His-tag Protein Expression and Purification

Materials and Supplies

- Champion *pET101* Directional TOPO Expression Kit (Invitrogen, *Catalog no. K101-01*)
- PfuUltra II HotStart DNA Polymerases (Stratagene, *Catalog no. 600670*)
- QIAquick Gel Extraction Kit (Qiagen, *Catalog no. 28704*)
- Agar Plate (Ampicillin)
- QIAprep Spin Miniprep Kit (Qiagen*, Catalog no. 27104*)
- PCR Master Mix (Promega, *Catalog no. M7502*)
- PCR tubes
- LB broth (Ampicillin)
- ProBond Purification System (Invitrogen, *Catalog no. K850-01*)
- Amicon Ultra Centrifugal Filter 10 kDa (Millipore, *Catalog no. UFC901008*)

Important Notes

- All work associated with bacterial culture should be conducted in a clean laminar flow. The working surfaces should be clean and wiped with 70% ethanol. Wear gloves and sterilize with 70% ethanol before working in the laminar flow, and wash hands before leaving lab.
- Gloves are essential when working with ethidium bromide. Try to minimize ethidium bromide contamination to surrounding work space and discard gloves when finished running the gel electrophoresis.
- Competent *E. coli* must be thawed and kept on ice.

Protocol

Gene Specific PCR Primer Design – To enable directional cloning, the forward PCR primer must contain the sequence, CACC, at the 5' end of the primer. The 4 nucleotides, CACC, base pair with the overhang sequence, GTGG, in the *pET101* vector. To design the reverse primer, omit the stop codon to clone the gene of interest in frame with the C-terminal His-tag.

High fidelity PCR Reaction – To amplify the gene of interest for insertion into *pET101* vector, select the appropriate initial parameters from the tables below.

Parameter	Targets: ≤10 kb (vector or genomic DNA)	Targets: >10 kb (vector or genomic DNA)	cDNA Targets
Extension time	15 seconds for targets ≤1 kb; 15 seconds per kb for targets >1 kb	30 seconds per kb	30 seconds for targets ≤1 kb; 30 seconds per kb for targets >1 kb
PfuUltra II fusion HS DNA polymerase	1 µl	1 µl	1 µl
Input template	100 ng genomic DNA; 5–30 ng vector DNA	200–250 ng genomic DNA; 5–30 ng vector DNA	1–2 µl cDNA from RT-PCR reaction (50–500 ng starting total RNA template)
Primers (each)	0.2 µM each primer	0.4 µM each primer	0.2 µM each primer
dNTP concentration	250 µM each dNTP (1 mM total)	500 µM each dNTP (2 mM total)	250 µM each dNTP (1 mM total)
Final reaction buffer conc	1.0×	1.0×	1.0×
Denaturing temperature	95°C	92°C	95°C
Extension temperature	72°C	68°C	72°C

Add appropriate concentration of each, and make up with $_{dd}H_2O$ to a final volume of 25µl. Put the reaction into a PCR machine and set it to run at the following parameters.

Segment	Number of cycles	Temperature	Duration (vector or genomic DNA)	Duration (cDNA)
1	1	95°C[b]	2 minutes	1 minute
2	30 cycles for vector or genomic DNA; 40 cycles for cDNA	95°C	20 seconds	20 seconds
		Primer T_m – 5°C[c]	20 seconds	20 seconds
		72°C	15 seconds for targets ≤1 kb 15 seconds per kb for targets >1 kb	30 seconds for targets ≤1 kb 30 seconds per kb for targets >1 kb
3	1	72°C	3 minutes	3 minutes

Post PCR Cleanup – After PCR is complete, run the reaction on an agarose gel electrophoresis and make sure a single distinct band of correct size is present. Cut the band out carefully using a surgical blade and put it into a 1.5ml micro-centrifuge tube. Refering now to QIAquick Gel Extraction Kit protocol, add 3x gel volume of solubilizing buffer QG and incubate in a 42°C water bath for 15 minutes to dissolve the agarose. After vortexing to homogenize the mixture, add the content into a QIAquick centrifuge column and centrifuge at maximum speed for 1 minute. Discard the flow through, add 750µl of wash buffer PE and again centrifuge at maximum speed for 1 minute. Discard to flow through, and centrifuge again at maximum speed for 1 minute to remove residual wash buffer. Place the column cartridge into a new 1.5ml microcentrifuge tube, and add 40µl of $_{dd}H_2O$. Incubate at room temperature for 5 minutes, and centrifuge at maximum speed for 1 minute. Label the tube, date and store at -20°C.

Setting up the TOPO Cloning Reaction – To clone the purified PCR product into the pET101 vector, add the reagents into a 1.5ml microcentrifuge tube as follows.

Reagents*	Chemically Competent *E. coli*
Fresh PCR product	0.5 to 4 µl
Salt Solution	1 µl
Dilute Salt Solution (1:4)	--
Sterile Water	add to a final volume of 5 µl
TOPO® vector	1 µl
Total Volume	**6 µl**

Mix the reaction gently and incubate for 15 minutes at room temperature.

Transformation of One Shot TOP10 Competent Cells – in a clean lamina flow, thaw a tube of competent cells on ice and add the TOPO cloning reaction. Gently tap the tube to mix, and incubate on ice for 15 minutes. Heat shock the tube in a 42°C water bath for 30 seconds and put immediately back on ice. Once the tube is cooled, add 250µl of S.O.C. medium and shake the tube horizontally (200 rpm) at 37°C for 1 hour. Spread the content evenly onto a usable ampicillin agar plate. Incubate upside down at 37°C for 16 hours.

Colony PCR – To select positive clones for sequencing, perform colony PCR using gene specific primers. Following protocols from Promega PCR Master Mix, add 0.6 µM of each forward and reverse primer to a final concentration of 1x PCR master mix in 85µl. Add 10 µl to each well of an 8-well strip, and place on ice. Using a clean and autoclaved pipette tip for each well, lift a bacterial colony and dip it into a well in the PCR tube. Either mark the colony or streak it onto a new ampicillin plate. Put the reactions into a PCR machine and run at the following parameters.

Step 1	95°C	10 minutes
Step 2	95°C	30 seconds
Step 3	55°C	30 seconds
Step 4	72°C	30 seconds/kb amplicon
Step 5	Back to Step 2	X 36 repeats
Step 6	72°C	10 minutes
Step 7	4°C	forever

Run the PCR products on an agarose gel electrophoresis. Look for distinct bands that are correct in size, those are the positive clones.

Isolate Recombinant Plasmid and Sequencing – Select 3 positive clones according to the colony PCR, and grow them in 3ml of LB with ampicillin at 37°C for 16 hours. Harvest the cells by centrifuge at 5000 x g for 6 minutes and remove the supernatant. There should be a white/yellow pellet at the bottom of the tube. Now refer to QIAprep Spin Miniprep Kit protocol, resuspend the cells using 250µl of chilled buffer P1 (With RNase added), and put the contents into a clean 1.5ml microcentrifuge tubes. Add 250 µl of buffer P2 and invert the tubes several times. Add buffer P3 and again mix the tubes by inverting it several times. There should now be white cloudy/viscous substance in the tubes. Centrifuge the tubes at maximum speed for 10 minutes. Pipette the clear supernatant into a new QIAprep centrifuge column, and centrifuge at maximum speed for 1 minute. Discard the flow through, add 750µl of wash buffer PE and again centrifuge at maximum speed for 1 minute. Discard the flow through, and centrifuge again at maximum speed for 1 minute to remove residual wash buffer. Place the column cartridge into a new 1.5ml microcentrifuge tube, and add 40µl of $_{dd}H_2O$. Incubate at room temperature for 5 minutes, and centrifuge at maximum speed for 1 minute. Label the tube, date and send it off for sequencing.

Transformation of BL21 Chemically Competent Cells – in a clean lamina flow, thaw a tube of competent cells on ice and add 4µl of purified plasmid. Gently tap the tube to mix, and incubate on ice for 15 minutes. Heat shock the tube in a 42°C water bath for 30 seconds and put immediately back on ice. Once the tube is cooled, add 250µl of S.O.C. medium and shake the tube horizontally (200 rpm) at 37°C for 1 hour. Spread the content evenly onto a usable ampicillin agar plate. Incubate upside down at 37°C for 16 hours. Perform the colony PCR as describe previously to identify positive clones.

Bacterial Culture, Induction for Recombinant Protein Expression and Purification of His-tagged Recombinant Protein – The pilot study for the expression of His-tagged recombinant proteins in the pathway of *P. trichocarpa* monolignol biosynthesis has been completed. The following section describes the specific protocol for each family of proteins.

PtrMYB156, PtrMYB167, PtrMYB 090, PtrMYB221

Recipes

(1) Lysis buffer: 6 M Guanidine Hydrochloride, 20 mM Sodium Phosphate, pH 7.8, 500 mM NaCl.
(2) Denature binding buffer: 8 M Urea, 20 mM Sodium Phosphate pH 7.8, 500 mM NaCl.
(3) Native washing buffer 1: 50 mM Tris, pH 7.5 with 20 mM imidazole.
(4) Native washing buffer 2: 50 mM Tris, pH 7.5 with 50 mM imidazole.
(5) Native washing buffer 3: 50 mM Tris, pH 7.5 with 75 mM imidazole.
(6) Elution buffer: 50 mM Tris, pH 7.5 with 250 mM imidazole.

Protocol

1. Grow 250 ml culture at 28 ^{o}C, OD600 to ~ 0.5, add IPTG to final concentration of 0.75 mM and further cultured for ~ 3 hours.
2. Harvest and cells by centrifuge 5000xg for 5 minutes
3. Add Lysis Buffer, rock the cells for 5–10 minutes at room temperature to ensure cell lysis.
4. Sonicate the cell lysate on ice with three 5 second pulses, 10 second rest for xxx minutes
5. Centrifuge the lysate at 3,000 × *g* for 15 minutes to pellet the cellular debris. Transfer the supernatant to a fresh tube.
6. Purify the protein based on the “Hybrid Conditions”. Column preparation follow vendor procedure.
7. 4 ml resin are used.
8. Add 8 ml lysate to a prepared Purification Column.
9. Bind for 15–30 minutes at room temperature using gentle agitation and keep the resin suspended in the lysate solution. Settle the resin by gravity or low speed centrifugation (800 × *g*) and carefully aspirate the supernatant.

10. Wash the column with 4 resin volume of Denaturing Binding Buffer by resuspending the resin and rocking for two minutes. Settle the resin by gravity.
11. Wash the column with 4 resin volume of Denaturing Wash Buffer (pH 6.0) by resuspending the resin and rocking for two minutes. Settle the resin by gravity.
12. Wash the column with 4 resin volume of Native Wash Buffer 1 by resuspending the resin and rocking for two minutes. Settle the resin by gravity.
13. Wash the column with 4 resin volume of Native Wash Buffer 2 by gravity flow.
14. Wash the column with 4 resin volume of Native Wash Buffer 3 by gravity flow.
15. Elution and collection: Elude the column with 2 resin volume of Native Wash Buffer 3 by gravity flow
16. Concentrate the protein to ~ 200 ul by Milliore Amicon Ultra Centrifugal Filter with 10 Kd cut off.
17. Quantity and precipitate the protein by addition of 5 volume of ice cold acetone to the protein eluded in 250 mM Imidazole and 50mM Tris pH 7.5, incubate at -20 ^{o}C for at least 2 hours, pelleted by centrifiguration at 14000xg and followed by 95% ethanol wash.

PtrCOMT2, PtrCCoAOMT1, PtrCCoAOMT2 and PtrCCoAOMT3 Recipes

(1) **Lysis buffer**: 50 mM Tris-HCL, pH 8.0; 500 mM NaCl; 20 mM imidazole, pH 8.0; 20 mM β-mercaptoethanol; 10% [v/v] glycerol; and 1% [v/v] Tween 20
(2) **Washing buffer**: 50 mM Tris-HCL, pH 8.0; 500 mM NaCl; 100 mM imidazole, pH 8.0; 20 mM β-mercaptoethanol; 10% [v/v] glycerol
(3) **Elution buffer**: 50 mM Tris-HCL, pH 8.0; 500 mM NaCl; 250 mM imidazole, pH 8.0; 20 mM β-mercaptoethanol; 10% [v/v] glycerol

Protocol

1. Grow 500 ml culture at 37 ^{o}C, OD600 to ~ 0.6, add IPTG to final concentration of 0.5 mM and culture at 25^{o}C for 6 hours.
2. Harvest and cells by centrifuge 5000xg for 5 minutes, remove supernatant.
3. Resuspend cell pellet in 10ml of lysis buffer.
4. Sonicate the cell lysate on ice with 6 second pulse, 15 seconds off for a total of 10 minutes.
5. Centrifuge the lysate at 10,000 × *g* for 20 minutes at 4^{o}C to pellet the cellular debris. Transfer the supernatant to a fresh tube.
6. Repeat step 5.
7. Aliquot 2 ml of ProBond Resin into a column at 4^{o}C, let it set by gravity.
8. Flow 20 ml of lysis buffer through the resin
9. Add the cleared lysate, let it flow through the resin.

10. Wash the column with 50 ml of lysis buffer.
11. Wash the column with 50 ml of wash buffer.
12. Elution and collection: Elude the column with 10 ml of elution buffer, collect in 1ml fractions.
13. Drop 20 µl of each fraction into 100 µl of Bradford buffer. Collect the fractions with proteins.
14. Concentrate the protein to ~ 200 ul by Milliore Amicon Ultra Centrifugal Filter with 10 kDa cut off by centrifugation at 4000 x g at 4 ^{o}C.
15. Aliquot and store the proteins at -80 ^{o}C.