

pETcoco™ System

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About the Kits

pETcoco™-1 System	1 kit	71131-3
pETcoco-2 System	1 kit	71149-3
pETcoco-1 DNA	10 µg	71129-3
pETcoco-2 DNA	10 µg	71148-3

Description

The pETcoco™ System is uniquely designed with “on command” amplification of vector copy number from a single copy to 40 copies per cell. This unique system in combination with the powerful T7 promoter is ideal for maximum expression of genes potentially toxic to *E. coli*. The single-copy state of pETcoco maximizes clone stability and minimizes background protein expression levels to 1/40 of the levels obtained with pET vectors (1). Additionally, cloning DNA sequences in the single-copy state is advantageous for reducing rearrangements, deletions and other mutations as demonstrated with BAC (Bacterial Artificial Chromosome) plasmids (2, 3). On command amplification from a single copy to 40 copies per cell permits easy plasmid isolation. Protein expression from the tightly regulated T7lac promoter is IPTG inducible from the single or multiple copy state in a standard λDE3 lysogen host expressing T7 RNA polymerase. The pETcoco vectors are also compatible with pETDuet™, pACYCDuet™, and pET vectors for coexpression of up to three target proteins in the same cell (4). The novel dual replicon design of pETcoco is ideal for cloning and robust expression of genes potentially toxic to *E. coli* (1).

The pETcoco vectors combine the replication elements of a single-copy genomic cloning vector and a medium-copy plasmid, with the expression elements of pET vectors. The single-copy state is controlled by the *oriS* origin of replication, *repE* gene and *parABC* partition determinants and is maintained by propagating the pETcoco vector in Luria Broth (LB) plus 0.2% D-glucose (5). Amplification of copy number is achieved by inducing the expression of the TrfA replicator under the control of the *araC-P_{BAD}* promoter system with 0.01% L-arabinose. The TrfA replicator activates the medium-copy origin of replication (*oriV*) and plasmid accumulates up to 40 copies per cell. The pET vector derived elements for cloning and target protein expression include a T7lac promoter, T7 terminator and the *lacI* gene. Cloning and propagation of pETcoco is accomplished in the NovaF[–] host strain. After the pETcoco construct is established in NovaF[–], it is maintained in the single-copy state except for amplification during plasmid preparation. pETcoco recombinants are transferred to a λDE3 lysogen expression host strain [e.g. Tuner™(DE3)]. Target protein expression is induced with IPTG from the single-copy state or from the medium-copy state after arabinose induction. pETcoco vectors encode all elements necessary for the single-copy to medium-copy transition and therefore does not require a special host strain for amplification (1–5). A wide variety of general or specialized λDE3 expression hosts can be utilized for optimal target protein expression.

Nova F⁻ cloning host strain

NovaF⁻ is the recommended high efficiency cloning strain for the pETcoco™ System and is ideally suited for generating high yields of plasmid DNA. This strain is derived from the K-12, *recA endA* NovaBlue strain and lacks an F episome. A cloning strain without an F episome is necessary to prevent recombination between pETcoco F factor elements (*repE*, *parABC*) and the host F factor. Cloning directly into a λDE3 host strain is not recommended because of 1) lower transformation efficiency, 2) poor plasmid preparations from *recA* and *endA* positive host strains [e.g. Tuner™(DE3)], and 3) the possibility for residual expression of the target gene during amplification of pETcoco for plasmid isolation. Note that without the F episome, NovaF⁻ does not have the *lacZΔM15* and *lacI^q* genes. Therefore, blue/white screening is not possible. Additionally, the absence of the *lacI^q* gene renders NovaF⁻ unsuitable for propagation of expression vectors that carry an *E. coli* promoter controlled by a *lacO* operator sequence (e.g. *tac*, *trc*, T5, etc.)

Expression host strains

For protein production, a recombinant pETcoco plasmid is transferred to an *E. coli* host containing a chromosomal copy of the gene for T7 RNA polymerase. Expression strains should not carry an F episome. The following table lists appropriate host strains. For a complete description of bacterial strain characteristics, see User Protocol TB009.

	Compatible Host Strains	Incompatible Host Strains
pETcoco™-1	Group A,	Groups B, C, D
pETcoco-2	Groups A, B	Group C

Group A	Group B	Group C	Group D
B834(DE3)	Rosetta™(DE3)	Origami 2(DE3)	pLysS strains
BL21(DE3)	Rosetta2(DE3)	NovaBlue(DE3)	pLysE strains
BLR(DE3)	Rosetta-gami™B(DE3)	RosettaBlue™(DE3)	pLacI strains
HMS174(DE3)		Rosetta-gami™ 2(DE3)	Any Cam ^R strain
Origami™ B(DE3)		Any strain carrying an F factor	
Tuner(DE3)			

Coexpression

pETcoco™ vectors can be used with pETDuet™, pACYCDuet™, and pET constructs that have compatible origins of replication and antibiotic resistance for coexpression (as indicated in the tables below). The combination of a “plain” T7 promoter plasmid with a T7lac promoter plasmid for coexpression is not recommended. See User Protocol TB340 for pETDuet and pACYCDuet vector information and User Protocol TB055 for pET vector information.

Note: The pETcoco vectors are not compatible with any pCDF or pRSF vectors.

Plasmid(s)	Replicon (source)	Compatible Replicons (Plasmid)
pETcoco™ (all)	Mini-F/RK2 (pBeloBAC11, RK2)	ColE1(pET, pETDuet), P15A (pACYCDuet)

Vector and Host Strain Compatibility

Vector 1	Vector 2	Compatible Host Strains	Incompatible Host Strains
pETcoco-1 (Cam ^R)	pETDuet-1 (Amp ^R)	Groups A, B	Groups C, E
pETcoco-1 (Cam ^R)	pET (Amp ^R)	Groups A, B	Groups C, E
pETcoco-1 (Cam ^R)	pET (Kan ^R)	Group A	Groups E, F
pETcoco-2 (Amp ^R)	pACYCDuet-1 (Cam ^R)	Groups A, B	Groups C, E
pETcoco-2 (Amp ^R)	pET (Kan ^R)	Groups A, D	Group F, NovaBlue(DE3)

Amp; ampicillin/carbenicillin, Kan; kanamycin, Cam; chloramphenicol

Strain Groups

Group A	Group B	Group C
B834(DE3)	Origami™ B(DE3)	NovaBlue(DE3)
BL21(DE3)		Origami 2(DE3)
BLR(DE3)		
HMS174(DE3)		
Tuner™(DE3)		
Group D	Group E	Group F
B834(DE3)pLysS	All pLysS	Origami 2(DE3)
BL21(DE3)pLysS	All pLysE	Origami B(DE3)
BLR(DE3)pLysS	All pLacI	Rosetta-gami 2(DE3)
HMS174(DE3)pLysS	Rosetta(DE3)	Rosetta-gami B(DE3)
Rosetta™(DE3)	Rosetta 2(DE3)	
Rosetta(DE3)pLysS	RosettaBlue™(DE3)	
Tuner(DE3)pLysS	Rosetta-gami™ 2(DE3)	
	Rosetta-gami B(DE3)	

Procedures

The following protocols outline the proper medium and growth conditions for maintaining pETcoco™ vectors in the single-copy state, amplifying the copy number prior to plasmid isolation and inducing target gene expression with or without amplification of plasmid copy number. The dual replication origins present in the pETcoco vectors are different than those found in Novagen's standard pET vectors and therefore require a separate set of growth and induction protocols.

Cloning procedures, including vector and insert preparation and ligation reactions can be found in the pET System Manual (User Protocol TB055). Note that cycling conditions for colony PCR from the single-copy state may require 35–40 cycles. For initial recovery of pETcoco recombinants, vector + insert ligation reactions should be transformed into the high efficiency NovaF[–] host strain or other high efficiency cloning strain lacking an F episome. For maximum insert stability, recombinants should be maintained in the single-copy until amplification of plasmid copy number is needed for plasmid isolation. Verified plasmids are then transformed into an expression host lacking the F episome [e.g. Tuner™(DE3)] and maintained in the single-copy for maximum stability and minimal background expression. Target protein induction can proceed with or without plasmid amplification according to plasmid stability, target gene toxicity and yield requirements.

Transformation Protocol

The NovaF[–] Competent Cells in the pETcoco System kit are provided in the Singles™ format. Each tube of NovaF[–] Competent Cells can be used for one transformation. The Tuner(DE3) Competent Cells in the pETcoco System kit are provided in 0.2 ml aliquots. The standard transformation reaction requires 20 µl cells, so each 0.2 ml tube of Tuner(DE3) Competent Cells contains enough cells for 10 transformations.

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.
4. To avoid multiple freeze-thaw cycles of the standard 0.2 ml cells, dispense the cells into aliquots after the initial thaw and store them at –70°C or below (note that Singles cells are provided as 50 µl aliquots, which are used “as is” and do not require dispensing). 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

Tip: Prepare LB agar plates with appropriate antibiotics for pETcoco™ and host strains ahead of time.

Glucose could be added to the agar (0.2%) to keep the copy number at 1 per cell and maximize stability. This may be most important for expression hosts [e.g. Tuner™ (DE3)].

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. If the standard cells are to be used, place the required number of empty 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill. Allow the cells to thaw on ice for ~2–5 min.
Note: Sterilize a 20% (w/vol) D-glucose solution in H₂O by filtration through a 0.22 micron filter or by autoclaving. Store sterile solution at room temperature. Add glucose to LB agar with antibiotics.
2. Visually check the cells to see that they have thawed and gently flick the cells 1–2 times to evenly resuspend the cells.
3. If standard cells are being used, pipet 20 µl aliquots of cells into the pre-chilled tubes.
4. (Optional) To determine transformation efficiency, add 0.2 ng (1 µl) Test Plasmid provided with Competent Cells to one of the tubes containing cells. Stir gently to mix and return the tube to the ice.
5. Add 1 µl of a ligation reaction or purified plasmid DNA (1–10 ng/µl plasmid) 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.
6. Place 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. For standard cells, add 80 µl of room temperature SOC Medium to each tube. For Singles cells, add 250 µl of room temperature SOC Medium to each tube. Keep the tubes on ice until all have received SOC.
10. Shake at 37°C (250 rpm) for 60 min prior to plating on selective media
11. Spread 50 µl of each transformation on LB agar plates containing antibiotics to select pETcoco and appropriate antibiotics for the host strain. A range of 10–100 µl may be spread to obtain the ideal number of colonies per plate. pETcoco-1 requires 12.5 µg/ml chloramphenicol (Cat. No. 220551). pETcoco-2 requires 50 µg/ml carbenicillin (Cat. No. 69101-3) or ampicillin.

Note: Plate a large volume of the transformation mix to obtain sufficient colonies. The larger size of the pETcoco plasmid reduces the transformation efficiency.

When using the Test Plasmid, plate no more than 5 µl (e.g., 5 µl of NovaF[–] cells at 1×10^8 efficiency) or 10 µl (e.g., 10 µl of cells at 1×10^6 efficiency) 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 bla gene).

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

Maintaining the single-copy state

pETcoco recombinants should be maintained in the single-copy state at all times to ensure maximum stability except during culturing for plasmid DNA isolation and culturing for protein expression (if desired). To maintain the pETcoco plasmid in the single-copy state, LB medium should contain a final concentration of 0.2% D-glucose from a 20% sterile stock. Also include 12.5 µg/ml chloramphenicol to select for pETcoco-1, or 50 µg/ml carbenicillin or ampicillin to select for pETcoco-2 and any appropriate antibiotics for the host strain. The medium inoculated with isolated colonies from the plates as well as all medium for subsequent passages should contain glucose to maintain the lowest copy number.

Amplification of pETcoco™ copy number for plasmid isolation

Purification of recombinant pETcoco™ plasmid DNA requires amplification of the copy number to obtain good plasmid yield. Amplification requires arabinose induction of the TrfA protein activating replication at the medium-copy *oriV*.

1. Culture NovaF⁻ transformed with a pETcoco plasmid overnight in LB + 0.2% glucose + antibiotics at 37°C.
2. Remove an aliquot and dilute 1:50 into prewarmed fresh medium (LB + antibiotics) without glucose. Glucose should be omitted because it prevents amplification.
3. Grow the culture at 37°C with shaking (250 rpm) until the OD₆₀₀ = 0.2–0.4 and then add L-arabinose (Cat. No. 178680) from a 2% stock solution to a final concentration of 0.01%.
4. Grow the culture for an additional 4–5 hours at 37°C with shaking prior to harvest for plasmid purification. Cultures of pETcoco fully amplified with arabinose yield similar quantities of plasmid DNA as compared to traditional pET vectors.

Target protein induction with or without amplification of plasmid copy number

After isolation and verification of a pETcoco recombinant plasmid from the NovaF⁻ strain, transform the plasmid into an appropriate expression host strain (DE3 lysogen, see page 4) on LB plates containing antibiotics for pET-coco and the host strain.

Target protein induction without prior plasmid amplification

1. Inoculate a single colony from a freshly streaked plate or a few microliters from a glycerol stock into LB containing vector and host strain antibiotics and a final concentration of 0.2% glucose from a 20% stock to maintain the pETcoco plasmid in the single-copy state. The presence of glucose also minimizes cAMP-CAP stimulation of the *lacUV5* promoter controlling the T7 RNA Polymerase gene (6-9) as well as the *P*_{BAD} and *P*_{cat} promoters.
2. Grow the culture to an OD₆₀₀ between 0.4–1.0 and add IPTG to induce target protein expression. Final IPTG concentrations between 25 µM and 1 mM should be tested to determine the optimal induction parameters with any of the λDE3 host strains.
3. Continue to grow the culture for > 1 h (typically 2–3 h) to allow target protein accumulation. Long inductions (> 3 h) may generate optimal yields because: 1) the accumulation of large amounts of target protein from single-copy plasmid cultures takes longer and 2) induced single-copy plasmid cultures that continue to grow without signs of stress may reach higher cell densities and in some cases higher target protein yields (Dr. Szybalski, Dr. Sektas, personal communication).

Target protein induction with prior plasmid amplification

1. Inoculate a single colony from a freshly streaked plate or a few microliters from a glycerol stock into LB containing antibiotics for pETcoco and host strain selection and a final concentration of 0.01% arabinose from a 2% stock. Glucose is omitted to allow the multiple copy origin of replication to be activated via arabinose induction.
2. Grow the culture to an OD₆₀₀ between 0.4–1.0 and then add IPTG to induce target protein expression. Final IPTG concentrations between 25 µM to 1.0 mM should be tested to determine the optimal induction parameters for a given target protein
3. After IPTG addition, continue to grow the culture for > 1 h (typically 2–3 h) to allow target protein accumulation. Highest expression levels will most often be obtained in the medium-copy state with a 3 h induction

Induction Controls

The induction control strains are included to allow convenient testing of performance. The strain is provided as a glycerol stock with an insert containing β -galactosidase. The size of the Induction Control P and Q fusion protein is 117.8 kDa and it has an N-terminal His•Tag[®]. Induction Control P is chloramphenicol resistant, and Induction Control Q is ampicillin resistant.

Induction analysis, protein purification, detection and quantification

For recommendations regarding induction analysis and optimization, sample preparation, purification, detection and quantification, review the pET System Manual (User Protocol TB055) and the following User Protocols as appropriate. All User Protocols are available at www.merck4biosciences.com.

	Cat. No.	User Protocol No.
His•Tag[®] detection		
His•Tag Monoclonal Antibody	70796-4 (3 µg) 70796-3 (100 µg)	TB283
His•Tag AP Western Reagents	70972-3	TB283
His•Tag AP LumiBlot™ Reagents	70973-3	TB283
His•Tag HRP LumiBlot Reagents	70974-3	TB283
HSV•Tag[®] detection		
HSV•Tag Monoclonal Antibody	69171-3 (40 µg) 69171-4 (200 µg)	TB067
S•Tag™ detection/assay		
FRETWorks™ S•Tag Assay Kit	70724-3 (100 assays) 70724-4 (1000 assays)	TB251
S•Tag Rapid Assay Kit	69212-3	TB082
S•Tag AP Western Blot Kit	69213-3	TB082
S•Tag AP LumiBlot Kit	69099-3	TB164
S•Tag HRP LumiBlot Kit	69058-3	TB145
S-protein FITC Conjugate	69060-3	TB143
S-protein AP Conjugate	69598-3	TB097
S-protein HRP Conjugate	69047-3	TB136
Extraction reagents		
BugBuster [®] Protein Extraction Reagent	70584-3 70584-3	TB245
BugBuster HT Protein Extraction Reagent	70922-3 70922-4	TB245
BugBuster 10X Protein Extraction Reagent	70921-3 70921-4	TB245
BugBuster (primary amine-free) Extraction Reagent	70923-3 70923-4	TB245

	Cat. No.	User Protocol No.
Extraction reagents		
PopCulture® Reagent	71092-3	TB323
rLysozyme™ Solution	71110-3 71110-4 71104-5	TB334 and TB323
Benzonase® Nuclease, Purity > 90%	70746-3 70746-4	TB245, TB323, and TB261
Lysonase™ Bioprocessing Reagent	71230-3 71230-4 71230-4	TB361
His•Tag® purification		
Ni-NTA His•Bind® Resin	70666-3 70666-4	TB273
Ni-NTA Superflow™	70691-3 70691-4	TB273
Ni-NTA Buffer Kit	70899-3	TB273
His•Bind Resin	69670-3 69670-4	TB054
His•Bind Columns	70971-3 70971-4	TB054
His•Bind Buffer Kit	69755-3	TB054
His•Mag™ Agarose Beads	71002-3 71002-4	TB054
His•Bind Purification Kit	70239-3	TB054
BugBuster® Ni-NTA His•Bind Purification Kit	70751-3	TB273
BugBuster His•Bind Purification Kit	70793-3	TB054
PopCulture His•Mag Purification Kit	71114-3	TB054
RoboPop™ His•Mag Purification Kit	71103-3	TB327
RoboPop Ni-NTA His•Bind Purification Kit	71188-3	TB346
S•Tag™ purification		
S•Tag rEK Purification Kit	69065-3	TB160

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Bacterial Strain Non-distribution Agreement

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3. Commercial customers must obtain a research license agreement from Brookhaven Science Associates before purchasing and using DE3 lysogens of host strains Origami 2, Origami B, Rosetta 2, RosettaBlue, Rosetta-Gami 2, or Rosetta-Gami B.

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