PART A: RELEVANT INFORMATION & PREPARING THE TARGET GENE

1. The pET Vectors:

* Transcription vectors are designed for expression of target genes that already carry their own ribosome binding site (RBS) and ATG start codon
* Translation vectors contain the highly efficient RBS from T7 major capsid protein
  + The translation vector names are distinguished from the transcription vector names by the addition of letter suffix following the name, which denotes the reading frame relative to the *Bam*HI cloning site recognition sequence, GGATCC. Vectors with “a” express from GGA, “b” express from GAT, and “c” express from the ATC triplet of the *Bam*HI recognition sequence.

**Fusion Tags**

An *Nde*I or *Nco*I site is available for cloning into the AUG start codon at the 5’ end of the insert coding sequence. Proteins without vector-encoded C-terminal fusions are obtained by including a translation stop codon in the insert.

Expression of the desired C-terminal fusions requires (1) the lack of a stop codon in the insert, and (2) the proper reading frame at the cloning junction.

Except for the pET-5 series, all of the pET vectors contain translation stop codons in all three reading frames following the cloning and tag regions, as well as a downstream T7 terminator sequence. If the T7 terminator is removed during cloning, B-lactamase is observed along with target protein because of read-through transcription. (B-lactamase gene is downstream of the T7 terminator; B-lactamase is responsible for ampicillin resistance.)

The T7 terminator is approximately 70% effective which causes a small amount of B-lactamase to be produced in an induced culture.

**Antibiotic Resistance**

Selective markers amp and kan are available in the pET vectors. Ampicillin selection tends to be lost in cultures as the drug is degraded by B-lactamase enzyme and by the drop in pH that usually accompanies bacterial fermentation. This can be avoided by replacing the medium with fresh ampicillin containing media or using the related drug carbenicillin, which is less sensitive to low pH

In ampR pET vectors, the B-lactamase promoter is located downstream of and in the same orientation as the T7 promoter. In kanR vectors, the gene is in the opposite orientation from the T7 promoter—so there should not be an increase in kan gene product due to read-through transcription.

2. Hosts for Expression

To produce protein, the recombinant plasmid is transformed into *E. coli* strains containing a chromosomal copy of the gene for the T7 RNA polymerase. The hosts are lysogens of a lambda bacteriophage derivative containing a DNA fragment with the *lacI* gene, *lacUV5* promoter, and T7 RNA polymerase gene. Lambda DE3 lysogens of the strain BL21 are the most widely used host for target gene expression. Transcription of T7 RNA polymerase is directed by the *lacUV5* promoter which is inducible by IPTG. Induction of T7 RNA polymerase results in transcription of the target DNA in the plasmid.

3. Media Containing Glucose

When lambda DE3 hosts are grown to stationary phase in media lacking glucose, cAMP mediated derepression of both wild type and *lacUV5* promoters occurs. Although growing to stationary phase is not recommended, derepression can be avoided in 16h cultures by growing the hosts in media containing 1.0% glucose. This is useful in the case of toxic target genes where it is beneficial to minimize the basal transcription. Addition of glucose is neither necessary nor recommended during the cloning steps in non-expression hosts.

4. The T7*lac* Promoter

Plasmids with a T7*lac* promoter contain a *lac* *operator* sequence just downstream of the T7 promoter, with a *lacI* gene oriented somewhere else in the opposite direction so that the T7*lac* promoter and *lacI* promoters do not converge.

In this layout, the *lac* repressor binds to both the *lacUV5* promoter in the host chromosome, and the T7*lac* promoter in the vector.

4. Induction controls

An induction control strain that matches the type of promoter, selective marker, and other vector elements is included with each pET vector and expression system. The strain is provided as a glycerol stock of an appropriate lambda DE3 lysogen containing a pET plasmid with an insert encoding B-galactosidase, which can be assayed spectrophotometrically.

5. pET System Process p.15 (flow chart)

6. Suitable Growth Media p.16

7. Vector Preparation

Use the restriction enzyme manufacturers recommended buffer and incubation conditions for the enzymes you are using. Various enzyme combinations are compatible when used together in the same buffer however there are caveats.

* Different restriction enzymes have different cutting efficiencies especially when two sites are close together. Enzymes with compatible buffers and whose sites are more than 10bp apart can be used together in the same reaction. In all other cases the digestions should be performed sequentially. Use the poorest cutter first and verify the digestion with an agarose gel before performing the second digestion.
* Some restriction enzymes may exhibit less stringent sequence dependence which alters specificity. This is called “star activity”. Conditions that can lead to star activity include high glycerol concentration >5%, high pH, and low ionic strength
* For cloning into a single site, dephosphorylate the vector following digestion to decrease self-ligation. This reduces the non-recombinant background. Calf intestinal phosphatase or shrimp alkaline phosphatase should be used according to manufacturer instructions.
* The same applies for vectors cut with two enzymes, especially when the sites are close together
* Following digestion, it is usually worthwhile to gel-purify the vector prior to insert ligation to remove residual nicked and supercoiled plasmid which transform efficiently relative to the desired ligation product. This step is optional but usually reduces the effort required to screen for the correct construct.
* Instructions for digesting and gel-purifying vector p.18

8. Insert Preparation

For inserts, preparation is straightforward. Use desired restriction enzymes to digest the insert and follow this with a gel purification. PCR can be used isolate genes of interest and modify them for functional insertion. One risk of PCR is the potential to introduce mutations. Here are some options to reduce the mutation rate.

* Use an enzyme with proofreading activity
* Limit number of PCR cycles
* Increase concentration of target DNA
* Increase primer concentration

9. Ligation protocol offered p.20

10. Non-expression host transformation

Novagen recommends that initial cloning is done in a *recA-* strain that lacks the T7 RNA polymerase. This ensures high percentage monomer plasmid yields for examination of the construct sequence, as well as separation of cloning from expression. This can be valuable for troubleshooting difficulties during later procedures.

Transformation of such a strain follows standard procedure. Protocols are provided. p.21

11. Analysis of pET recombinants

There are usually more colonies produced from the plasmid containing the insert than the negative control. Sometimes cloning can be successful even though the number of colonies on the two plates are roughly equivalent. Transformants can be analyzed by colony PCR, plasmid miniprep, restriction analysis, sequencing, and *in vitro* transcription and translation assays.

Transcription/Translation analysis STP3 (protocol) p.23,24; Direct colony PCR p.24; Plasmid miniprep p.25; Sequencing p.26

PART B: EXPRESSING THE TARGET GENE & PURIFYING PROTEIN

1. Expression host transformation

Use 1µl of a 50-fold dilution (approx. 1ng) of plasmid in sterile water or TE buffer and follow the transformation procedure on p.20. Streak transformants for single colonies and prepare glycerol stocks as described p.17

2. Inducing expression

Expression of the target DNA is induced by adding IPTG to a growing culture. For pET constructs with the standard T7 promoter, a final concentration of 0.4mM IPTG is recommended. For T7*lac* promoters (what we have) use a final concentration of 1mMM IPTG.

Induction protocol p.27

3. Optimizing expression

Before induction, it is recommended to test the culture to determine the fraction of cells that carry the target plasmid (plasmid stability test). If appropriate attention is paid, more than 98% of the cells in the culture will usually contain expressible target plasmid.

Solubility of the protein is important as each is unique. Temperature is a key factor in determining how the protein will be have upon expression. Lysis buffer also plays a role in determining the partitioning of the protein into the soluble or insoluble fraction. See p.28 for various explanations and examples. In addition, target proteins with highly charged domains may also associate with other cellular components. In theory, they may be dissociated by adding salt to the lysis buffer.

Inclusion bodies can be taken advantage of in purification. They are easily isolated by centrifugation, and inclusion body formation protects the protein from proteolytic attack. There are also kits available and protocols which can be optimized for refolding protein.

When inclusion bodies are formed, some portion of the target protein is usually soluble within the cell. There may be a significant amount of soluble protein even then most of the target protein aggregates. Soluble protein is not necessarily folded properly. If soluble protein fraction is folded properly, the induction can be scaled up and affinity chromatography can be used to purify and concentrate the soluble fraction.

4. Difficult target proteins

Refer to the novagen manual on this section. It provides various suboptimal situations and troubleshooting/solutions to the proposed situations including secondary structure in the mRNA, excessive rare codon usage, unexpected stop codons and more. p.31-32

Many proteins are made in equivalent amounts whether the T7 transcription terminator is present in the vector or not. In some cases, having the terminator behind the target gene increases production of the target protein. This is observed when the target gene carries its own translation initiation signals.

5. Detecting and quantifying target proteins

Protein expression can be tested with SDS-PAGE followed by staining with Coomassie blue which can reveal the target protein as a unique band when run adjacent to an uninduced extract. Western blotting is also used as a more sensitive and specific method for identification and estimation of purification levels.

6. Purifying target protein

There are a lot of variables that dictate the method of protein purification to use. Generally, conditions that decrease the rate of protein synthesis, such as low induction temperatures or growth in minimal media, tend to increase the percentage of target protein found in soluble form.

A variety of affinity purification methods take advantage of peptide fusion tags listed on p.15

Prior to purification or activity measurements, preliminary analysis of expression levels, cellular localization, and solubility of the target protein should be performed.

Small scale analysis protocols p.34-40

7. Large scale induction and fractionation

After the various parameters of growth, induction, and localization are established on a small scale, these can be applied to larger scale cultures for production of target proteins.

Large scale protocols p. 40-43

8. Solubilization of inclusion bodies and refolding proteins

There are a variety of methods available to deal with this. Most protocols isolate insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Optimal refolding conditions must be empirically determined. Optimal folding conditions can be rapidly determined on a small scale by a matrix approach in which variables such as protein concentration, reducing agent, redox treatment, divalent cations, etc. are tested.

Novagen has a Protein refolding kit available.

Proteins solubilized from washed inclusion bodies may be >90% homogenous and may not require further purification. Purification under full denaturing conditions is possible using His-Tag fusions and metal chelation chromatography. Refolded fusion proteins can be affinity purified under native conditions using His-tag and others.

9. Induction Control: B-galactosidase Recombinant

All pET systems include an induction control which is usually a glycerol stock of an appropriate pET vector containing the *E. coli* B-galactosidase gene as an insert. Useful control assays with the induction control are on p.45.