNA isolation are available from a number of commercial suppliers. The NovaBlue host strain carries an F' and is therefore suitable for helper phage infection.

References

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