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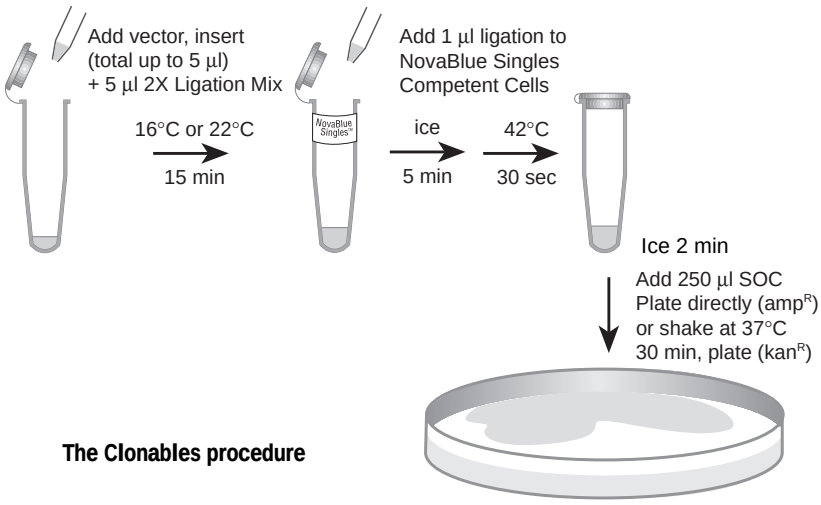
About the Kit

Clonables Ligation/Transformation Kit	11 rxn	70526-3

Description

The Clonables Kit simplifies and streamlines the fundamental cloning procedures of ligation and transformation (1). The kit provides a standardized set of reagents that enables convenient, dependable, high efficiency ligation and transformation with virtually any type of plasmid vector and insert. The kit features a unique, universal ligation premix containing ligase, buffer and cofactors that supports rapid, optimal ligation of any type of DNA "sticky" end as well as blunt end. Ligations are designed for a 10 µl volume containing 5 µl of the Clonables 2X Ligation Premix plus vector, insert and water (if needed). Transformation is performed using the NovaBlue Singles™ Competent Cells, which are optimized for a streamlined protocol that takes less than 6 minutes to perform with ampicillin-resistant plasmids and 36 minutes using kanamycin selection. The kit works with virtually any plasmid vector and resistance marker, except for tetracycline, which cannot be used for selection due to its presence on the F' in the NovaBlue strain. NovaBlue allows blue/white screening by α-complementation with appropriate vectors, supports high yield of plasmid monomers, and is well-suited for plasmid DNA preparation as well as for single stranded DNA production from plasmids containing the f1 origin of replication by infection with helper phages. A control vector and insert mix are included in the kit to verify performance of the entire system, and a separate test plasmid is provided to test the competent cells independently.



The Clonables procedure

Components

The kit contains components sufficient for 11 ligation and transformation reactions.

- 55 µl Clonables 2X Ligation Premix
- 10 µl Clonables Positive Control
- 1.5 ml Nuclease-free Water
- 11 × 50 µl NovaBlue Singles Competent Cells
- 2 × 2 ml SOC Medium
- 1 Test Plasmid

Storage

Store at –70°C.

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Related products/available separately	Size	Cat. No.
Carbenicillin	5 g	69101-3
Chloramphenicol	5 g	220551
Clonables 2X Ligation Premix	11 rxn	70573-3
IPTG, 100 mM Solution	10 × 1.5 ml	70527-3
Kanamycin	25 g	420311
NovaBlue Singles Competent Cells	11 rxn 22 rxn	70181-3 70181-4
Pellet Paint™ Co-precipitant	125 rxn	69049-3
Perfectly Blunt™ End Conversion Mix	100 µl	70179
Tetracycline	25 g	58346

Vector and Insert Preparation

Vector

For plasmid vector preparation, use the restriction enzyme manufacturer's recommended buffer and incubation conditions for the enzymes you are using. Many combinations of enzymes are compatible when used together in the same buffer. Note that the digestion efficiency varies with the enzyme, reaction conditions and relative proximity of the restriction sites. In general, enzymes with compatible buffers and sites that are more than 10 bp apart can be used together in the same reaction. If one of the enzymes is a poor cutter, if the buffers are incompatible, or if the sites are separated by 10 bp or less, the digestions should be performed sequentially. The first digestion should be done with the enzyme that is the poorest cutter. The second enzyme should be added after the first digestion has been verified by running a sample of the reaction on an agarose gel.

Note that some restriction enzymes may display "star activity", a less stringent sequence dependence that results in altered specificity. Conditions that can lead to star activity include high glycerol concentration (> 5%), high pH, and low ionic strength.

If cloning into a single site, dephosphorylate the vector following digestion to decrease the background of non-recombinants due to self-ligation of the vector. Molecular biology grade calf intestinal or shrimp alkaline phosphatase should be used according to the manufacturer's instructions. It is also useful to dephosphorylate vectors cut with two enzymes, especially when the sites are close together or if one of the enzymes is a poor cutter. This decreases the non-recombinant background caused by incomplete digestion with one of the enzymes, which is undetectable by gel analysis.

Following digestion it is usually worthwhile to gel-purify the vector prior to insert ligation to remove residual nicked and supercoiled plasmids, which transform very efficiently relative to the desired ligation products. This step is optional, but it usually reduces the effort required to screen for the correct construction.

Insert

Sticky ends

Inserts prepared by restriction enzyme digestion possess phosphorylated 5' ends. Preparing inserts by restriction digestion from existing vector constructs is straightforward. Note, however, that in order to minimize the possibility of the original vector interfering with the subsequent cloning steps, the insert should be gel purified.

PCR can be used to add convenient restriction enzyme sites onto the ends of target genes. In general, primers should contain a minimum of 15 (preferably 18–21) nucleotides complementary to the sequence of interest with a GC content of about 50%, and restriction sites should be

flanked by 3–10 (depending on the enzyme) "spacer" nucleotides at the 5' end to allow for efficient digestion. Following amplification, the PCR product should be purified prior to restriction enzyme digestion. Note that the small terminal fragments generated by digestion possess complementary overhangs that can participate in the subsequent ligation reaction resulting in decreased cloning efficiency. Fragments such as this (smaller than 50 bp) can be quickly and efficiently removed by precipitation in the presence of Novagen's Pellet Paint™ Co-precipitant.

Blunt ends

Blunt ends are created by employing blunt-cutting restriction enzymes, filling in 5´ overhangs, polishing ("chewing back") 3' overhangs or by amplifying PCR fragments with thermostable enzymes possessing 3'→5' proofreading activity. Vectors having blunt ends on both sides of the insertion site are typically dephosphorylated to prevent recircularization of the vector without an insert. Therefore, inserts should possess 5' phosphorylated ends. PCR generated inserts must be amplified with 5´ phosphorylated primers or treated with polynucleotide kinase following PCR using standard conditions. Novagen's Perfectly Blunt™ End Conversion Mix accomplishes 5' phosphorylation as well as fill-in and polishing reactions all in one convenient reaction.

Single 3' dA overhangs

PCR products amplified with thermostable DNA polymerases lacking 3'→5' proofreading activity are known to leave single 3' deoxynucleoside extensions due to a terminal transferase activity of the enzymes. The frequency of addition and type of base added are influenced by the base composition at and near the 3'end (2, 3). The most frequently added 3'deoxynucleoside is a dA residue. The addition of this nucleotide forms the basis for T-vector cloning. T-vectors are not typically dephosphorylated and therefore the use of 5' phosphorylated PCR primers is not required.

Clonables Ligation Reaction

For maximum ligation and transformation efficiency, the vector and insert DNA should be free of phenol, ethanol, salts, protein and detergents, and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) or in water. The standard ligation is performed in a total volume of 10 µl and contains 50 ng (0.025 pmol) of linearized, dephosphorylated vector (see Note "a", below), target insert and 5 µl of 2X Ligation Premix. Assemble the reaction according to the type of ends being ligated as indicated in the table below.

	Type of ends being ligated[b]		
	Sticky (2–4 nt)	**Blunt**	**T/A (single nt)**
Vector[c]	x µl (0.025 pmol)	x µl (0.025 pmol)	x µl (0.025 pmol)
Insert[d]	y µl (0.05–0.125 pmol)	y µl (0.0625 pmol)	y µl (0.125–0.25 pmol)
Nuclease-free Water	z µl (5–x–y)	z µl (5–x–y)	z µl (5–x–y)
2X Ligation Premix	5 µl	5 µl	5 µl
Total volume	10 µl	10 µl	10 µl
Insert:Vector ratio	2–5:1	2.5:1[e]	5–10:1
Incubation temperature	16°C	22°C	16°C
Incubation time	15 min	15 min	15 min–2 h[f]

Add the 2X Ligation Premix last, and stir gently with the pipet tip to mix. Incubate at the indicated temperature and time. Following incubation, the reaction can be stored at 0–4°C until used for transformation.

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Notes:

a) The need for dephosphorylation depends on the cloning strategy being employed. See *Vector and Insert Preparation* for details.

b) Ligation of "mixed" end types should be set up as follows: for blunt + sticky, use sticky conditions; for blunt + T/A, use T/A conditions.

c) pmol DNA = mass of DNA (pg) ÷ [DNA length (bp) × 650 pg/pmol/bp]
e.g., 50 ng of a 3 kbp plasmid = 0.025 pmol

d) e.g., 32.5 ng of a 500 bp insert = 0.1 pmol

e) Higher molar ratios of insert:vector may be used for blunt ends; however, the frequency of recombinants with multiple inserts will increase.

f) With T/A ends, hundreds of clones are typically obtained when a 15 min ligation is performed and 90 µl of the transformation reaction is plated. For higher T-vector based cloning efficiencies, the ligation time can be extended to 2 h at 16°C.

g) Vector and insert concentrations can be determined by A_{260} readings (where 1 A_{260} unit = 50 µg/ml DNA)., or estimated by agarose gel electrophoresis of a DNA sample along side known amounts of DNA of similar sizes in adjacent wells. Dilute solutions of DNA can be concentrated easily and with high recovery using Pellet Paint™ Co-Precipitant. Pellet Paint is compatible with ligation and transformation.

Control reactions

Positive control: The Clonables Positive Control consists of a mixture of a blunt, dephosphorylated blue/white screening vector and a blunt 52 bp insert at an insert:vector molar ratio of 2.5:1. Combine 5 µl of Clonables Positive Control and 5µl of 2X Ligation Premix and incubate at 22°C for 15 min. When transformed and plated as described below, the Clonables Positive Control should produce hundreds of colonies (90 µl plated) with > 95% white phenotype on X-gal/IPTG indicator media.

Negative control: As a negative control, assemble a parallel reaction containing the same amount of prepared vector, while omitting the target insert (substitute water or TE buffer to bring the reaction volume to 10 µl). This reaction will determine the "background" of non-recombinants due to residual circular plasmid and/or recircularization of the plasmid due to incomplete digestion with a second enzyme, incomplete dephosphorylation, or reinsertion of the small vector fragment between two restriction sites used for cutting.

Abbreviated Transformation Protocol for Experienced Users

Note: *See the following section for a detailed protocol.*

1. Thaw the required number of tubes of cells on ice and mix gently to ensure that the cells are evenly suspended.
2. Add 1 µl of the ligation reaction directly to the cells. Stir gently to mix.
3. Place the tubes on ice for 5 min.
4. Heat the tubes for exactly 30 sec in a 42°C water bath; do not shake.
5. Place the tubes on ice for 2 min.
6. Add 250 µl of room temperature SOC medium to each tube.
7. If selecting for ampicillin resistance, plate 10 µl and 90 µl cells on separate LB agar plates containing 50 µg/ml carbenicillin or ampicillin (plus 15 µg/ml tetracycline + IPTG/X-gal for blue/white screening). If selecting for kanamycin resistance, shake at 37°C (250 rpm) for 30 min prior to plating on LB agar containing 30 µg/ml kanamycin (plus tetracycline and IPTG/X-gal for blue/white screening). See note "d" on p. 7 for preparation of IPTG/X-gal plates.

Transformation - Detailed Protocol

Singles Competent Cells are provided in 50 µl aliquots, which are used "as is" for single 50 µl transformations. If the vector and insert DNA used in the ligation reaction are sufficiently pure, the mixture can be added directly to the competent cells. Inactivation of the ligase is not required prior to transformation.

Handling tips

1. 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.
2. 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.
3. To mix cells, flick the tube 1–3 times. *NEVER* vortex the competent cells.

Transformation reaction

1. Remove the appropriate number of Singles tubes from the freezer (include one extra tube 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 1–3 times to evenly resuspend the cells. The cells are then ready for the addition of the DNA.
3. (Optional) To determine transformation efficiency, add 1 µl (0.2 ng) Test Plasmid to one of the Singles tubes. Gently flick the tube to mix and return the tube to the ice.
4. Add 1 µl of a ligation reaction 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. Incubate the tubes on ice for 5 min.
6. Heat the tubes for exactly 30 sec in a 42°C water bath; do not shake.

Tip: *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 with tubes on ice.*

7. Place the tubes on ice for 2 min.
8. Add 250 µl of room temperature SOC medium to each tube.
9. If selecting for β-lactamase ($carb^R$/amp^R), no outgrowth (shaking incubation) step is required; proceed to step 10. If selecting for the expression of aminoglycoside 3'-phosphotransferase (kan^R), shake at 200–250 rpm at 37°C for 30 min.

Tip: *The outgrowth incubation is conveniently performed in a shaking incubator using a test tube rack anchored to the shaking platform. Place each transformation tube in an empty 13 mm × 100 mm glass test tube in the rack. The snap-caps on the transformation tubes prevent them from falling to the bottom of the test tubes, and all transformation tubes remain vertical.*

10. Spread 10 µl and 90 µl of each transformation on separate LB agar plates containing appropriate antibiotics and indicators (see notes below). When plating less than 25 µl, apply transformation mixture to a 50 µl pool of SOC before spreading (see *Plating technique* for details).

Important: *The number of colonies produced varies with the efficiency of both the ligation and the competent cells. As little as 5 µl will typically yield several hundred transformants with Clonables ligation of sticky ends. By plating two different amounts (10 µl and 90 µl) it is likely that at least one of the plates will contain an appropriate number of colonies.*

***When using the Test Plasmid or the Clonables Positive Control**, plate 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 and Positive Control vector carry the bla gene).*
Recommended plating volumes: Test Plasmid, 2–5 µl; Clonables Positive Control, 10 µl and 90 µl.

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

Tip: *Following overnight incubation, plates can be placed at 4°C for a few hours to enhance color development when performing blue/white screening.*

Notes on antibiotics and indicators:

a) For recombinants expressing β-lactamase ($carb^R$/amp^R), use plates containing 50 µg/ml carbenicillin or ampicillin (plus 15 µg/ml tetracycline and IPTG/X-gal when blue/white screening).

b) For recombinants expressing aminoglycoside 3'-phosphotransferase (kan^R) use plates containing 30 µg/ml kanamycin (plus 15 µg/ml tetracycline and IPTG/X-gal when blue/white screening).

c) The use of tetracycline is not necessary, but it ensures that the selectable F'-containing *lacZΔ M15* is maintained and thus eliminates the small background of non-recombinant white colonies that have lost the F' (when performing blue/white screening).

d) For blue/white screening with appropriate vectors, IPTG and X-gal can be pre-spread on the plates and allowed to soak in for about 30 min 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.

Plating technique

1. Optimal results are usually obtained using prewarmed plates. During the outgrowth (step 9, or earlier if omitting outgrowth), place the plates at 37°C. If the plates contain a lot of moisture, place them cover-side up and open the cover ~1/3 of the way to allow the plates to dry for 30–45 min. If the plates do not need drying, keep them closed and place them cover-side down in the 37°C incubator for ~20 min prior to plating.
2. Remove the plates from the incubator. If plating less than 25 µl of the transformation, we recommend plating onto a pool of SOC, which facilitates even colony distribution on the plate surface. Using a sterile pipet tip, place 50 µl of SOC in the center of a plate for a plating cushion.
3. To remove the transformation sample, flick the transformation tube 5–8 times, open the cap and immediately remove the sample volume from the middle of the transformation reaction.
4. Transfer the sample to the plate by dispensing the sample volume into the SOC cushion. After the sample is out of the pipet tip, use the same tip to pipet up the sample volume's worth of SOC from the cushion edge and dispense that SOC back into the cushion. (This effectively rinses out your pipet tip.)
5. Immerse the plating spreader (bent glass rod or equivalent) into ethanol and flame to sterilize. After the flame is extinguished, allow the spreader to cool ~10 seconds prior to placing the spreader on the plate. Place the spreader on the LB agar at the outside of the plate (not touching the pool of cells). This further cools the spreader on the LB agar before spreading the cells.
6. Slowly turn the plate while supporting the weight of the spreader.

Important: *Do not press down on the spreader – use just enough pressure to spread the cells.*

7. 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 spread until the sample and cushion have absorbed completely into the plate, as overspreading can decrease transformation efficiency. Instead, after spreading briefly, allow the plates to sit upright at room temperature for ~15 min prior to placing them in the 37ºC incubator. This will allow excess moisture to absorb into the plates before the plates are inverted and placed in the incubator.

References

1. Novy, R., Kolb, K., and Mierendorf, R. (1999) *inNovations* **9**, 6–8.
2. Brownstein, J.M., Carpten, J.D., and Smith, J.R. (1996) *BioTechniques* **20**, 1004–1010.
3. Magnuson, V.L., Ally, P.S., Nylund, S.J., Karanjuwala, Z.E., Rayman, J.B., Knapp, J.I., Lowe, A.L., Ghosh, S., and Collins, F.S. (1996) *BioTechniques* **21**, 700–709.

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