

## Troubleshooting Guide for Molecular Cloning

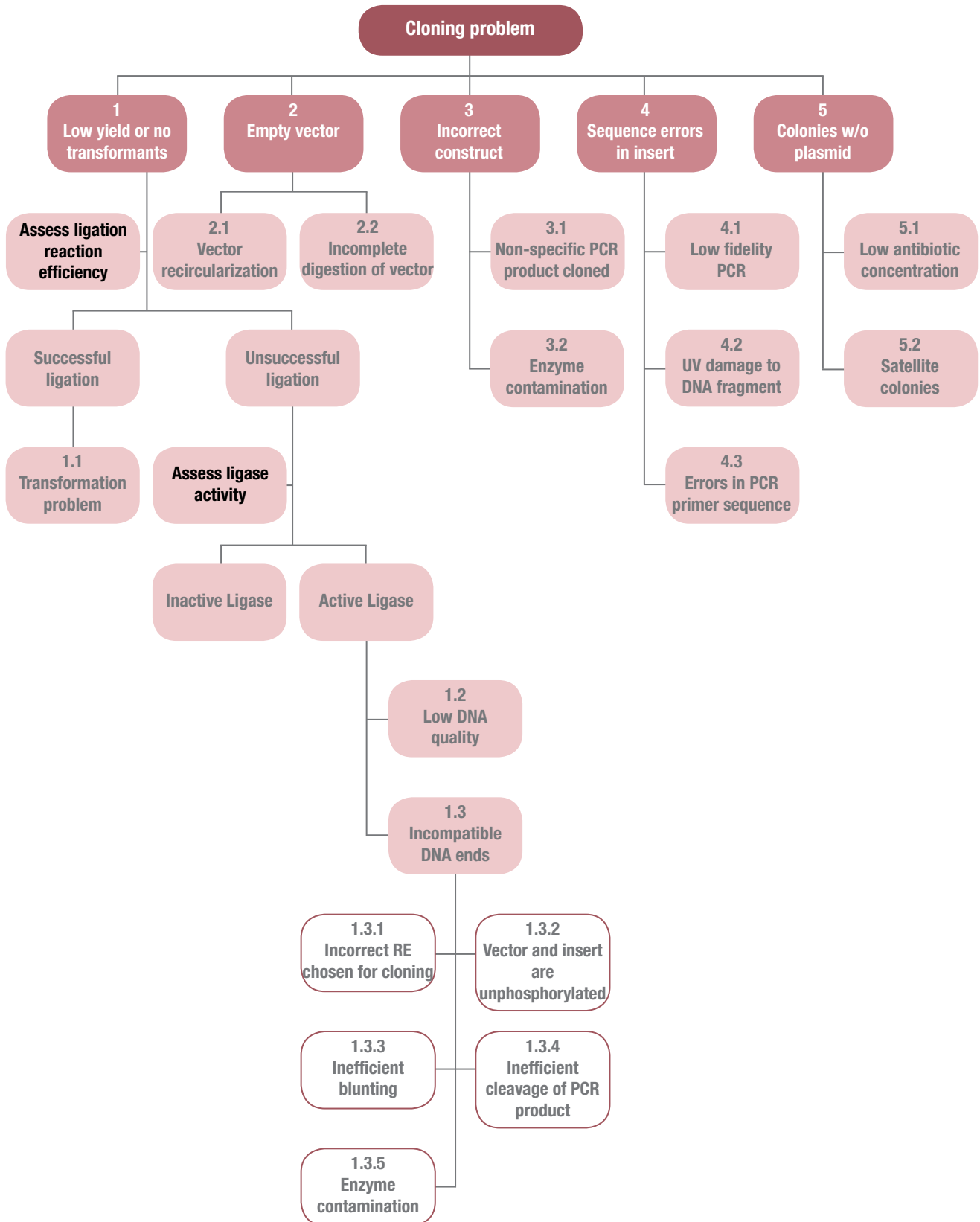
**4**
**4. MOLECULAR CLONING**


Table 4.1. Troubleshooting Guide for Molecular Cloning.

Problem	Possible cause and recommended solution
<b>1. Low yield or no transformants</b>	<p><b>1.1. Inefficient transformation.</b></p> <p><b>1.1.1. Low transformation efficiency of competent <i>E. coli</i> cells.</b> Check transformation efficiency with 0.1 ng of a supercoiled vector DNA, e.g. pUC19 (#SD0061). The competent cells should yield at least <math>1 \times 10^6</math> transformants per <math>\mu\text{g}</math> of supercoiled DNA, which corresponds to 100 colonies, when 0.1 ng of plasmid had been used for transformation.</p> <p><b>1.1.2. Ligase and/or PEG were not removed prior to electroporation.</b> When electroporation is used for transformation of blunt-end ligation reaction mixture, chloroform extraction instead of heat inactivation prior to electroporation is recommended.</p> <p><b>1.1.3. Ligase was not heat inactivated.</b> Heat inactivation of ligase improves the yield of transformants.</p> <p><b>1.1.4. Excessive amount of ligation mixture used for transformation.</b> Do not use more than 5 <math>\mu\text{l}</math> of ligation mixture for 50 <math>\mu\text{l}</math> of chemically competent cells and 1 <math>\mu\text{l}</math> for electrocompetent cells.</p> <p><b>1.1.5. Cloned sequence is not tolerated by <i>E. coli</i>.</b> Check the target sequence for strong <i>E. coli</i> promoters or other potentially toxic elements, as well as inverted repeats. In cases where the product of a cloned gene is toxic to the host, use promoters with a very low expression background or choose a low copy plasmid as cloning vehicle.</p> <p><b>1.1.6. Excessive amount of ligase used for ligation.</b> Do not use more ligase than it is recommended in the protocol (<i>see</i> p.334) relative to the vector and insert concentration. For easier manipulation Fermentas provides T4 DNA Ligase at 1 <math>\text{u}/\mu\text{l}</math> (#EL0015).</p>
	<p><b>1.2. Low DNA quality.</b></p> <p><b>1.2.1. DNA contains contaminants</b> (check according to p.335). Ensure DNA is free of contaminants (e.g. excess salts, EDTA, proteins, phenol, etc.) that may inhibit ligation. Gel purify and/ or phenol/chloroform extract the vector and insert prior to ligation.</p> <p><b>1.2.2. DNA was damaged by UV light during excision from the agarose gel.</b> Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit DNA exposure to UV to a few seconds. Keep the gel on a glass or plastic plate during UV illumination. Alternatively, use dyes visible in ambient light to visualize DNA in standard agarose gels (1, 2). Another method to avoid exposure to UV is to load your sample in two or more lanes and then cut and stain only one lane with ethidium bromide after electrophoresis. Use this stained lane as a reference for excising the DNA from unstained lane that is not exposed to UV light.</p>
	<p><b>1.3. Incompatible DNA ends.</b></p> <p><b>1.3.1. Incorrect restriction enzyme chosen for DNA digestion.</b> Incompatible vector and insert ends. Recheck the cloning strategy and choose restriction enzymes generating compatible overhangs for ligation.</p> <p><b>1.3.2. Vector and insert are nonphosphorylated.</b> If using dephosphorylated vectors, make sure the insert possesses phosphates. PCR products generally lack phosphate groups and need to be phosphorylated with T4 Polynucleotide Kinase (#EK0031) prior to ligation. The CloneJET™ PCR Cloning Kit (#K1231) is compatible with both phosphorylated and dephosphorylated DNA fragments.</p> <p><b>1.3.3. Inefficient blunting of DNA ends.</b> Use the appropriate DNA blunting method for the type of DNA fragment ends. <i>See</i> p.336 for DNA blunting protocols and recommendations.</p> <p><b>1.3.4. Inefficient cleavage of PCR product.</b> When introducing restriction enzyme sites into primers for subsequent digestion and cloning, refer to the Table 1.9. "Cleavage Efficiency Close to DNA Ends" on p.162 to define the number of extra bases required for efficient cleavage. Prior to digestion remove the active thermophilic DNA polymerase from the PCR mixture. DNA polymerases may alter the ends of the cleaved DNA and reduce the ligation yield. After digestion, gel-purify the PCR product to remove short DNA fragments, which compete with the insert in the ligation reaction.</p> <p><b>1.3.5. Contaminated enzymes used for cloning.</b> Use only the highest quality enzymes for cloning, e.g LO-tested enzymes (<i>see</i> p.2) and exclude any possibility of endo-, exo-nuclease and phosphatase contamination in enzyme preparations.</p>

(continued on next page)

**Table 4.1.** Troubleshooting Guide for Molecular Cloning.

Problem	Possible cause and recommended solution
<b>2. Empty vector (no insert)</b>	<p><b>2.1. Vector recircularization.</b>            Dephosphorylate the vector with FastAP™ Thermosensitive Alkaline Phosphatase (#EF0651), CIAP (#EF0341) or SAP (#EF0511) prior to ligation (<i>see</i> protocol on p.337). Vector dephosphorylation is recommended in all cases, including cloning strategies where the vector ends are incompatible for recircularization. Ensure the phosphatase is completely inactivated or removed after dephosphorylation.</p> <p><b>2.2. Incomplete cleavage of the vector.</b>            Check the cleavage efficiency on an agarose gel. If it is difficult to achieve complete cleavage, gel-purify the linear form of the vector using the DNA Gel Extraction Kit (#K0513).</p>
<b>3. Incorrect constructs</b>	<p><b>3.1. Non-specific PCR product cloned.</b></p> <ul style="list-style-type: none"> <li>• Gel-analyze the PCR product prior to ligation. If non-specific PCR products or primer-dimers were generated during the PCR reaction, gel-purify the target PCR product. Smaller DNA fragments present in the PCR mixture are ligated more efficiently with the cloning vector and out-compete the target PCR products.</li> <li>• Gel-purify the PCR product if the PCR template encodes β-lactamase to avoid background colonies on LB-ampicillin agar. If the template and expected PCR product are of similar size, digest the template within the ampicillin resistance gene following the PCR reaction, e.g. with PdmI.</li> </ul> <p><b>3.2. Truncated insert due to contaminating endo- or exonucleases.</b>            Use only the highest quality enzymes for cloning, e.g. LO-tested enzymes (<i>see</i> p.2) and exclude any possibility of endo-, exonuclease and phosphatase contamination in enzyme preparations.</p>
<b>4. Sequence errors in insert</b>	<p><b>4.1. Low fidelity DNA polymerase was used in PCR.</b>            If PCR product will be used for cloning it is always recommended to use high fidelity DNA polymerase with proofreading activity, such as <i>Pfu</i> DNA Polymerase (#EP0671).</p> <p><b>4.2. DNA was damaged by UV light during the excision from agarose gel.</b>            Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When a short-wavelength (254-312 nm) light-box is used, limit DNA exposure to UV to a few seconds. Keep the gel on a glass or on plastic plate during UV illumination. Alternatively, use dyes visible in ambient light to visualize DNA in standard agarose gels (1, 2).</p> <p><b>4.3. Errors in PCR primers.</b>            If the cloned PCR product contains sequence errors or is missing 5' bases and the same error persists in more than one clone, re-order the PCR primers from a reliable supplier and repeat the procedure starting from the PCR step.</p>
<b>5. Colonies without plasmid</b>	<p><b>5.1. Insufficient amount of antibiotic in agar medium.</b>            Use 100 µg/ml of ampicillin in LB-ampicillin agar plates. Allow the LB medium to cool to 55°C before adding the antibiotic. Ampicillin is sensitive to light – long-term exposure to light can lead to low ampicillin concentration in plates.</p> <p><b>5.2. Satellite colonies.</b>            Some fast growing <i>E.coli</i> strains (e.g. C600) degrade ampicillin faster, which leads to formation of smaller satellite colonies around transformants after &gt;16 hours of incubation. Use shorter incubation times and do not use small satellite colonies for clone analysis.</p>

**References**

1. Rand, K.N., Crystal Violet can be used to Visualize DNA Bands during Gel Electrophoresis and to Improve Cloning Efficiency, Elsevier Trends Journals Technical Tips Online, T40022, 1996.
2. Adkins, S., Burmeister, M., Visualization of DNA in agarose gels and educational demonstrations, Anal. Biochem., 240 (1), 17-23, 1996.