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Selection of mRNA 5′-untranslated region sequence with high translation efficiency through ribosome display

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ABSTRACT

The 5′-untranslated region (5′-UTR) of mRNAs functions as a translation enhancer, promoting translation efficiency. Many in vitro translation systems exhibit a reduced efficiency in protein translation due to decreased translation initiation. The use of a 5′-UTR sequence with high translation efficiency greatly enhances protein production in these systems. In this study, we have developed an in vitro selection system that favors 5′-UTRs with high translation efficiency using a ribosome display technique. A 5′-UTR random library, comprised of 5′-UTRs tagged with a His-tag and *Renilla* luciferase (R-luc) fusion, were in vitro translated in rabbit reticulocytes. By limiting the translation period, only mRNAs with high translation efficiency were translated. During translation, mRNA, ribosome and translated R-luc with His-tag formed ternary complexes. They were collected with translated His-tag using Ni-particles. Extracted mRNA from ternary complex was amplified using RT-PCR and sequenced. Finally, 5′-UTR with high translation efficiency was obtained from random 5′-UTR library.

In vitro translation systems provide a powerful tool for protein research. These systems commonly use lysates from *Escherichia coli*, wheat germ or rabbit reticulocytes, which include essential molecules necessary for translation with the exception of template RNA. Compared to in vivo expression methods, in vitro translation systems can express proteins rapidly. This is suitable for a myriad of applications including the synthesis of biologically important proteins. However, the amount of protein produced may be limited by many factors [1–3], including starvation of substrate molecules and degradation of template RNA. Two methods, continuous-flow and continuous-exchange, overcome these problems by supplying substrates continuously and removing by-products inhibit translation efficiency [4]. However, these methods extend the reaction time to increase the amount of synthetic protein and require excessive amounts of reagents as well as specific equipment. For these reasons, we have created an alternative in vitro translation method to increase protein synthesis by utilizing the 5′-UTR of mRNA.

In living cells, the 5′-UTR of mRNA regulates translational efficiency [5]. To increase protein synthesis of eukaryotic in vitro translation, a translation enhancer sequence is frequently added to the RNA templates. For example, the 5′-leader sequence (omega) of tobacco mosaic virus (TMV) has been incorporated into the wheat germ in vitro translation system [6–8]. Addition of this sequence increases the translational expression of reporter genes by 2- to 10-fold. Similarly, the Kozak consensus sequence (ACCAAUGG) in addition to translation enhancer sequences, such as the 5′-UTR of *Xenopus* β-globin, are essential for efficient in vitro translation initiation in rabbit reticulocyte lysate, the most widely used in vitro translation system [9–11]. Addition of these translational enhancer sequences leads to an increase in protein expression by 10- to 300-fold as compared to other endogenous UTR sequences.

A method to select sequences with high translation efficiency has been recently developed using the eukaryotic in vitro translation system [12,13]. In these studies, the translation efficiency is defined as the number of recruited ribosomes to an RNA template, which affects the amount of polyribosomal RNAs that are fractionated by sucrose density gradient centrifugation. Repetition of this polysome-mediated selection cycle enriches for higher translation efficiency RNAs. From a random library of mRNA 5′-UTRs, several sequences that are as efficient as the omega sequence from TMV have been identified by polysome-mediated selection in a wheat embryo in vitro translation system [12]. Additional studies show similar results [13].

In this study, we have developed a novel selection method by using ribosome display, a technique by which the ternary ribosomal complexes, consisting of mRNA, ribosome, and nascent proteins, are formed in vitro. These ternary complexes are selected by the activity of the translated proteins. This method has been successfully utilized by our laboratory to identify and subsequently study the function of various proteins, such as enzymes and

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DNA-binding proteins [14–16]. The strategy of our selection system is shown in Fig. 1. Our modified method differs from the general ribosome display method in that the translation time is shortened. This favors the identification of 5′-UTRs with higher translation efficiency. In order to determine the effectiveness of this modified method, we tested whether 5′-UTRs with high translation efficiency sequences could be selected from mixtures containing competitive sequences. In addition, we utilized this method to select 5′-UTRs with high translation efficiency sequences from a random 5′-UTR library.

Materials and methods

Construction of plasmids and random library. The plasmids, pBS-UTK68-6HisRLuc (UTK) and pBS-UTK26-BS42-6HisRLuc (UTK-BS), were constructed as follows. *Renilla* luciferase (R-luc) fragment was amplified by PCR from pRL-CMV vector (TOYO-ink) using primer sets 6HisRluc_fwd (AAAAGATCTAAACCATGGAACATCATCACCA TCATCACAGCGCTGGTGGAATGACTTCGAAAGTTTATG) and Rluc_rev (TTTTCCTTTTGCGGCCGCTAGTACTGTTCATTTTTGAGAAC). Amplified Rluc fragment with His-tag was digested with NotI and BglII, and then inserted into pBS5LAlucPS which has constructed in our laboratory previously. To construct pBS-UTK68-6HisRluc, resulting plasmid pBS-6HisRluc was digested with BglII and Aor51 HI, followed by insertion of synthesized fragment (UTK68_fwd; GATCTA ATACGACTCACTATAGGGAATACAAGCTTGCTTGTTCTTTTTGCAGAAG CTCAGAATAAACGCTCAACTTTGGCAGATCTAC and UTK68_rev; CAT GGTAGATCTGCCAAAGTTGAGCGTTTATTCTGAGCTTCTGCAAAAAGA ACAAGCAAGCTTGTATTCCCTATAGTGAGTCGTATTA). UTK68 sequence was chimeric sequence of 5′-UTR of *Xenopus* β-globin mRNA and optimal initiation sequence [11]. As same as pBS-UTK68-6HisRluc, pBS-BS-6HisRluc was constructed by insertion of synthesized BS fragment (BS68_fwd; GATCTAATACGACTCACTATAGGGCG AATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTG ATATCGAATTCCTC and BS68_rev; CATGGAGGAATTCGATATCAAGC TTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCC TATAGTGAGTCGTATTA). BS68 sequence was derived from pBluescript II sk(+) located under the T7 promoter. To construct pBS-UTK26-BS42-6HisRLuc (UTK-BS), pBS-BS-6HisRluc was digested with BglII and XhoI followed by insertion of synthesized oligonucleotide which encode upstream of UTK (26 bp) and T7 promoter (BS-UTR-s; GATCTAATACGACTCACTATAGGGAATACAAGCTTGCTTG TTCTCCC and BS-UTR-a; TCGAGGGAGAACAAGCAAGCTTGTATTCCC TATAGTGAGTCGTATTA).

Random library construction. Random library was constructed by PCR using primer sets UTR-rand 2 (AGATCTAATACGACTCACTATAG GGAATACAAGCTTGCTTGTTCTNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNACCATGGAACATCATCAC) and Rev2 Xba (GGCCGCTCTAGAACTGTTCATTTTTGAGAACTCGC). UTR-rand 2 was including 42 bp of random sequence between T7 promoter and His-tag sequences. For PCR reaction, pBS-UTK68-6HisRluc was used as a template. After construction, sequences of random library were analyzed. Random library fragments were cloned into pBluscript II after digestion with HindIII and XhoI. Finally, each sequence was analyzed by using CEQ2000XL (Beckman Coulter).

In vitro transcription. For the first cycle of the competitive experiment, the constructed plasmids were linearized by digestion with ScaI which is inserted before the stop codon. For additional cycles of the competitive experiment and all cycles of the random library experiment, 1 μg of amplified cDNA fragments was used. Template RNA was transcribed from these DNA templates. mRNA transcripts were produced in vitro using the MEGAscript T7 kit (Ambion) in the presence of m7G(5′)ppp(5′)G (CAP) as described by the manufacturer. RNA transcripts were purified by phenol/chloroform extraction and ethanol precipitation and quantified using UV spectroscopy.

In vitro translation and selection cycles. In vitro translation was performed using a Flexi rabbit reticulocyte lysate system (Promega). 1.5–3.0 μg of template RNA was added to rabbit reticulocyte lysates containing 50 mM KCl, 2 mM DTT, 20 μM Complete Amino Acid Mixture (promega), and 0.8 U/μl RNasin Ribonuclease inhibitor (Promega). A total volume of 30 μl from this reaction mixture was incubated for 12–60 min at 30 °C. To stop the translation reac-

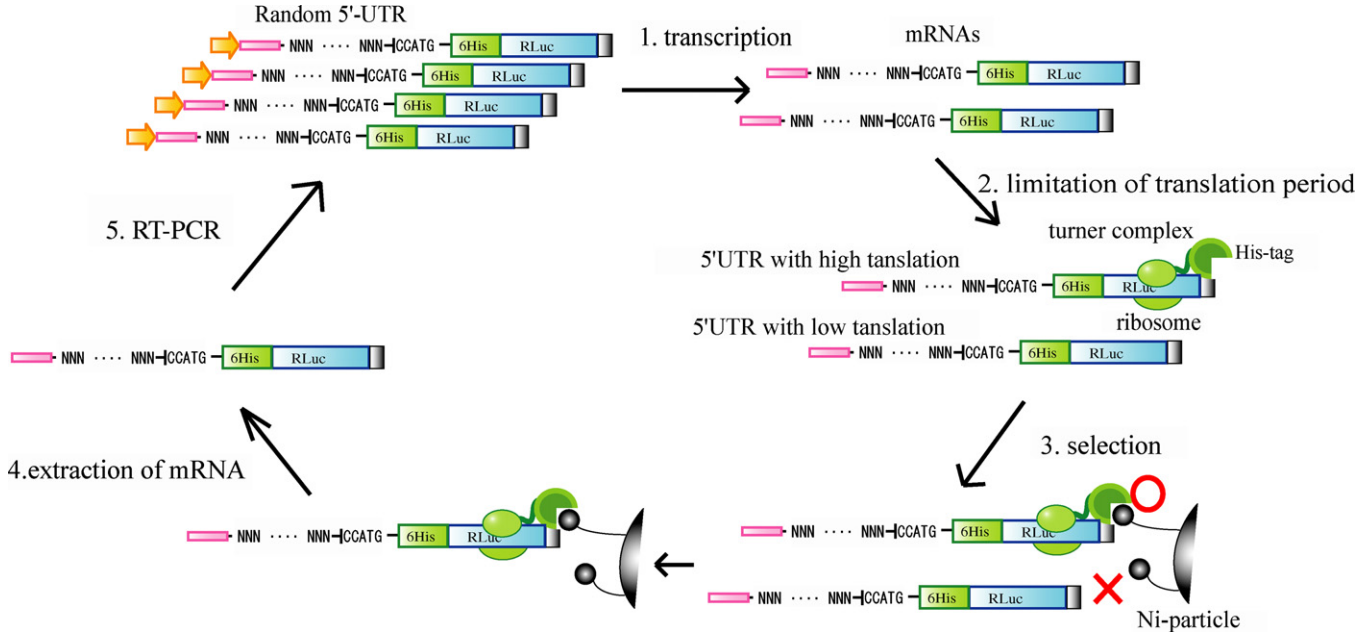

Fig. 1. Schematic drawing of the selection system using ribosome display. (1) A random 5′-UTR DNA library was used, 5′-UTR sequences followed by a His-tag and the *Renilla* luciferase gene were transcribed to mRNA. (2) Transcribed mRNAs were translated in rabbit reticulosyte lysates. Ternary complexes, containing ribosomes, translated protein, and mRNA progenitors, were made. Translation periods were limited to select for 5′-UTR sequences with high translation activity. (3) Ternary complexes expressing the His-tag were selected by Ni-particles. (4) mRNAs were extracted from selected ternary complexes. (5) Selected mRNAs were pooled and used as templates for RT-PCR.

tion and to form protein–ribosome–mRNA (PRM) complexes, 75 μl of ice-cold stop buffer (50 mM HEPES–NaOH (pH 7.5), 50 mM $MgCl_2$, 1 μl RNasin Ribonuclease inhibitor, 1 μg/ml cycloheximide, 1 mM DTT, 2% Block Ace (DainipponSeiyaku)) was added and incubated on ice. MagneHis Ni-Particles (Promega) were added and mixed gently at 4 °C to bind the PRM complex to Ni-particles via the translated His-tag. After 2 h of mixing, Ni-particles were washed five times with wash buffer (100 mM HEPES–NaOH (pH 7.5), 1 M NaCl, 50 mM $MgCl_2$, 1 μg/ml cycloheximide, 50 mM imidazole, 2% Block Ace, 0.1% Tween 20) to remove non-specific unbound molecules from Ni-particles. To collect the PRM complexes, 110 μl of elution buffer (100 mM HEPES–NaOH (pH 7.5), 500 mM NaCl, and 200 mM EDTA) was added to Ni-particles. After 5 min incubation on ice, RNA was purified from the supernatant using the RNeasy Mini Kit (QIAGEN). Purified RNA was reverse-transcribed by Super Script III reverse transcriptase (Invitrogen) with a RLuc_rev2 primer, according to the manufacturer's protocol, and (amplified by?) PCR. The PCR reaction was performed using Pyrobest DNA polymerase (Takara) with T7-add and RLuc_rev2 primers. (PCR conditions were as follows: 98 °C for 5 min, 45 cycles of 98 °C 10 s, 63 °C 30 s and 68 °C 1 min.) Amplified cDNAs were purified and transcribed as described above.

Evaluation of selection efficiency. To identify the ratio between UTK and UTK-BS in the competitive experiment, amplified cDNA was digested with BglII after each PCR cycle. Only the UTK sequence has a BglII recognition site. After digestion, DNA fragments were separated by agarose gel electrophoresis.

Evaluation of Renilla luciferase activity. For the random library experiment, R-luc activities were measured to confirm selection of higher translation sequences. Before and after each selection cycle, template RNA (9 μg) was added to 81 μl of rabbit reticulocyte lysate solution and incubated at 30 °C. Every 5 min, 60 μl of phosphate buffered saline (PBS) was added to 20 μl of translation solution and incubated on ice to stop the reaction. R-luc activity was measured using a *Renilla* luciferase assay system (Promega) as described by the manufacturer.

Evaluation of selected sequence. In the random library experiment, after 2 cycle selections, RT-PCR products were cloned into a pUC18 vector and their sequence was confirmed. Plasmids (0.5 μg) were translated using the TNT T7 Coupled Reticulocyte Lysate System (Promega). R-luc activity was measured by chemiluminescence to evaluate translation efficiency.

Results and discussion

Competitive selection

A competitive selection was performed to demonstrate the effectiveness of this method. As shown in Fig. 1, our selection method was based on ribosome display. 5′-UTRs of mRNAs were fused to a luciferase linker sequence as well as a His-domain sequence. Using in vitro translation, ternary complexes, consisting of mRNA, ribosome and nascent polypeptide, were formed in rabbit reticulocytes. His-tagged proteins were produced that were selected with Ni-affinity particles. Nascent polypeptides of R-luc fused to a His-affinity tag were also in vitro translated. By limiting the translation period, mRNAs with 5′-UTR sequence of higher translation efficiency were selected from the library. Other selection systems have been previously reported [12,13]. In these systems, polysomes were fractionated by sucrose density gradient centrifugation depending on the number of recruited ribosomes. In these studies, the initiation rate of ribosome to mRNA is regarded as the translation efficiency. A recent report, using eukaryotic non-capped mRNAs in a wheat germ cell-free translation system, suggests that the efficiency of translation is mainly reflected by the initiation rate [17]. However, the factors that affect the translation efficiency, are speculated not only to include the initiation rate of ribosomes but also to include the stimulation of elongation. Therefore, translated product was used for selection in our selection system.

The different translation efficiencies of 5′-UTR sequences were compared to the R-luc activities of UTK (the 5′-UTR of *Xenopus* β-globin linked with Kozak consensus sequence (ACCAAUGG)). Previous studies have shown that replacing endogenous leader sequences with UTK dramatically improves the translation of a variety of polypeptides in reticulocyte lysates [11]. UTK-BS was used as a negative control. To construct this sequence, 42 bp of the 5′-UTR of *Xenopus* β-globin sequence was replaced with sequence derived from pBluescript II. R-luc activity was measured after 20 min of translation of each sequence (Fig. 2A). Although the UTK-BS showed some activity due to the presence of the Kozak consensus sequence, the R-luc activity of UTK was about 4-fold higher. Using these sequences, competitive selection experiments were performed. After transcription of each plasmid, purified RNAs were mixed at various ratios of UTK:UTK-BS including 1:1, 1:10, 1:100, 1:1000, and 1:10,000. These samples were translated for 20 min and selected. After every cycle, the ratio between UTK and UTK-BS was evaluated by electrophoresis (Fig. 2B). At a ratio of 1:1, the amount of UTK was larger than UTK-BS regardless of the selection cycle. At a ratio of 1:10, UTK expression was higher than UTK-BS after the second selection cycle, suggesting that at these ratios, even in the presence of UTK-BS, UTK was enriched. Higher ratios depleted the UTK pool. Based on this preliminary competition screen, we determined that our method would be useful for the selection of high translation efficiency 5′-UTR sequences from a random library of mRNA 5′-UTRs.

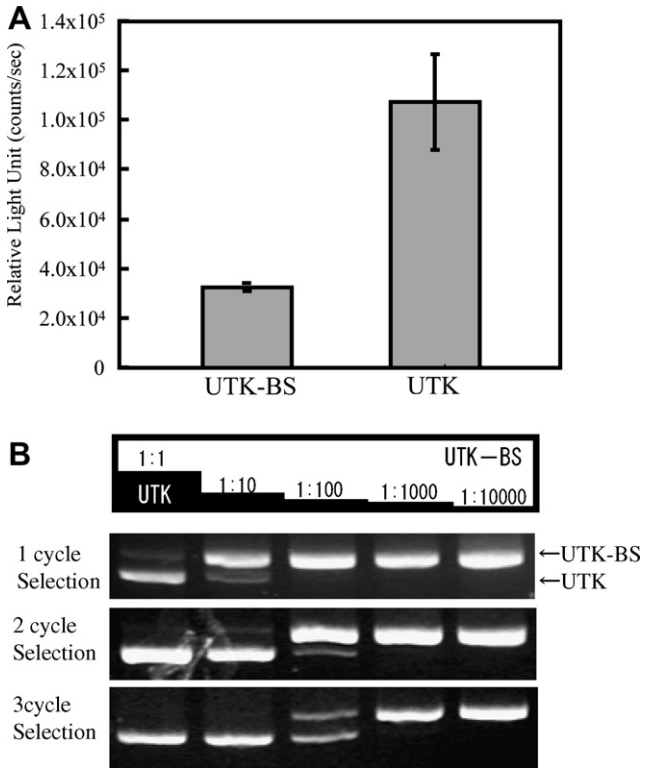

Fig. 2. A competitive selection experiment between UTK and UTK-BS. (A) Representation of the differences in translation levels between UTK and UTK-BS as measured by R-luc activity. (B) UTK and UTK-BS were mixed at different ratios. Expression levels were measured after each selection cycle. Amplified DNAs were digested and separated by agarose gel electrophoresis.

Selection from the random library

A random library was constructed by PCR using random primer sequences. Prior to translation, sequences were confirmed. These sequences included a considerable number of UTK (data not shown) as well as completely random sequences (around 40% of colonies). After transcription, three rounds of selection cycles were performed. In our system, translation speed was regarded as the translation efficiency. To concentrate high translation efficiency sequences, the translation time was altered during every cycle. For the first cycle, the translation time was 20 min. Subsequently, the translation time was shortened by 4 min per additional cycle. After each cycle, RNAs were obtained and amplified by RT-PCR. After the first and second cycle selection, a single band was detected which subsequently disappeared after the third selection cycle (data not shown). Therefore, selection cycles were discontinued after the third cycle.

After each cycle, R-luc activities were measured to evaluate the selection efficiency. After the first and second selection cycles, the libraries were translated for 0–20 min and measured for R-luc activity (Fig. 3). Although the library included a considerable number of UTK sequences, R-luc activity increased by 5-fold after the first selection cycle and continued to increase after the second selection cycle, indicating that sequences with high translation efficiency were selected from the random library.

Analysis of selected sequences

Selected sequences were confirmed and analyzed. A primary analysis was performed on 18 sequences obtained from the second selection cycle. The majority of these candidates were the UTK sequence (14 samples). However, additional sequences were also obtained (4 samples) (Table 1). One particularly interesting sequence contained a single mutation in the UTK sequence, while other candidates varied considerably from the UTK sequence. The translation efficiencies of these samples were evaluated by R-luc activity. Candidate samples and control sequences (Table 1) were translated using the TNT T7 Coupled Reticulocyte Lysate System (Fig. 4). In this system, the coupling of transcription and translation was facilitated by the addition of a linearized plasmid. Therefore, the incubation time was extended compared to the selection cycles. The reaction was allowed to proceed for 20–60 min upon which R-luc activities were measured. R-luc activities increased during the initial 40 min of the reaction time at which point it stabilized. This occurred for all samples except sample 4. At 40 min, the activity of UTK was about five times higher than UTK-BS. This result was similar to Fig. 2A, suggesting that these results specifically reflected only translational efficiency. Sample 1, which contains a single mutation in the UTK sequence (underlined in Table 1), showed 80% more activity than UTK, suggesting that the single mutation did not affect its translation. In addition, its predicted RNA secondary structure was similar to that of UTK (data not shown). Surprisingly, sample 4 showed lower activity than UTK-BS even though the selection conditions were similar to those used in the competitive experiment. One possible explanation is that the Kozak consensus sequence (ACCAAUGG) is sufficient to initiate the translation of every sequence. Therefore, even low activity sequences can be detected. This background can most likely be reduced by increasing the number of selection cycles. A second possibility is that sample 4 is acting as an aptamer, capable of binding to other oligonucleotides or peptide molecules. A similar problem was observed in our previous experiment [16] and is unavoidable in a genotype–phenotype linkage selection system such as ribosome display. A "negative" selection cycle, in which translation is not performed, should reduce the effect of this problem.

Samples 2 and 3 showed considerably higher activity than UTK-BS. For sample 3, both the nucleotide sequence and RNA secondary structure prediction differed considerably from UTK. A BLAST search indicated that there are no sequences with high similarity to that of sample 3. Although the sequence of sample 3 contains an additional ATG before the Kozak sequence, this ATG was neither flanked by a His-tag or R-luc, nor was it located downstream of an additional Kozak consensus sequence, indicating that it would not work as an efficient start codon. Sample 3, along with our other candidates, indicates that the method we have developed can be successfully used to obtain 5′-UTRs with high translation efficiency from a random library.

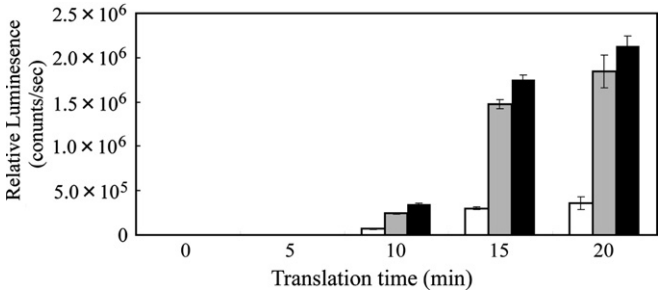

Fig. 3. Evaluation of the selection efficiency from the random 5′-UTR library. Before and after selection cycles, R-luc activities were evaluated every 5 min during translation. White bars: before selection, Gray bars: after first cycle selection, Black bars: after second cycle selection.

Table 1
Selected sequences from random 5′-UTR library after second cycle selection

Name	Sequence
UTK	TTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGCAGATCT
UTK-BS	CCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCt
Sample 1	TTTTGCAGAAGCTCAGCATAAACGCTCAACTTTGGCAGATCT
Sample 2	GGGCTCTAGGAAGTTGGTGCTGTTCTTAGGGCATCCGCTCGG
Sample 3	TCGTCTGCATGCCTTGTGAGGGTCTTCCTTGTCCTTTTCGTT
Sample 4	CCCTCGAGGTCGACGGTATCGATAAGCTAGATATCGAATCCT

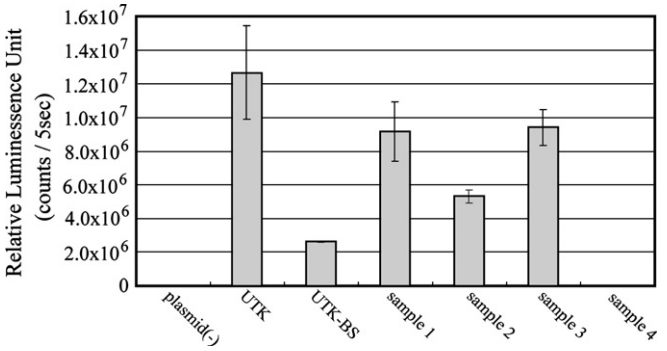

Fig. 4. Translation activities of selected 5′-UTRs. R-luc activity of selected sequences after second cycle selection, including UTK and UTK-BS, were measured by chemiluminescence.

Conclusion

We have developed a novel selection system which incorporates ribosomal display to detect 5′-UTRs with higher translation efficiency sequences from a 5′-UTR random library. Although the efficiency of these enhancer sequences was lower than UTK, an increase in cycle number may identify 5′-UTRs with much higher

efficiency from the random library. Optimization of this technique may provide a useful tool in the production of protein within the in vitro translation system.

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