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Direct facile screening of recombinant DNA vector constructs

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Abstract

Direct efficient facile screening of bacterial transformants with the goal of selecting, retrieving, and using recombinant DNA is exemplified by simple visual-based colorimetric inspections or fluorescent protein-based assays. We describe pRedScript, which introduces the constitutive expression of a very bright red fluorescent protein into transformants. On agar plates, red colonies are simply visualized in ambient white light in stark contrast to recombinant transformants that are white. In addition, the bright red fluorescence of the reporter protein can also be harnessed as a sensitive signal for screening bacterial promoters during the development of optimized fermentation conditions.

Keywords

Recombinant DNA; Screening; mCherry; Red color

The advent of a colorimetric-based technology that provided a means to visually discriminate recombinant DNA transformants from empty vector transformants within a lawn of thousands of bacterial colonies [1–3] was a great advancement for molecular biology because it allowed for rapid unambiguous screening and use of the recombinant bacteria. Subsequent innovative modifications were reported [4–6], culminating in the generation of a very versatile cloning/colony screening vehicle designated pBlueScript [7]. pBlueScript's colorimetric selection is based on the expression of a portion of the α -subunit of β -galactosidase in *Escherichia coli* strains lacking this portion of the enzyme. This results in an intact active β -galactosidase, which in the presence of X-gal, an analog of lactose, generates a blue dye, that is, blue colonies. However, the insertion of an investigator's sequence of choice into the multiple cloning site (MCS)¹ that is embedded in the α -subunit's coding sequence disrupts production of a native α -subunit. As a result, recombinant bacterial transformants do not produce an active β -galactosidase, cannot metabolize X-gal, and do not exhibit blue color, with colonies remaining white. pBlueScript

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¹Abbreviations used: MCS, multiple cloning site; cDNA, complementary DNA; GFP, green fluorescence protein; UV, ultraviolet; PCR, polymerase chain reaction; SAGE, serial analysis of gene expression.

has been in use for more than 25 years and is a very well proven and robust technology. However, this technology requires special bacteria engineered with appropriate α -subunit gene deletions and X-gal, and ambiguous results are often observed when small inserts are being cloned.

Here we describe a simple modification to pBlueScript that provides an analogous color-based screening for recombinant transformants but requires no additional reagents or specially engineered bacteria. pRedScript (Fig. 1) was generated by cloning the complementary DNA (cDNA) of a red fluorescent protein (mCherry) from pRSET_B-mCherry (from Roger Tsien's laboratory) into pBlueScript using the *Bam*HI and *Eco*RI restriction sites, which placed it in-frame and fused to the short β -galactosidase α -subunit sequence. The resulting vector construct retains constitutive reporter messenger RNA (mRNA) expression from the *lac* promoter as well as the universal sequencing primer sites for M13 reverse and T3 primers and eight commonly used unique restriction sites at the MCS (Fig. 1). As shown in Fig. 2, this resulted in a vector that can be used as a colorimetric-based screening vehicle. Fig. 2A shows the bright reddish-pink colonies that resulted from the protein expression of the very bright mCherry in empty vector transformants. In addition, these bright red colonies are very distinct from the white colonies that resulted from successful insertion of a sequence of choice at the MCS, which disrupts mCherry's reading frame. The red color is readily seen in ambient room light and does not require any instrumentation for visualization. The plate is simply examined in white light because this is an adequate source for the excitation of this fluorescent protein and generation of the red signal. The best contrast and visualization of the white colonies is observed when viewing the plate against a black background. We also noted an enhancement in color intensity after

1 h of storage at 4 °C, which is similar for the blue color development when using pBlueScript. Fig. 2B presents an example demonstrating the use of the vector during the screening of recombinant transformants. In this case, using standard molecular biology protocols, an approximately 665-bp cDNA was cloned at the *Not*I and *Bam*HI sites of pRedScript and DH-5 α transformants were grown on LB- Amp (ampicillin) agar plates overnight (Fig. 2A). Three red colonies and three white colonies were selected and inoculated into overnight cultures. A diagnostic of vectors isolated (plasmid mini-preps) from these cultures was performed using *Sac*II and *Bam*HI, and the resulting digests were resolved on a 1% agarose gel. As can be seen in Fig. 2B, only DNA isolated from white colonies (lanes 1– 3) released the inserted cDNA as two fragments of approximately 200 and 500 bp. Several other clonings have provided similar results, and inserts as small as 80 bp resulted in white colonies (not shown). Thus, pRedScript provides for rapid unambiguous screening of recombinant transformants without the added cost of X-gal or need for special instrumentation.

A previous report [8] described a modification of pBlueScript in a manner similar to that described here. The authors used the expression, or lack thereof, of a mutated green fluorescence protein (GFP) that emitted yellow fluorescence as their reporter. Two main drawbacks to this system were that (i) the yellow color (empty transformants) in ambient light was nearly completely indistinguishable from the normal white/ocher color of the

recombinant transformants and (ii) visualization of the yellow fluorescence required exposure of the samples to potentially harmful ultraviolet (UV) excitation.

We are also aware of three other reports that describe similar fluorescent protein reporter-based prokaryotic screening vectors [9–11]. pZK18S/T [9] was introduced as a fluorescence reporter version of a TA cloning strategy. However, generation of pZK18S/T is a relatively complex endeavor when compared with the simple single-step cloning of mCherry cDNA described above. Thus, generation of this closed circular vector as the first step in generating pZK18T (i.e., the linear “T” overhang form of the vector) is a multistep process. In brief, pZK18S/T is generated as follows: (i) synthesis of complementary oligonucleotides that, when annealed, generate a polylinker with appropriate restriction site overhangs; (ii) polymerase chain reaction (PCR) generation of KillerRed from a commercial vendor (Evrogen); (iii) digestion of the PCR product and its ligation to the polylinker; and (iv) purification of the ligation product and its cloning at the *EcoRI* and *HindIII* sites of pUC18. Thus, this is a labor-intensive and costly process that includes the use of two commercial vectors and PCR reagents. Moreover, pZK18S/T has been generated at the cost of pUC18’s versatile MCS. Thus, TA cloning with this vector, which first requires the generation of pZK18T (i.e., linearization and purification of pZK18S/T), results in inserts that are flanked only by an *EcoRI* site and a *BamHI* site that greatly limits further subcloning strategies. pZK18S [10] has been designed for facile screening of SAGE (serial analysis of gene expression) libraries. This technology is designed to be a very efficient screening tool, with irradiation of transformants with white light eliminating empty vector transformants, which is due to a byproduct of KillerRed’s fluorescence that is toxic to cells. The MCS of this vector has been limited to four restriction sites, with two specifically designed to be complementary to SAGE inserts; thus, the vector’s use as a general screening tool is somewhat limited. p24MGFPm [11] is another example of a relatively complicated alternative to the cloning/screening vector described here. In this case, to identify transformants, a special amber suppressor bacterial strain is required and the insertion of a cDNA occurs at only two restriction sites and must be in-frame with the GFP cDNA. Thus, the transformants are identified as fluorescent GFP fusion proteins under UV light. In addition, protein expression requires induction with arabinose. The requirement of an insert that generates an in-frame fusion construct implies that this strategy generally adds a subcloning (PCR) step. Thus, overall this technology is labor- and time-intensive relative to the screening vector described here, which is based on a simple loss of color.

We also point out that pRedScript could provide a quantifiable fluorescent assay that would be useful for screening bacterial promoters for strength/optimization [12–15]. In such screenings, promoters, combinations of promoters, promoter libraries, or mutated promoter constructs are inserted at the MCS. mCherry expression, or the lack of its expression, would result in a continuous range of fluorescent intensities from no fluorescence to high fluorescence, providing a sensitive readout of promoter characteristics over a range of any selected conditions. The advantage over current technologies is that the signal intensity of mCherry [16] increases the dynamic range of the assay beyond GFP in use at this time, and its $\lambda_{\text{excitation}} = 587 \text{ nm}$ is nonmutagenic.

In summary, we have noted here a simple modification of a well-tested technology (i.e., pBlueScript) that generates a versatile cloning/screening vehicle that we have designated as pRedScript. pRedScript provides a simple red versus white visual screening method for recognizing white colony DNA transformants relative to red colony empty vector transformants without the need for added reagents or special instrumentation. Selection is simply done in ambient room light. However, the bright fluorescence of the reporter can be adapted to quantitative studies, including assays that provide screenings at the industrial level of bacterial promoters during their optimization for robust production of fermentation products or pharmaceuticals.

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Fig. 1.

Schematic representation of pRedScript. (A) Positions of functional sequences of the vector backbone relative to the first base of the *lacZ* promoter (*P_{lac}*): open reading frame (ORF), which includes the MCS that is bounded by *SacI* and *BamHI* restriction sites immediately upstream of the reporter cDNA (mCherry), f1 origin (f1 ori), ampicillin resistance gene (*Amp^R*), and ColE1 origin of replication (ColE1 ori). (B) Linear sequence details of the reporter gene construct indicating the positions of *P_{lac}*, the universal primer sequences (M13 reverse and T3), translational start site of the ORF, restriction sites of the MCS, and the first four and last codons of the mCherry cDNA sequence.

Fig. 2. pRedScript's red (empty) versus white (recombinant) colonies screening/selection utility. (A) Agar plate with a lawn of red and white colonies. The plate was photographed in visible ambient room light. (B) Diagnostic analysis of vectors isolated from overnight cultures of three white colonies (lanes 1–3) alongside three red colonies (lanes 4–6). All six vectors were digested with *Sac*II and *Bam*HI for 1 h at 37 °C, and the digests were resolved in a 1% agarose gel containing ethidium bromide. The molecular weight (bp) ladder used was 1 Kb Plus (Life Technologies, Grand Island, NY, USA). DNA bands were visualized under UV illumination in a G:Box gel documentation imager (Syngene, Frederick, MD, USA).