DNA product from the 1 µg of DNA template was between 4 and 25 µg. This was approximately 5–30 times higher than the yield of labeled DNA synthesized from the same template using the digoxigenin labeling mixture from the nonradioactive DNA labeling kit. After labeling, the 20-µL reaction was precipitated with 2.5 µL of 4 M LiCl and 75 µL of 100% ethanol at -20°C for 1 h, after which the centrifugation pellet was washed twice in 70% ethanol, then dried. We find that the addition of the carrier tRNA to the precipitation and the purification of the probe to remove unincorporated dNTPs are absolutely unnecessary. Probe was dissolved in 400 µL of hybridization buffer to a final concentration of approximately 10 ng/µL. At this point, the probe can be stored at -20°C for at least 2 years. Hybridization buffer consists of 50% formamide, 10% dextran sulfate, 200 µg/mL of sheared salmon sperm DNA and 4× standard saline citrate with phosphate. The composition of 1× standard saline citrate with phosphate is: 15 mM sodium citrate, 20 mM sodium phosphate, 120 mM NaCl, 1 mM EDTA, pH 7.0.

Salivary gland squashes were heated in prewarmed 2× standard saline citrate with phosphate in a Coplin jar for 30 min at 68°C. After rinsing for a few seconds in water at room temperature, slides were denatured for 2 min in 70 mM NaOH, washed in 2× standard saline citrate with phosphate (twice, 3 min each), twice in 100% ethanol (5 min each) and then air-dried.

Probe was denatured by heating for 7 min at 80°C in a heating block, and then the denatured probe (50 µL) was placed on top of a microscope slide with the salivary gland squash. Each slide was covered with a Parafilm® coverslip (Oncor, Gaithersburg, MD, USA). We find that these coverslips do not need to be sealed and are very easy to remove after the hybridization. All slides were placed horizontally in a humidified chamber (containing Whatman paper [Clifton, NJ, USA] soaked in distilled water), covered with plastic wrap and hybridized overnight at 37°C.

After the hybridization, the Parafilm coverslips were removed gently and the slides washed in a Coplin jar at 68°C in 2× standard saline citrate with phos-

phate (3 times, 20 min each). After rinsing in phosphate-buffered saline (PBS) at room temperature, the slides were mounted in DAPI/Antifade (Oncor) and viewed in a fluorescence microscope using the combination of DAPI and fluorescein filters (Figure 1). Slides can be kept at 4°C for a few weeks without any noticeable decrease in DAPI or fluorescein signal.

The method described above has a very high level of sensitivity, which allowed us to detect the hybridization signal with probes directed not only against rDNA, which is a multicopy gene and a 30-kb fragment of the *Drosophila* chromosome, but also against a 4-kb single-copy gene (Figure 1).

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Activity Screening of Bacteria Containing *Renilla* Luciferase Plasmids

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Chemiluminescence- and bioluminescence-based detection are rapidly becoming the systems of choice for the analysis of reporter assays (1). Firefly (*Photinus pyralis*) luciferase (FL) has been the most extensively used of the bioluminescence reporters to date. Its high expression level, sensitivity and signal-to-noise ratio make it the reporter gene of choice in most situations. While many other bioluminescence genes have been identified in other species of fireflies and the coelenterates, their utility as bioluminescence reporter genes has not been fully exploited. Recently, a bioluminescence gene from the sea pansy (*Renilla reniformis*) has become commercially available (Promega, Madison, WI, USA). It is difficult to state without scientific data which bioluminescence reporter is the most advantageous; rather, it is dependent on the particular experimental conditions. *Renilla* luciferase (RL) offers all the advantages of FL, but it possesses some unique features of its own. These features include no requirements for divalent cations or ATP and the potential for in vivo monitoring of expression in cells (3,5). We describe a simple procedure to accelerate cloning of the RL gene into functional reporter plasmids. This technique will allow for the rapid identification of bacteria containing RL plasmids in the correct orientation and therefore reduce the time and cost of performing extensive miniprep analysis.

RL is a 36-kDa monomeric protein that, like FL, is functionally active immediately following translation. RL reacts with its substrate, coelenterazine, in the presence of oxygen to yield oxyluciferin, carbon dioxide and blue light (3). In addition, RL has a similar kinetic profile to FL when assayed in the Dual-Luciferase™ Reporter Assay System (4). This system takes advantage of the similarities of these enzymes to rapidly analyze reporter studies following transfection of FL as the reporter plas-

Benchmarks

mid and RL expression plasmid as the control. A plasmid containing only the RL gene (pRL-Null) is also available (Promega), which allows the RL gene to be used as a primary reporter when cloned into the appropriate system.

Low-level expression of FL independent of a bacterial promoter and selection of *E. coli* cultures containing the FL gene have been previously described (2,6). We believe that the comparable activities of these enzymes might allow for similar results in RL cloning experiments. We decided to clone, analyze and express a plasmid containing the RL gene to test this hypothesis.

An in vitro expression plasmid containing the RL gene was constructed as follows. The *NheI/BamHI* fragment from pRL-Null containing the RL gene was blunt-ended by treatment with DNA polymerase I (Klenow) and ligated into pT7Blue-2 (Novagen, Madison, WI, USA) digested with *MscI* and treated with calf intestinal alkaline phosphatase. The resulting construct, pT7 β RL, contains the T7 promoter and the *Xenopus* β -globin 5' untranslated region (UTR) for enhanced in vitro translation upstream of the RL gene (Figure 1A). The ligation reaction was transformed into DH5 α TM *E. coli* (Life Technologies, Gaithersburg, MD, USA) and plated onto LB agar plates supplemented with ampicillin (100 μ g/mL). Eight individual *E. coli* colonies were selected and grown separately overnight in 2 mL LB (also supplemented with ampicillin [100 μ g/mL]) at 37°C. Each bacterial culture (100 μ L) was transferred to a MicroliteTM 96-well plate (Dynatech Laboratories, Chantilly, VA, USA). Stop & GloTM reagent containing the RL substrate coelenterazine was prepared according to the manufacturer's specifications (Promega). The Stop & Glo reagent was added at 100 μ L per well, and the samples were mixed briefly on a plate shaker. Luminescence output readings were determined using a Model ML3000 luminometer (Dynatech) set on integrate flash mode for two seconds per well. Out of the eight cultures analyzed, only clones 3, 5, 6 and 8 showed marked luminescence at levels $>10^8$ relative light units (RLUs), while clones 1, 2, 4 and 7 showed levels only slightly above the broth control

(Figure 1B). This constitutes a luminescence activity range of approximately 1×10^4 between the two sample populations. While this window is quite large, the results will vary depending on the culture density and volume, *E. coli* strain, amount of substrate added and the equipment used to monitor the bioluminescent signal. Qualitatively, similar results were generated using photographic film exposure to the microplate (data not shown). These data suggest that the cultures generating a high luminescent signal contain the RL gene. Following bacterial activity analysis, the remaining overnight cultures can

now be used for restriction analysis.

All eight clones were subject to miniprep DNA isolation using the QIAprepTM Spin Miniprep Kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA, USA) and examined by restriction analysis. Plasmid DNA containing the RL gene in the proper orientation will produce a 459-bp *XbaI* fragment. The same plasmid, but with the RL gene in the opposite orientation, will produce an 1154-bp fragment (Figure 1A). Only the clones that exhibited RL activity (Nos. 3, 5, 6 and 8) produced the *XbaI* fragment of the correct size while the

Figure 1. Cloning strategy and RL activity of minipreps of plasmid pT7 β RL. (A) Schematic detail illustrating the cloning sites used to generate pT7 β RL and the location of the *XbaI* restriction enzyme sites used for screening miniprep plasmid DNA. The *XbaI* site at nucleotide 1041 represents the correct orientation (5' $\rightarrow$ 3') of the insert while nucleotide 346 shows the opposite orientation (3' $\rightarrow$ 5'). The *XbaI* site at nucleotide 1500 is present in the vector sequence and used to orient the insert DNA. (B) The RL activity of bacterial cultures obtained from transformation plates. RLUs are recorded as the signal generated by the luciferase-coelenterazine reaction. LB alone was included as a negative control.

Benchmarks

remaining clones (Nos. 1, 2, 4 and 7) contained the RL gene in the opposite orientation (Figure 2A). This demonstrates that only plasmid DNA containing the RL gene in the proper orientation will express RL at measurable levels in *E. coli* cultures containing pT7 β RL.

Finally, to confirm the plasmid DNA for expression of RL, we expressed all eight plasmid DNAs in a rabbit reticulocyte in vitro system. Briefly, 5 μ L of miniprep plasmid DNA (ca. 0.5–1.0 μ g per reaction) were added to the TNT[®] T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's specifications along with ³⁵S methionine. Following a 90-min reaction at 30°C, the proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE). As demonstrated previously in the above experiments, only clones 3, 5, 6 and 8 containing the RL gene in the correct orientation produced a 36-kDa protein corresponding to the correct size of the RL protein (Figure 2B). These reactions were also run in an identical fashion with unlabeled methionine and tested for RL activity in the luminometer assay. Only the positive clones (Nos. 3, 5, 6 and 8) produced values $>10^8$, comparable to the *E. coli* LB samples (data not shown).

In conclusion, we describe a procedure for preselection of *E. coli* miniprep cultures containing the RL gene. Only the bacterial clones generating a light signal contained the functional RL gene in the proper orientation and could be subsequently expressed in vitro to produce the RL protein. Preliminary characterization of overnight cultures for RL activity greatly reduces the number of minipreps required to select a functionally active plasmid, because only the plasmids generating a light signal need to be checked by restriction analysis. The RL activity was easily determined using the Stop & Glo reagent, but coelenterazine prepared in other appropriate buffers could also be used (3,5).

The promising utility of this bioluminescence gene is just now becoming apparent. Gene expression studies in *Candida albicans* cannot be performed using FL as a reporter because of expression problems associated with codon usage (5). The RL gene can circumvent this dilemma because it does not contain these rare codons. RL reporter systems offer an efficient bioluminescence substitute without compromising sensitivity. Similar to what has been reported for FL, we recognize that use of this technology will greatly expedite the cloning of the RL gene into new and existing reporter-based assay systems, perhaps increasing the use of this effective bioluminescence reporter.

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Isolation of Recombinant Secretory Proteins by Limited Induction and Quantitative Harvest

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Vectors with features that direct recombinant proteins to the periplasm are used extensively for the expression of heterologous secretory products in *Escherichia coli*. Many of these vectors encode bacterial secretion signals that facilitate the membrane translocation of the recombinant protein, with removal of the secretion signal occurring concomitantly. When exported to the periplasm, heterologous secretory proteins are more likely to be isolated

Figure 2. Confirmation of RL gene orientation and in vitro expression of plasmid pT7 β RL. (A) The *Xba*I restriction digests of the eight miniprep DNA samples were resolved on a 0.9% TBE gel, stained with ethidium bromide and photographed. Arrow A shows the 459 bp present in clones containing the RL gene in correct orientation as illustrated in Figure 1. Arrow B shows the 1154 bp produced by the RL gene in the opposite orientation. (B) In vitro expression analysis of the same miniprep DNA samples separated on a 15% SDS polyacrylamide gel. The commercial plasmid RL-TK (Promega) was included as a size standard for the RL gene.