Chapter 2

Basic DNA Electrophoresis in Molecular Cloning: A Comprehensive Guide for Beginners

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Abstract

Presented here is a complete molecular cloning protocol consisting of a number of separate but interconnected methods such as preparation of *E. coli* competent cells; in vitro DNA digestion and ligation; PCR; DNA agarose gel electrophoresis and gel extraction; and screening transformants by colony PCR, analytical restriction digests, and sequencing. The method is described in a lot of details so that it can be easily followed by those with very little relevant knowledge and skills. It also contains many tips that even experienced researchers may find useful.

Key words Cloning, Agarose gel electrophoresis, DNA gel extraction, Competent cells, Clone screening, Restriction enzymes, Ligation

1 Introduction

A protocol for molecular cloning involves several basic molecular biology techniques such as in vitro enzymatic reactions, DNA agarose gel electrophoresis, bacterial transformations, and PCR which form a core set of skills for a qualified researcher. Gaining experience in any or all of these techniques is very common among early career scientists. The introduction below is a short summary of knowledge essential for using the method. Additional reading on the subject can be found in ref. 1.

1.1 Cloning: Vectors, Inserts, and Enzymes

Cloning is the construction of a DNA molecule of a novel nucleotide sequence generated by bringing together two or more existing DNA fragments. Like many construction projects, it involves cutting things at designed sites and gluing them together to assemble novel structures. Restriction endonucleases, the enzymes that cut DNA at specific nucleotide sequences, play the role of molecular scissors in cloning. Another enzyme, the T4 DNA ligase (originally isolated from the bacterophage T4) is used to "glue" DNA fragments together.

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In any cloning experiment there are two major DNA components—a vector and an insert—that are to be joined into a single DNA molecule (Fig. 1). The vector plays the role of a transport vehicle that is used to move its passengers—the cloned genes—between cells and cell-free environments in which they are manipulated by researchers. In most cases, the vector is a plasmid that can be selected for and maintained in the bacterium *Escherichia coli*. Vectors always contain a selective marker, a gene for resistance to an antibiotic such as ampicillin, kanamycin, or chloramphenicol, and a bacterial origin of replication required for plasmid propagation. The frequency of replication initiation varies in different vectors depending on the origin sequence (some fire more often than others) and results in a different plasmid copy number per bacterial cell, from 1 to ~500. High-copy-number vectors are good for plasmid DNA amplification but can be toxic to bacteria if a gene cloned in the vector is highly over-expressed as a result of plasmid amplification. To overcome this problem, genes are either cloned into low-medium-copy-number vectors or placed under the control of inducible promoters so as to keep bacteria viable until the gene expression is required.

If the expression of a cloned gene is designed to occur in an organism other than *E. coli*, so-called shuttle vectors are used. These can be maintained in *E. coli* during cloning or plasmid amplification but have additional features allowing them to be introduced into a different type of cell once the plasmid is constructed and isolated from *E. coli*. For example, budding yeast shuttle vectors contain yeast genetic markers, such as *URA3*, *LEU2*, *TRP1*, *HIS3*, and *KAN*, used to select for the plasmids upon transformation into yeast. In addition, the vectors need to be replicated and segregated inside a yeast cell. This can be achieved in several ways. Integration vectors are designed to insert the plasmid DNA, via homologous recombination, into a locus of the yeast genome that shares homology with the plasmid. The plasmid DNA is then replicated and segregated as a part of the chromosome in which it has been integrated. In contrast, CEN/ARS vectors contain a yeast centromere DNA sequence (CEN) and an origin of replication (ARS for autonomous replication sequence), which allow them to be maintained in vivo as a circular molecule of a single copy per cell. Another series of yeast vectors is based on the yeast endogenous 2 μ plasmid that replicates autonomously and has variable copy number (10–40 per cell) as it segregates through diffusion between daughter cells. It is mainly used for gene overexpression in yeast.

The source of a gene or DNA fragment you want to clone is, normally, either genomic DNA or cDNA. The DNA sequence of interest represents just a tiny fraction of the genomic DNA and therefore would be hard to clone using genomic DNA directly. PCR is used to enrich for the desired sequence to simplify cloning

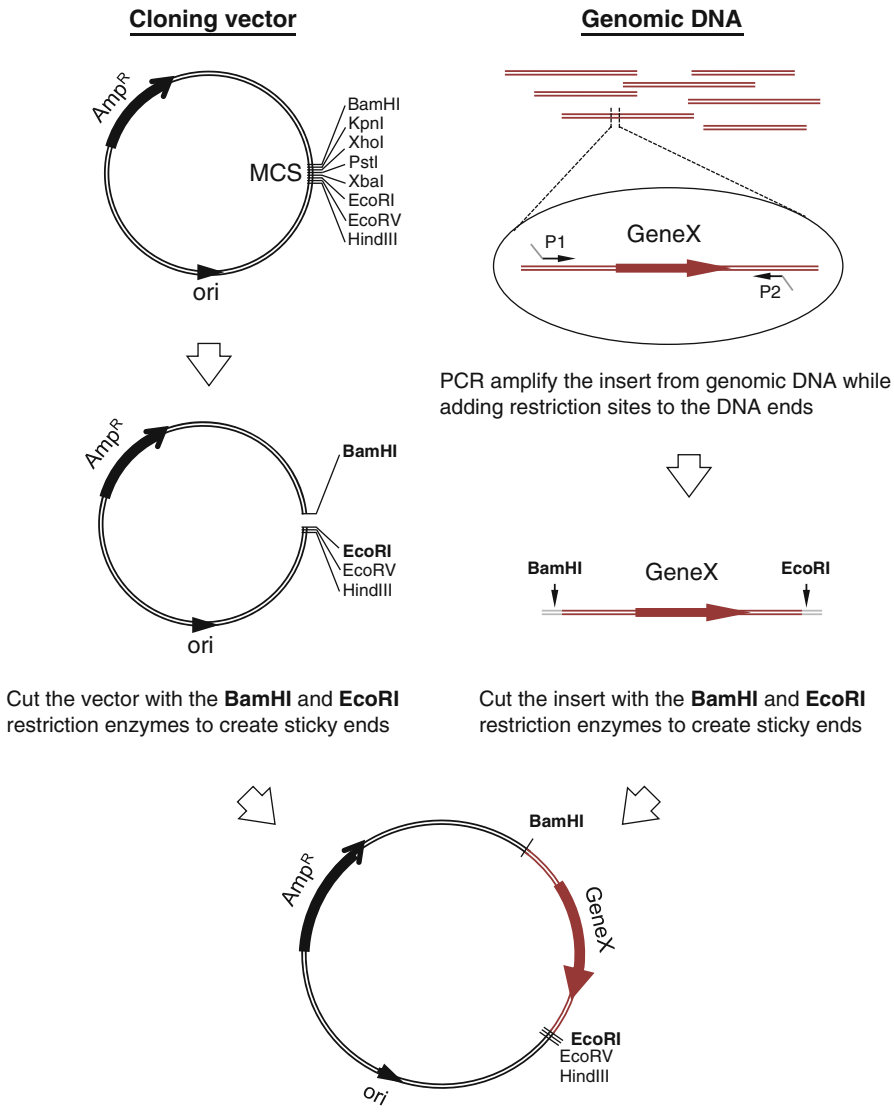

Fig. 1 A schematic diagram of a cloning experiment in which the insert (GeneX) is PCR-amplified using genomic DNA. The PCR primers P1 and P2 are designed so that their 5′ ends contain sequences which, when double-stranded, are recognized by BamHI and EcoRI, respectively. When both vector and insert are cleaved using these two restriction enzymes, their ends can be joined together by DNA ligase, to produce a recombinant plasmid with GeneX cloned in to the vector. MSC, multiple cloning sequence (sites for eight different restriction enzymes are shown); *AmpR* β-lactamase gene coding for resistance to ampicillin, *ori* origin of replication

(Fig. 1). PCR products have either blunt ends or overhangs of a single adenosine at the 3′ end. Though such ends can be ligated, the ligation efficiency is higher if longer overhangs are present on both ends or, at least, on one end of a PCR fragment. This is easily

achieved by placing recognition sequences for restriction enzymes at the 5′ ends of the primers used to PCR amplify the DNA sequence of interest. When the PCR product is generated, it can be digested with the appropriate restriction enzymes to generate the overhangs for the ligation step.

Once you have decided which gene you are cloning and into which vector, you can begin designing your cloning experiment. Each vector contains a multiple cloning sequence (MCS) sometimes called polylinker. This is a short ~100 bp region containing a cluster of unique restriction sites, the position of which is indicated on a plasmid restriction map. One or more often two of these sites are used to "open up" the vector, i.e., to convert the circular molecule into a linear DNA fragment, so that another DNA fragment, the one you want to clone, can be inserted into the created gap. The two fragments are "glued" together by ligase that essentially reverses the cleavage reactions performed by restriction enzymes. For joining of the end of the vector and the fragment to be cloned to happen, the DNA ends have to be compatible, i.e., the ssDNA overhangs have to be 100 % complementary to each other. The easiest way to achieve this is to use the same pair of restriction enzymes to cut both vector and insert DNA. When vector and insert cannot be cut with the same enzyme, the incompatible sticky ends generated by different enzymes can be converted into compatible, though harder to ligate, blunt ends. This is achieved by using either nucleases to remove overhangs or polymerases to convert 5′ single-stranded overhangs into blunt-end dsDNA. Having one pair of ends sticky and the other pair blunt works reasonably well in a ligation reaction. However, relying on ligation of completely blunt-ended fragments is not advisable.

During ligation, any DNA fragments with compatible DNA ends can be randomly joined by ligase, whether those are two vector fragments, two inserts, or one of each. To maximize the probability of the desired ligation, i.e., a vector fragment joined to an insert, the ends of a linearized vector are dephosphorylated by a phosphatase so that vector ends cannot be ligated back to each other or to the ends of another vector molecule. Ligation of multiple inserts to each other without a vector molecule involved is less of a problem as they do not result in transformants due to the lack of the vector-encoded drug marker in the recombinant molecule and the inability to be maintained in bacteria.

Ideally, only linearized, dephosphorylated vector molecules and insert fragments digested on both ends should be present in the ligation reaction. Using agarose gel electrophoresis to separate linearized vector from residual uncut supercoiled molecules is particularly important as even very small amounts of the latter would result in an extremely high level of unwanted transformants with the "empty" vector rather than a recombinant plasmid.

Having the right DNA fragments with intact compatible ends going into ligation is the most critical step in any cloning experiment.

Transformants containing putative recombinant plasmids are then screened for having the desired plasmid. The first screen employs colony PCR as it allows screening a larger number of colonies reasonably fast. Then, PCR-positive candidates are further screened by restriction digest of the plasmid DNA they contain. Finally, the clones that are positive after the first two screens are sequenced to confirm the sequence of the cloned fragment and to make sure no mutations were introduced during the cloning procedure.

1.2 DNA Agarose Gel Electrophoresis

DNA electrophoresis in agarose gel is one of the essential molecular biology techniques routinely used in many biological, medical, forensic, and other research laboratories dealing with the analysis of DNA samples from a variety of sources: PCR products, genomic DNA or DNA from mitochondria or chloroplasts, plasmid DNA, to name the most common ones.

DNA molecules are negatively charged due to phosphoric acid in the DNA sugar-phosphate backbone. Because of this charge, DNA moves in an electric field as an anion, from a cathode to an anode (marked black and red, respectively, on most gel electrophoresis tanks). The DNA electrophoresis technique separates DNA molecules of different sizes and shapes in agarose gels that are subjected to an electric field. This is possible due to the molecules' mobility in a gel being dependent on their size and shape. The agarose gel matrix works like a net, trapping DNA molecules as they move through the gel. Larger and/or extended molecules are affected by the trapping more than smaller and/or compact ones. The higher the agarose concentration is, the smaller the holes in the net are, and the slower the DNA molecules move.

Agarose gels normally run at a constant voltage that is stated in volts per centimeter (V/cm) of distance between the electrodes in a gel tank, to unify the running conditions for gel tanks of different designs. However, one has to remember that factors other than voltage and agarose gel concentration have an effect on gel-run progress. The temperature of the gel running buffer is one such factor. The buffer temperature may increase as the gel runs, particularly if a small tank is used and/or the voltage is high; this leads to faster DNA migration. Often the buffer warming up is uneven through the gel tank leading to gel "frowning" if the temperature is higher on the sides of the gel than in the middle or to gel "smiling" if the temperature is higher in the middle than on the sides of the gel. Using a pump to recirculate/mix the buffer or running your gel at a lower voltage can help if even mobility of all the samples is critically important for an experiment. Otherwise, loading DNA size marker into both side wells and the one in the

middle helps estimate the size of the analyzed DNA fragments more accurately, even if the migration is somewhat uneven. Another factor that influences gel runs is the amount of buffer in the gel tank. One can have either just enough buffer to cover the gel on the top or quite a bit more. In the first case the gel would run considerably faster as additional buffer increases resistance in a system with set voltage and, as a result, the current and the rate of DNA migration decrease.

The DNA itself is not visible with the naked eye but when bound by ethidium bromide, it can be easily detected by visualizing the ethidium in the UV spectrum. Adding ethidium to agarose when casting a gel is sufficient for visualizing DNA; having ethidium in the running buffer is normally not required. However, ethidium will diffuse out of the gel left in running buffer for a few hours or longer. DNA also diffuses in agarose, both during gel runs and afterwards. Therefore, the best gel images are taken right after the run. For the same reason, DNA bands for gel extraction should be excised as soon as the run is complete. The gel slices can then be stored at −20 °C.

In the cloning protocol described in this chapter, agarose gel electrophoresis is used at several steps, each time for a different purpose: to purify an insert PCR product, to separate linearized vector molecules from the uncut circular ones, to assay PCR products when screening transformants by colony PCR, and to analyze the composition of recombinant plasmids which have been digested with restriction enzymes. All these are simple agarose gel electrophoresis experiments ideal for training early career molecular biologists.

2 Materials

All reagents should be made using deionized water (dH_2O). Using sterile double-deionized water (ddH_2O) is recommended for setting up PCR, restriction digests, and ligation reactions.

2.1 Cloning Design

1. New England Biolabs catalogue.
2. Software for DNA sequence analysis and manipulation, for example, Serial Cloner.

2.2 Preparing E. coli Competent Cells

1. L-broth: Dissolve 10 g Bacto Tryptone, 5 g Bacto yeast extract, and 10 g NaCl in 900 mL dH_2O, adjust pH to 7.0 using 1 M NaOH. Bring the volume to 1 L, aliquot in screw cap glass bottles, autoclave at 121 °C for 15 min, and store at room temperature, away from light.
2. LB-agar plates with antibiotics. Dissolve 10 g Bacto Tryptone, 5 g Bacto yeast extract, and 10 g NaCl in 900 mL dH_2O,

adjust pH to 7.0 using 1 M NaOH. Pour the broth into a 1 L graduated glass bottle with a stir bar inside. Add 20 g Bacto agar powder and bring the volume to 1 L. Autoclave at 121 °C for 20 min. Take the bottle out of the autoclave and place it on a stirring plate. Stir until the agar media cools down for you to hold the bottle in your bare hand (*see* **Note 1**). Add desired antibiotic, stir briefly, and pour agar into Petri dishes, ~25 mL per plate. Once the agar has set, turn the plates upside down, stack them in plastic bags (to prevent drying), and store in this position at 4 °C away from light for up to several months. On the day of the experiment, dry the number of plates to be used (*see* **Note 2**).

3. Antibiotic for selecting the plasmid vector used in the cloning experiment. Most commonly used antibiotics come in a powder form, which is then used to prepare stock solutions.
   - Ampicillin: prepare stock at 100 mg/mL in dH_2O, make 1 mL aliquots in microcentrifuge tubes, and store them at −20 °C. Use in media at a final concentration of 100 μg/mL. When transforming low-copy-number plasmids, decrease the concentration to 20–50 μg/mL for the initial plasmid selection.
   - Chloramphenicol: stock solution 10 mg/mL in 50 % ethanol, store at 4 °C; final concentration 20 μg/mL.
   - Kanamycin or streptomycin: stock solution 10 mg/mL in dH_2O, store at 4 °C; final concentration 20–50 μg/mL.
   - Rifampicin: stock solution 10 mg/mL in methanol, store at 4 °C; final concentration 50 μg/mL.
   - Tetracycline: stock solution 10 mg/mL in 50 % ethanol, wrap in foil, and store at 4 °C in the dark; final concentration 10 μg/mL. The solution should be light yellow. When the color turns dark yellow, a new stock solution should be made.
4. 1 M $CaCl_2$: Dissolve $CaCl_2$ in dH_2O, adjust the volume, filter sterilize or autoclave, and keep at room temperature.
5. 50 % (w/w) glycerol stock solution: To prepare a ~200 mL solution, pour 100 mL dH_2O into a 250 mL glass beaker. Place the beaker on a scale and press the TARE button. Slowly pour 100 g of glycerol while the beaker is on the scale. Put a stir bar into the beaker and stir on a stirring plate until the solution looks well mixed (no glycerol swirls in water should be visible). Filter-sterilize and keep in the dark at room temperature.
6. Sterile dH_2O.
7. 0.1 M $CaCl_2$, 20 % glycerol: In a 50 mL sterile conical tube, mix 5 mL $CaCl_2$, 20 mL of 50 % glycerol, and 25 mL sterile dH_2O.

8. Sterile flasks of various volumes: 50 or 100 mL and 2 L.
9. Sterile 50 mL conical tubes.
10. Shaking platform with flask holders, set at 37 °C.
11. Roller drum for microcentrifuge tubes or rocker set at 37 °C (*see* **Note 3**).
12. Spectrophotometer (visible light spectra) and cuvettes.
13. Refrigerated tabletop centrifuge.
14. Water bath at 42 °C (*see* **Note 4**).
15. Ice bucket large enough to accommodate a 2 L flask.
16. Sterile 1.5–1.7 mL microcentrifuge tubes (*see* **Note 5**).
17. Saran Wrap or any other cling film.
18. Dry ice.
19. *E. coli* DH5α or *E. coli* XL1-Blue (*see* **Note 6**).
20. Plasmid DNA sample of known DNA concentration.

2.3 Generation of Insert DNA Fragment by PCR Amplification

1. DNA template for PCR amplification (genomic DNA or cDNA).
2. Primers designed in Subheading 3.1.
3. High-fidelity DNA polymerase with a reaction buffer, such as Pfu DNA polymerase (Promega, M7741) or Herculase II Fusion Enzyme (Stratagene, 600677).
4. Stock of dNTPs, 10 mM each.
5. QIAquick Gel Extraction kit (Qiagen) or equivalent.
6. ddH_2O.

2.4 Agarose Gel Electrophoresis

1. Gel tank with a casting tray and a set of combs with different well sizes. For simple applications such as analyzing diagnostic PCRs, diagnostic restriction digests, and cloning experiments, I would recommend using a mini-gel electrophoresis apparatus which is basically a 10 cm × 10 cm gel box with two electrodes on opposite sides (Fig. 2). There are two gaskets that when inserted into the box generate a 10 cm × 8 cm compartment in the middle, used to cast an agarose gel. Therefore, there is no casting tray as such; the gel box is used both as a casting tray and a gel tank. Either one or two combs can be used to cast a single gel and up to 40 samples can be run at a time. Once the gel is polymerized, the gaskets are removed and the box is filled with 1× TBE and is ready to be used. Because the box is made of UV-transparent material, the gel can be photographed while in the box filled with buffer. This setting is very economical with respect to the amount of running buffer (50 mL) and gel volume (40–50 mL) required as well as very convenient for gel handling.

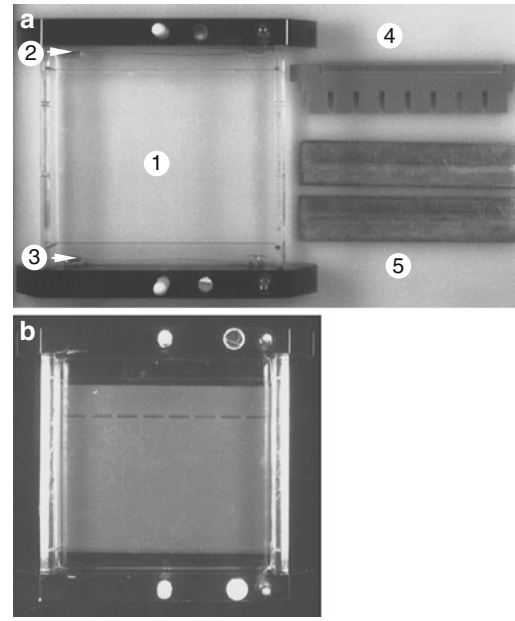

Fig. 2 Agarose gel electrophoresis equipment for a 10 × 8 cm mini-gel. (**a**) On the *left* is a 10 cm × 10 cm gel tank (1) with the electrodes, a cathode (2), and an anode (3), indicated by *white arrows*. On the *right* is a set of matching accessories for gel casting: an 8-well comb (4) and a pair of gaskets (5). (**b**) The same gel tank as in **a** but with an agarose gel inside

2. 10× TBE: 890 mM Tris base, 890 mM boric acid, and 20 mM EDTA. For 1 L, dissolve 108 g Tris and 55 g boric acid in 900 mL water, add 40 mL of 0.5 M EDTA, pH 8.0, and adjust the final volume to 1 L. Do not pH (*see* **Note 7**).
3. Agarose.
4. Ethidium bromide 10 mg/mL (*see* **Note 8**).
5. 6× sample buffer (gel-loading dye): 15 % Ficoll 400, 0.25 % bromophenol blue. Dissolve 1.5 g of Ficoll 400 in a total volume of 10 mL of dH_2O. Pinch in some bromophenol blue (*see* **Note 9**). Filter sterilize, aliquot into microcentrifuge tubes, and store at 4 °C (*see* **Note 10**).
6. DNA size markers: 1 kb and 100 bp DNA ladders (New England Biolabs).
7. Ready-to-load DNA size markers: mix 40 μL a DNA size marker, 40 μL 10× NEB 3 buffer (NEB), 320 μL dH_2O, and 80 μL 6× sample buffer (*see* **Note 11**). Mix well and store at −20 °C. When needed, defrost, mix, and load 3–5 μL per well.

2.5 Digestion and Ligation of DNA Fragments

1. Vector plasmid DNA of high purity.
2. Insert DNA generated by PCR and gel-purified (*see* Subheading 3.3).

3. Restriction enzymes with 10× reaction buffers and 100× BSA, provided by the enzyme suppliers (New England Biolabs, Roche, Promega, Fermatas, etc.).
4. Calf intestinal phosphatase (New England Biolabs) (*see* **Note 12**).
5. T4 DNA ligase with 10× reaction buffer (New England Biolabs) (*see* **Note 13**).
6. Agarose gel electrophoresis equipment and reagents (*see* Subheading 2.3).
7. Scalpels (preferred) or razor blades.
8. Long-wave (366 nm) UV box.
9. UV-protecting goggles or face mask.
10. Qiagen Gel Extraction kit or equivalent.
11. Qiagen MinElute PCR purification kit.
12. Water baths at 37 °C (possibly other temperature, depending on the choice of restriction enzymes) and 55 °C.

2.6 Recovery of Recombinant Plasmids in Bacteria: E. coli Transformation

1. *E. coli* competent cells (from Subheading 3.2).
2. Water bath at 42 °C (*see* **Note 4**).
3. L-broth and LB agar plates with an antibiotic to select for transformants (*see* Subheading 2.2 for media recipes).
4. Roller drum for microcentrifuge tubes or rocker set at 37 °C (*see* **Note 3**).

2.7 Colony Screening by PCR

1. 10 mM NaOH.
2. 0.2 mL thin wall PCR tubes, eight tube strips, and eight cap strips.
3. Thermocycler (PCR machine).
4. PCR reagents: Taq polymerase with 10× reaction buffer and 10 mM dNTPs.
5. Primers designed so that the PCR runs across one of the ligation sites (*see* Subheading 3.7 for more explanations).
6. L-broth with plasmid-selecting antibiotic (*see* Subheading 2.2 for recipes).
7. Agarose gel electrophoresis equipment and reagents (*see* Subheading 2.3).

2.8 Plasmid Diagnostics by Restriction Digests

1. Tabletop centrifuge.
2. Plasmid DNA mini-prep kit (Qiagen), Wizard Plasmid DNA Purification kit (Promega), or similar (*see* **Note 14**).
3. Restriction enzymes with 10× reaction buffers and 100× BSA, provided by the enzyme suppliers (New England Biolabs, Roche, Promega, Fermatas, etc.).
4. Agarose gel electrophoresis equipment and reagents (*see* Subheading 2.2).

2.9 Plasmid Verification by Sequencing

1. Primers for sequencing (*see* Subheading 3.9 for design).
2. Sequencing analysis software, for example FinchTV.

3 Methods

Your local Institution health and safety rules and regulations should be observed, particularly when working with potent carcinogens such as ethidium bromide and UV light. Wear gloves, lab coat, and UV protective goggles or a face mask.

3.1 Cloning Design

1. Download the complete sequence of your plasmid vector, and use it to create a file in one of the programs to manipulate and analyze DNA sequences, for example, Serial Cloner.
2. Using the software, generate and print out a restriction map of your vector in which only unique restriction sites are shown. Identify the multiple cloning site (MCS).
3. Download the sequence of the DNA fragment you want to clone, for example, the gene of interest, with its promoter and transcriptional terminator if desired. Create another DNA sequence file using the software for sequence analysis as above.
4. Scan the fragment sequence against the enzymes in the MCS and identify restriction sites that are present in MCS but absent in the fragment to be cloned.
5. Use a New England Biolabs catalogue to find out (a) which of the four reaction buffers are optimal for each of the enzymes selected in **step 4** and (b) how many base-pairs overhang they produce upon cutting. Identify a pair of enzymes that are active 75–100 % in the same buffer, preferably at the same temperature and generate 4 bp overhangs (*see* **Note 15**).
6. Decide if you want to clone the fragment in one orientation rather than the other. This will determine which restriction site will be placed upstream of the coding sequence and which one downstream.
7. Design a pair of primers to PCR amplify the fragment to be cloned, with the addition of four cytosines (*see* **Note 16**) and the corresponding restriction site at the 5′ end. For example, if in **step 5** you chose EagI and SalI to use for cloning your DNA fragment, then your oligos will look as follows:

   5′-ccccCGGCCGnnnnnnnnnnnnnnnnnn-3′,

   5′-ccccGTCGACnnnnnnnnnnnnnnnnnn-3′,

   where poly-n is the sequence specific for your fragment. Since the ten nucleotides at the 5′ ends are not homologous to the PCR template, exclude them when calculating annealing temperature for the PCR.

8. Order the oligos and the two restriction enzymes if you do not have them in your lab collection.
9. Using the software for sequence manipulation, create a file with a reconstituted sequence corresponding to the plasmid you are constructing. This will be useful in Subheadings 3.7–3.9 when performing clone screening.

3.2 E. coli Competent Cells

In most single-gene cloning experiments, competent cells with the efficiency of ~10^7 transformants per 1 μg of DNA allow to recover sufficient number of clones for further screening. The described protocol for preparing chemically competent *E .coli* cells is simple and very reproducible. In just a day, a batch of up to 100 aliquots can be easily generated and stored frozen for at least 1 year without loss of transformation efficiency.

The rules of sterility should be observed throughout the procedure to avoid culture contamination.

3.2.1 Preparing Competent Cells

1. Pick a single colony of a *recA E. coli* strain (*see* **Note 6**) and start a 10 mL L-broth culture in a 50 or 100 mL conical flask. Grow cells overnight on a shaker at 37 °C.
2. The next morning, dilute the overnight culture 1:100 in 200 mL of fresh L-broth in a 2 L flask. Grow the culture at 37 °C with vigorous aeration (*see* **Note 17**).
3. After 1 h of culture growth, start taking culture OD_{600} every 30 min. When the OD_{600} approaches 0.3–0.4, do the measurements more often.
4. While the culture is growing, precool a tabletop centrifuge to 4 °C. Also, prechill on ice four 50 mL conical tubes, 150 mL of sterile 0.1 M $CaCl_2$, and 15 mL of sterile 20 % glycerol in 0.1 M $CaCl_2$.
5. Place open microcentrifuge tubes (*see* **Note 5**) in a tube rack with 12 places × 8 rows using every other row. Normally, the tube tops from the rows filled later overlap with the tube bases from the previous row. When four rows are filled with 48 tubes, wrap the rack with the tubes with Saran Wrap and place it into a −80 °C freezer. Repeat this procedure with another rack, bringing the total number of tubes to 96.
6. When the bacterial culture OD_{600} reaches 0.4–0.5, chill the culture rapidly by placing the flask in an ice-water bath for 5 min. Swirl the flask every 10–20 s while it is in the bath to facilitate cooling.
7. Transfer the culture to the four prechilled 50 mL conical tubes and harvest cells in the prechilled tabletop centrifuge (from **step 4**) at 2,500 × *g* for 10 min.

8. Carefully pour off the supernatants and place the tubes on ice. Use a 1 mL pipetman to remove as much of the remaining media as possible (*see* **Note 18**).
9. Add 25 mL of cold 0.1 M $CaCl_2$ to each tube and resuspend the cells by vortexing. No cell clumps should be seen.
10. Incubate cells on ice for 1 h.
11. Combine the four 25 mL aliquots in two tubes and harvest the cells in the prechilled tabletop centrifuge at 2,500×*g* for 10 min.
12. Carefully pour off the supernatant and place the tubes on ice. Use a 1 mL pipetman to remove as much of the remaining $CaCl_2$ as possible.
13. Add 5 mL of ice-cold 20 % glycerol in 0.1 M $CaCl_2$ to each tube and resuspend the cells by vortexing. No cell clumps should be visible.
14. Combine the two aliquots in one tube and place the cells on ice.
15. Pour some dry ice into an ice tray. Take one of the two racks with prechilled microcentrifuge tubes out of the −80 °C freezer, remove the Saran Wrap, and place the rack on dry ice.
16. Quickly pipette 100 μL of cells into each tube and close the tubes (*see* **Note 19**). Transfer the tubes to a −80 °C freezer.
17. Repeat the aliquoting with the rest of the tubes.
18. Store the competent cell aliquots at −80 °C until needed.

3.2.2 Testing Competent Cells

It is important to test each newly made batch of competent cells before using them in any sort of experiments. One should test the cells for competency, i.e., score the efficiency of transformation, as well as test for the presence of any contamination in the culture.

1. Take two aliquots out of the −80 °C freezer and place them on ice for ~5 min.
2. Gently flick the tubes to make sure that the aliquots are defrosted.
3. Label one of the aliquots with the name of the plasmid to be transformed and use the other as a negative, "no DNA" control.
4. Add 10 ng of plasmid DNA to the first aliquot. Flick the tube to mix. Incubate on ice for 30 min.
5. Heat-shock both aliquots of cells by placing the tubes into a 42 °C water bath.
6. Transfer the tubes back on ice for 1 min.
7. Add 0.9 mL L-broth to each tube.

8. Incubate the tubes at 37 °C with aeration (on a roller drum or a rocker) for 1 h or without aeration for 1.5 h.
9. Make tenfold culture dilutions in LB-broth, and plate 0.1 mL from each dilution onto plates with LB-agar with an antibiotic to select for the transformants. Incubate the plates at 37 °C overnight.
10. First, check the plates with the dilutions for the "no DNA" control aliquot. There should be no colonies as no plasmid was added to the cells. If there are any colonies, then either the culture was contaminated or your antibiotic is not working for some reason.
11. If the control plates from the previous step have no colonies but the ones for the plasmid transformation do, then your colonies are the expected transformants. Count colonies on a plate that has between 50 and 500 colonies and calculate the number of transformants per 1 μg of DNA (efficiency of transformation, *E*) using the following formula:

$$E = N \times A \times 1{,}000,$$

where *N* is the number of colonies on a plate and *A* is the dilution factor for that plate (*see* **Note 20**).

3.3 Generation of Insert DNA Fragment by PCR Amplification

Cloning a PCR amplified fragment has the advantage of having plenty of DNA to work with, as a result of PCR amplification. However, DNA synthesis during PCR is error-prone, and one has to address this issue by using high-fidelity polymerases and running as few amplification cycles as possible to generate sufficient DNA. For obvious reasons, amplifying longer fragments, mutation-free, presents a more difficult challenge than working with shorter ones.

1. Make threefold serial dilutions of your DNA template (normally genomic DNA or cDNA) and use them for three or four PCR reactions with various amounts of the template.
2. Set up PCR reactions, each in a total volume of 50 μL. Use a high-fidelity DNA polymerase and add the other components into the reactions according to the manufacturer's recommendations (*see* **Note 21**).
3. While the PCR is running, prepare a small agarose gel as described in Subheading 3.4. Choose a comb to generate wells holding ~60 μL (*see* **Note 22**).
4. When the PCR is finished, add 10 μL of sample buffer to each reaction and mix well (*see* **Note 23**). Load each reaction into a separate well. Load a DNA size marker on one or both sides of the gel, next to the PCR samples.

5. Run the gel at ~10 V/cm until the bromophenol blue migrates 6–8 cm from the wells (*see* **Note 24**).
6. Visualize the gel using a gel documentation system (*see* **Note 25**).
7. Identify the required PCR product and compare its amount in the samples with the different amount of the template used. Identify the sample with the highest amount of the template which generated a highly visible amount of product.
8. Place the gel in the casting tray on top of a long-wave (normally 366 nm) UV box. Use a clean scalpel or a razor blade to cut out a gel slice with the PCR product you chose in the previous step. Work fast to minimize the DNA exposure to UV.
9. Extract the DNA from the gel slice using QIAquick Gel Extraction kit or equivalent according to manufacturer's recommendations (*see* **Note 26**).
10. Keep the purified DNA fragment in the freezer until needed.

3.4 Agarose Gel DNA Electrophoresis: Casting, Loading, and Running a Gel

When casting a gel, one has to think of what is to be achieved by electrophoresing a given set of DNA samples through agarose. For example, if the purpose is to test samples from a PCR run for the presence/absence of a DNA product of the expected size, then the DNA migration distance does not have to be long, 2–3 cm would be sufficient in most cases. In contrast, when DNA fragments close in size are analyzed, long, up to 20 cm, migration may be required. The migration distance is one of the factors that should be taken into consideration when choosing the gel apparatus for your experiment. The other factor is the number of samples to be run on a gel. The gel comb should contain a sufficient number of teeth to provide wells for all the samples as well as a DNA size marker, preferably loaded on both sides of the sample set.

The agarose concentration in a gel depends on the size of DNA fragments electrophoresed. 0.4–0.5 % gels are used to separate longer, 4–12 kb fragments as well as for separating supercoiled and linear DNA of the same or very similar size in the range of 4–12 kb (*see* more on this in Subheading 3.5). For resolving short, 100–500 bp DNA fragments, 2 % agarose gels work very well. Even shorter dsDNA molecules (40–100 bp) can be resolved when run in a 3–4 % gel made with low melting point agarose.

When working in the presence of ultraviolet light, protect your skin from exposure by wearing gloves, long-sleeve clothing, and a UV protective face mask or goggles. When handling bottles with hot agarose, use heat protective gloves.

1. Assemble a gel-casting tray on a bench. Use a levelling table if casting a larger gel (15 cm × 20 cm or larger).
2. Choose an agarose concentration appropriate for the expected size of the DNA fragments to be analyzed. To prepare 100 mL of agarose gel, weigh the appropriate amount of agarose (e.g., 1 g

for 1 % gel) and place it into a 200–250 mL heat-resistant screw-top glass bottle (or a flask with a sponge inserted at the top) with 100 mL of 1× TBE and a small stirring bar.

3. Stir on a stirring plate on a lower setting for a few seconds to disperse the agarose.
4. Using a marker pen, mark the top of the liquid level on glass, screw the bottle top slightly loose, and place the bottle into a microwave. Heat on "high" with occasional swirling until the solution starts boiling. Then, lower the power and simmer until the agarose is completely dissolved (*see* **Note 27**).
5. Carefully take the bottle out of the microwave and place it inside a container with cold water. Place the container on a stirring plate and stir until the bottle is cool enough that you can hold it in your hand (*see* **Note 28**).
6. Remove the bottle from the container and place it directly on a stirring plate. Open the bottle and add ethidium bromide to a final concentration of 5 μg/mL.
7. Stop the stirrer and check if the volume of the TBE has dropped below the mark on the bottle and, if needed, add some water to bring it back to the original volume and stir briefly.
8. Pour the gel solution into the casting tray and use a plastic tip to move any bubbles to the sides of the casting tray. Let the gel solidify (*see* **Note 29**).
9. When the gel is set, place it into the gel tank and pour just enough 1× TBE running buffer to cover the gel with a 2–3 mm layer of liquid (*see* **Note 30**).
10. Carefully remove the comb. Place a strip of dark paper or plastic on the bench under the gel tank; the wells are much easier to see against a dark background.
11. For sample loading, choose a pipette with the lower volume range (e.g., to load 20 μL sample use P2–20 rather than P20–200) as its gentler spring allows more controlled, gentle loading. Pipette a sample into the pipette tip and while holding the pipette in one hand, rest the index finger of the other hand on the middle part of the pipette to keep it steady. Lower the tip into a well, about halfway through and slowly load the sample. If the well is going to be fully loaded, slowly pull the pipette out of the well while loading so that the tip is always above the loaded sample.
12. Repeat the loading with the rest of the samples and size marker(s).
13. Attach the leads to the gel tank and the power supply, set the voltage, and start the run.
14. Once the run has begun, look at the electrodes in the gel tank and make sure more bubbles comes from the electrode closest

to the wells (cathode). If this is not the case, stop the run and check if the leads are connected to the gel tank and the power supply in the right order.

3.5 Digestion and Ligation of DNA Fragments

Set up restriction digests of the vector plasmid and the PCR product to be cloned into the vector.

1. For the vector digest, mix 0.5–1 μg of DNA, 3 μL of 10× restriction buffer, and 5–20 U of each restriction enzyme (*see* **Note 31**) and 5 U of calf intestinal phosphatase (*see* **Note 32**) in a total volume of 30 μL.
2. In parallel, set up three control reactions in 10 μL each (*see* **Note 33**). The control reactions should be in the same reaction buffer and have the same concentration of vector DNA and the same concentration of each restriction enzyme as in the 30 μL digest. The first two should each have one of the two restriction enzymes used in the 30 μL digest (two single-enzyme digests), and the third one should have no restriction enzymes at all (undigested DNA control).
3. For the DNA fragment digest, mix 0.2–0.5 μg of DNA (*see* **Note 34**), 3 μL of 10× restriction buffer, and 5–20 U of each restriction enzyme in a total volume of 30 μL (*see* **Note 31**).
4. Incubate all the digests at 37 °C (*see* **Note 35**).
5. During the digestion, prepare a small or medium (8 cm or slightly longer) 0.5 % agarose gel in 1× TBE with at least six 10–12 mm wells (*see* **Notes 36** and **37**) as described in Subheading 3.4.
6. After the DNA has been digested for 1 h, remove all of the four plasmid reactions (three controls plus the 30 μL double digest) and leave the DNA fragment digest for further incubation while you perform **steps 7–12**.
7. Add 2 μL of the 6× sample buffer to each of the control reactions and 6 μL of the 6× sample buffer to the double digest, mix well, and load on the 0.5 % gel in the following order:
   (a) 1 kb DNA ladder size marker.
   (b) Single-enzyme digest 1.
   (c) Single-enzyme digest 2.
   (d) Undigested DNA.
   (e) Double-enzyme digest.
   (f) 1 kb DNA ladder size marker.
8. Run the gel at 5–6 V/cm until the bromophenol blue migrates at least 6–7 cm away from the wells.
9. Take a picture of the gel using a gel documentation system.

10. From the gel picture assess the following:
    (a) If each of the enzymes cut the vector close to completion. This can be found out from comparing the lanes with single digests against the lane with uncut DNA. Circular DNA is predominantly in a supercoiled form that runs faster than linear DNA molecules of the same size, but a minor fraction of nicked circular molecules is often present and runs slower than the linear molecules (Fig. 3). If each of the single digests ran as a single band corresponding to the linear form, then the digests are close to completion.
    (b) If the double digest and supercoiled vector are well separated during the run. Estimate the run distance between the supercoiled DNA in lane (d) and the linear DNA in lane (e). If the difference is less than 3 mm, continue running the gel until the two forms of DNA are separated clearly (*see* **Note 38**).
11. Place the gel in the casting tray on a long-wave UV box and use a clean scalpel or a razor blade to cut out a gel slice with the linear double-digested vector DNA from lane (e). Cut right around the band and stay away from lane (d) as well as from the area in lane (e) in which uncut vector molecules are expected to run.
12. Purify the DNA using Qiagen Gel Extraction kit. At the elution step, elute the DNA with 30 μL of pre-warmed (55–60 °C) deionized water and leave at room temperature for at least 15 min before spinning down the column in the centrifuge.
13. Measure the volume of the eluate and split the sample into two equal aliquots, normally 14–15 μL each.
14. Recover the DNA fragment digest from 37 °C and use Qiagen minElute PCR purification kit to purify the DNA from the restriction enzymes and buffer components. Follow the manufacturer's protocol until the elution step. At this point, instead of using water for eluting the DNA fragment, use one of the linearized vector aliquots from the previous step. Pipette 14–15 μL of the vector DNA solution right in the center of the purple column and let it stand for about 15 min until recovering the DNA by centrifugation. The tube contains 14–15 μL mixture of the vector and insert, while the other tube from **step 13** contains vector DNA only, at the same concentration and in the same volume as the tube with the vector/insert mix.
15. Set up ligation reactions. Add 2 μL of 10× T4 DNA ligase buffer to each of the two tubes from the previous step. Flick the tubes to mix. Add 200–400 U of T4 DNA ligase to each tube. Mix well by flicking the tubes. Spin briefly in the microcentrifuge to collect the liquid at the bottom of the tube. Incubate on the bench for 1–16 h (*see* **Note 39**).

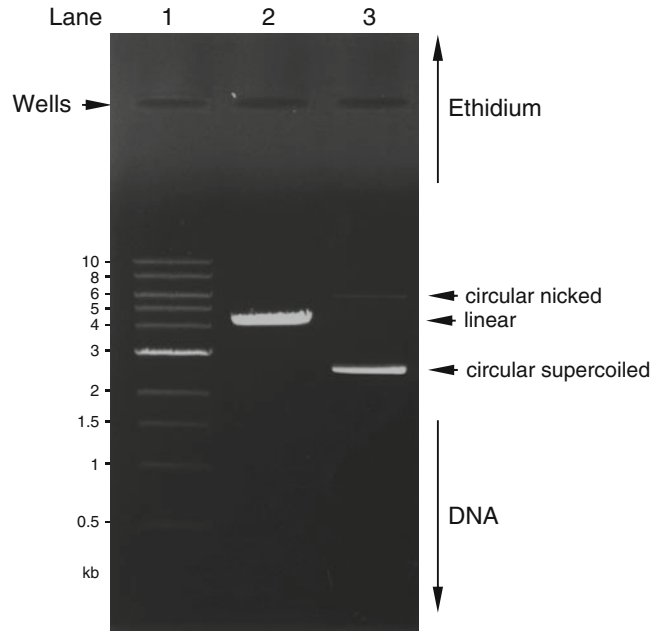

Fig. 3 Different isoforms of the plasmid vector pRS404 (4,274 bp) electrophoresed in 0.5 % agarose gel at 5 V/cm. *Lane 1* 1 kb DNA ladder (NEB). Sizes of individual bands are shown on the *left*. *Lane 2* pRS404 linearized with the EagI restriction enzyme. *Lane 3* undigested pRS404 contains two isoforms. The majority of plasmids in the sample are supercoiled circles but some nicked circles are present, as indicated by *horizontal arrows* on the *right*. Compare the *lanes 2* and *3* supercoiled circles move through the gel faster than linear DNA molecules, which in turn migrate faster than *nicked circles* of the same size. Notice that the band of linear DNA in *lane 2* looks brighter than the sum of bands in *lane 3* (the same amount of DNA was loaded in the *two lanes*) because ethidium binds relaxed DNA better than supercoiled DNA. On the *right*, *vertical arrows* indicate the directionalities of gel migration for DNA and ethidium. During electrophoresis, ethidium still remains in the *upper part* of the gel which looks brighter on the image

3.6 Recovery of Recombinant Plasmids in Bacteria: E. coli Transformation

1. Defrost on ice two 100 μL aliquots of *E. coli* competent cells.
2. Add the same volume of the "vector control" and "vector plus insert" ligation reactions, up to 10 μL, to the separate aliquots of competent cells.
3. Incubate on ice for 30 min or longer.
4. Place the tubes in a 42 °C water bath for exactly 2 min to heat-shock the bacteria and return the samples on ice for 1 min.
5. Add 900 μL of L-broth to each sample.
6. Place the tubes on a rotating weal at 37 °C for 1 h.

7. During the incubation, prepare (dry, pre-warm, or both) two plates with LB-agar and the antibiotic for plasmid selection (most commonly ampicillin at 100 μg/mL).
8. Spin the cells for 1–2 min at 2,000 × *g* in a microcentrifuge and remove ~900 μL of the supernatant by pouring it out of the open tubes.
9. Vortex to resuspend the cells in the remaining liquid and plate each sample onto a Petri dish prepared in **step 7**.
10. Incubate plates at 37 °C overnight.
11. Compare the number of colonies on the "vector control" plate and the "vector plus insert" plate. If the number of colonies on the two plates looks more or less similar or the "vector plus insert" plate has fewer colonies than the control plate, use colony PCR to screen 15–30 clones, as described in the next section. If the "vector plus insert" plate has at least three times more colonies than the control plate, then inoculate 6–10 random colonies from the "vector plus insertion" plate, each into 5 mL LB with the plasmid-selecting drug in a 15 mL conical tube and grow at 37 °C overnight with aeration. Then proceed to Subheading 3.8.

3.7 Colony Screening by PCR

It is reasonable to screen 15–30 colonies by colony PCR to identify candidate clones to be further analyzed by plasmid restriction digests as described in Subheading 3.8. In the colony PCR screen, one primer should hybridize to the vector sequence and the other one to the insert sequence so that a PCR product of 0.2–1 kb is generated when the expected plasmid is constructed. Use the plasmid sequence file you generated in Subheading 3.1 to design the PCR primers.

1. Use a Sharpie pen to number each colony to screen on the back of the plate.
2. Aliquot 10 mM NaOH to 0.2 mL PCR tubes in 8 tube strips, 3 μL per tube. The number of tubes should be equal to the number of colonies you are screening plus a tube for a negative control. Number the tubes.
3. Use a tip for 200 μL pipettes to pick a tiny bit of cells from each colony on the plate (*see* **Note 40**) and resuspend the bacteria in the NaOH in the PCR tube with the corresponding number.
4. Repeat the previous step for all the colonies to be screened. As a negative control, use a colony from the "vector only" plate or your bacterial strain without any plasmid transformed.
5. Close the PCR tubes with strips of caps and lyse the cells by heating the samples in the PCR machine for 10 min at 99 °C.

6. While lysing the cells, prepare a PCR master mix in a micro-centrifuge tube. For N reactions (*see* **Note 41**), mix:

   $(N+1)\times 2.5$ µL of 10× Taq polymerase reaction buffer.

   $(N+1)\times 0.5$ µL of 10 mM dNTPs.

   $(N+1)\times 0.5$ µL of primer 1.

   $(N+1)\times 0.5$ µL of primer 2.

   $(N+1)\times 18$ µL of ddH_2O.

   Mix all the components well, using a 1 mL pipette, then add $(N+1)\times 0.25$ µL of Taq polymerase and mix again by pipetting (*see* **Note 42**).

7. Aliquot the master mix into fresh PCR tube caps, 22 µL per cap.
8. When the cell lysis (**step 4**) is finished, take the tubes out of the PCR machine, remove the caps, and replace them with the caps filled with the PCR master mix (**step 7**).
9. Spin the strips of tubes in a mini-centrifuge. Flick the samples to mix the lysed cells with the PCR mix and spin again.
10. Place the tubes in the PCR machine and run a colony PCR program:

1 cycle of	94 °C for 2 min
30–40 cycles of	94 °C for 30 s
	45–60 °C for 30 s (*see* **Note 43**)
	72 °C for 15–60 s (*see* **Note 44**)
1 cycle of	72 °C for 2 min

    Keep samples cold afterwards.

11. While the PCR is running, pour a 1.5–2 % agarose gel (as described in Subheading 3.4) to accommodate all the PCR samples along with a DNA size marker. We normally use a small (7 cm × 10 cm) gel with two rows of wells, 20 wells per row.
12. When the PCR run is finished, add 5 µL of 6× sample buffer to each sample (*see* **Note 23**).
13. Set your pipette at 15 µL, pipet up and down each sample and load 15 µL on a gel. When loading samples leave the first and the last lane in each row empty.
14. Load 5 µL of 100 bp ladder mix on either side of the set of lanes with the PCR samples.
15. Run the gel at 10 V/cm for 10–20 min, or until the bromophenol blue has migrated 2–3 cm into the gel.
16. Take an image of the gel and identify the lanes with the PCR product of the expected size.

17. Mark the corresponding *E. coli* colonies as PCR-positive on the plate from the cloning experiment.
18. Inoculate 4–8 positive colonies into L-broth with a drug selecting for the plasmid, each colony into 5 mL of media in a 15 mL conical tube.
19. Grow the cultures overnight at 37 °C with aeration.

3.8 Plasmid Diagnostics by Restriction Digests

Plasmid diagnostic by restriction digest allows testing if the transformants contain the desired plasmid by purifying and cleaving the analyzed plasmids with restriction enzymes and estimating the sizes of the generated fragments using agarose gel electrophoresis. The most straightforward analytical digest employs the same restriction enzymes that were used in the cloning. These kinds of digests should generate the linear fragments that were taken into the ligation reaction, i.e., one fragment corresponding to the linearized vector and the other one to the insert. As a control, cut vector plasmid DNA should be included in the analysis as its presence in the gel alongside the analyzed plasmids helps verifying the sizes of the DNA fragments.

When a DNA fragment is inserted into a vector using a single restriction enzyme, additional digests are required to find out the orientation of the insert in the vector. For this purpose, use the plasmid sequence you built earlier (Subheading 3.1, **step 9**) to choose a pair of unique site restriction enzymes so that one of them cuts within the insert and the other one in the plasmid backbone, both should cut away from the middle of the corresponding DNA regions, i.e., considerably closer to one side versus the other. Calculate the expected fragment sizes for each orientation and make sure that the two cases will be easily distinguishable in gel electrophoresis. For example, 5 + 1 kb is different enough from 4.5 + 1.5 kb, whereas 3.4 + 2.6 kb is not easy to distinguish from 3.8 + 2.2 kb, particularly if only one kind of clones is present on a gel.

1. Spin down the 5 mL bacterial cultures in a tabletop centrifuge and purify plasmid DNA from *E. coli* cells using a commercial kit, for example, Plasmid DNA mini-prep kit (Qiagen), Wizard Plasmid DNA Purification kit (Promega), or similar. At the elution step, use deionized water (*see* **Note 45**) pre-warmed to 55–60 °C and allow the elution step to proceed for 15 min or longer (*see* **Note 46**) before collecting the plasmid DNA solution by centrifugation. When not used, plasmid DNA samples should be kept at −20 °C.
2. Digest the analyzed plasmids as well as the vector plasmid used for the cloning using the restriction enzymes utilized in the cloning. For each digest, use 0.5–1 μL of the DNA mini-prep (*see* **Note 47**) in a total volume of 15 μL per digest (*see* **Note 48**). Incubate at the temperature optimal for the restriction enzymes for 1 h.

3. During the plasmid digestion, prepare a small (10 cm × 8 cm) 0.5–1 % agarose gel as described in Subheading 3.4. Use a comb with small-medium size wells (3–5 mm wide), enough wells for all the digests plus, at least, two wells for the size markers. The exact agarose concentration depends on the size of the smallest fragment expected for the positive colonies.
4. Once the 1 h digest incubation is finished, add 3 μL of 6× sample buffer to each reaction, mix, and load samples on the gel, starting from the second well. Load 5 μL of 1 kb DNA ladder into the first lane; into the last lane—either the same marker or 5 μL of 100 bp ladder mix if fragments smaller than 1 kb are expected.
5. Run the gel at 7–10 V/cm until the bromophenol blue is 1 cm from the bottom of the gel. You can monitor the gel running using a UV box.
6. Take an image of the gel using a gel documentation system.
7. Analyze each lane for the presence of the DNA fragments of the expected size and mark your positives. The corresponding plasmid samples can now be verified by sequencing.

3.9 Plasmid Verification by Sequencing

When cloning involves PCR, the cloned DNA should be thoroughly analyzed by sequencing as a single base pair mutation can result in a change of gene expression and/or protein structure and function. When designing primers for sequencing, make sure that you will be able to read the sequence from one restriction site that you used in your cloning all the way to the other one. Depending on the sequencing service available, 500–800 bases of DNA sequence can be read in one reaction. Design your first primer ~100 bp upstream of the cloned fragment with the 3′ end towards it. Then design more primers on the same strand, every 500 bp starting from the first one and until it is less than 500 bp to the end of the end of the cloned fragment.

In present days, sequencing is outsourced to private companies or university run services. The sequencing reactions should be set up/run according to the requirements of the sequencing service provider available to you.

When the sequencing data are received, the files usually can be opened by FinchTV, a freely available program. The sequencing data are presented as a linear set of colored peaks, each peak labelled with A, G, C, or T. Each of the four bases is color coded, and the base present at a given position in the DNA sequence results in a peak of density of the corresponding color.

1. For each sequencing reaction, check the density tracks to identify the region in which the sequencing went well and the data can be trusted. This region will have a clear single-color

peak per position. Normally, the readable sequence starts from ~40th base and, depending on the quality of sequencing, may extend all the way to 800–850 bases.

2. Select the DNA sequence from the "trusted" region, copy and paste it in a new window in Serial Cloner or another program for sequence analysis.
3. Use the Alignment tool to align the sequence against the expected plasmid sequence you generated in the Subheading 3.1.
4. Carefully scroll through the alignment and see if there are any mismatches. If there is a mismatch, go back to the sequencing file and find the color peak corresponding to the position. If this is a clear one-color peak, then there is a mismatch in the cloned sequence. If there are two overlapping peaks, then it could be a sequencing artifact and the sequencing information can be clarified by re-sequencing.
5. Mark the region of the plasmid that is covered by the sequencing reaction and has no mismatches.
6. Repeat **steps 1–5** for each sequencing reaction.
7. Check if all the sequence from one restriction site to the other is covered by the sequencing and if there are any mismatches in the sequence. Once you have identified a clone satisfying both requirements, the analysis of sequencing reactions you may have for other clones in the experiment is no longer required.

4 Notes

1. Most antibiotics are heat sensitive and therefore should be added when a medium has been cooled down after autoclaving. Measuring the temperature directly without breaking the sterility is somewhat problematic; instead, using your hand touch as a sensor is convenient and works well enough, assuming reasonable precautions are taken to avoid skin burns.
2. Drying plates can be done at any temperature in the range from room temperature (on the bench) to 50 °C (in special ovens or incubators), but the time required is longer at lower temperatures. It should be done in clean environment to preserve the media sterility. While drying, the plates should stay open or half-open to allow moisture evaporation. Turn all the plates upside down and slide the agar-containing bases halfway over the lids and rest them on the plate lids. Leave plates in this position until you can see a marble-like pattern on the agar surface.
3. These pieces of equipment are not essential. They provide aeration for bacteria to speed up their recovery after transformation as well as the expression of drug resistance genes from

the plasmid transformed. The latter is required prior to the cells being plated on drug-containing agar plates. The same can be achieved by incubating cultures in 37 °C water bath for additional 15–30 min.

4. For the 42 °C heat-shock, a water bath, not a heat block, must be used as the heat transfer properties of water and the metal in the block are different. The time of the heat-shock in this protocol is optimized for a water bath.
5. Autoclaving of microcentrifuge tubes is not necessary. For making competent cells, I just open a brand new box of RNase/DNase-free tubes but handle them with special care by avoiding touching the edges of the tube as well as the inner side of the lids, i.e., the parts that are likely to be in contact with bacterial cultures.
6. *E.coli recA*-mutant strains should be used for cloning or plasmid amplification experiments to avoid plasmid dimers, trimers, tetramers, etc., produced via homologous recombination in a RecA-dependent manner.
7. 10× TBE tends to precipitate, particularly during cold nights in labs with no overnight heating. Once precipitated, it is practically impossible to get back into solution, even at a lower concentration. 5× TBE is less prone to precipitation. Filtering the stock solution after it has been prepared lowers the chances of precipitation as it removes tiny particles of undissolved chemicals that seed precipitation.
8. Ethidium bromide, as any other DNA-binding agent, is highly toxic and carcinogenic. Be particularly careful and use gloves when handling it. I prefer using it over the less toxic new generation type of DNA-binding agents (SYBR Green and similar) as ethidium can be present in the gel during the electrophoresis and therefore allows monitoring of the DNA migration during the gel run. Please notice that ethidium is a cation, and during electrophoresis it migrates in the direction opposite to that for DNA and therefore the bottom part of the gel may be depleted of ethidium (*see* Fig. 3). If proper staining of this part of the gel is essential, the gel can be stained in 1× TBE with ethidium bromide after electrophoresis.
9. Bromophenol blue and often xylene cyanol are added to the sample buffer to monitor the progress of electrophoresis. The mobility of the dyes in agarose gels depends on agarose concentration, but bromophenol blue always runs faster than xylene cyanol. Having too much of a dye in a sample has a negative effect on the visibility of DNA fragments that have the same mobility as the dye. To avoid this problem, I pinch just enough bromophenol blue to make the 6× sample buffer greenish and do not use xylene cyanol at all.

10. In cloning experiments, it is very important to preserve ligatable DNA ends after they have been generated by restriction enzymes. Even a trace of nuclease or phosphatase activities will make the ends unligatable. Therefore, the 6× sample buffer should be made and handled as a sterile solution. For the same reason, I recommend using Ficoll as a density-increasing agent rather than the commonly used glycerol and sucrose, which are good carbon sources for microbes, should any contamination of the buffer occur. Bromophenol blue is also a pH indicator and turning the color of your buffer from greenish to bluish-purplish is an indication of contamination. Avoid using commercially made sample buffers that come with some kits as they are not sterile, normally kept at room temperature (along with the kits) for extended amount of time, and in my experience have led to DNA degradation.
11. DNA size markers come as DNA solutions in water, whereas most DNA samples we load in a gel are in some kind of reaction buffer, such as a restriction digest and PCR. Having some salt in the reaction buffers helps loading samples into agarose gel wells; otherwise the samples may float out of the wells despite the presence of the sample buffer. To prevent this problem, I add 10× NEB3 to the size marker samples, to a final concentration of 1×. In general, if your samples float out of the wells, it means that the density of your sample is lower than that of the buffer in the well. The possible reasons are (a) the presence of residual ethanol in your sample (normally after DNA purification), (b) not enough salt in your sample, (c) not enough sample buffer or the concentration of Ficoll in your sample buffer is lower than it should be, or (d) the concentration of salt in the wells is too high. This often happens when a gel tank with a gel in it is left open for hours and water from the running buffer surface evaporates leading to an increase in the concentration of TBE on the surface of the gel and the wells. Mixing the buffer and washing the wells easily solve this problem.
12. Other phosphatases, such as Antarctic phosphatase or shrimp alkaline phosphatase, can be used instead. These alternative phosphatases can be inactivated by heat but since my protocol does not require enzyme inactivation, calf intestinal phosphatase has no disadvantage in this case.
13. The 10× ligase buffer contains ATP, which is required for the ligation reaction to occur. ATP is sensitive to thaw-freeze cycle and therefore the original aliquot of 10× buffer should be defrosted on ice, pipetted into 0.2 mL PCR tubes as 10 μL aliquots, and stored at −20 °C. When needed, an aliquot should be defrosted on ice, the amount of buffer needed taken out of the tube, and the rest should be disposed of.

14. There is a great variety of kits on the market for purifying plasmid DNA from *E. coli*. While most of them rely on the same alkaline lysis protocol, the purity of the DNA after using kits from different suppliers can vary dramatically. DNA purity is particularly important for the sequencing step. Therefore, before you commit to a given supplier, test if plasmids purified using their kit can be sequenced.
15. It is very important to pick the "right" pair of restriction enzymes for cloning. Carefully study the profiles for each potential enzyme using the New England Biolabs (NEB) catalogue and take into consideration the following aspects.

    Firstly, ligation and recutting, or what fold over-digestion, leaves 90–95 % of ends ligatable and re-cleavable. The higher the allowed over-digestion is, the lower the presence of contaminating nucleases that affect the intactness of the cleaved ends.

    Secondly, if the enzyme activity is affected by the Dam or Dcm methylation. Dam and Dcm are two *E. coli* resident methylases, and if a recognition sequence for a restriction enzyme contains a methylated base(s), some of the enzymes will be inhibited but others will not be. Only DNA purified from *E. coli* cells, such as plasmids, are methylated while in vitro synthesized PCR products are not. Use the NEB catalogue to find out if the enzymes you are considering are sensitive to methylation and, if so, check the target sequence in the plasmid to see if there are sequences that would be methylated.

    Thirdly, if there is a common reaction buffer for the pair of enzymes you plan to use. Sometimes, when such buffer is not available from the list of four NEB buffers, it is worth checking if such buffer can be found among the Roche five buffers. For example, NEB recommends using a sequential digest for BamHI and HindIII while both enzymes can be used in the Buffer B from Roche. Enzymes from different manufacturers can be used in the same reaction but keep in mind that the enzyme unit definitions can be different for different companies. Also, an enzyme from one manufacturer can be used in a buffer from another one if the activity of this particular enzyme (not of its Isoschizomers!) is known.

    Fourthly, give the priority to the enzymes that generate longer, normally four base, overhangs as this will increase the efficiency of ligation. Be creative with your cloning design by taking advantage of some restriction enzymes generating compatible ends. Use Cross Index of Recognition Sequences in the NEB catalogue to find out which enzymes produce compatible ends. For example, the enzymes SpeI, AvrII, NheI, and XbaI each recognize a different 6 bp sequence (ACTAGT, CCTAGG,

GCTAGC, and TCTAGA, respectively) but all generate the same four base 5′ overhang 3′-CTAG-5′. Therefore, if you use any of the four enzymes to cut the vector you can use any of the four to cut you insert.

And finally, check if the enzymes cut at the same temperature. Most restriction enzymes are active at 37 °C, but some nucleases from thermophilic bacteria require higher temperatures (50–65 °C), while an enzyme such as SmaI is quickly inactivated at temperatures above 25 °C. If you need to digest DNA with two enzymes that cut at different temperatures, first add the one that requires higher temperature and proceed with the reaction. Then add the second enzyme and incubate the digest at the lower temperature.

Here are some pairs of enzymes that worked well in my hands in cloning experiments: EagI and SalI in NEB3, PstI and SalI in NEB3, PstI and BglII in NEB3, Acc65I and SalI in NEB3, BamHI and HindIII in Roche Buffer B, and XhoI and HindIII in NEB2.

16. The cccc is added at the 5′ end to facilitate the cleavage by restriction enzymes as the enzyme activity often goes down if the recognition sequence is too close to the DNA end. Having strong C–G base pairing is also advantageous over A–T as it is less prone to DNA end breathing (strand unpairing) and therefore keeps the DNA end double-stranded. While the same can be achieved by having four Gs in the primers, it is not recommended as G-quartet DNA can be formed by primer molecules with stretches of guanines.
17. The rate of aeration/rate of cell growth is very important for cell competency and therefore the volume of the culture should not exceed 1/10 of the volume of the flask.
18. The cells become competent due to their starvation in calcium and therefore it is important to remove as much of the remaining medium as possible.
19. Aliquoting the cells fast is much easier when done by two people. One person starts pipetting cells into tubes in the order reverse to filling up the rack, i.e., the last row of tubes is used first. The other person closes the tubes with cells right away.
20. Count the tube with 1 mL bacterial culture as undiluted (dilution factor of 1), the first tenfold dilution should have the dilution factor of 10, the second tenfold dilution the factor of 100, and so on. The 1,000 in the equation comes from 100× and 10× adjustments that should be taken into account. Because only 100 μL out of 1 mL (i.e., 1/10) is plated, multiplying by 10 corrects for the plating volume. Also, 10 ng of DNA is used per transformation whereas the Efficiency E is

calculated per 1 μg and therefore the difference of 100-fold requires additional multiplication by 100.

21. If running more than two reactions, use tubes linked into strips as those are more convenient to handle. It is recommended to make a master mix as it simplifies pipetting of small volumes and ensures the uniformity of the PCR reactions within the experiment. When making a master mix, always make a bit more than needed. For four reactions, make a master mix sufficient for 4.5 reactions. When mixing the PCR components, add everything except the enzyme and mix well. Then add the enzyme and mix by pipetting up and down with a pipetman set at least half the volume of the mix. Never vortex enzyme-containing solutions as vortexing causes proteins to denature.
22. To find out how much you can load into a well before casting the actual gel, measure the base of a tooth in a comb in mm and multiply the comb thickness by the tooth width. Multiply the resulting number by 5 as a well in a 6 mm thick agarose gel is about 5 mm deep. The resultant number is the well volume. For example, a 4 mm tooth of a 1 mm thick comb will generate a well of 20 mm loading capacity ($4 \times 1 \times 5$). If you want to load more you either need to use a different comb or pour a thicker gel.
23. I keep an 8 strip of PCR tubes filled with 6× sample buffer and use a multichannel pipette to add sample buffer to any samples in PCR tubes, such as PCR reactions and restriction digests.
24. The purpose of this gel run is (a) to evaluate the results of the PCR runs, i.e., to see if a PCR product of the expected size is present, and (b) to separate the PCR product from any non-specific products and PCR components (primers, dNTPs, buffer, DNA polymerase). Therefore, if a nonspecific PCR product of a size close to the desired product is present, make sure you resolve them well from each other on the gel.
25. DNA exposure to UV causes DNA damage that, in turn, increases the chance of mutations in cloned DNA. Minimize the gel exposure to UV while taking a gel image. It is advantageous to have long-wave UV bulbs in your gel documentation system.
26. Often there is an optional wash step in the DNA purification protocols from a gel to get rid of residual agarose. Include this step as residual agarose may inhibit restriction enzymes later in the experiment. At the very last step, when the DNA is eluted from the DNA binding matrix, use water rather than elution buffer. Pre-warm water to 55–60 °C and let the elution proceed for 15 min, particularly if the DNA fragment is longer than 1 kb. This will increase the DNA recovery.

27. Hold the bottle by the cap and look at it against light while gently swirling the agarose. There should be no particles visible when agarose is melted completely.
28. A handful of ice can be added to the water in the container to speed up agarose cooling.
29. Casting the gel in a cold room decreases the time needed for the agarose to solidify.
30. The amount of buffer in the gel tank affects the speed of DNA migration. At a constant voltage, increasing the volume of the buffer will increase resistance in the system and lower the current, resulting in slower migration of DNA samples.
31. Many restriction enzymes show a loose-site recognition specificity (also called star activity) when glycerol concentration in the reaction is higher than 5 %. Because enzyme stocks normally come in 50 % glycerol, one has to be particularly careful when calculating the total volume of enzyme stocks that can be added to a reaction. Not more than 1/10 of the total volume of a reaction should come from enzyme stocks, e.g., if a restriction digest is set up in 30 μL, the combined volume of restriction enzymes and phosphatases added should not exceed 3 μL.

    When adding two (or more) restriction enzymes to a reaction, their relative amounts should be balanced. Each enzyme stock comes at a certain concentration in U/μL, written on a stock tube. For most restriction enzymes from NEB, 1 U is defined as the amount of enzyme required to digest 1 μg of phage λ DNA in 1 h, and therefore the number of target sites in λ for each enzyme (can be found in the NEB catalogue) should be taken into account when calculating the relative amount of enzymes for your digest. The formula reflecting this balance for a pair of enzymes A and B as follows:

$$C_A \times N(\lambda)_A \times V_A / N(S)_A = C_B \times N(\lambda)_B \times V_B / N(S)_B,$$

    where C is the enzyme stock concentration; $N(\lambda)$ and $N(S)$ are the numbers of restriction sites for a given enzyme in phage λ and your sample DNA, respectively; and V is a volume of the enzyme to be added to digest the sample DNA. To calculate the unknown V_A and V_B, a second equation is required:

$$V_A + V_B = V_T,$$

    where V_T is the total volume of the two enzyme stocks to be added to the reaction.

    Let's consider an example in which a plasmid with single sites for BamHI and EagI is to be digested and dephosphorylated in a reaction volume of 30 μL. Because of the "not more than 1/10" rule, and to be on a safe side, 2.5 μL will be allowed for all the enzymes. 0.5 μL of Phosphatase will be

added, leaving 2 μL for the two restriction enzymes. BamHI has five sites in λ and comes at 20 U/μL, while EagI has two sites in λ and comes at 10 U/μL. Therefore, our first equation will look as:

$$20 \times 5 \times V_{\text{BamHI}}/1 = 10 \times 2 \times V_{\text{EagI}}/1, \text{ or } 5 \times V_{\text{BamHI}} = V_{\text{EagI}}.$$

Because the total volume of the two restriction enzymes in the reaction is 2 μL,

$$V_{\text{BamHI}} + V_{\text{EagI}} = 2.$$

Solving the system of two simple equations, $V_{\text{BamHI}} = 0.33$ μL and $V_{\text{EagI}} = 1.67$ μL.

32. If your digest requires NEB1 buffer you have to use Antarctic phosphatase as CIP is not active in this buffer.
33. When both restriction sites are close to each other, the products of a single and a double digest are indistinguishable from each other on a gel. To assay the activity of each enzyme two single digests are required. The uncut control is useful to monitor the mobility of undigested DNA during electrophoresis.
34. It is very important not to overload the digest with DNA as under-digestion will result in most of the DNA fragments being cut on one end or the other while only the fragments cleaved on both sides will contribute to the productive ligation at the next stage. In contrast, single-end digested fragments will have an inhibitory effect on the productive ligation by competing for the vector molecules. When deciding how much DNA to take into a digest, remember that 1 μg of 100 bp DNA fragment has ten times as many DNA ends (and therefore restriction sites) as 1 μg of 1 kb DNA fragment. Therefore, using DNA amounts in moles rather than DNA mass will produce more accurate calculations.
35. Special care should be taken to prevent water evaporation from the reaction to tube walls or lids as this will increase the amount of salt and glycerol in the reaction and may result in loss of enzyme activity or encouragement of star activity. Reactions set up in small volumes are particularly sensitive to the problem. Use water bath with a lid or an incubator so that tube lid are heated too and do not stimulate condensation. I avoid sample evaporation by setting up digests in a thermocycler, with the lid heated to at least 70 °C.
36. The agarose concentration of 0.5 % is suitable for separating supercoiled and linear molecules of 4 kb and larger. If your vector is smaller, increase the agarose concentration to 0.6–0.7 %.
37. Loading DNA into larger wells helps keeping bands thinner and sharper, which results in better separation of species of similar mobility in a gel.

38. The gel can be run further even if the bromophenol blue migrates out of the gel completely but the run progress should be monitored more carefully not to lose the DNA due to over-running of the gel.
39. Sticky ends are ligated rather fast and the ligation reactions transformed into bacteria after 1 h normally produce enough colonies to screen. However, when a blunt end is involved, ligating for longer time periods might be needed for the ligation to occur. A sensible approach is to transform half of the reaction after 1–2 h ligation and leave the rest to proceed overnight. The next morning, if the number of transformants is not satisfactory the rest of the ligation reactions can be transformed.
40. It is important not to use too many cells in the colony PCR reaction as some cell lysate components, such as cell wall and lipids, inhibit amplification. Touch a side of a colony with a pipette tip to pick just enough cells to see with the naked eye.
41. When making a master mix, always make some extra to ensure sufficient amount of the mix is made for all the samples.
42. Always add enzymes last to master mixes to ensure they get diluted into the appropriate buffer conditions to prevent enzyme denaturation or precipitation.
43. The exact temperature depends on the primer melting temperature. Calculate the melting temperature of your primers in the PCR buffer and use a temperature 1–2 °C lower than the lower of the two melting temperatures of the primers.
44. The amplification time is dependent on the length of the expected PCR product. Allow 1 min per 1 kb of DNA to be amplified by the Taq polymerase.
45. Using water for elution allows using the plasmid samples for sequencing. If TE is used, EDTA will inhibit the sequencing reactions.
46. Pre-warming water and increasing elution time result in higher plasmid yields as they both promote DNA release from the column matrix. The larger the plasmids are, the harder it is to elute them.
47. If the insert is very small (shorter than 1 kb) more DNA may need to be digested to visualize the short DNA fragment. Also, because ethidium migrates in the direction opposite to DNA, the bottom of the gels in which smaller fragments run become depleted of the stain by the end of the run. Stain the gel in 1× TBE with 5 μg/mL ethidium for 30 min before visualizing the gel.
48. Making a master mix and setting up the digests in eight PCR tube strips help pipetting multiple digests faster and more accurately.

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