

# Chapter 9

## High Throughput Cloning with Restriction Enzymes

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The systematic structural analysis of many target proteins involves generating expression clones in high throughput. This requires robust laboratory procedures and benefits from laboratory automation and data management systems. This chapter gives an overview of the Protein Structure Factory, a structural genomics project focusing on human proteins, and presents the authors' method for cloning bacterial expression clones with the restriction enzymes BamHI and NotI and compatible enzymes. PCR amplification, product purification and digestion and vector ligation were adapted to the 96-well microtiter plate format.

### 1. Introduction

The Protein Structure Factory (PSF) is a structural genomics project focusing on human proteins (1,2). To increase the throughput, standardized and automated procedures have been introduced to establish a pipeline from target selection to structure determination.

Targets were mainly selected according to biophysical criteria and availability of cDNA clones. Proteins were expressed as His-tag and StrepII-tag (3) fusion proteins in *Escherichia coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris*. Procedures adapted to the 96-well microplate format were established for cloning and characterization of expression clones by small-scale expression and purification (4,5).

Protein production for crystallization is performed by a standardized procedure of affinity chromatography and His-tag removal by TEV protease digest, followed by ion exchange chromatography and gel filtration (6). Purified proteins are concentrated, to avoid aggregation, in a stepwise manner. The particle size is controlled by dynamic light scattering at each concentration step.

Protein crystallization at the PSF is highly automated (7). Crystallization screens are set up by nanoliter pipetting robots. The screening buffers are automatically prepared from stocks in deep well microplates by a pipetting robot. This robot allows setting up standard screens as well as fine screens for optimization of crystallization conditions. X-ray diffraction experiments are performed at the beamlines established by the PSF at the BESSY synchrotron storage ring.

The PSF pipeline consists of the following steps:

1. Target selection and design of expression constructs
2. High throughput cloning and clone sequence verification
3. Characterization of expression clones by small scale expression and purification
4. Automated protein crystallization
5. X-ray crystallography

Standard cloning methods based on restriction enzymes and DNA ligase can be adapted to the microplate format to achieve a moderately high throughput. Ligation-based cloning (LIC) using the T4 DNA polymerase to generate overhangs (8) or recombination-based methods such as the Invitrogen Gateway system are popular alternatives to the traditional approach (9,10).

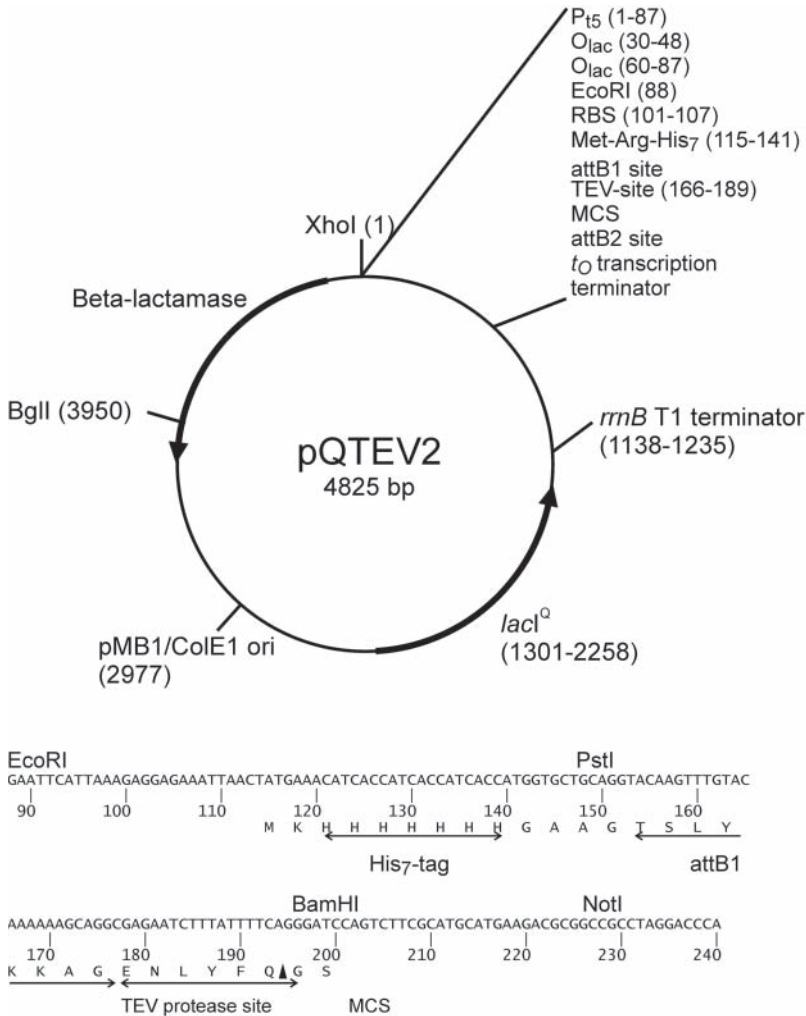
A large number of Gateway Entry clones available from academic and commercial sources allow to produce expression clones by a simple recombination reaction that avoids PCR amplification (11). All other cloning approaches require a PCR step with primers that have tails of functional sequences, such as restriction or recombination sites. Restriction enzyme recognition sites are generally shorter than the sequences required for LIC and recombination methods. Therefore, restriction enzyme-based cloning allows for shorter PCR primers that are less expensive and less likely to contain mutated byproducts. A six-base pair recognition site only introduces two additional amino acids. This is advantageous when designing N-terminal fusion proteins. On the other hand, restriction enzyme-based cloning of PCR products involves more steps, including DNA purification, which makes it more difficult to set up in a robust way with high throughput. Moreover, restriction sites can occur within the target sequences to be cloned, demanding to choose alternative enzymes for these targets.

At the PSF, expression clones were generated with the restriction enzymes BamHI and NotI and other enzymes that generate compatible overhangs. The vectors used for cloning are pQTEV (1), pQTEV2 (Fig. 9.1), pQLinkH, pQLinkG, pGEX-6P1 and pEntryTEV (Table 9.1). All these are *E. coli* expression vectors except for pEntryTEV, which is used to generate Entry clones with a TEV site for the Gateway recombination cloning system (Invitrogen). pQLinkH and pQLinkG plasmids can be easily combined into co-expression plasmids (19).

For templates containing internal BamHI or NotI sites, compatible overhangs are produced by alternative enzymes or by the hetero-stagger cloning method (12). Alternative enzymes are BglII for BamHI and the type II enzymes BpiI, Eco31I, and Esp3I, which can replace both BamHI and NotI. Type II enzymes cut outside their recognition sequence and can produce arbitrary overhangs.

The *E. coli* strain SCS1 carrying the helper plasmid pRARE was used for cloning of expression clones and protein expression experiments. pRARE is a plasmid of the Novagen Rosetta strain; it carries genes for overexpression of rare tRNAs and a chloramphenicol resistance marker (13).

To control the correctness of new clones, their insert size was verified. Clones produced by PCR may contain mutations even if their insert size is correct. These mutations are mostly caused by byproducts of PCR primer synthesis in the authors' experience. It is advisable to test several transformants for protein expression in case some contain a mutation. Alternatively, clones can be verified by sequencing. This chapter describes how the Staden sequence analysis package can be used for this purpose.



**Fig. 9.1** Map and sequence of the multiple cloning site (MCS) of the pQTEV2 vector.

The generation of many clones in parallel requires electronic data management of some sort. A list of the constructs to be produced containing identifiers of the target proteins, the template cDNA clones, the start and end position of the construct with respect to the template cDNA clone sequence, and PCR primer identifiers should be available (see [Note 1](#)).

The aim of the high throughput cloning method described here is to adapt common laboratory procedures to allow processing of many cloning experiments in parallel. It uses a common restriction-ligation design. No special instrumentation is needed except for a 96-pin inoculation gadget. However, a pipetting robot may facilitate preparation of primer plates, purification of PCR products and reformatting of samples in microtiter plates (see [Note 2](#)). The procedure consists of the following steps:

**Table 9.1** Vectors for cloning with BamHI and NotI.

Vector	N-terminal affinity tag, protease site	C-terminal affinity tag	Gateway att sites	Accession No.	Comment
pQTEV	His <sub>7</sub> , TEV	—	—	AY243506	See ref. (1)
pQTEV2, pQLinkH	His <sub>7</sub> , TEV	—	attB1, attB2	EF025688	Insert can be shuttled into a Gateway Donor vector (Fig. 9.1).
pQLinkG	GST, TEV	—	attB1, attB2	EF025689	
pGEX-6P1	GST, PreScission	—	—		
pEntryTEV	none, TEV	—	attL1, attL2		Gateway Entry clones

1. Preparation of template clone plates and diluted primer plates. Preparation of restriction digested and dephosphorylated vector (1–2 days).
2. PCR and evaluation of PCR products by gel electrophoresis. If necessary, rearranging of successful reactions (1–3 days).
3. Purification of PCR products and quantification with gel electrophoresis, set up of ligations (2 days).
4. Transformation and plating: 96 to 192 transformations can be processed in parallel (1 day).
5. Picking of transformed bacteria clones (0.5 day; needs overnight incubation).
6. Verification of clones by colony PCR (1–3 days).
7. Picking of correct clones for further analysis (0.5 day, needs overnight incubation).

## 2. Materials

### 2.1. Preparation of Competent Cells

1. SCS1 *E. coli* cells (Stratagene) carrying the pRARE plasmid (see Introduction).
2. DMSO.
3. SOB broth (14): Dissolve 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl and 0.186 g KCl in 1 liter demineralised water and autoclave. Add 5 ml of sterile 2 M MgCl<sub>2</sub> just before use.
4. Inoue Transformation Buffer (15): 10 mM PIPES, 55 mM MnCl<sub>2</sub>, 15 mM CaCl<sub>2</sub>, 250 mM KCl. Mix all components except for the MnCl<sub>2</sub>. Adjust the pH with KOH and add the MnCl<sub>2</sub>. Sterilize by filtration.

### 2.2. Preparation of Inserts

1. PCR primers to amplify the open reading frames of interest (see Note 3).
2. Polystyrene microtiter plates with lids for bacterial cultures and glycerol stocks (Nunc, No. 260895).

3. Expand High Fidelity PCR kit (Roche Applied Science).
4. 10mM of each dATP, dCTP, dGTP, dTTP in TE buffer (Fermentas, R0151).
5. Thermowell 96 well plates (Costar).
6. 96-pinned replicators. Plastic and steel replicators are available from Genetix or Nunc.
7. Adhesive sealing sheets for microtiter plates (Abgene, Epsom, UK).
8. 96-well PCR product cleaning kit. We use an in-house magnetic beads procedure that is commercially available from Bruker (Genopure ds kit).
9. Restriction enzymes with buffers (NEB).

### 2.3. Preparation of the Vector

1. Chromaspin 1,000 size exclusion columns (Clontech, Mountain View, CA) for DNA purification.
2. Shrimp alkaline phosphatase (SAP, 1 unit/ $\mu$ l, Roche Applied Science, Indianapolis, IN).
3. NEBuffer BamHI: 10mM Tris-HCl, 150mM NaCl, 10mM MgCl<sub>2</sub>, 1mM Dithiothreitol, pH 7.9 at 25°C.
4. Dephosphorylation buffer: 0.5 M Tris-HCl, 50mM MgCl<sub>2</sub>, pH 8.5 at 20°C.

### 2.4. Ligation and Transformation

1. T4 DNA ligase with buffer (NEB).
2. 2×YT broth: Add 16 g bacto-tryptone, 10 g bacto-yeast extract and 5 g NaCl per liter demineralised water, autoclave.
3. 2×YT agar: Add 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl and 15 g agar per liter, autoclave. Add supplements such as antibiotics and glucose after cooling to 50°C.
4. QTray with divider (Genetix, New Milton, UK) to prepare agar plates with 48 wells.
5. 10×HMFM: Dissolve 72 g KH<sub>2</sub>PO<sub>4</sub> and 188 g K<sub>2</sub>HPO<sub>4</sub> (Mw 174) in 800 ml water and autoclave. Dissolve 3.6 g MgSO<sub>4</sub>×7H<sub>2</sub>O, 18 g Na<sub>3</sub>-citrate×2H<sub>2</sub>O, 36 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 1,760 g glycerol (1,645 ml of 87% glycerol) in 3.2 L water, autoclave and add the 800 ml phosphate solution.
6. 40% (w/v) glucose: dissolve 400 g D+glucose monohydrate in distilled water to 1 liter and sterilize by filtration through a 0.2- $\mu$ m pore size filter.
7. Polypropylene deep well plates (96 wells of 2.2 ml, non-sterile, Wertheim, Germany).

### 2.5. Colony PCR

1. Taq DNA polymerase, prepared according to Engelke et al. (16).
2. 10× PCR buffer: 0.5 M KCl, 1% Tween 20, 15 mM MgCl<sub>2</sub>, 350 mM Tris-base, 150 mM Tris×HCl.
3. 5 M betaine.

### 2.6. Clone Sequence Verification

1. The Staden package (<http://staden.sourceforge.net>).

### 3. Methods

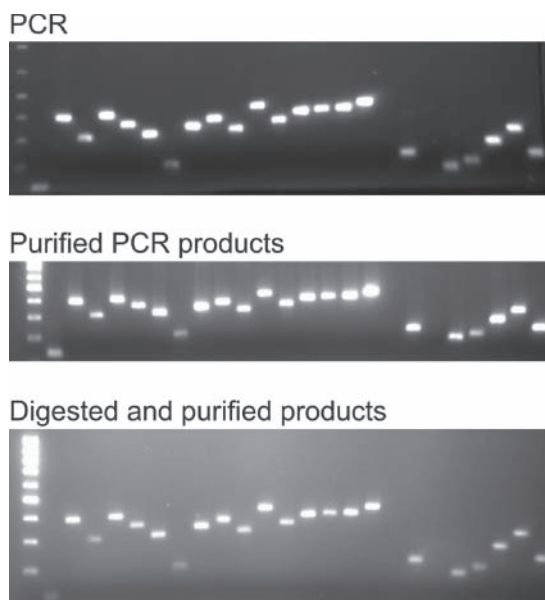
#### 3.1. Preparation of Competent Cells

The preparation of competent cells follows the method of Inoue et al. (15) with slight modifications.

1. Grow a 5-ml starter culture of *E. coli* SCS1/pRARE overnight at 37°C in SOB broth supplemented with 34 mg/l chloramphenicol.
2. Inoculate 250 ml of the same broth with 2 ml overnight culture and grow to  $OD_{600} = 0.6$  at 18°C.
3. Cool on ice for 10 min.
4. Transfer to two sterile, prechilled 250-ml polypropylene centrifuge bottles and spin down at 2,500 g for 10 min.
5. Remove the supernatant and gently resuspend the pellet with 40 ml ice-cold Inoue Transformation Buffer.
6. Pool both suspensions and place them on ice for 10 min. Centrifuge again as above and resuspend the cells in 20 ml Inoue Transformation Buffer, incubate for 10 min on ice and add 1.4 ml DMSO.
7. Dispense the cell suspension into 900- $\mu$ l aliquots, freeze them in liquid nitrogen and store at -80°C.
8. The quality of the cells is tested by transformation with 50 pg pUC19 DNA. The expected yield is around  $10^7$  colonies/ $\mu$ g DNA.

#### 3.2. Preparation of Inserts

1. Arrange diluted primers (10  $\mu$ M, see [Note 3](#)) and glycerol stocks of template cDNA clones in three different polystyrene microtiter plates, such that templates and their corresponding primers are located on corresponding positions. If different pairs of restriction enzymes are necessary for different targets, try to place targets with the same enzymes together.
2. Prepare master mixes of polymerase, buffer and dNTPs for 100  $\mu$ l. Expand High Fidelity PCRs on ice according to the manufacturer's instructions. Dispense 100  $\mu$ l per well of a Thermowell 96 well plate on ice. Dispense 1  $\mu$ l of each primer. "Inoculate" the reactions with the template bacteria from cultures in a 96-well microtiter plate using a sterile 96-pinned replicator. Cover the Thermowell plate with an adhesive sealing sheet and run the following PCR program: 5 min at 96°C followed by 25 cycles of 1 min at 94°C, 0.5 min 55°C, and 1 min per 1,000 bp of maximum expected product size at 72°C followed by a final extension step of 15 min at 72°C.
3. Check a 5- $\mu$ l aliquot of each reaction on an agarose gel. If all or most reactions are successful, proceed. Otherwise, consider repeating failed reactions using different parameters ([Fig. 9.2](#)).
4. Purify PCR products using a suitable 96-well PCR purification system (see [Fig. 9.2](#)).
5. Set up restriction digests with 30  $\mu$ l of the purified DNA in a new Thermowell plate for 3 hours using 1 unit of each enzyme in 50  $\mu$ l total volume according to the manufacturer's instructions. Purify again as above.
6. Separate 5  $\mu$ l on a gel and estimate the concentration by comparison with control samples of known concentrations. Molar concentrations are obtained by automatically determination of product amounts with a gel image



**Fig. 9.2** PCR amplification, product purification, and digestion. The *upper panel* shows the results of PCR amplification of a number of targets on an agarose gel stained with ethidium bromide. The *middle panel* shows the same PCR products upon cleaning with magnetic beads. Note that the product amounts are more uniform after cleaning due to the limited amount of beads used for cleaning. The *lower panel* shows the PCR products after restriction digest and a second purification.

evaluation software and multiplication of DNA amounts with molecular weights stored in a database. We have used the software Phoretix 1D (now totallab, Nonlinear Dynamics, Inc., Newcastle upon Tyne, UK) and our in-house database for this purpose (see [Note 1](#)).

### 3.3. Preparation of the Vector

1. Linearize 5  $\mu\text{g}$  vector DNA by digestion with 3  $\mu\text{l}$  of BamHI (20 units/ $\mu\text{l}$ ) and 3  $\mu\text{l}$  NotI (10 units/ $\mu\text{l}$ ) in 100  $\mu\text{l}$  of NEBuffer BamHI containing 100  $\mu\text{g}/\text{ml}$  bovine serum albumin (BSA) overnight at 37°C. Add one more microliter of each enzyme, mix gently and incubate for another 3 hours. Inactivate enzymes by heating to 65°C for 20 min. Purify the linearized vector with a Chromaspin 1,000 column.
2. Dephosphorylate 44  $\mu\text{l}$  of the linearized vector DNA by adding 1  $\mu\text{l}$  shrimp alkaline phosphatase and 5  $\mu\text{l}$  10 $\times$  dephosphorylation buffer at 37°C for 1 hour. Inactivate the phosphatase by heating to 72°C for 20 min. Purify the linearized and dephosphorylated DNA with another Chromaspin 1,000 column.

### 3.4. Ligation and Transformation

1. Set up ligations according to PCR product concentration. Usually it is sufficient to distinguish low and high product concentrations and to use 6.5 or 2  $\mu\text{l}$  product, respectively, for the ligation. In the wells of a Thermowell plate, add PCR product and water to 6.5  $\mu\text{l}$  and a mix of 2  $\mu\text{l}$  (5 ng) linearized

- vector DNA, 0.5 µl T4 ligase (400 units/µl), 1 µl 10× ligase buffer. Cover the Thermowell plate with a sealing sheet and incubate the samples for 1 hour at 20°C in the PCR cycler. Heat inactivate the samples for 20 min at 65°C.
2. Thaw chemical competent *E. coli* SCS1/pRARE cells on ice. Meanwhile, transfer 5 µl of each ligation reaction to a well of a new prechilled Thermowell plate on ice. Add 100 µl bacteria to each well and incubate on ice for 5 min. Heat shock the cells at 42°C for 30 sec using a PCR cycler. Immediately put the plate back on the ice and incubate for 1–5 min. Recover cells with 500 µl 2×YT broth supplemented with 20 mM MgCl<sub>2</sub> and 20 mM glucose for 45 min at 37°C in a polypropylene deep well plate. Spin down cells at 2,000 g for 10 min at 4°C. Discard 300 µl of the supernatant, resuspend the remainder, and plate on agar.
  3. Agar plates are prepared in large QTray plastic dishes with 48 wells. The dishes are filled with 300 ml 2×YT agar supplemented with 100 mg/l ampicillin, 34 mg/l chloramphenicol and 2% glucose. 200 µl transformed bacteria are pipetted into each well. The liquid is spread by gently moving the dishes and dried in a sterile hood without the lid, followed by overnight incubation of the closed dishes at 37°C.
  4. Fill a polystyrene microtiter plate with 200 µl of 2×YT broth containing 1×HMF<sub>2</sub>M, antibiotics and 2% glucose. Pick six colonies of each agar dish well with sterile tooth picks into individual wells of the microtiter plate. Wrap the plate in plastic foil and incubate at 37°C until all wells are grown evenly (approx. 16 h).

### 3.5. Colony PCR

To check the presence of inserts of the new clones, a colony PCR is performed. Enhancers such as betaine should be added to the PCR to improve the amplification of difficult templates (17).

1. Prepare a PCR master mix for 30 µl reactions of 80 units/ml Taq polymerase, dNTPs (65.5 µM each), 1.25 M betaine, and vector primers pQE65 and pQE276 (0.3 µM of each) in PCR buffer. Dispense 30-µl volumes in a Thermowell 96-well plate. ‘Inoculate’ the reactions with the cultures of the picked transformants with a sterile 96-pinned replicator. Cover the Thermowell plate with sealing sheet and run the following PCR program: 5 min at 95°C followed by 30 cycles of 0.5 min at 95°C, 0.5 min at 60°C, and 1 min per 1,000 bp of maximum expected product size at 72°C followed by a final extension step of 15 min at 72°C.
2. Check an aliquot of each reaction on an agarose gel. Choose two clones per transformation that had PCR products of the expected size for further protein expression and purification. Again, a gel analysis software in combination with a database is useful, as it allows determination of product sizes and comparison with expected sizes stored in the database.
3. Inoculate two wells of another polystyrene microtiter plate with 2×YT broth and supplements with the selected clones using sterile tooth picks. A pipetting robot is helpful in this step to avoid the error prone reformatting by hand. Incubate the wrapped plate at 37°C as before and store at –80°C until testing the clones by small scale expression and purification (4,5).

### 3.6. Clone Sequence Verification

Mutation may be introduced by PCR during cloning. In the authors' experience, most errors are found in the sequences of the PCR primers and are probably caused by primer synthesis byproducts. The Staden package (18) is a useful tool to compare DNA sequencing results with the expected sequence. It is available for Unix/Linux, Windows and Mac computers. For each cloning experiment, a database has to be created with the *gap4* program of the package, according to the following procedure, that can be automated by a suitable script.

1. Create an empty database named "gap4" with the *gap4* program.
2. Use *pregap4* to create two configuration files. Choose the modules "Estimate Base Accuracies," "Initialize Experiment Files," and "Quality Clip" and deselect all other modules. Choose "Save all Parameters To": and save as *pregap4-init.conf*. Then select only the module "Gap4 Shotgun Assembly," set the database name to *gap4*, and save as *pregap4-assembly.conf*.
3. For each cloning experiment, copy the empty *gap4* database files, the expected clone sequence in Staden text format, and the chromatogram files with the sequencing results into a new directory. The files should have the suffixes *.sdn* and *.abi*, respectively.
4. In the same directory, run *pregap4* with the following parameters to create experiment files (*.exp*) for each chromatogram:

```
pregap4 -config [path]/pregap4-init.conf -nowin
- *.abi
```

5. To include all experiment files and the expected sequence into the *gap4* database, run *pregap4* again with the other configuration file:

```
pregap4 -config [path]/pregap4-assembly.conf -
nowin - *.exp *.sdn
```

6. The steps 3.–5. can be combined into a script to facilitate the analysis of many clones. The *gap4* database are examined manually with *gap4* to verify agreement of sequencing results with the expected sequence.

## 4. Notes

1. Simultaneously handling larger amounts of samples requires an appropriate bookkeeping method. To record high-throughput cloning experiments, we implemented a sample management system, which is embedded into the authors' projectwide database structure. A brief description of this system can be found here:

<http://www.proteinstrukturfabrik.de/tp03page/lims.shtml>

The system contains a batch clone management module that allows creation, handling, and evaluation of large numbers of HTP cloning experiments simultaneously. For each cloning step, all necessary objects (PCR reactions to generate templates, microtiter plates for storage, agarose gels for evaluation, and new clones with respective data) are created automatically. Different kinds of experimental observations such as PCR results (product amount and size, success or failure of cleaning steps) can be entered interactively and are automatically assigned to the objects to which they belong. Results are always

- judged by the user, but the system supplies several kinds of support: Prediction of DNA and protein sequences of affinity tag fusion constructs and calculated fragment sizes.
- Most steps of the cloning procedure are performed with a multichannel pipet but there are some reformatting and “cherry picking” steps which benefit from a pipetting robot. The authors’ computer-based storage and sample management system (see [Note 1](#)) can create robot command files. A robot is especially useful to prepare the primer and template plates and to reformat successful candidate clones at the end of the procedure. The authors also use the robot to assemble restriction digests with different enzymes.
  - The primers should have a common annealing temperature of about 63°C. The authors’ use the program ORFprimer to design primers. The program adjust the lengths of the primers to obtain similar annealing temperatures. Forward primers should have a BamHI site (GGATCC) tail encoding glycine, serine in frame with the amplified gene (see [Fig. 9.1](#)). Reverse primers should have a NotI site (GCGGCCGC) tail adjacent to the stop codon. For inserts including BamHI or NotI sites, choose alternative enzymes (see Introduction). For efficient cleavage, two nucleotides should be added to the 5' end before the BamHI site and four nucleotides before the NotI site. Here is an example:

Forward primer

5'-CAGGATCCGCTTGTGCTGAGTTTTCTTTTCATG -3'

G S A C A E F S F H

Reverse primer

5'-GACTGCGGCCGCTCAATCTCGCCAATTGAATGCG-3'

Stop

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