Virtual Elimination of False Positives in Blue-White Colony Screening

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Blue-white screening is one of the most popular methods for the identification of bacterial colonies that harbor recombinant plasmids. Many vectors in current use, such as the pUC series or M13mp series of plasmids, carry short segments of *E. coli* DNA that contain the regulatory sequences and coding information for the first 146 amino acids of the β -galactosidase (*lacZ*) gene. In a phenomenon known as α -complementation (1,2), this N-terminal segment, known as the α fragment, forms an active complex with an inactive C-terminal fragment of β -galactosidase. This C-terminal fragment, termed the omega (ω) fragment, is borne by a Lac(-) strain of bacteria that contains any of a number of deletions in the 5' end of the *lacZ* gene. As well as being integrated into the bacterial chromosome (as in DH5 α and TOP 10 strains of *E. coli*), the complementing ω fragment will often be carried on an F' episome (such as that found in Stratagene's XL series and Novagen's Nova Blues). This requires the inducer IPTG to inactivate the lac repressor, allowing synthesis of both the ω peptide and the vector-encoded α complement (3). When intact, the enzymatic activity of β -galactosidase cleaves the chromophore, X-gal (Sigma, St. Louis, MO, USA), causing dimerization and nonenzymatic oxidation of the indoxyl monomer sidegroup, leading to the formation of a blue precipitate (4,5). The result is a bacterial colony with a medium- to deep-blue pigmentation.

Polyc cloning sites containing multiple restriction enzyme recognition sites can be inserted in the N-terminus of the β -galactosidase gene present on a plasmid, resulting in only a harmless interpolation of amino acids (2). Cloning of a DNA insert into this polyc cloning region interrupts the β -galactosidase gene, leading to inactivation of the gene with resultant white colonies. This phenomenon is exploited in standard blue-white colony screening, in which colonies containing plasmids without inserts, ostensibly medium to deep blue in

color, can be immediately recognized and passed over in subsequent miniprep assays, while the white ones are selected for further processing. Nevertheless, this technique is far from 100% effective in eliminating false positives. While insertion of any fragment of foreign DNA into the polyc cloning site of a plasmid almost invariably results in the production of an amino-terminal fragment incapable of α -complementation (2), an insert smaller than 500 bp and in frame with the α complement of the β -galactosidase gene can lead to a certain degree of leakiness in the system, resulting in pale-blue colonies or white colonies with light-blue centers on LB Amp agar plates containing 40 μ L (40 mg/mL in dimethylformamide) X-gal and (where required) 4 μ L (200 mg/mL) IPTG (Reference 6 and personal observation based on empirical data). Furthermore, out-of-frame re-ligation of plasmids with slightly damaged ends following restriction endonuclease digestion can result in interruption of β -galactosidase gene expression, leading to the growth of white colonies containing plasmid with no insert. However, we have found that the vast majority of false positives result from incomplete color development of colonies following overnight incubation on various antibiotic-containing agar plates with X-gal or X-gal/IPTG. When a significant proportion of white colonies selected turn out to be false positives, this can represent an unnecessarily expensive and wasteful means to screen for recombinant plasmids containing desired inserts, especially when hundreds of minipreps are being processed. The task of screening numerous colonies has been rendered far less onerous by the availability of excellent commercially produced kits designed to rapidly yield high-quality, clean DNA minipreps that can be subsequently screened for insert, sequenced, etc. However, these systems are not inexpensive, as key kit components such as individual spin columns can approach a cost of \$1/column. Alterna-

Product Application Focus

tively, the use of colony PCR to check for insertion is a reasonably economical means for high-throughput screening, but this process can also be tedious and time consuming.

In a recent study, PCR products of 500, 750, and 1000 bp, generated in separate runs of 35–40 cycles of PCR using *Taq* DNA polymerase under standard conditions (7,8), were gel purified using a QIAquick™ gel extraction kit (Qiagen, Valencia, CA, USA) and ligated into pCR 2.1 TOPO® vector by means of the TOPO TA Cloning system (Invitrogen). Four microliters (80–120 ng) of each recombinant vector were used to transform 50 µL chemically competent aliquots of TOP10 *E. coli*, which were incubated for 1 h in 250 µL SOC medium in a shaker (250 rpm at 37°C) and then grown overnight at 37°C on LB agar plates containing 100 µg/mL ampicillin and overlaid with 40 µL 40 mg/mL X-gal in dimethylformamide (TOP 10 *E. coli* do not require IPTG for blue-white screening). These TOPO cloning reactions yielded numerous colonies (generally more than 100) ranging in color from white to pale blue to very dark blue. This pigmentation was further enhanced by several hours to overnight incubation at approximately 4°C to allow full development of the blue color. Colonies containing active β-galactosidase are lighter blue in the center and dense blue at

their periphery. White colonies occasionally show a faint blue spot in the center to appear pale blue but are colorless at the periphery (2). For demonstration purposes, several sets of 10–20 white or pale-blue colonies were picked and grown up in LB medium containing 50 µg/mL ampicillin or kanamycin for further analysis. In an effort to enhance the efficiency of standard blue-white screening, we incorporated a range 50, 100, or 150 µg/mL (final concentration) of X-gal directly into overnight broth cultures as well. The presence and extent of any color development were noted the following morning. After preparing minipreps and conducting restriction enzyme digests of each sample, we consistently observed that approximately 10% to as high as 40% or 50% of the white or pale colonies, putatively positive for recombinant plasmid, was actually found to contain parental plasmid with no insert. While these had appeared as white or pale colonies on the antibiotic-containing/X-gal agar plates, the overnight selective broth cultures turned greenish to dark blue in color (depending on the concentration of chromophore that had been added), confirming negative for recombinant plasmid. This problem does not lie with a faulty pCR 2.1 TOPO cloning system but with incomplete or misleading color development of colonies on

the X-gal plates. Furthermore, the greater the number of colonies on the plate, the more pronounced this problem will be as X-gal applied to the surface of the agar is depleted in areas of dense colony growth. By incorporating X-gal (as little as 50 µg/mL X-gal was sufficient to observe a color change) into the LB overnight broth cultures, it was found that a significant percentage of false positives could be identified and eliminated before the laborious step of processing and analyzing minipreps.

Based on these findings, InBios International has developed a new product called RecombSelect™, for enhanced efficiency of standard blue-white screening. Several formulations of RecombSelect (default antibiotic = ampicillin, no IPTG) are available for rapid, sure screening of colonies that putatively contain plasmid with insert. The end user simply adds 3 mL sterile water to each tube, allows media pellet to go into solution (30–60 s), and inoculates the tube with a single white or pale blue colony from a selective agar plate. After overnight growth in a shaker (250 rpm at 37°C), tubes containing blue broth are discarded and only straw-colored broth tubes containing true positives for plasmid with insert are processed. In six independent experiments, 10 randomly selected sets of white colonies were grown overnight in RecombSelect. The mean number of false positives (white colonies that turned the medium blue) was determined to be $28.3 \pm 4.8\%$, suggesting that, on average, nearly 30% of the colonies that are randomly

Figure 1. Secondary screening. Ten white TOP 10 *E. coli* colonies, grown at 37°C overnight on an LB/Amp agar plate overlaid with 40 µL (40 mg/mL in dimethylformamide) X-gal, were randomly selected for secondary screening. These were inoculated into 3-mL aliquots of rehydrated RecombSelect and incubated overnight on a shaker (250 rpm at 37°C). Digital photographs were taken using a Nikon Coolpix 990 system. The upper panel shows the colonies as they appeared on the agar plate. The lower panel shows the vivid color development after overnight growth in RecombSelect.

selected and processed in standard blue-white screening do not contain recombinant plasmid. Every blue colony inoculated into RecombSelect turned overnight broth culture blue, but no bacterial growth was ever observed in RecombSelect inoculated with non-transformed bacteria or with bacteria bearing non-AmpR[®] plasmids. Figure 1 shows the results of growing 10 white (putatively positive for insert) colonies from a standard LB/Amp/X-gal plate overnight in RecombSelect. In this particular case, 40% of the cultures turned blue, indicating a negative result for recombinant plasmid. Upon processing all 10 miniprep cultures, tubes 3, 5, 7, and 8 (Figure 1) were found to contain the 3.9-kb pCR 2.1-TOPO parental plasmid without the 750-bp PCR insert used in this particular ligation/transformation experiment. The other straw-colored cultures all yielded recombinant plasmid with the desired insert. Minipreps were generally found to yield 1.0 ± 0.25 μg plasmid DNA/ μL (50 μL total volume) whether or not X-gal is present in the medium. Only 1 in 200–300 plasmid minipreps made from positive (straw-colored) X-gal-containing broth cultures were found to yield plasmid with no insert or no significant insert (unpublished observations). Sequencing of some of these vectors revealed the presence of damaged sequences at the TOPO cloning site that re-ligated without insert or, more rarely, an insertion of a small cassette of 10–25 bp (presumably a PCR artifact), both of which resulted in frame shifts in the *lacZ* gene and interrupted β -galactosidase expression. Similar results to those described above were obtained in experiments using other β -galactosidase blue-white screening vectors, such as pBluescript[®] (Stratagene, La Jolla, CA, USA) (data not shown). Based on these observations, it is concluded that by simply growing overnight miniprep broth cultures in the presence of chromophore (and inducer where required), the number of false positives ultimately processed in blue-white recombinant plasmid screening can be markedly reduced without expending any extra time between plating and results. RecombSelect provides a very economical and time-saving tool to accomplish this.

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