The Single Step (KRX) Competent Cells: Efficient Cloning and High Protein Yields

ABSTRACT | We describe a new strain of *Escherichia coli* that has advantageous features for cloning and screening plus engineered attributes for tightly controlled protein expression. This new KRX strain is compatible with blue/white screening and can be made highly competent. In addition, KRX allows T7 RNA polymerase-based protein expression, one of the most widely used expression systems due to its well-defined promoter and the rapid elongation rate of the polymerase. In this strain, the T7 RNA polymerase gene is controlled by a rhamnose promoter. When we used the KRX strain to express firefly luciferase protein, the precise control of the rhamnose operon resulted in a dramatic induction ratio of 1,700-fold upon addition of rhamnose, whereas the leaky IPTG-inducible T7 RNA polymerase-based system in BL21(DE3)-derived strains only showed an 8- to 43-fold induction ratio, primarily due to high pre-induction levels of protein expression. Protein expression levels in KRX for three additional proteins were shown to be as high, or higher than, levels in BL21(DE3)-derived strains.

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The Single Step (KRX) cells are highly competent, allow blue/white screening, and have mutations that make them *end*A–, *rec*A–, *omp*T– and *omp*P–.

INTRODUCTION

Escherichia coli remains the first choice of many researchers for producing recombinant protein for functional studies due to its ease of use, well established protocols, rapid cell growth and low cost of culturing. BL21(DE3) is a popular strain for recombinant protein expression because it can produce relatively high yields of recombinant proteins. Unfortunately, BL21(DE3) is notorious for low transformation efficiency and leaky expression under uninduced conditions. Researchers often need to clone using an *E. coli* host with good transformation characteristics first, then transfer the desired clone to the expression host. We have developed a new *E. coli* host, KRX, that provides protein yields comparable to those of BL21(DE3) but with much higher transformation efficiencies. This new KRX strain allows cloning and protein overexpression using a single strain.

KRX is an *E. coli* K12 derivative that can be made highly competent and has salient features associated with cloning and screening strains. The strain has engineered attributes to optimize controlled protein expression. Attributes that make this a good cloning strain are partially defective restriction systems (*hsd* and e14), a lack of the most common nuclease that copurifies with plasmid DNA isolated from *E. coli* (*end*A–), and a preventive mutation to minimize undesirable recombination events (*rec*A–). KRX can also be used for blue/white screening, as it is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the *lac*Z gene. The partial deletion in the episomal (F´ factor) copy of the *lac*Z gene (Δ(*lac*Z)M15) can be complemented by adding a functional α-peptide encoded by a plasmid cloning vector. Bacterial colonies are blue on X-Gal medium if α-complementation occurs, but white if α-complementation does not occur. This blue/white screening identifies recombinant colonies.

KRX also has attributes that make it a good protein expression strain. The *omp*T– and *omp*P– mutations eliminate one source of proteolysis of overexpressed protein in *E. coli*. KRX incorporates a chromosomal copy of the T7 RNA polymerase gene driven by a rhamnose promoter (*rha*P_{BAD}) to provide dramatic control of recombinant protein expression via a T7 promoter. T7 RNA polymerase-based systems (3) are one of the most widely used expression systems by virtue of the well-defined promoter, which is completely independent of *E.coli* RNA polymerase promoters, and the rapid elongation rate, about five times that of *E.coli* RNA polymerases. Since these systems have been in use for two decades, many vectors are commercially available to overproduce proteins in *E. coli* using the T7 promoter, including the Flexi® Vectors.

The T7 RNA polymerase gene replaces the *rha*BAD gene such that its expression is controlled by the *rha*P_{BAD} promoter. This promoter is subject to catabolite repression by glucose and activated by addition of rhamnose to the medium. This provides precise control of T7 RNA polymerase abundance and thereby precise control of recombinant protein production. *rha*P_{BAD}-driven expression is positively controlled through a regulatory cascade of two activators. Rhamnose induces the activator RhaR, which induces RhaS, which then activates transcription from the rhaP_{BAD} promoter (1,2). Since the isomerase (RhaA), kinase (RhaB) and aldolase (RhaD) are deleted and replaced with the gene for T7 RNA polymerase in KRX, rhamnose is not metabolized by the cell and is not consumed during growth.

HIGH TRANSFORMATION EFFICIENCY

To test the transformation efficiency of KRX, three independent competent cell preparations were made according to a modified procedure of Hanahan (4), dispensed into 50µl and 200µl aliquots and stored at –70°C. We then measured transformation efficiency using pGEM®-3Z Vector (Cat.# P2151). The transformation procedure was different for the two dispensed sizes. For the 50µl size, 10pg of plasmid DNA was added directly to a 1.5ml microcentrifuge tube containing thawed competent KRX cells. The DNA and cells were incubated for 30 minutes on ice, then heat shocked for 25 seconds at 42°C. The cells were placed on ice for 2 minutes, then 450µl of ice-cold SOC medium was added to allow cells to recover. Recovery was done by taping tubes on their sides to a 37°C incubator platform and incubating with shaking at 225rpm for one hour. For competent cells in the 200µl size, a total of 100µl of thawed competent KRX cells was added to an ice-cold 17 × 100mm polypropylene tube, and 100pg of plasmid DNA was added. The DNA and cells were incubated for 10 minutes on ice, then heat shocked for 47 seconds at 42°C. The cells were placed on ice for 2 minutes, then 900µl of ice-cold SOC medium was added. Cells were allowed to recover in a 37°C incubator, with shaking at 225rpm and tubes at a 30° angle, for one hour. The cells were plated on ampicillin-containing LB plates and incubated overnight at 37°C. Ampicillin-resistant colonies were counted to determine transformation efficiency. For the 50µl and 200µl sizes of Single Step (KRX) Competent cells, efficiencies were 3.36 ± 0.27 × 10^8cfu/µg and 3.21 ± 0.71 × 10^8cfu/µg, respectively. This is comparable to other commercially available highly competent cells, such as JM109 Competent Cells (Cat.# L2001).

KRX Genotype

[F′, *tra*D36, Δ*ompP*, *proA*$^+$B$^+$, *lacI*^{q}, Δ(*lacZ*)M15] Δ*ompT*, *endA1*, *recA1*, *gyrA96* (Nal^r), *thi-1*, *hsdR17* (r_k^-, m_k^+), e14$^-$ (McrA$^-$), *relA1*, *supE44*, Δ(*lac-pro*AB), Δ(*rha*BAD)::T7 RNA polymerase

The pF1K T7 Flexi® Vector allows expression of native (untagged) proteins using the T7 RNA polymerase promoter. The vector contains the kanamycin-resistance gene for selection in *E. coli*.

LOW PRE-INDUCTION EXPRESSION LEVELS WITHOUT SACRIFICING FINAL YIELD

BL21(DE3)-derived expression strains use the L8-UV5 *lac* promoter (5,6), which has three point mutations relative to wildtype *lac* promoters. All three mutations contribute to a stronger promoter; two of these mutations reduce dependence on cAMP levels, and the remaining mutation reduces the promoter's sensitivity to glucose. While these mutations lead to high levels of T7 RNA polymerase production upon IPTG addition, they also contribute to a significant basal expression of T7 RNA polymerase, which can lead to problems with cloning and expressing genes toxic to *E. coli* in vectors with T7 promoters (7). One way to overcome this problem is to reduce basal expression of T7 RNA polymerase by expressing an inhibitor, T7 lysozyme, carried on the pLysS plasmid (8).

To analyze transcriptional control in KRX, we measured expression of firefly luciferase under the control of the T7 promoter in the pF1K T7 Flexi® Vector (Cat.# C8451). We compared KRX performance to that of BL21(DE3) and its derivatives: BL21(DE3)pLysS and Rosetta™ 2 pLysS. Starter cultures were grown overnight at 37°C and diluted 1:100 in Terrific Broth the following morning. When cultures reached an O.D.$_{600}$ of 0.8–1.0, they were moved to a 25°C incubator, and when the cultures reached an O.D.$_{600}$ of 1.0–1.5, they were induced with either 0.1% rhamnose (KRX strain) or 1mM IPTG [BL21(DE3) derivatives] and grown overnight at 25°C. Samples were removed for luciferase assays prior to and after induction. Luciferase activity was measured, and the results are presented in Figure 1 and Table 1, with the luminescence (in relative light units, RLU) normalized to cell density (O.D.$_{600}$).

The inset of Figure 1 shows pre-induction protein expression levels in KRX compared to levels in BL21(DE3) strains. Pre-induction expression of luciferase was 7- to 20-fold lower in KRX using T7 RNA polymerase under rhamnose control than that in BL21(DE3) strains. The stringent control provided by the rhamnose-driven T7 RNA polymerase may allow cloning of proteins toxic to *E. coli*. Furthermore, the post-induction levels of luciferase were higher in KRX, with 2- to 29-fold higher production of active luciferase compared to strains using T7 RNA polymerase under IPTG control. The precise control of the rhamnose operon resulted in a dramatic induction ratio of 1,702-fold for luciferase, whereas the leaky IPTG-inducible systems only showed an 8- to 43-fold induction ratio (Figure 1). More importantly, KRX did not sacrifice total protein yield to achieve this low pre-induction level of protein expression.

HIGH PROTEIN YIELDS

We compared expression levels of three additional proteins, each with mammalian codon usage, using KRX and the same panel of BL21(DE3)-derived strains. hRL is a synthetic version of *Renilla* luciferase with codons modified from those of the sea pansy to those frequently used in human genes. PKRACA is the human α catalytic subunit of protein kinase A (GenBank® accession number NM_002730), and CRBP2 is the human cellular retinol binding protein II (GenBank® accession number U13831). As with firefly luciferase, hRL and CRBP2 protein-coding regions were cloned in the pF1K T7 Flexi® Vector. PKRACA was cloned in pFN6K (HQ) Flexi® Vector (Cat.# C8521), which appends an HQ, metal-affinity tag to the amino terminus of PKRACA. The Flexi® Vectors all use the T7 promoter to control transcription and protein expression. Cell cultures were grown overnight at 25°C and protein expression induced as described for firefly luciferase. The cell density of each culture (O.D.$_{600}$) was measured, and an equal number of cells were centrifuged. Cell pellets were resuspended in TE buffer and lysed in a Mini-BeadBeater-1™ (BioSpec Products, Inc.).

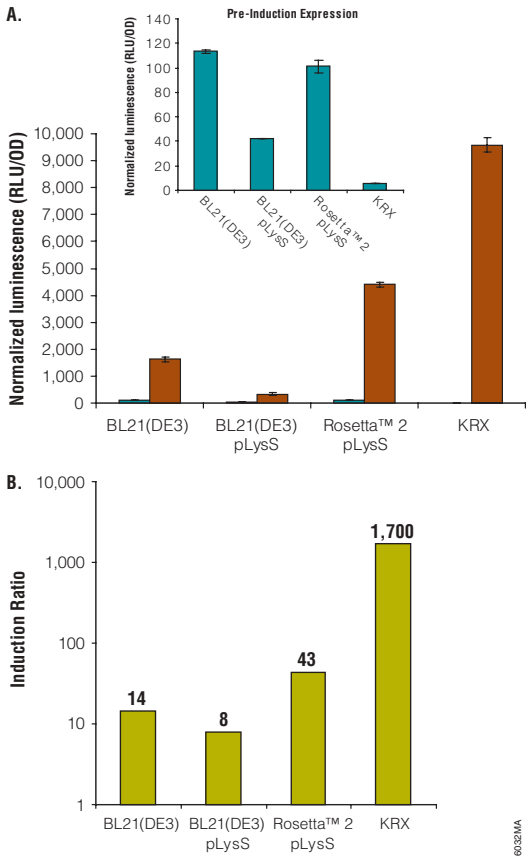

Figure 1. Pre-induction and post-induction expression levels of firefly luciferase. Cells were transformed with the pF1K T7 Flexi® Vector containing the firefly luciferase gene. Starter cultures were grown overnight in LB medium supplemented with 0.2% glucose and 30µg/ml kanamycin at 37°C with shaking at 275rpm. Overnight cultures (30µl) were inoculated into 3ml of Terrific Broth containing 30µg/ml kanamycin. Cultures were grown at 37°C with shaking at 275rpm until they reached an optical density ($O.D._{600}$) of 0.8–1.0. Cultures were then moved to a 25°C incubator shaker. When cultures reached an $O.D._{600}$ of 1.0–1.5, protein expression was induced by adding either 0.1% rhamnose or 1mM IPTG. Cultures were grown overnight at 25°C with shaking at 275rpm. Samples were removed prior to induction ($O.D._{600}$ of 1.04–1.15) and after induction ($O.D._{600}$ of 9.64–10.88) for luciferase assays. **Panel A.** The level of firefly luciferase expression was determined by combining 2µl of culture with 18µl of water and 20µl of Bright-Glo™ Luciferase Assay Reagent. Light output was measured for 10 seconds on a TD-20/20 luminometer. Pre-induction and post-induction firefly luciferase expression levels were normalized to cell number by dividing luminescence by the $O.D._{600}$ (n = 3). **Panel B.** Induction ratios were calculated by dividing the post-induction luminescence values by the pre-induction values.

Insoluble debris was removed by centrifugation, and the soluble supernatant fractions were analyzed by SDS-PAGE. The results are shown in Figure 2.

The level of firefly luciferase protein expression observed by SDS-PAGE (Figure 2, Panel A) is similar to that seen by measuring luciferase enzyme activity (Figure 1) with one exception. The 61kDa firefly luciferase protein was clearly visible prior to induction in BL21(DE3) and Rosetta™ 2 (DE3)pLysS. After induction, KRX exhibited the highest yield of luciferase, while BL21(DE3)pLysS had such poor yield that the protein band was barely visible. The BL21(DE3) post-induction levels were much higher than expected based on the luciferase activity measurements shown in Figure 1. This indicates that most of the luciferase expressed in BL21(DE3) is nonfunctional. For the 31kDa hRL protein, some of the BL21(DE3)-derived strains had a clearly visible 36kDa band visible prior to induction, whereas KRX had little visible expression, yet KRX provided comparable expression to BL21(DE3) and BL21(DE3)pLysS after induction (Figure 2, Panel B). Surprisingly, Rosetta™ 2 (DE3)pLysS showed little expression of hRL. The 16kDa CRBP2 protein showed excellent expression in KRX but very poor expression in the BL21(DE3)-derived strains (Figure 2, Panel C). The HQ-tagged 42kDa PKRACA showed good expression in both KRX and Rosetta™ 2 (DE3)pLysS, but poor expression in the other BL21(DE3)-derived strains (Figure 2, Panel D).

As we performed these comparative protein expression studies, we noted another difference between KRX and the BL21(DE3)-derived strains. The lag phase after dilution of the overnight culture to regain exponential growth was shorter for KRX. We measured the time required from dilution to attain an $O.D._{600}$ of 0.8 for the four strains containing firefly luciferase cloned in the pF1K T7 Flexi® Vector. The lag phase was about 3.25 hours for KRX. BL21(DE3)pLysS had a lag phase more than twofold longer at 7.5 hours, while Rosetta™ 2 (DE3)pLysS required 10 hours, and BL21(DE3) required 11 hours. The length of this lag phase makes evaluating protein expression in a reasonable workday a significant issue for the BL21(DE3)-derived strains.

During lag phase of bacterial growth, there is no apparent increase in cell numbers. Cells are adjusting to the new nutrient-rich medium and have a high rate of metabolic activity as they synthesize the components necessary for cell division.

SUMMARY

The new KRX protein expression strain is designed for efficient transformation and tightly controlled protein expression. Transformation efficiencies are $>10^8$cfu/µg, similar to other highly competent cells. Single Step (KRX) Competent Cells are available in both single transformation (50µl) and multiple transformation (200µl) sizes. KRX lacks a major nuclease responsible for degrading plasmid DNA preparations (*end*A–), minimizes unwanted recombination events (*rec*A–), and is compatible with blue/white screening, making it useful for general cloning applications. For overexpression of cloned proteins, KRX provides dramatic control of expression of protein-coding regions expressed from a T7 promoter. Pre-induced expression levels are significantly lower (7- to 20-fold) than those of BL21(DE3)-derived expression strains, yet the fully induced levels are similar to, or exceed, those of BL21(DE3)-derived strains. The KRX strain also lacks the *omp*T and *omp*P proteases, eliminating one source of over-expressed protein in *E. coli*. This one strain consolidates the best attributes of a good cloning strain and a good protein expression strain, avoiding the need to transfer to another strain for expression. Using KRX also saves time as it more rapidly exits lag phase and enters exponential growth than BL21(DE3)-derived strains, such that induction protocols fit more easily into the workday.

Pre-induction levels of firefly luciferase expression were 7- to 20-fold lower than in BL21(DE3)-derived strains and post-induction levels were 2- to 29-fold higher.

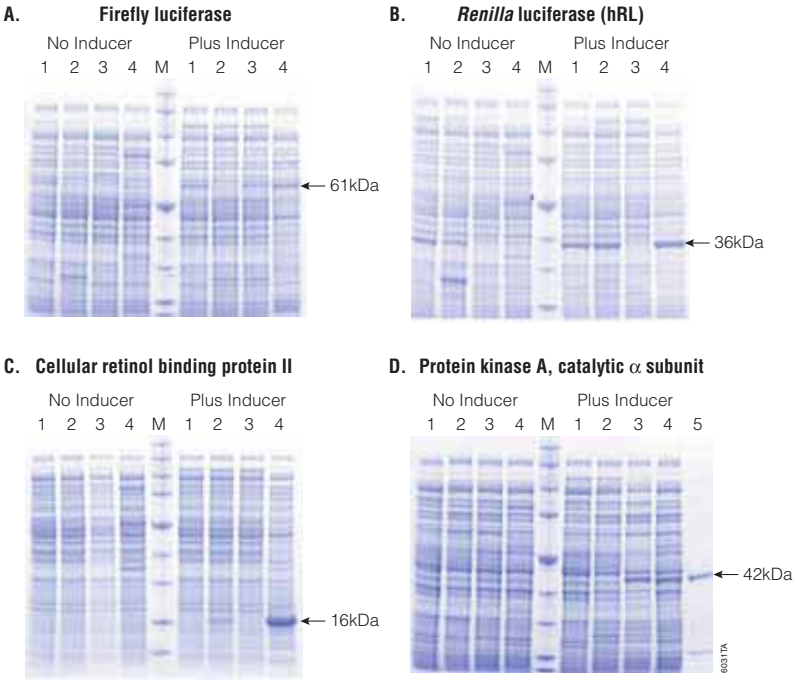

Figure 2. Comparison of proteins overexpressed in KRX and BL21(DE3)-derived strains. Starter cultures were grown overnight in LB medium supplemented with 0.2% glucose and 30µg/ml kanamycin at 37°C with shaking at 275rpm. Overnight cultures (30µl) were inoculated into 3ml of Terrific Broth containing 30µg/ml kanamycin. Cultures were grown at 37°C, with shaking at 275rpm until they reached an optical density ($O.D._{600}$) of 0.8–1.0. Cultures at that stage were moved to a 25°C incubator shaker. When the cultures reached an $O.D._{600}$ of 1.0–1.5, protein expression was induced with either 0.1% rhamnose or 1mM IPTG. Cultures were grown overnight at 25°C with shaking at 275rpm. The cell density of each culture ($O.D._{600}$) was measured the next morning to normalize the number of cells. Cultures were centrifuged at 10,000rpm for 10 minutes in a microcentrifuge. Cell pellets were resuspended in 450µl of TE buffer and lysed in a Mini-BeadBeater-1™ (twice at 5,000rpm for 20 seconds). Insoluble debris was removed by centrifugation at 14,000rpm for 5 minutes in a microcentrifuge. Soluble fractions were analyzed on a 4-12% NuPAGE® gel (Invitrogen), and the gels were stained with SimplyBlue™ Safestain (Invitrogen). Lane 1, BL21(DE3); lane 2, BL21(DE3)pLysS; lane 3, Rosetta™ 2 (DE3)pLysS; lane 4, KRX; lane 5, HQ-tagged protein kinase A, catalytic (alpha) subunit purified using the MagneHis™ Protein Purification System according to *MagneHis™ Protein Purification System Technical Manual* #TM060; lane M, protein molecular weight marker.

REFERENCES

1. Egan, S.M. and Schleif, R.F. (1993) *J. Mol. Biol.* **234**, 87–98.
2. Haldimann, A., Daniels, L. and Wanner, B. (1998) *J. Bacteriol.* **180**, 1277–86.
3. Studier, F.W. and Moffat, B.A. (1986) *J. Mol. Biol.* **189**, 113–30.
4. Hanahan, D. (1985) *DNA Cloning, Vol. 1*, Glover, D., ed., IRL Press, Ltd., 109.
5. Grossman, T.H. *et al.* (1998) *Gene* **209**, 95–103.
6. Pan, S.H. and Malcolm B.A. (2000) *Biotechniques* **29**, 1234.
7. Studier, F.W. (1991) *J. Mol. Biol.* **219**, 37–44.
8. Moffat, B.A. and Studier, F.W. (1987) *Cell* **49**, 221–7.

ORDERING INFORMATION

Products	Size	Cat.#
Single Step (KRX) Competent Cells	5 × 200µl	L3001
	20 × 50µl	L3002