pSTBlue-1 AccepTor™ Vector Giga Kits

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Description

The pSTBlue-1 **A**ccep**T**or Vector Giga Kits feature NovaBlue GigaSingles™ Competent Cells for transformation efficiency (>1 × 10^9 cfu/µg). pSTBlue-1 is a general purpose vector for archiving, subcloning, and sequencing PCR products. The vector contains dual opposed SP6/T7 promoters for *in vitro* transcription, ampicillin and kanamycin resistance, and a wide array of restriction sites, including dual *Eco*R I sites that flank the cloning site.

The **A**ccep**T**or Vector Giga Cloning Kits are designed for simplified cloning of PCR products generated non-proofreading thermostable DNA polymerases, such as Nova*Taq*™ DNA polymerase, that leave single 3'-dA overhangs on their reaction products (1). The linearized **A**ccep**T**or vector contains single 3'-dU overhangs that are compatible with direct ligation of these products without the need for intermediate reactions. The kits contain the Clonables™ 2X Ligation Premix, a unique, universal ligation cocktail that contains ligase, buffer and cofactors to support rapid, optimal ligation. Ligations are designed for a 10 µl volume containing 5 µl of the Premix plus vector, insert and water (if needed). Following transformation, the dU residues are replaced with dT residues as the bacteria replicate the plasmid. Target inserts having other types of ends, such as reaction products of KOD HiFi or KOD Hot Start DNA Polymerase or restriction fragments, can be easily cloned using Novagen's Perfectly Blunt® Cloning Kits.

Components

Component	AccepTor Vector Kits	
	20 rxn	40 rxn
Accep**T**or Vector, 50 ng/µl	2 × 0.5 µg	4 × 0.5 µg
Positive Control Insert, 15 ng/µl	10 µl	10 µl
Clonables 2X Ligation Premix	2 × 55 µl	4 × 55 µl
Nuclease-free Water	1.5 ml	1.5 ml
NovaBlue GigaSingles™ Competent Cells	22 × 50 µl	44 × 50 µl
SOC Medium	4 × 2 ml	7 × 2 ml
Test Plasmid for Transformation, 0.2 ng/µl (amp^R)	10 µl	10 µl

Storage

Store Competent Cells and SOC Medium at –70°C. Store all other components at –20°C.

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Preparation of the Insert

Direct Cloning without Purification

The need for PCR product purification is determined by the quality of the amplified material. If the PCR is very clean (i.e., the gel shows a clear, distinct band of the expected size with no extraneous bands), a small sample (≤ 2 µl) of the reaction can be added to the ligation reaction after extracting the PCR reaction with chloroform. The chloroform extraction inactivates the *Taq* DNA polymerase. 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 top aqueous phase is added to the ligation reaction. Precipitation is not necessary unless the PCR product is at a low concentration.

Notes: *Using more than 2 µl of the extracted PCR product will reduce ligation efficiency. Up to 5-fold higher cloning efficiencies may be attained by purifying the PCR product by gel purification with SpinPrep™ Gel DNA Kit or Pellet Paint® Co-Precipitant methods (see below).*

Purification of PCR Product

Pellet Paint® Co-Precipitant procedures are presented below. Gel purification with SpinPrep™ Gel DNA Kit is found in Technical Bulletin 274. Other methods of partial purification, such as spin columns, may be substituted.

PCR 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 Na Acetate. Invert Pellet Paint to mix; do not vortex.
2. Add 2 µl Pellet Paint and 0.1 volume Na Acetate to the nucleic acid sample and mix.
3. Add 2 volumes of ethanol or 1 volume 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 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 $A_{555} \times$ (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 $A_{260} \times$ dilution factor $\times$ 50 µg/ml.

Note: *The lot specific A_{260}/A_{555} ratio is given on the Pellet Paint 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.*

Ligation

For a standard reaction, 1 µl (50 ng; 0.02–0.022 pmol) **A**ccep**T**or Vector is ligated with 0.15 pmol amplified product (50 ng of a 500 bp fragment) in a total volume of 10 µl.

1. Assemble the following components in a microcentrifuge tube:

1 µl	**A**ccep**T**or Vector (50 ng/µl)
0.5–4.0 µl	PCR product (or 2 µl **A**ccep**T**or Control Insert; *see note below)
X µl	Nuclease-free Water to a total of 10 µl
5.0 µl	Clonables™ 2X Ligation Premix
10 µl	total volume

Mix gently by stirring with a pipet tip.

* Set up a positive control to test the efficiency of the vector: substitute 2 µl (30 ng = 0.22 pmol) of the **A**ccep**T**or Vector Control Insert provided with the kit in place of the amplified product in the above ligation reaction. The **A**ccep**T**or Vector Control Insert is a 212 bp PCR product amplified with *Taq* DNA polymerase.

Also prepare a negative control, omitting the PCR product or control insert.

2. Incubate the ligation reaction at 16°C for 30 min.

Notes: *Incubation can be performed from 15 min to 2 h; the number of recombinants can be increased 2–3 fold by incubating for 2 h vs. 30 min.*

Up to 4 µl of a purified insert can be added to the ligation reaction. However, more than 2 µl of a chloroform-extracted PCR reaction tends to inhibit ligation and/or transformation. If the insert to vector molar ratio of 5:1 to 10:1 cannot be obtained by adding 2 µl or less of PCR reaction, then precipitate the PCR product and resuspend in a smaller volume of TE buffer or water. See the Pellet Paint protocol on the previous page for quick precipitation.

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Transformation

DNA in ligation reactions containing high-quality reagents is suitable for direct addition to Novagen's NovaBlue GigaSingles™ Competent Cells. Inactivation of the ligase is not required prior to transformation. 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 the competent cells.

1. Remove the appropriate number of tubes of NovaBlue GigaSingles 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 check the cells to see that they have thawed and gently flick the cells 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 (amp^R) to one of the tubes containing cells. Gently flick the tube to mix and return the tube to the ice. Plate only 5 µl of the final transformation mix (on LB + 50 µg/ml carbenicllin; see below).
4. Add 1 µl of a ligation reaction or purified plasmid DNA (~1ng/µl) directly to the cells. Stir gently to mix and return the tube to the ice, making sure that the tube is surrounded by ice except for the cap. Repeat for additional samples.
5. Place tubes on ice for 5 min.
6. Heat the tubes for exactly 30 sec 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 half of the tubes exposed. Hold the rack in the water bath so that the lower halves of the tubes are submerged for 30 sec, 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
9. Shake at 200–250 rpm at 37°C for 60 min prior to plating to obtain 1×10^9 cfu/µg efficiency.

Notes: ***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.*

When selecting for the kanamycin resistance marker*, substitute 30 µg/ml kanamycin for carbenicillin in Step 10.*

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).*

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 20–100 µl transformation mixture directly on LB agar media containing 50 µg/ml carbenicillin, 15 µg/ml tetracycline, 70 µg/ml X-gal and 80 µM IPTG. Tetracycline ensures that the selectable F'-containing *lacZ*ΔM15 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.

Notes: *The appropriate amount of transformation mixture to plate will vary with the efficiency of both the ligation and the competent cells. It is generally recommended to plate 20 µl and 100 µl of the transformation mixture to ensure that one of the plates will contain a sufficient number of isolated colonies for screening.*

ColiRollers™ Plating Beads (Cat. No. 71013) are specially treated glass beads that eliminate the use of the spreader and alcohol flame while evenly and consistently distributing cells without the possibility of damage.

Using ColiRollers Plating Beads, 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. After the moisture is absorbed, do not continue spreading. If the plates are wet, spread until the sample is evenly distributed. ***Do not spread until the sample and cushion have absorbed completely into the plate, because overspreading is lethal to the cells****.*

11. Let the plates sit on the bench for several minutes to allow excess liquid to be absorbed, then invert and incubate overnight at 37°C (preferably 15–18 h).

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.*

Screening

Blue/White Phenotype

The **A**ccep**T**or 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 10–50 fold more white 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 number 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 **A**ccep**T**or vector typically destroys the EcoR V site: therefore, EcoR V is not a useful enzyme for restriction mapping of recombinant plasmids.*

The following rapid PCR screening method also can be used to distinguish religated vector from vector containing insert.

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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 followed by agarose gel electrophoresis. 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 table on the next page) 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 pSTBlue-1 vector in both the presence and absence of the 212 bp Positive Control Insert are listed in the following table.

		Expected PCR Product	
AccepTor Vector	**Vector Primers (5' + 3')**	**No Insert**	**+ 212 bp Insert**
pSTBlue-1	T7 promoter + U-19-mer	231 bp	443 bp
	R-20mer + U-19mer	258 bp	470 bp

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 get as many cells as possible. If a *copy* of the colony is desired, touch the pipet tip to a plate before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a tube containing 50 µl of sterile water. Vortex to disperse the pellet.
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. Leave on ice until use.

Note: As an optional step, a hot start procedure can be used in which the cell lysate samples are prewarmed to 80°C before the addition of the master mix.

6. Make a master reaction mix, as follows:

Per reaction:	
31.8 µl	Nuclease-free water
1 µl	dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
1 µl	5' primer, approx. 5 pmol/µl
1 µl	3' primer, approx. 5 pmol/µl
5 µl	10X Nova*Taq* Buffer with $MgCl_2$
0.25 µl	(1.25 U) Nova*Taq*™ DNA polymerase
40 µl;	total volume

Note: If using NovaTaq Buffer without $MgCl_2$, add 25 mM $MgCl_2$ to a final concentration of 1.5–2.5 mM and decrease the volume of water added to compensate.

Mix together the master reaction components in a single tube using amounts corresponding to the number of reactions desired. (It is convenient to multiply the amounts by X.5, where X is the number of reactions. This accounts for pipetting losses.)

7. Add 40 µl master mix to each sample, mix gently, add 2 drops (~ 40 µl) mineral oil if appropriate, cap the tubes and put the samples in a thermal cycler (Perkin-Elmer).

   Process for 35 cycles, as follows:
   94°C 1 min
   55°C 1 min
   72°C 2 min
   Include a final extension of 72°C for 5 min
   To analyze the reaction products, first remove the oil overlay (if one was used) by adding 100 µl of chloroform. Add 5 µl of 10X loading dye to the top aqueous phase and load 10–25 µl per lane on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

DNA Isolation and Sequencing

After positive clones are identified, the high-copy pSTBlue-1 **A**ccep**T**or Vector plasmid 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 isolated with Mobius™ or UltraMobius™ Kits are essentially RNase-free. However, plasmid DNA isolated with SpinPrep™ Plasmid Kits or other manufacturers kits may require an additional phenol:CIAA extraction to eliminate RNases.

Plasmid Kit	Scale	DNA Yield	Cat. No.	Size
Mobius 1000 Plasmid Kit	100 ml	> 1 mg	70854-3 70853-3 70853-4	2 rxn 10 rxn 25 rxn
UltraMobius 1000 Plasmid Kit	100 ml	> 1 mg	70907-3 70906-3 70906-4	2 rxn 10 rxn 25 rxn
Mobius 200 Plasmid Kit	35 ml	> 200 µg	70970-3	25 rxn
UltraMobius 200 Plasmid Kit	35 ml	> 200 µg	71090-3	25 rxn
SpinPrep Plasmid Kit	1–3 ml	5–10 µg	70957-3 70851-3	20 rxn 100 rxn

If the sequencing template is precipitated, the addition of Pellet Paint or Pellet Paint NF Co-Precipitant helps make the pellet visible after precipitation. Use Pellet Paint NF (Cat. No. 70748-3) with rhodamine based labeling methods (e.g. PE Applied Biosystems automated sequencers) and Pellet Paint (Cat. No. 69049-3) with Cy5-based automated sequencers.

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.novagen.com.

It is possible to prepare single stranded DNA template from PCR products with the Strandase™ Kit (Cat. No. 69202-3). Also, because the pSTBlue-1 vector 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

1. Clark, J.M. (1988) *Nucleic Acids Res.* **16**, 9677–9686.

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