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Development and Application of Detection Methods for Capture and Transcription Elongation Rate of Bacterial Nascent RNA*

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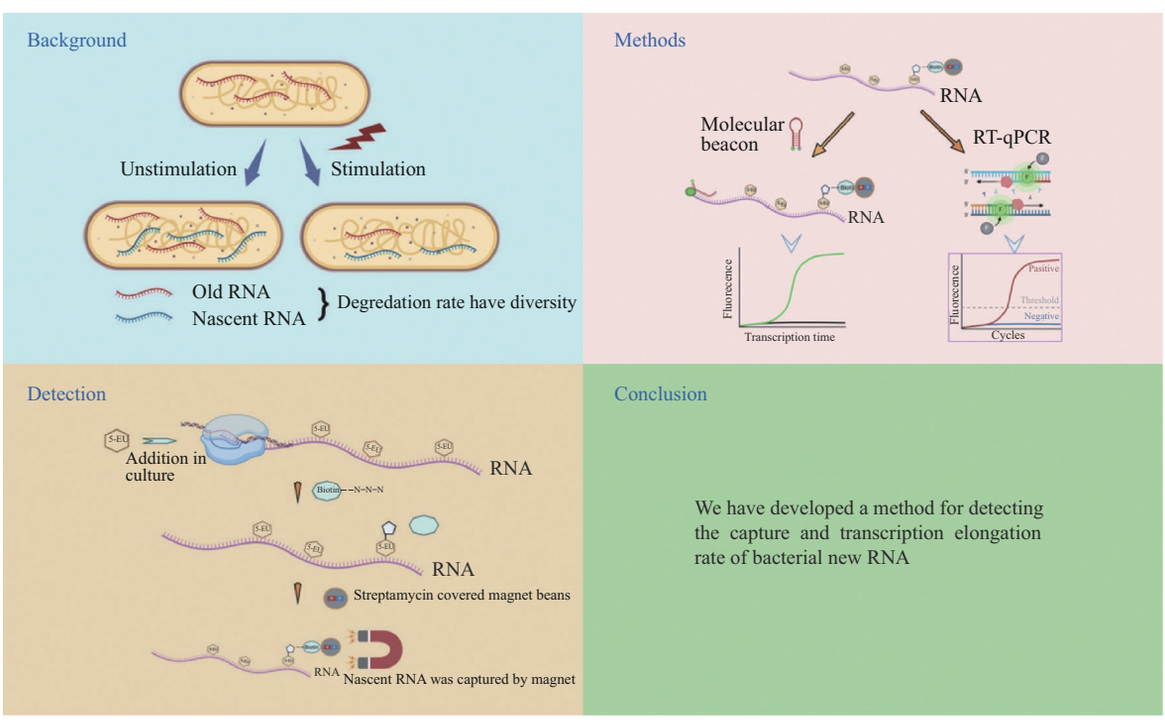
Graphical abstract



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Abstract **Objective** Detection and quantification of RNA synthesis in cells is a widely used technique for monitoring cell viability, health, and metabolic rate.After exposure to environmental stimuli, both the internal reference gene and target gene would be degraded. As a result, it is imperative to consider the accurate capture of nascent RNA and the detection of transcriptional levels of RNA following environmental stimulation. This study aims to create a Click Chemistry method that utilizes its property to capture nascent RNA from total RNA that was stimulated by the environment. **Methods** The new RNA was labeled with 5-ethyluridine (5-EU) instead of uracil, and the azido-biotin medium ligand was connected to the magnetic sphere using a combination of "Click Chemistry" and magnetic bead screening. Then the new RNA was captured and the transcription rate of 16S rRNA was detected by fluorescence molecular beacon (M.B.) and quantitative reverse transcription PCR (qRT-PCR). **Results** The bacterial nascent RNA captured by "Click Chemistry" screening can be used as a reverse transcription template to form cDNA. Combined with the fluorescent molecular beacon M.B.1, the synthesis rate of rRNA at 37℃ is 1.2 times higher than that at 15℃. The 16S rRNA gene and *cspI* gene can be detected by fluorescent quantitative PCR,it was found that the measured relative gene expression changes were significantly enhanced at 25℃ and 16℃ when analyzed with nascent RNA rather than total RNA, enabling accurate detection of RNA transcription rates. **Conclusion** Compared to other article reported experimental methods that utilize screening magnetic columns, the technical scheme employed in this study is more suitable for bacteria, and the operation steps are simple and easy to implement, making it an effective RNA capture method for researchers.

Key words nascent RNA selection, Click Chemistry, fluorescence molecular beacon
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Bacteria, when stimulated by the environment, such as nutrition starving, cold shock, heat shock, drug inhibition, and acid or base stimulation, will induce gene response expression[1-4]. Analyzing the expression response of nascent RNA transcriptomics could lead to a more precisely understanding of the mechanisms by which these genes mediate regulation[5-6]. Furthermore, this approach could provide insights into the molecular processes underlying bacterial adaptation to various environmental stimuli[7-9]. However, in most cases of quantitative transcription or transcriptomics experiments for gene expression, bacterial total RNA was used as a template for reverse transcription[10] . The generated cDNA template encompasses all RNA molecules during the two distinct periods preceding and succeeding the stimulation. In instances where RNA degradation rates exhibit variations prior to and during the bacterial stress response, utilizing total RNA as a template to detect the expression level of stress genes may lead to inaccuracies[11].

Hence, there is a pressing requirement for an experimental approach that can effectively capture nascent transcripts from bacterial total RNA[12].

To achieve the experimental purpose of capturing nascent RNA from bacterial total RNA, this study requires the separation of transcripts before and after stimulation[13-14]. Transcription inhibitor rifampicin is used to bind firmly to the β subunit of RNA polymerase, preventing the binding of polymerase to DNA and inhibiting the initiation of transcription[15-17]. Bacteria are then incubated continuously for a certain period, allowing all RNA polymerases that have initiated transcription to continue until transcription is complete and the template DNA is separated. At this point, the phage transcription can be synchronized to the uninitiated stage. The addition of 5-ethyl-uridine (5-Eu), which can replace native uridine, at this time point labels the ensuing nascent transcript with an ethynyl group[18-19]. Ethynyl and azide can be simply and stably combined in a reaction called "Click Chemistry"[20]. The "Click Chemistry" reaction has several characteristics: it is simple to operate, has a mild reaction, is insensitive to the presence of water or oxygen, and the reaction product is relatively easy to purify, with a high recovery rate of the product and good selectivity to the substrate[21-22].

Since the introduction of the "Click Chemistry" method, it has been widely used in the field of chemical synthesis and has become a research hotspot in life sciences[23-24]. It has been used for the establishment of biosensors in the fields of supramolecular chemistry, functional materials, proteomics, cell markers, compound synthesis, and DNA analysis[25-27]. A typical RICK (capture of the newly transcribed RNA interactome using click chemistry） experimental procedure takes only 1 d, excluding the steps of cell preparation, 5-Eu labeling, validation (silver staining, western blotting, quantitative reverse transcription PCR (qRT-PCR) or

RNA sequencing (RNA-seq)) and proteomics and more[28]. However, it is rarely used in the field of pathogen detection, which presents a great opportunity for the development of new detection methods.

1 Materials and methods

1.1 Materials

1.1.1 Bacterial Strains

E. coli D7 rrnp strain was storied in Laboratory of Camerino, Italy, in which all 7 ribosome RNA gene (rrn) operons were deleted and a pKK3535 plasmid containing ribosome RNA B (rrnB) sequence was transformed to support ribosomal DNA transcripting to rRNA.

1.1.2 Fragment sequence

Fluorescence molecular beacons (M. B. s) and complimentary oligo fragments sequence were shown in Table 1.

Table 1 Fluorescence molecular beacons and complimentary oligo fragments sequence

	Fluorescence molecular beacons		Oligo fragments
M.B.1	5'-6-FAM-CCGCGCATCTCGGTTGATTTCTTTTCCTCGGGCGCGG-Dabcyl-3'	O.F.1	5'-CCGAGGAAAAGAAATCAACCGAGAT-3'
M.B.2	5'-6-FAM-CCGCGCGGTTAAGCCTCACGGTTCATTAGTAGCGCGG-Dabcyl-3'	O.F.2	5'-TACTAATGAACCGTGAGGCTTAACC-3'
M.B.3	5'-6-FAM-CCGCGCTCTGAGCCATGATCAAACTCTTCAATTTGCGCGG-Dabcyl-3'	O.F.3	5'-AAATTGAAGAGTTTGATCATGGCTCA-GA-3'

1.1.3 Buffer

M.B. buffer (100 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris HCl , pH 8.0), TNE 0.2 buffer (10 mmol/L Tris HCl, pH 7.4; 1 mmol/L EDTA, pH 8.0; and 200 mmol/L NaCl), TNE 2.0 buffer (10 mmol/L Tris HCl, pH 7.4; 1 mmol/L EDTA, pH 8.0; and 2 mol/L NaCl).

1.1.4 Biotin selection magnet beans

EasySep™ Biotin Positive Selection Kit II (Stem cell Technologies) was used for selection.

1.2 Methods

1.2.1 Fluorescent molecular beacons

(1) Synthesis of fluorescent M.B. and detection.

Fluorescence M. B. s and oligonucleotide fragments were purchased from ComateBio. The 5' end of the M. B. is linked to a fluorescent dye (6-FAM), while the 3' end is coupled to a quencher molecule (Dabcyl). The region which base-pairs with the target (oligo or RNA) is indicated in a gray frame (text in Table 1).

(2) Fluorescence M.B.s melting curve test.

The fluorescence emitted by the M. B. s in the presence or the absence of the complementary target oligonucleotide(s) was measured as a function of increasing temperature from 15℃ to 80℃ . All reactions were performed in a spectrofluorometric thermal cycler at 488 nm in 20 µl solution containing 50 nmol/L M.B., 100 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L Tris HCl (pH 8.0), and 300 nmol/L target DNA, when present. The temperature was increased in steps of 1℃, with each step lasting 2 min.

(3) Fluorescence M.B. binding gradient concentration of oligonucleotide target fragments assay.

The M.B. was incubated at 90℃ for 1 min and immediately chilled on ice. The denatured M.B. (final concentration 50 nmol/L) was then incubated with the indicated increasing amounts of complementary oligonucleotide target in 20 µl of M.B. buffer at 40℃ for 10 min. At the end of the incubation, samples were transferred to a Black and White Wallac plate, and fluorescence emission was measured in a FLUOstar Omega apparatus with a gain of 1 000, excitation of 485 nm, and emission of 520 nm.

(4) M.B.'s specialized binding *E. coli* rRNA.

rRNA extraction: 500 µl of purified *E. coli* 30S subunits were treated twice with buffer-saturated phenol and 0.1% SDS, twice with 1∶1 (*v*/*v*) phenol/chloroform, twice with chloroform and precipitated by addition of 0.1 volume of 3 mol/L sodium acetate (pH 5.4) and 3 volume of absolute ethanol. The rRNA was then dried and dissolved in 400 µl sterile H_2O.

The M.B. was incubated at 90℃ for 1 min and then immediately placed on ice to denature. The denatured M. B. (final concentration of 50 nmol/L) was then incubated with increasing amounts of the indicated target rRNA or unrelated rRNA, used as a control, in 20 µl of M.B. buffer at 40℃ for 10 min (or at other temperatures as indicated). At the end of the incubation, the samples were transferred to a Black and White Wallac plate, and the fluorescence emission

was measured.

(5) M. B. detection of rifampicin inhibits *in vitro* transcription assay.

Add a gradient increase in the concentration of rifampicin solution from 0 μg/L to 20 μg/L in a total of 16 *in vitro* transcription samples, which followed an *E. coli* rrnB rDNA as a template. The transcription reaction temperature is 37℃ and incubated for 30 min. Subsequently, the transcripts were mixed with M. B. 2, incubate at 95℃ denature for 2 min then annealed for 10 min at 45℃. Transfer the reactant into a black 96-well plate and measure the fluorescence excitation amount using a fluorescence enzyme-linked immunosorbent assay.

1.2.2 “Click Chemistry” capture nascent transcripts

The modified EasySep™ Biotin Positive Selection Kit II (Stem cell Technologies) protocol has been used in the streptavidin pull-down step[29-30] (Figure 1, 2). Denature biotinylated RNA at 65℃, 5 min, then quickly chill on ice. To eliminate RNase, use 1 μl of RNasin Plus RNase inhibitor in each sample. Transfer each 50 μl sample to a 96-well plate, mix with 5 μl Selection Cocktail mix, and incubate for 15 min. After incubation, add 3.75 μl RapidSphers

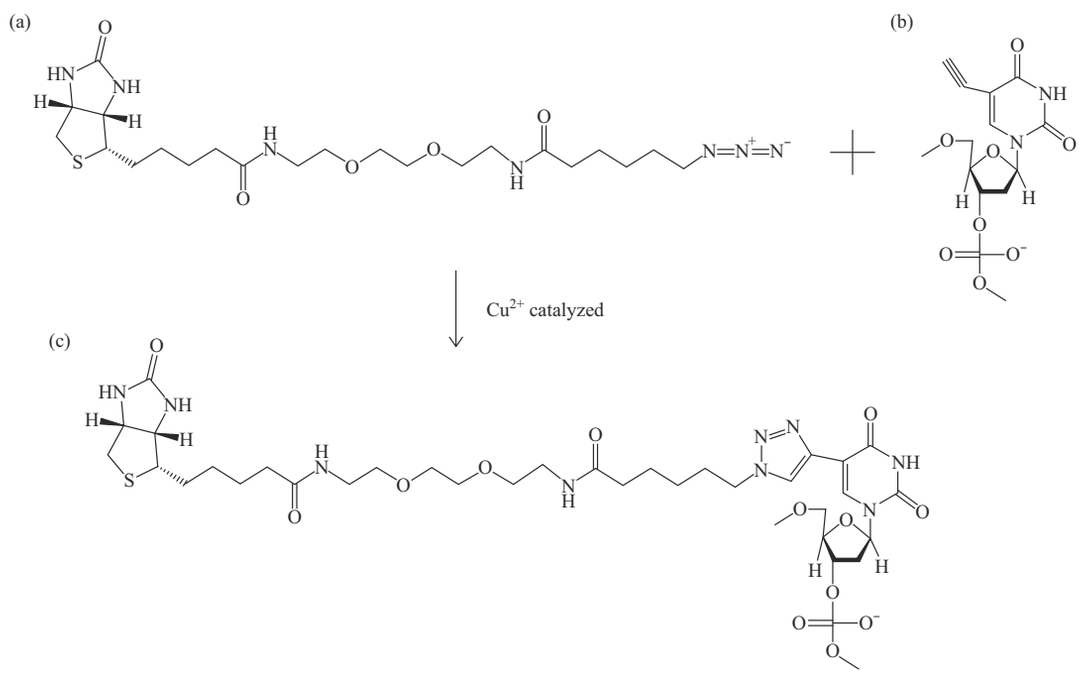

Fig. 1 “Click Chemistry” reaction of Biotin−PEG2−C6−azide azidoacetylene binding with 5−ethyluridine (5−EU)

(a) Structure of biotin-PEG2-C6-azide; (b) structure of 5-ethyluridine. (5-EU); (c): (a) and (b) chemical compounds azidoacetylene cyclization (CuAAC) by “Click Chemistry” reaction.

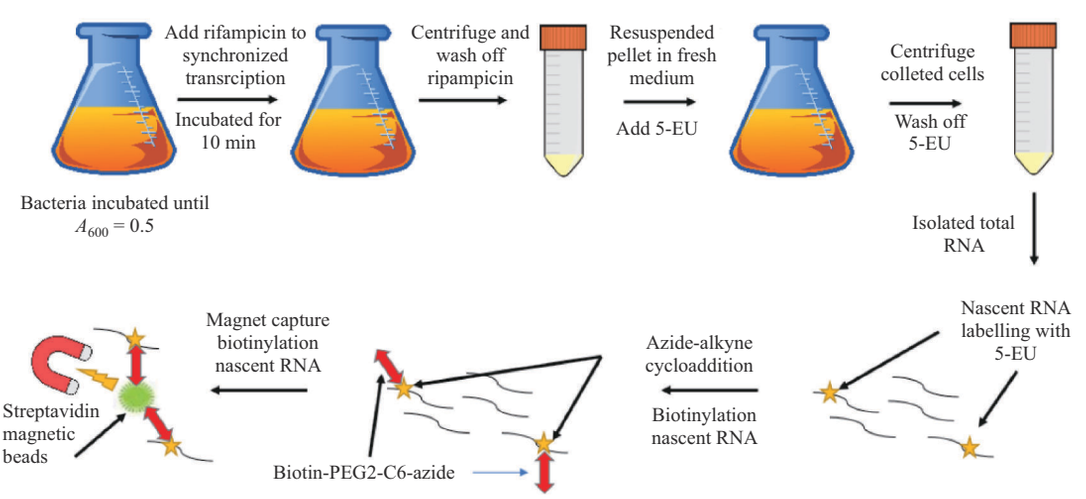

Fig. 2 Process of biotinylated nascent RNA captured by magnet RapidSphers

beads to the mixed sample and incubate for another 10 min. After this step, the biotinylated RNA could bind to RapidSphers. The RapidSphers could be held by a magnet plate (EasyPlate™ EasySep™) on the bottom of a 96-well plate. Wash the wells twice with TNE 2.0 buffer and once with TNE 0.2 buffer to eliminate unbiotinylated RNA. Resuspend RapidSphers-biotinylated RNA with 10 μl of TNE 0.2 buffer and collect to Eppendorf tubes after removing the magnet plate. The isolated RapidSphers-biotinylated nascent RNA is ready to detect transcriptional rate by M.B. assay.

(1) Synchronize transcript initiation by rifampicin test.

The *E. coli* D7 strain was incubated in 10 ml of LB medium in 25 ml flasks at 37℃ and 160 r/min. When the culture's A_{600}=0.5, synchronized transcription was achieved using 10 mg/L of rifampicin (Solarbio Life Science), which specifically binds to the RNA polymerase β-subunit, inhibiting the binding of RNA polymerase to DNA and blocking transcription initiation. After a 10 min incubation with rifampicin, all transcript products would complete the elongation procedure. The cultures were then centrifuged at 3 000 r/min for 5 min at 4℃. The bacterial cells were resuspended in a physiological solution to wash off the rifampicin. In order to establish the inhibition efficacy of rifampicin, a negative control group without the presence of rifampicin should be established alongside a positive control group that is inhibited by rifampicin but not washed off. The A_{600} was continued measured every 30 min, to make the growth curve.

(2) Metabolic labeling of cultured bacterial with 5-EU.

The centrifuged cell pellet from the previous step was resuspended in 10 ml of fresh LB medium containing 400 μmol/L 5-EU to label the nascent RNA. The cultures were grown at both 37℃ and 15℃. After a time course, samples were collected to label the nascent transcripts with 5-EU. The 5-EU medium was discarded, and the cells were washed once with a physiological solution. Bacterial cells were collected by centrifugation and resuspended in 1 ml Trizol. The samples were flash-frozen in liquid nitrogen and stored at −80℃. Total RNA extraction was performed using the standard Trizol RNA extraction procedure (Takara RNAiso plus). At the end of the procedure, the dried RNA pellet was resuspended in 44 μl of nuclease-free water. DNase was removed using DNase I reagent, and DNase I was inactivated by adding 2.5 μl of 0.5 mmol/L EDTA. The mixture was heated at 80℃ for 2 min, followed by purification of the RNA using sodium acetate and ethanol. The RNA was then resuspended in 25 μl of nuclease-free water.

(3) Biotinylation of 5-EU-labeled RNA with "Click Chemistry".

To biotinylate the RNA sample (1−5 μg), follow the steps below. Prepare a 60 μl biotinylation reaction containing: 100 mmol/L Tris HCl, pH 7.5, 10% acetonitrile, 250 mmol/L sodium L-ascorbate (prepared freshly), 0.5 mmol/L Biotin-PEG_2-C_6-azide (Confluore), 100 mmol/L PMDTA, and 1 mmol/L $CuSO_4$. Mix well and incubate in the dark at 45℃ for 30 min with shaking at 750 r/min in a thermomixer. Clean up the reaction by ethanol, precipitation and resuspend the pellet in 50 μl of TNE 0.2 buffer Check the yield using NanoDrop. Biotinylated RNA samples can be stored at −80℃ for up to a week until they are used in the streptavidin pull-down step. Long-term storage is not recommended.

1.2.3 "Click Chemistry" *E. coli* nascent rRNA selection and fluorescence M. B. -based transcription elongation rate

(1) Fluorescence M. B. detection of synchronized transcription *E.coli* total rRNA.

Two groups of *E. coli* culture were incubated at 37℃ and 15℃. Took the samples at 0, 1, 2, 3, 5, 8, 10, 12, 15, and 20 min for 37℃ group before synchronization. And took samples every 30 min from 0 min to 210 min for 15℃ group before synchronization. Prepare total RNA by triazole RNA extraction procedure. Total RNA samples binding with 1.5 μmol/L M.B.s in M.B. buffer (total volume was 20 μl) in each Eppendorf tube. After incubating at 90℃ for 1 min, switch the tubes to 45℃ and incubate for 10 min. Transfer all incubated samples to a black 96-well plate, which each sample well containing 180 μl 10 mmol/L Tris HCl pH 9.0. And the fluorescence emission was measured.

(2) Fluorescence M.B. detects nascent RNA and total RNA transcription level.

The *E. coli* samples were incubated at a temperature of 15℃ and were subjected to periodic

sampling every 30 min until a total time of 210 min was reached. Total RNA and nascent RNA samples were prepared using the previously described procedure (**1.2.2**). The nascent RNA samples were then mixed with 1.5 μmol/L M. B. s in M. B. buffer, with a total volume of 20 μl in each Eppendorf tube. Subsequently, the RNA was denatured by incubation at 90℃ for 1 min and then switched to 45℃ for 10 min to allow for the M. B. s to anneal to RNA. Finally, all the incubated samples were transferred to a black 96-well plate, with each sample well containing 180 μl of 10 mmol/L Tris HCl pH 9.0. The fluorescence emission was then measured for each sample.

1.2.4 Nascent RNA and total RNA transcription level detection by real-time PCR

The *E. coli* K12 strain was subjected to a controlled experiment, wherein it was cultured into two distinct groups, each containing three repeats. The culture was grown to the logarithmic growth stage (A_{600}=0.5) at a temperature of 37℃. Following this, the samples were subjected to a series of gradient temperature incubations, ranging from 37℃ to 16℃, under 160 r/min shaking. One of the groups was treated with 5-EU, while the other group served as the negative control. After incubating for another 30 min, the cell culture was collected *via* centrifugation and total RNA was extracted using the TransZol Up reagent. The test group was operated using the magnet nascent RNA capture method, which was described in detail in this study, while the negative control group was subjected to traditional operation method. Subsequently, after reverse transcription, cDNA was formed, and real-time PCR was performed to detect the amplification cycles. The fold-changes of the expression of cold shock protein I (*cspI*) gene were determined using the $2^{-\Delta\Delta C_t}$ method.

2 Results

2.1 Detection of fluorescent molecular beacon

2.1.1 Fluorescence M.B.s melting curve test

Figure 3 exhibits the melting curve obtained using a spectrofluorometric thermal cycler at 488 nm, with temperature ranging from 15℃ to 80℃ in 1℃ increments. The curve demonstrates that the fluorescence emission of M. B. 2 (pink) and M. B. 3 (green) is negligible at temperatures below 50℃ in the absence of the target oligonucleotide with a perfectly complementary sequence, respectively. However, as the temperature increases, fluorescence also increases owing to the denaturation of the secondary structure. In the presence of the target complementary oligonucleotide, the fluorescence of M. B. 2 (pink) and M. B. 3 (green) increases immediately and reaches its maximum at approximately 45℃, due to the formation of target-M. B. hybrids that disassociate the quencher from the fluorophore. Nevertheless, with increasing temperatures, fluorescence decreases again as the fraction of hybrids reduces. Finally, when the temperature exceeds 65℃, the M.B. denatures even in the presence of the complementary target.

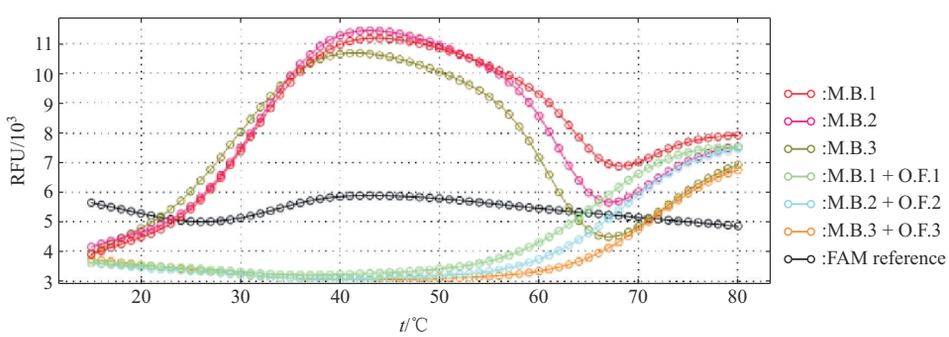

Fig. 3 Fluorescence molecular beacons melting curve

Melting curves of M.B. 1 alone (red), M.B. 2 alone (pink), and M.B. 3 alone (green) or in the presence of a complementary target oligo fragment 1 (light green), 2 (cyano), 3 (orange). The black trace is a control of a FAM-labeled oligonucleotide without a quencher.

2.1.2 Fluorescence M. B. binding gradient concentration of oligonucleotide target fragments assay

To establish the range of concentrations of the target required for obtaining a proportional liner fluorescent emission, fluorescence was measured after mixing the M. B. with increasing oligo target concentrations. The result of these experiments (Figure 4) indicates that the fluorescent signal is linearly related to the concentration of the target when this latter is less than the concentration of the M. B. Furthermore, the emission reaches a plateau when the target is present in about a 3-fold molar excess concerning the M.B.s.

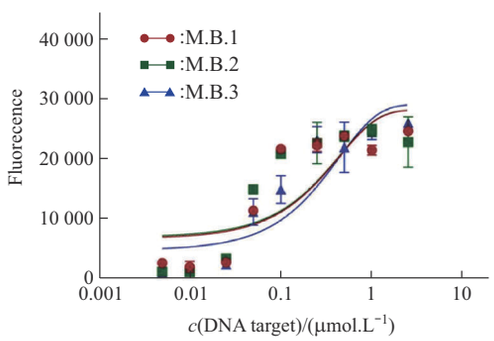

Fig. 4 Fluorescence molecular beacon binding gradient concentration of oligonucleotide target fragments

M.B.1 (red line), M.B.2 (green line), and M.B.3 (blue line) binding with each gradient concentration of DNA target oligonucleotide fragments. The M. B. excitation fluorescence value depends on the DNA target concentration.

2.1.3 Fluorescence M. B. detecting inhibition of *in vitro* transcription by rifampicin

In this study there applied M. B. 2 to detect a series of *in vitro* transcription which was inhibited by gradient concentration of rifampicin. The result in the *in vitro* transcript reactions (Figure 5) shows that the M. B.' s signal is inversely according to rifampicin concentration. The results reflect the inhibition of rRNA production by the transcription inhibitor rifampicin in the *in vitro* transcription system.

2.2 Synchronized *E. coli* transcription by rifampicin test

The *E. coli* D7 strain culture was treated with 10 g/L rifampicin at A_{600}=0.5, after incubating for 10 min, and then wash off rifampicin by centrifuge.

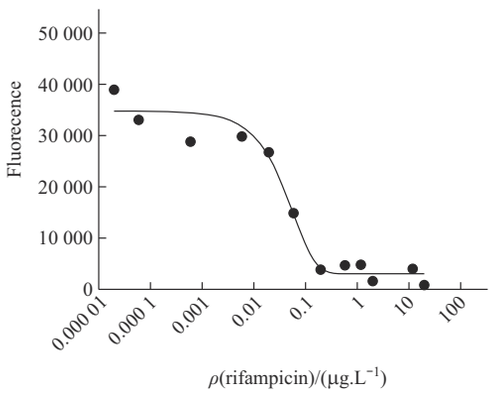

Fig. 5 Detection of rifampicin inhibiting *in vitro* transcription using M.B.2

The growth curve of the samples had been interrupted by rifampicin treatment. But a few hours after it was been washed off, the curve was back on the truck. After 10 h growth, the growth curve reached to plateau. For the positive control samples without being treated with rifampicin, the result shown in the Figure 6 is a standard growth curve. And the negative control continue treatment with rifampicin from the beginning to the test end, and the growth curve shows bacteria strain stops growth after being treated with a 10 g/L inhibitor. The results show that rifampicin can detect bacterial transcription synchronously.

2.3 Fluorescence molecular beacon detection of synchronized transcription *E.coli* nascent rRNA

The experimental findings presented in Figure 7 demonstrate that the quantity of rRNA templates detectable by the M. B., prior to transcription synchronization, exceeds the amount that can be detected after synchronization. This suggests that a certain proportion of RNA undergoes degradation and loss, consequent to the blocking of transcription initiation by rifampicin and its subsequent removal. The signal intensity of rRNA displays an increasing trend post-synchronization, indicating a resumption of the transcription process. Beyond the 120-min incubation, a decline in the fluorescence signal is observed, signifying the degradation of rRNA. This transcription rate detection method accurately reflects the temperature-mediated transcription rate in bacteria, as evidenced by the time required for the transcripted products to reach a plateau between 37℃ and 15℃.

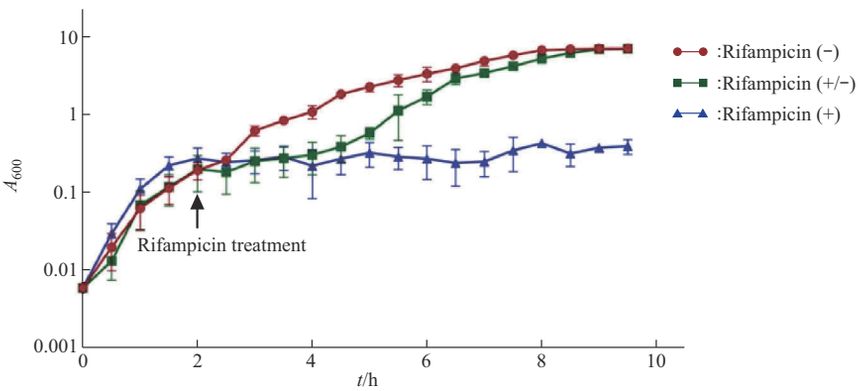

Fig. 6　Inhibition curve of transcription inhibitor rifampicin on growth of *Escherichia coli*

The red line in the figure represents the group without rifampicin treatment, the green line represents the group with rifampicin removed after a period of treatment, and the blue line represents the group with rifampicin treatment.

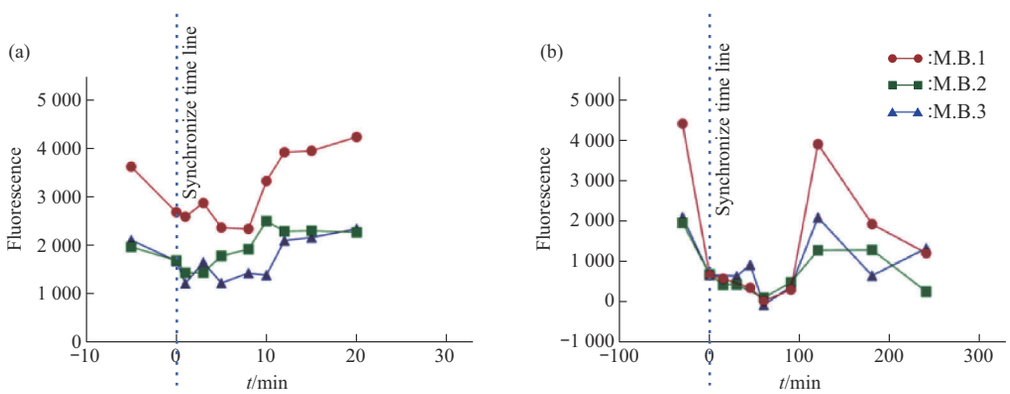

Fig. 7　Fluorescence molecular beacon detection after synchronization and *E.coli* nascent rRNA transcription rate

(a) *E. coli* was insulated at 37℃; (b) *E. coli* was insulated at 15℃. The disconnected line at 0 min separates before and after synchronized by rifampicin. The red symbol represents the M.B.1 signal, the green symbol represents the M.B.2 signal, and the blue symbol represents the M.B.3 signal.

2.4　Fluorescence molecular beacon detects nascent rRNA transcription level

The binding curve depicted in Figure 8 provides evidence that the emission signal generated by M.B.s binding with total RNA is higher than that of those binding with nascent rRNA. This observation suggests that the total RNA contains old rRNA, which was

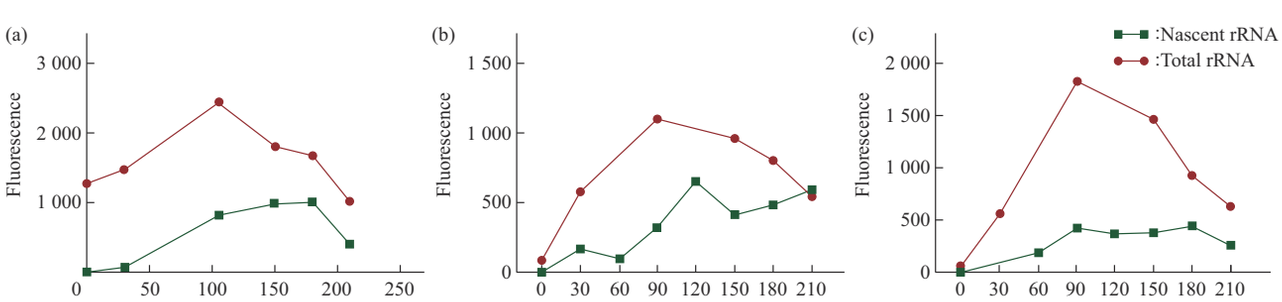

Fig. 8　The binding curve of fluorescence molecular beacon with nascent rRNA to detect transcription rate

(a) M.B.1 binding curve, (b) M.B.2 binding curve, (c) M.B.3 binding curve. The green symbols represent M.B. binding to nascent rRNA which was selected by magnet RapidSphers, The red symbols represent M.B. binding to total RNA.

synthesized prior to the synchronization of transcription. On the other hand, the nascent rRNA is a constituent of the total RNA, which was separated from it after synchronization and labeled with 5-EU using magnet RapidSphers. By employing this selection procedure, it becomes possible to eliminate old rRNA residues. It is worth noting that the application of nascent RNA sorted after transcriptional synchronization for transcriptional expression detection is likely to yield more accurate results in terms of reflecting the regulatory changes of transcripts caused by environmental changes. This is primarily due to the exclusion of the influence of old RNA that existed before regulation.

2.5 Nascent RNA and total RNA transcription level detection by real−time PCR

The present study aims to compare the real-time PCR data obtained from the *cspI* gene using the magnet nascent RNA selection method and the traditional RNA extraction method. The obtained results are presented in Figure 9. Under traditional methods, total RNA was extracted for quantitative analysis. The gene expression level did not exhibit any significant variation when cultured at 30/25℃ and the control group at 37℃. However, under 16℃ conditions, the gene expression level significantly increased, revealing a significant trend. On the other side, utilizing “Click Chemistry” to extract nascent RNA for quantitative analysis, the gene expression level did not exhibit any significant variation under 30℃ conditions, and the difference was not statistically significant. However, the gene expression level was significantly increased under 25/16℃ conditions. Relative expression ($\Delta\Delta C_t$) of the *cspI* gene was compared with and without a treatment thought to affect bacterial environment limitation. For many transcripts, the change in relative gene expression measured was dramatically enhanced when nascent RNA was used for the analysis rather than total RNA.

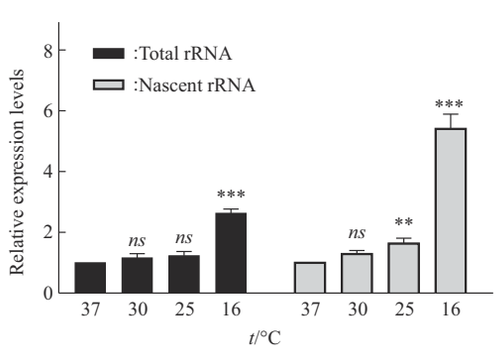

Fig. 9 Effect of cold shock on *cspI* gene relative expression ($\Delta\Delta C_t$)) in *E. coli*

Black columns represent the total RNA obtained from traditional RNA extraction; the gray columns represent nascent RNA obtained from “Click Chemistry” of RNA selection. **P<0.01; ***P<0.001; *ns*: P>0.05.

3 Discussion

The experimental work presented in this study is divided into two main parts. Firstly, a preliminary experiment was conducted to detect the growth curve of the *E. coli* D7 strain by adding rifampicin and washing it off. It was observed that the strain was significantly inhibited in its growth after the addition of rifampicin. However, the growth of the strain was limited for a short duration after washing off rifampicin, but it did not hinder reaching the plateau. This clearly demonstrated that rifampicin could effectively inhibit transcription, which laid the foundation for the subsequent experiment.

The other part of the study involved the design and synthesis of three fluorescent M. B. s, namely, M.B.1, M.B.2, and M.B.3, based on the *E. coli* rRNA sequence. These M. B. s were prepared for the detection of transcription rates at a later stage. The dissolution curves of the three fluorescent M.B.s and their binding ability to complementary oligonucleotides were analyzed separately. It was observed that the fluorescence signals of all three groups increased with the target nucleotide concentration, and their binding ability to the total RNA of *E. coli* demonstrated that the three fluorescent M. B. s could bind well to their complementary oligonucleotides. The fluorescence signals increased with reaction time, with no significant difference.

Subsequently, *E. coli* 16S rRNA and *P. Berghei* apical body 16S rRNA were employed as detection objects to verify the specific binding ability of the three fluorescent M.B.s to *E. coli* 16s rRNA, and the results indicated that they were successfully bound. In the *in vitro* transcription experiment, an increase in rifampicin concentration led to the inhibition of transcription and a corresponding reduction in transcription rate. The rifampicin concentration exhibited an inverse proportion to the transcription rate. Based on the detection results of our fluorescent M. B. 2, the fluorescence signal decreased with the

increase of rifampicin concentration, which further established the credibility of the fluorescent M.B..

Based on the above experiments, fluorescent M. B. s were used to detect nascent RNA from synchronously transcribed *E. coli*. The time required for the transcript to reach the plateau was found to be different at 15℃ and 37℃, respectively, with 37℃ being more conducive to transcription. It was also observed that the number of rRNA templates in total RNA detectable by M. B. s before transcriptional synchronization was slightly higher than the number of rRNA detectable after transcriptional synchronization, suggesting that a certain number of RNA was degraded and lost during the use of rifampicin to block transcription. Similarly, when the time exceeded 120 min, the fluorescence signal was attenuated, indicating that rRNA had been degraded.

The following text describes an experiment aimed at investigating the transcription rate of temperature-mediated bacteria. The study employed the "Click Chemistry" method to verify its superiority in detecting the transcription rate of 5-EU labelled group strains. The use of three fluorescent M. B. s enabled the detection of the transcription rate of each strain. However, the total RNA of the unlabeled group strains contained old rRNA synthesized before synchronous transcription, leading to a detected fluorescence signal. To eliminate the effects of old rRNA, the treatment group was separated by magnetic beads RapidSphers. This selection process facilitated the detection of sorted nascent RNA after transcriptional synchronization, providing a more accurate reflection of external factors' effects on the transcription rate. The study also compared real-time PCR data obtained from the *cspI* gene using magnet nascent RNA selection methods and traditional RNA extraction methods. The investigation yielded valuable insights into the differences in total RNA and nascent RNA expression results and their potential impact on downstream gene expression analysis. The gene expression level of total RNA did not exhibit any significant variation when cultured at 30/25℃ and the control group at 37℃ . Conversely, the "Click Chemistry" selected nascent RNA showed a significant increase in gene expression level under 25/16℃ conditions. The comparison between the two methods demonstrated the "Click Chemistry" method's precision in excluding the background noise of old RNA. Additionally, the experiment illustrated how specific environmental stimuli could cause the transcription of bacteria to deviate from the experimental results due to the degradation of transcription products. In summary, the study provides an important experimental technique for detecting RNA synthesis in cells to measure the total transcription rate. The detection and quantification of RNA synthesis in cells are widely used techniques to monitor cell viability, health, and metabolic rate.

4 Conclusion

This study presents a novel approach for capturing nascent *E. coli* rRNA utilizing the "Click Chemistry" technique and detecting the transcription elongation rate of nascent rRNA with a specialized binding fluorescence M. B.. The experimental procedure involved first synchronizing *E. coli* culture's transcription *via* rifampicin shock, followed by labeling nascent RNA with 5-EU. The ethyl chemical group facilitated the binding of nascent RNA with the biotin-PEG2-C6-azide ligand's azide group through a "Click Chemistry" reaction. The ligand's biotin group was then able to join with streptavidin-covered magnet beads through an immunoaffinity reaction, allowing for the capture of nascent rRNA *via* magnet selection. The transcriptional synthesis efficiency of nascent rRNA was then independently reflected by three fluorescent M. B. s that could bind at the beginning, middle, and end of rRNA. The combination of these three M. B. s enabled the detection of the transcription rate of rRNA, accurately reflecting the synthesis rate of rRNA under 37℃ and 15℃ conditions. Finally, qRT-PCR detection after reverse transcription of the sorted newborn RNA correctly reflected the content of 16s rRNA and the content of *cspI* gene, indicating that the sorting method developed in this study has a broader application range and can be used to analyze the changes in bacterial transcriptome following condition stimulation in greater detail. Overall, this research provides a new and promising methodology for studying transcriptional changes in regulated bacteria, opening up new avenues for future research in this field.

References

[1] Brandi A, Giangrossi M, Paoloni S, *et al*. Transcriptional and post-transcriptional events trigger *de novo* infB expression in cold stressed *Escherichia coli*. Nucleic Acids Res, 2019, **47**(9): 4638-4651

[2] Brandi A, Piersimoni L, Feto N A, *et al*. Translation initiation factor IF2 contributes to ribosome assembly and maturation during cold adaptation. Nucleic Acids Res, 2019, **47**(9): 4652-4662

[3] Brandi A, Pietroni P, Gualerzi C O, *et al*. Post-transcriptional regulation of CspA expression in *Escherichia coli*. Mol Microbiol, 1996, **19**(2): 231-240

[4] Kehl-Fie T E, Skaar E P. Nutritional immunity beyond iron: a role for manganese and zinc. Curr Opin Chem Biol, 2010, **14**(2): 218-224

[5] Roberts J W, Shankar S, Filter J J. RNA polymerase elongation factors. Annu Rev Microbiol, 2008, **62**: 211-233

[6] Shikalov A B, Esyunina D M, Pupov D V, *et al*. The σ^{24} subunit of *Escherichia coli* RNA polymerase can induce transcriptional pausing *in vitro*. Biochemistry, 2019, **84**(4): 426-434

[7] Wang M, Bissonnette N, Laterrière M, *et al*. Genome-wide DNA methylation and transcriptome integration associates DNA methylation changes with bovine subclinical mastitis caused by *Staphylococcus chromogenes*. Int J Mol Sci, 2023, **24**(12): 10369

[8] Du Q, Campbell M, Yu H, *et al*. Network-based feature selection reveals substructures of gene modules responding to salt stress in rice. Plant Direct, 2019, **3**(8): e00154

[9] Jacobs A, Elmer K R. Alternative splicing and gene expression play contrasting roles in the parallel phenotypic evolution of a salmonid fish. Mol Ecol, 2021, **30**(20): 4955-4969

[10] Garbacz W G, Huang J T J, Higgins L G, *et al*. PPARα is required for PPARδ action in regulation of body weight and hepatic steatosis in mice. PPAR Res, 2015, **2015**: 927057

[11] Mokry M, Hatzis P, Schuijers J, *et al*. Integrated genome-wide analysis of transcription factor occupancy, RNA polymerase II binding and steady-state RNA levels identify differentially regulated functional gene classes. Nucleic Acids Res, 2012, **40**(1): 148-158

[12] Sloutskin A, Itzhak D, Vogler G, *et al*. A single DPE core promoter motif contributes to *in vivo* transcriptional regulation and affects cardiac function. bioRxiv, 2023. DOI: 10.1101/2023.06.11.544490

[13] Jacobson E C, Perry J K, Long D S, *et al*. Migration through a small pore disrupts inactive chromatin organization in neutrophil-like cells. BMC Biol, 2018, **16**(1): 142

[14] Kirkconnell K S, Paulsen M T, Magnuson B, *et al*. Capturing the dynamic nascent transcriptome during acute cellular responses: the serum response. Biol Open, 2016, **5**(6): 837-847

[15] Howlett R, Read N, Varghese A, *et al*. *Streptomyces coelicolor* strains lacking polyprenol phosphate mannose synthase and protein O-mannosyl transferase are hyper-susceptible to multiple antibiotics. Microbiology (Reading), 2018, **164**(3): 369-382

[16] Garg S, Kumar R, Kunimoto D, *et al*. Anti-mycobacterial activity of flavonoid and pyrimidine compounds. Molecules, 2022, **27**(19): 6714

[17] Lavania M, Singh I, Turankar R P, *et al*. Enriched whole genome sequencing identified compensatory mutations in the RNA polymerase gene of rifampicin-resistant *Mycobacterium leprae* strains. Infect Drug Resist, 2018, **11**: 169-175

[18] Kingston E R, Bartel D P. Global analyses of the dynamics of mammalian microRNA metabolism. Genome Res, 2019, **29**(11): 1777-1790

[19] Naro C, Jolly A, Di Persio S, *et al*. An orchestrated intron retention program in meiosis controls timely usage of transcripts during germ cell differentiation. Dev Cell, 2017, **41**(1): 82-93.e4

[20] Kumari N, Hassan M A, Lu X, *et al*. AFF1 acetylation by p300 temporally inhibits transcription during genotoxic stress response. Proc Natl Acad Sci USA, 2019, **116**(44): 22140-22151

[21] Regis J E, Renteria A, Hall S E, *et al*. Recent trends and innovation in additive manufacturing of soft functional materials. Materials (Basel), 2021, **14**(16): 4521

[22] Odrobińska J, Neugebauer D. PEG grafted polymethacrylates bearing antioxidants as a new class of polymer conjugates for application in cosmetology. Materials (Basel), 2020, **13**(16): 3455

[23] Kinnane C R, Wark K, Such G K, *et al*. Peptide-functionalized, low-biofouling click multilayers for promoting cell adhesion and growth. Small, 2009, **5**(4): 444-448

[24] Mantovani G, Ladmiral V, Tao L, *et al*. One-pot tandem living radical polymerisation-Huisgens cycloaddition process (“click”) catalysed by *N*-alkyl-2-pyridylmethanimine/Cu(i)Br complexes. Chem Commun, 2005(16): 2089-2091

[25] Qiu S, Miao M, Wang T, *et al*. A fluorescent probe for detection of histidine in cellular homogenate and ovalbumin based on the strategy of click chemistry. Biosens Bioelectron, 2013, **42**: 332-336

[26] Guo X, Tariq M, Lai Y, *et al*. Capture of the newly transcribed RNA interactome using click chemistry. Nat Protoc, 2021, **16**(11): 5193-5219

[27] Tay H, Du Cheyne C, Demeyere K, *et al*. Depletion of embryonic macrophages leads to a reduction in angiogenesis in the *ex ovo* chick chorioallantoic membrane assay. Cells, 2020, **10**(1): 5

[28] Cheng J, Cao X, Wang X, *et al*. Dynamic chromatin architectures provide insights into the genetics of cattle myogenesis. J Anim Sci Biotechnol, 2023, **14**(1): 59

[29] Asai T, Condon C, Voulgaris J, *et al*. Construction and initial characterization of *Escherichia coli* strains with few or no intact chromosomal rRNA operons. J Bacteriol, 1999, **181**(12): 3803-3809

[30] Liu J, Feldman P, Chung T D Y. Real-time monitoring *in vitro* transcription using molecular beacons. Anal Biochem, 2002, **300**(1): 40-45

细菌新生RNA捕获及转录延伸速率检测方法的开发与应用*

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摘要 **目的** 检测和定量细胞中RNA的合成是一种广泛应用于监测细胞活力、健康和代谢率的技术。在环境刺激下，内参基因和靶基因都会发生一定程度的降解。因此，在受到环境刺激后，必须考虑对新生RNA的精确捕获和RNA转录水平的检测。本研究的目的是创建一种点击化学方法，利用其特性从环境刺激的总RNA中捕获新生RNA。**方法** 使用5-乙基尿苷（5-EU）对新生RNA进行标记，利用“点击化学”和磁珠筛选相结合的方法，将叠氮化物-生物素介质配体与磁球连接，随后捕获新生RNA并利用荧光分子信标（M.B.）和定量反转录PCR（quantitative reverse transcriptase-mediated PCR，qRT-PCR）方法检测16S rRNA转录速率。**结果** 经过“点击化学”筛选捕获得到的细菌新生RNA可以被用作反转录模板进行反转录形成cDNA，结合荧光分子信标M.B.1准确反映37℃条件下rRNA的合成速率是15℃条件下的1.2倍，通过荧光定量PCR对16S rRNA基因和*cspI*基因进行检测，得到在25℃和16℃条件下用新生RNA而不是总RNA进行分析时，测量的相对基因表达的变化显著增强，实现RNA转录速率的精确检测。**结论** 本研究采用的技术方案较其他方法更适合细菌，操作步骤简单，易于实现，是适于研究人员使用的有效的RNA捕获方法。

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