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Evaluation of NanoLuc and GFP reporter-labeled control strains for Shiga toxin-producing *Escherichia coli* (STEC) Assays

Jodie Y. Lee, M.S. and Dev Mittar, Ph.D.

ATCC, Manassas, Virginia, USA

Introduction

Positive controls are essential for establishing assay performance and equipment efficacy. Yet, some food testing laboratories refrain from using bacterial strains as positive controls for fear of cross-contaminating their samples. Under the Food and Drug Administration (FDA) Food Safety Modernization Act[1], laboratories face an increasing number of regulations to expand testing for objectionable organisms. Control strains with unique, easily detectable traits which distinguish positive control strains from actual food contaminants can help differentiate true contamination from control strain cross-contamination.

In this study, we introduced shuttle vectors encoding either green fluorescent protein (GFP, Life Technologies) or NanoLuc® (Promega)[2] into *Escherichia coli* strains, including Shiga toxin-producing O157 (*stx1*$^{+}$, *stx2*$^{+}$, *eaeA*$^{+}$) and non-Shiga toxin-producing O157 (*stx1*$^{-}$, *stx2*$^{-}$, *eaeA*$^{-}$), for use in food pathogen detection assays (Table 1). Both reporters can be easily visualized without specialized detection equipment; GFP fluoresces when excited by UV light, while bacteria engineered with NanoLuc emit a strong light signal in the presence of a chemical substrate. Upon establishing the detectability of NanoLuc in the *E. coli* O157 strains, the reporter was transformed into the "Big Six" non-O157 *E. coli* strains (serogroups: O26, O45, O103, O111, O121, and O145) for use as reporter-labeled positive controls (Table 1).

Table 1: ATCC reporter-labeled strains

Serotype	Genotype	NanoLuc (ATCC® No.)	Designation	GFP (ATCC® No.)	Designation
O157:H7	*stx1+, stx2+, eaeA+*	BAA-2586™	*E. coli* O157:H7-Nanoluc	BAA-2586GFP™	*E. coli* O157:H7-GFP
O157:H7*	*stx1-, stx2-, eaeA-*	BAA-2587™	*E. coli* NT O157:H7-Nanoluc	BAA-2587GFP™	*E. coli* NT O157:H7-GFP
O26:H11	*stx1+, stx2+, eaeA+*	BAA-2580™	*E. coli* O26:H11-Nanoluc	--	--
O45:H2	*stx1+, stx2-, eaeA+*	BAA-2581™	*E. coli* O45:H2-Nanoluc	--	--
O103:H11	*stx1+, stx2-, eaeA+*	BAA-2582™	*E. coli* O103:H11-Nanoluc	--	--
O111	*stx1+, stx2+, eaeA+*	BAA-2583™	*E. coli* O111-Nanoluc	--	--
O121:H9	*stx1-, stx2+, eaeA+*	BAA-2584™	*E. coli* O121:H9-Nanoluc	--	--
O145	*stx1-, stx2+, eaeA+*	BAA-2585™	*E. coli* O145-Nanoluc	--	--

*Non-toxinogenic (NT)

Results

Reporter Signal Detection

A plasmid bearing either the *nanoluc* or *gfp* gene was transformed into both a toxinogenic and non-toxinogenic strain of *E. coli* O157:H7 to create reporter-labeled strains. The NanoLuc reporter was visualized by exposing cells directly to a 1:49 mixture of Nano-Glo® Luciferase Assay Substrate and Nano-Glo® Luciferase Assay Buffer (Promega), respectively. Three scaffold formats of NanoLuc detection were explored: 1) cotton swab, 2) filter paper, and 3) pelleted broth culture. In each of these methods, cells were subsequently lysed and visualized in a single step to detect the presence of the reporter. Colonies that expressed *gfp* were visualized by exposing the plate to UV light. Each detection method produced a strong, clear signal that was visible to the naked eye (Figure 1).

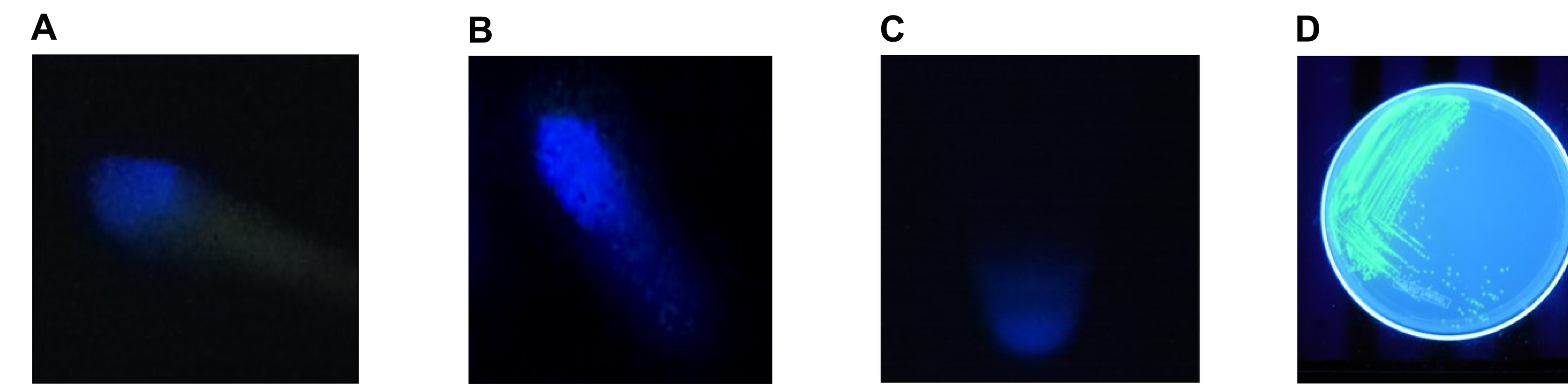

Figure 1: Visualization of the reporters-labeled strains. Toxinogenic *E. coli* O157:H7 transformed with a plasmid bearing *nanoluc* (*E. coli* O157:H7-Nanoluc) or *gfp* (*E. coli* O157:H7-GFP) were grown at 37°C on Tryptic Soy Agar (TSA). Visualization was realized by A) swabbing the surface of the *E. coli* O157:H7-Nanoluc plate with a cotton swab to collect 10-15 colonies, then soaking the swab in 50 µL of detection reagent, B) scratching individual *E. coli* O157:H7-Nanoluc colonies on filter paper soaked with 50 µL of detection reagent, C) pelleting 1 mL of an *E. coli* O157:H7-Nanoluc broth culture and resuspending the pellet in 50 µL of detection reagent, or D) exposing the *E. coli* O157:H7-GFP plate to UV light (302 nm).

Chromogenic Medium

Chromogenic media may be used to assist in the identification *E. coli* serotypes; the color of colonies from reporter-labeled strains and their progenitor strains should be in the same color family. Reporter-labeled strains bearing *nanoluc* or *gfp* plasmids were compared with their progenitor strains to identify phenotypic changes on Rainbow® Agar (Biolog). Color differences between reporter-labeled and progenitor strains were minimal (Figure 2).

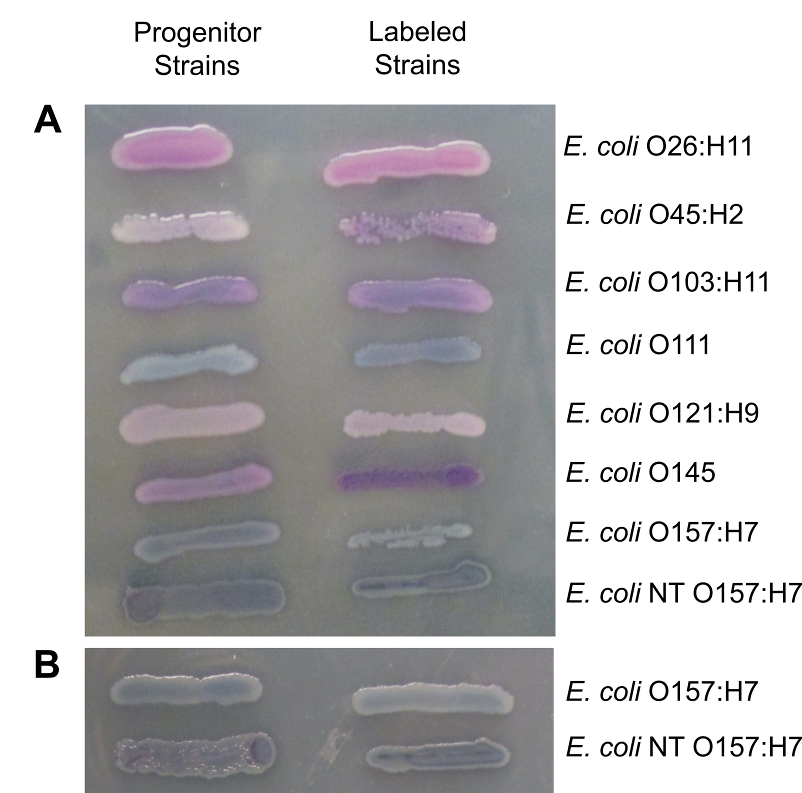

Figure 2: Chromogenic phenotype on rainbow agar. Rainbow Agar was prepared according to manufacturer specifications. The progenitor strains (left) and the reporter-labeled strains (right) were streaked on a plate and incubated for 16h at 37°C. The chromogenic properties of progenitor strains were compared to those of reporter-labeled strains containing A) *nanoluc* and B) *gfp*.

Growth Effects

To determine whether *gfp* (data not shown) or *nanoluc* expression affects the growth and viability of the reporter-labeled *E. coli*, growth studies were performed (Figure 3). The growth constant (k) varied from 1.2±0.1 to 2.5±0.2 generations/hour in the absence of the plasmid and from 1.3±0.0 to 2.1±0.0 generations/hour in the presence of the plasmid. The doubling time (g) varied from 23.7±1.5 to 48.5±2.7 minutes in the absence of the plasmid and from 29.3±0.5 to 46.5±0.1 minutes in the presence of the plasmid. The maximum decrease in the growth constant was a 28.6% drop for *E. coli* O42:H2-Nanoluc. The maximum increase in doubling time was 46.8% for *E. coli* O121:H19-Nanoluc. A paired Student's t-test was performed (data not shown); with the exception of *E. coli* O111 (k, p=0.003; g, p=0.003), the changes were not statistically significant. All changes were within an acceptable range as these strains were intended for qualitative assays that simply require visible growth after overnight culturing on plates.

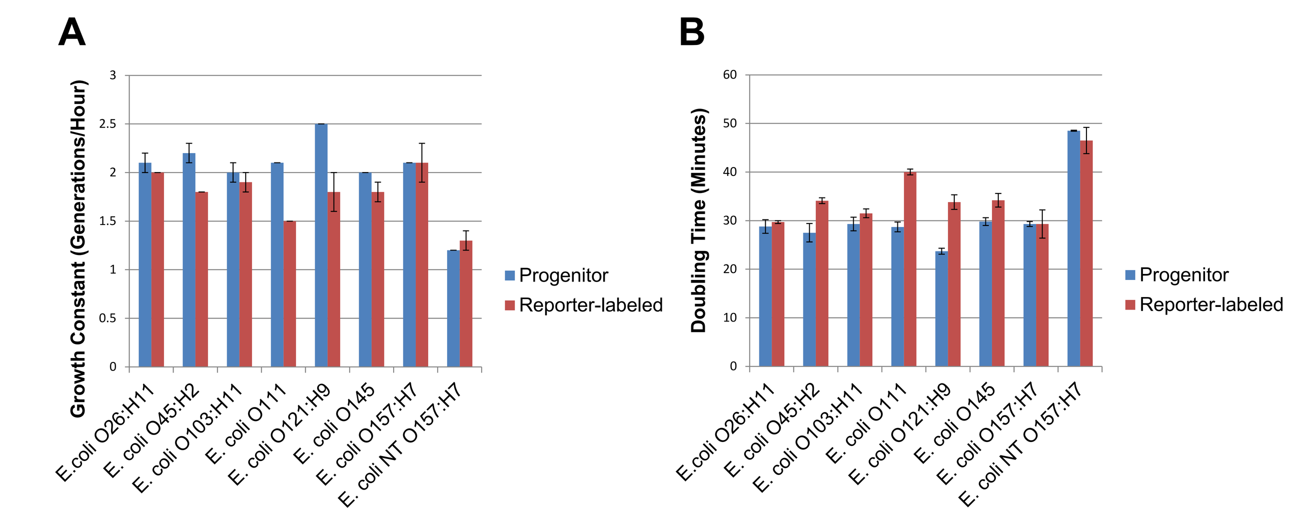

Figure 3: Growth rate of progenitor and reporter-labeled strains of *E. coli*. Growth curves were performed in triplicate to determine the A) growth constant (k) and B) doubling time (g). Using the Bioscreen C MBR (Oy Growth Curves Ab Ltd.), 200 µL cultures were prepared with a 1:100 inoculum from an overnight culture and were incubated at 37°C in Tryptic Soy Broth (TSB) with constant shaking.

Plasmid Stability

To determine the stability of the *nanoluc* plasmid, the engineered reporter-labeled strains were passaged once every 24h under temperature stress at 42°C. The percentage of NanoLuc positive colonies varied depending on the strain, representing between 67-100% of the population after two days (Figure 4). This level of plasmid stability is within an acceptable range for the intended qualitative testing workflow.

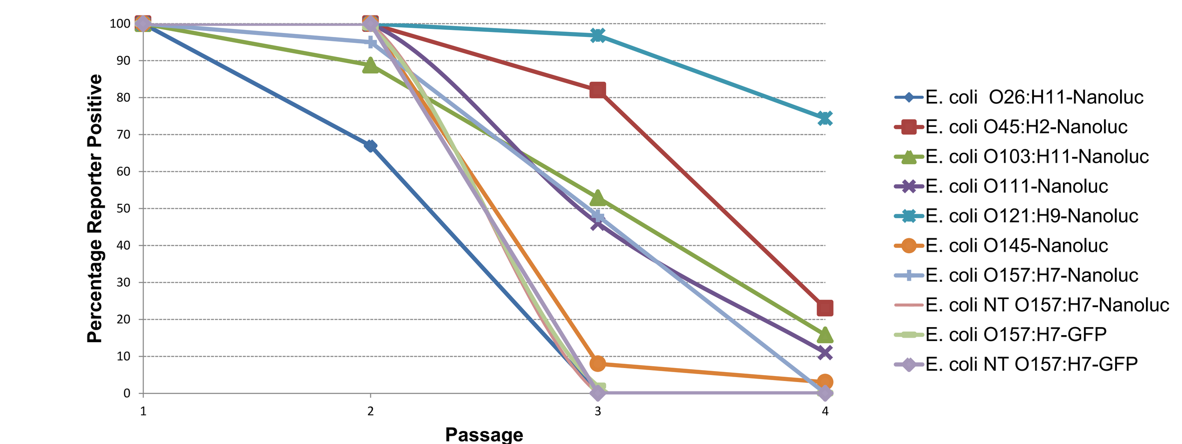

Figure 4: Plasmid stability. Reporter-labeled strains were grown in 5 mL of TSB at 42°C and passaged 1:100 into fresh TSB once every 24h. A serial dilution was performed to obtain a countable number of colonies. 100 µL of an appropriate dilution was plated on TSA and incubated overnight at 37°C. The percentage of colonies expressing the reporter was recorded daily over four days.

Conclusions

In this study, multiple serotypes of *E. coli* were engineered with NanoLuc and GFP reporters. Phenotypic changes between the progenitor and reporter-labeled strains were minimal on chromogenic medium. As expected, growth rate differences between the progenitor and reporter-labeled strains were present in liquid culture, but were acceptable for the qualitative assays for which the strains were designed. The *nanoluc* plasmid was stable in bacterial populations for ≥2 days. These reporter-label bacteria strongly emit light and can be detected in as little as 15 minutes, eliminating uncertainty about cross-contamination. This study demonstrates that the NanoLuc and GFP reporter-labeled QC strains can be routinely used as positive controls to increase reliability in food testing assays.

References

1. http://www.fda.gov/food/guidanceregulation/fsma/ucm239907.htm
2. Promega. *Technical Bulletin: Luciferase Assay System*. 2011.

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ATCC 10801 University Boulevard, Manassas, Virginia 20110-2209 phone: 800.638.6597 email: tech@atcc.org www.atcc.org