

**TITLE:** *In vitro* transcription assays and their application in drug discovery

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**SHORT ABSTRACT:**

In this manuscript, we describe a protocol to functionally examine transcription and the inhibitory activity of antibacterial agents targeting bacterial transcription.

**LONG ABSTRACT:**

*In vitro* transcription assays have been developed and widely used for many years to study the molecular mechanisms involved in transcription. This process requires multi-subunit DNA-dependent RNA polymerase (RNAP) and a series of transcription factors that act to modulate the activity of RNAP during gene expression. Sequencing gel electrophoresis of radiolabelled transcripts is used to provide detailed mechanistic information on how transcription proceeds and what parameters can affect it. In this paper we describe the protocol to study how the essential elongation factor NusA regulates transcriptional pausing, as well as a method to identify an antibacterial agent targeting transcription initiation through inhibition of RNAP holoenzyme formation. These methods can be used as a platform for the development of additional approaches to explore the mechanism of action of the transcription factors which still remain unclear, as well as new antibacterial agents targeting transcription which is an underutilized drug target in antibiotic research and development.

## INTRODUCTION:

Transcription is the process in which RNA is synthesized from a specific DNA template. In eukaryotic cells there are three distinct RNAPs: RNAP I transcribes rRNA precursors, RNAP II is responsible for the synthesis of mRNA and certain small nuclear RNAs, and the synthesis of 5S rRNA and tRNA is performed by RNAP III. In bacteria, there is only one RNAP responsible for the transcription of all classes of RNA. There are three stages of transcription: initiation, elongation and termination. Transcription is one of the most highly regulated processes in the cell. Each stage in the transcription cycle represents a checkpoint for the regulation of gene expression<sup>1</sup>. For initiation, RNAP has to associate with a sigma factor to form holoenzyme, which is required to direct the enzyme to specific sites called promoters<sup>2</sup> to form an open promoter complex. Subsequently, a large suite of transcription factors are responsible for the regulation of RNAP activities during the elongation and termination phases. The transcription factor examined here is the highly conserved and essential protein, NusA. It is involved in regulating transcription pausing and termination, as well as anti-termination during rRNA synthesis<sup>3-5</sup>.

*In vitro* transcription assays have been developed as powerful tools to study the complex regulatory steps during transcription<sup>6</sup>. In general, a linear fragment of DNA including a promoter region is required as the template for transcription. The DNA template is usually generated by PCR or by linearizing a plasmid. Purified proteins and NTPs (including one radiolabelled NTP for detection purposes) are then added and product analysed following the required period of incubation. Using appropriate templates and reaction conditions, all stages of transcription have been examined using this approach which has enabled detailed molecular characterization of transcription over the last half century<sup>7</sup>. In combination with information on the 3-dimensional structure of RNAP it has also been possible to probe the molecular mechanism of transcription inhibition by antibiotics and antibiotic leads, and use this information in the development of new, improved drugs<sup>8-10</sup>.

In this work we provide examples of how transcription assays can be used to determine the mechanism of regulation by transcription elongation/termination factor NusA, and how the mechanism of action of a novel transcription initiation inhibitor lead can be determined.

## PROTOCOL:

Caution: Experiments involve the use of the radioactive isotope <sup>32</sup>P and no work should be undertaken until all appropriate safety conditions have been met. Generally personnel are required to attend a safety course and undergo supervised practice **prior** to experiments using radioactive reagents. Please wear personal protection equipment (thermoluminescent dosimeter, safety glasses, gloves, radiation room lab coats, full length pants, closed-toe shoes) when performing the reaction.

### 1. Preparation of assay materials

### **1.1) Obtain the proteins required for transcription assay.**

1.1.1) For the experiment described here, overproduce and purify *E. coli* RNAP in the laboratory as described<sup>11</sup>, or obtain from a commercial source (Table of Materials). Note: Both provide similar results. Other methods for purification of native or affinity labelled RNAP can also be used<sup>12, 13</sup>. Sigma/NusA factors can be obtained as described in previous work<sup>14, 15</sup>.

### **1.2) Preparation of diethyl pyrocarbonate (DEPC) treated water**

Note: Alternatively, RNase-free water can be obtained from commercial sources (Table of Materials).

1.2.1) Add 1 ml diethyl pyrocarbonate (DEPC) to 1 L of purified water (0.1% v/v) and mix overnight.

1.2.2) Autoclave DEPC water and tips twice.

### **1.3) DNA template preparation**

1.3.1) Purify plasmid pSG1154<sup>16</sup> and/or pIA226<sup>17</sup> using a plasmid purification kit (Table of Materials) according to manufacturer's instructions.

1.3.2) To amplify the template for run-off transcription assays (a 360 bp fragment encompassing the *P<sub>xyI</sub>* promoter region), assemble a PCR reaction on ice including 25 µl 2X PCR mix (Table of Materials), 2 µl each of the primers (5 µM): 5'-CAAAGCCTGTCGGAATTGG-3' (forward) and 5'-CCCATTAACATCACCATC-3' (reverse), 1 µl pSG1154 plasmid preparation (250 µM) and 20 µl purified water).

1.3.2.1) Or alternatively, for single round transcription assays, amplify a 447 bp fragment encompassing the  $\lambda$  *P<sub>R</sub>* promoter region from pIA226<sup>17</sup>. Set up a PCR reaction on ice using 25 µl 2X PCR mix, 2 µl each of the primers (5 µM): 5'-GTGCTCATACGTAAATCT-3' (forward) and 5'-CAGTTCCTACTCTCGCATG-3' (reverse), 1 µl pIA226<sup>17</sup> plasmid preparation (250 µM) and 20 µl purified water).

Note: This template lacks cytosine until the +26 nt position, which is the 26<sup>th</sup> nucleotide from the transcription start site. Therefore, transcription using this template will allow initiation, promoter clearance and stalling at the +26 nt to form a stable transcription elongation complex (EC) when only ATP, UTP and GTP are present in the reaction.

1.3.3) Set up a PCR reaction with the following conditions: 95°C for 30 s, 50°C for 30 s, 72°C for 30s, repeating for 30 cycles.

1.3.4) Purify the product using a PCR clean-up kit (Table of Materials) according to manufacturer's instructions and elute in 100 µl transcription buffer for the transcription assay.

1.3.5) Determine the template DNA concentration using a fluorospectrometer (Table of Materials).

#### **1.4) NTP preparation**

Note: NTPs can be obtained from different commercial sources (Table of Materials)

1.4.1) Dissolve NTPs ( $\geq 99\%$  pure) in DEPC-treated water to make 1 M stock, aliquot 1 ml of the solution into 1.5 ml tubes (50 µl in each tube) and store at  $-20^{\circ}\text{C}$ .

1.4.2) Order  $\alpha$ - $^{32}\text{P}$  UTP (3000 Ci/mmol) **prior** to the experiment.  $^{32}\text{P}$  has a short half-life of 14.29 days.

Note: Using alpha-labelled UTP avoids comparing the intensity of bands with different length.

#### **1.5) RNA ladder preparation**

1.5.1) Assemble the following reaction at room temperature: 0.5 µg RNA Template Mix (Table of Materials), 80 mM HEPES buffer pH7.5, 12 mM  $\text{MgCl}_2$ , 10 mM NaCl, 10 mM DTT, 2 mM ATP/CTP/GTP, 0.5 mM UTP, 2 µl  $\alpha$ - $^{32}\text{P}$  UTP, 0.5 µl T7 RNAP (Table of Materials), DEPC-treated water to 20 µl.

1.5.2) Allow reaction to proceed at  $37^{\circ}\text{C}$  for 1 hour. Use immediately or store at  $-80^{\circ}\text{C}$ .

### **2. Preparation of RNA sequencing gel and RNA ladder**

#### **2.1) Gel preparation**

2.1.1) Clean the plates from a sequencing gel apparatus (Table of Materials) twice with 70% ethanol and purified water, and assemble the cell with a comb and two spacers.

Note: One plate can be coated with siliconizing reagent (Table of Materials) to facilitate gel removal in Step 4.2.

2.1.2) Dissolve 33.6 g urea in 22 ml water and 16.4 ml of 5x Tris/Borate/EDTA (TBE) buffer (54 g tris(hydroxymethyl)aminomethane; 27.5 g boric acid; 10 ml of 1 M ethylenediaminetetraacetic acid (EDTA), pH 7.4 in 1 L water) using a magnetic stirrer.

2.1.3) Add 16 ml of 40% Acrylamide/bis-acrylamide solution (19:1) to the urea solution and filter through a 0.45  $\mu$ m filter.

2.1.4) Add 514  $\mu$ l 10% (w/v) freshly prepared ammonium persulfate in water followed by 51.4  $\mu$ l N,N,N',N'-Tetramethylethylenediamine (TEMED) to the filtered solution.

2.1.5) Gently invert the gel solution to avoid excessive aeration and inject immediately and smoothly into the horizontally positioned pre-packed sequencing cell from the bottom injection hole. Take care to avoid making bubbles during injection.

2.1.6) Allow 1-1.5 h until the gel is completely set before use in a transcription assay. The gel can be stored at room temperature for two or three days when wrapped with water-wet tissue on the top of the sequencing cell.

### **3. Transcription Reaction**

#### **3.1) Transcription Elongation Pausing assay**

##### **3.1.1) Formation of a transcription elongation complex**

3.1.1.1) Add 1  $\mu$ l of RNAP core enzyme (1  $\mu$ M stock in transcription buffer) to a 1.5 ml tube.

3.1.1.2) Add 1  $\mu$ l of purified sigma-70 factor ( $\sigma^{70}$ ; 3  $\mu$ M stock in transcription buffer) to the RNAP and incubate on ice for 10 min to form RNAP holoenzyme.

3.1.1.3) Add 3  $\mu$ l 500 nM purified template DNA ( $\lambda$   $P_R$  promoter from the pIA226 PCR product) in transcription buffer to the RNAP holoenzyme and incubate at 37 °C for 5 min to form the open complex.

3.1.1.4) Prepare a mixture of reaction components in another 1.5 ml tube using 5  $\mu$ l of 10 $\times$  transcription buffer (Table of Materials: 400 mM Tris-HCl, 1.6 M KCl, 100 mM MgCl<sub>2</sub>, 10 mM DTT, 50% glycerol, pH 7.9 in DEPC treated water), 2  $\mu$ l of 250  $\mu$ M each GTP and UTP, 1  $\mu$ l of 1 mM ATP, and 1  $\mu$ l of  $\alpha$ -<sup>32</sup>P UTP (3000 Ci/mmol). Add DEPC treated water to 41  $\mu$ l.

Note: RNase inhibitor (Table of Materials) can be used to minimize the RNase contamination.

3.1.1.5) Transfer the mixture solution to the tube containing the open complex and incubate at 37 °C for 15 min to form the transcription elongation complex stopped at +26 nt.

3.1.1.6) Move the reaction to room temperature (~21°C) and add 1  $\mu$ l of 2.5 mg/ml rifampicin to a final concentration of 50  $\mu$ g/ml.

3.1.1.7) Add 1  $\mu$ l of 0.1 mM purified NusA to the reaction mixture and incubate for 5 minutes at

room temperature.

3.1.1.8) Add 2  $\mu\text{l}$  of 250  $\mu\text{M}$  CTP into the reaction mixture to make a final volume of 50  $\mu\text{l}$  and measure the reaction time using a timer.

3.1.1.9) Transfer 5  $\mu\text{l}$  of the reaction solution into 2  $\mu\text{l}$  of RNA gel loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) at 30, 60, 120, 240, 480, and 1200 seconds to quench the reaction.

3.1.1.10) Dispose the  $^{32}\text{P}$  UTP tips and tubes in a radioactive waste container.

### **3.2) Transcription Initiation Assay**

#### **3.2.1) Formation of the open complex**

3.2.1.1) Dilute purified  $\sigma^{70}$  to 0.5  $\mu\text{M}$ .

3.2.1.2) Repeat Steps 3.1.1.1 to 3.1.1.3 using 0.5  $\mu\text{M}$  (instead of 3  $\mu\text{M}$ ) purified  $\sigma^{70}$ , and the DNA template (the  $P_{xyI}$  promoter region from pSG1154 PCR product).

#### **3.2.2) Inhibition of transcription initiation assay**

3.2.2.1) Add the inhibitor designed to prevent RNAP holoenzyme formation to RNAP core enzyme prior to addition of  $\sigma^{70}$  (after step 3.1.1.1), followed by Steps 3.1.1.2 and 3.1.1.3 to examine whether the inhibitor is able to disrupt holoenzyme formation.

3.2.2.2) Alternatively, after Step 3.1.1.2, add the inhibitor to examine whether it is able to competitively inhibit  $\sigma^{70}$  binding to RNAP.

3.2.2.3) Use the same amount of DMSO, the solvent of the inhibitor, instead of the inhibitor stock in control reactions.

#### **3.2.3) Completion of the reaction**

3.2.3.1) Dissolve heparin in water to a stock concentration of 1 mg/ml and stock at  $-20\text{ }^{\circ}\text{C}$ .

Note: Heparin is a competitor of DNA that can sequester RNAP that has not formed an open complex. Once the open complex is formed, heparin cannot enter the DNA binding site of RNAP and inhibit transcription from pre-formed open complex. Therefore, heparin is only required for single-round assays; it is not needed for multi-round assays.

3.2.3.2) Prepare the reaction mixture using 5  $\mu\text{l}$  of 10 $\times$  transcription buffer (Table of Materials), 1  $\mu\text{l}$  of 1 mg/ml heparin solution, 1  $\mu\text{l}$  of 10 mM each ATP, GTP, and CTP, 2.5 mM UTP, and 1  $\mu\text{l}$

of  $\alpha$ -<sup>32</sup>P UTP (3000 Ci/mmol). Add DEPC treated water to 45  $\mu$ l.

3.2.3.3) Mix the reaction mixture with the transcription open complex, pulse spin and incubate at 37 °C for 20 min.

3.2.3.4) Transfer 10  $\mu$ l of the reaction solution into 5  $\mu$ l of RNA gel loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) to quench the reaction.

#### **4. RNA gel running and development**

##### **4.1) Running the denaturing polyacrylamide gel**

4.1.1) Run the gel at 50 °C and 50 W in 1× TBE buffer for approximately 1.5 hours until the temperature of the gel and the buffer is stable at 50 °C.

4.1.2) Heat samples at 90 °C for 30 seconds and move them onto ice prior loading onto the gel.

4.1.3) Load samples into the wells on the top of the gel alongside one lane containing 2  $\mu$ l RNA ladder (1:100 dilution).

4.1.4) Run the gel at 50 W and 50 °C in 1× TBE buffer until the bromophenol blue dye reaches ~2/3 down the gel.

##### **4.2) Gel treatment**

4.2.1) Open slowly and carefully separate the upper plate from the sequencing cell. Take great care to avoid breaking the gel.

4.2.2) Cut chromatography paper (Table of Materials) to match the gel size from the top to the bromophenol blue dye. Use a Geiger counter to confirm the approximate position of radioactively labelled RNA transcripts.

4.2.3) Carefully cover the gel with the filter paper, and press firmly till the gel adheres to the filter paper. Cut off and discard the bottom of the gel to radioactive waste as the unincorporated NTPs run to the bottom of the gel, which can be very hot.

4.2.4) Place a similar sized piece of filter paper on the drying bed of a vacuum-drying machine, and carefully lay the gel-coated paper onto it with the gel side up.

4.2.5) Cover the gel with a plastic wrap and dry at 60 °C for 1.5 h

4.2.6) Wrap the other side of the gel coated filter paper with plastic wrap, and place on a photostimulable phosphor plate in the imaging case, with the appropriate exposure time, which

can vary from 1h to overnight depending on the reaction efficiency.

4.2.7) Image the phosphor plate using an imager scanner according to manufacturer's instructions. Determine the relative band intensity using ImageJ software according to the software manual.

4.2.8) Use a Geiger counter to check the radioactive contamination in the work area, wipe the suspected items and area with tissue and a cleaning agent and dispose the tissue in the disposal container.

### REPRESENTATIVE RESULTS:

Transcription efficiency can be determined by measuring the level of radiation in the bands at different time points. The pausing assay to test the function of NusA factor enabled visualization of the pause, termination, and run-off products (Figure 1A). In the presence of the N-terminal domain of NusA (NusA NTD; amino acid residues 1-137), appearance of the RNA products was significantly delayed compared to the control experiment lacking NusA. Deletion of amino acid residues 104-137 (Helix 3) of the NusA fragment, or alteration to alanine of a series of amino acids (residues K36, K37, R104, Q108, K111, Q112, Q116, and R119) resulted in the complete loss of NusA pause activity. Helix 3 residues R104 and K111 were shown to be essential for NusA NTD pause activity and with the R104A and K111A constructs giving similar pause half-life values to the control where NusA NTD was absent (Figure 1B). It should be noted that deletion of Helix 3 or alteration of these two amino acid residues has no effect on NusA binding to RNAP<sup>15</sup>. The result suggested that R104 and K111 were the key amino acids required for regulation of NusA pause activity.

Similarly, as shown in Figure 2A, the dose-response curve demonstrated the inhibition of *in vitro* transcription (multi-round) using different concentrations of a newly identified bacterial transcription inhibitor C5<sup>14</sup>. C5 was identified following an *in silico* screen from project to rationally target the interaction of  $\sigma^{70}$  with its major binding site on RNAP called the Clamp-Helix region<sup>18</sup>. With addition of C5 into the transcription reaction, this compound can compete against  $\sigma^{70}$  for binding to RNAP, and thus inhibited transcription. Mechanistically, addition of C5 to RNAP followed by  $\sigma^{70}$  factor to the reaction mixture was significantly more efficient for transcription inhibition than the experiment using C5 after the formation of RNAP holoenzyme (Figure 2B). These results demonstrated that  $\sigma^{70}$  couldn't displace C5 bound to RNAP, or that C5 could not efficiently displace  $\sigma^{70}$  bound to RNAP in a single-round assay.

### Figure Legends:

**Figure 1: Effects of Mutant NusA NTD in transcription.** A) Transcription pause assays without NusA, using NusA NTD (+ NusA), NusA NTD with helix 3 deleted ( $\Delta$  Helix), and NusA NTD with alanine substitutions at residues K36, K37, R104, Q108, K111, Q112, Q116, and R119 (All Ala). P, pause site; T, termination site; RO, run-off. Wedges above the gel demonstrate the time points sampled (0.5, 1, 1.5, 2, 5 and 10 min)<sup>15</sup>; B) Pause activity of half-lives of mutant NusA NTD relative



to wild-type protein. Average of 3 experiments with SD. This figure has been modified from *Nucleic Acids Res.* **43** (5), 2829-2840 (2015).

**Figure 2: Transcription inhibition by compound C5.** A) Inhibition curve of *E. coli* RNAP transcription by compound C5 in a multi-round assay<sup>14</sup>. Percent inhibition of transcription was determined by the RNA product relative to a control (no C5 present) against the concentration of C5 used in the reaction. B) Percent inhibitory activity of compound C5 in single-round transcription assays with addition of C5 before (complex +  $\sigma^{70}$ ; gray) and after (Holoenzyme + C5; black) holoenzyme formation. This figure has been modified from *ACS Infect. Dis.* **2**, 39-46 (2016).

## DISCUSSION:

In all organisms, transcription is a tightly regulated process. *In vitro* transcription assays have been developed to provide a platform for testing the effects of transcription factors, small molecules and transcription inhibitors. In this method paper, an assay for general bacterial transcription was described. Transcription assays combined with sequencing gel electrophoresis of transcripts are very important for mechanistic studies as they allow visualization of all the transcription products along a timeline. As a result, this method is capable of identifying the influence of minor changes in reaction conditions and revealing a plausible mechanism of action. Furthermore, it becomes feasible to design specific transcription assays with regard to the function of transcription factors with unknown mechanisms of action.

In this paper we also described the method to confirm the mechanism of action of an inhibitor of RNAP holoenzyme formation. The method described here can display the change of each RNA transcript with time, and with appropriate modification can enable the testing of antibacterial agents against each step of transcription as appropriate. The multi-round assay can be used as a relative simple screening method to identify new transcription targeting inhibitors, while the single round assay can be used to study the mechanism of inhibitors, such as competitive/non-competitive properties. Note that small molecular aggregation can cause false positive hits as non-competitive inhibitors in the drug discovery process and the single round mechanistic study may provide a comprehensive approach for hit-to-lead evaluation. As a result, this controlled transcription process is capable of precisely revealing how an inhibitor affects transcription, which can help with rationalized *de novo* drug design. Together with high-resolution information on inhibitor binding sites by X-ray crystallographic studies, structure-based rational drug design will be highly amenable for application in drug discovery targeting transcription.

Although high-throughput results cannot be obtained with *in vitro* transcription assays, they are extremely useful in dissecting the precise mechanisms associated with the control of transcription. The components and assay conditions could be readily modified for individual needs. Various factors (e.g. NusA)<sup>4, 15</sup>, small molecules (e.g. ppGpp)<sup>19</sup> and/or transcription inhibitors (e.g. rifampicin)<sup>20</sup> of interest can be added to test their functions in transcription regulation. The DNA template should be individually designed, with careful choice of strong promoters, pause sequence and terminators. In order to achieve high reproducibility of the assay, proteins must be highly purified and free from residual RNase activity, and the transcription

buffer, DNA template, and stock solution of NTP substrates must be made in DEPC treated water. The use of radioactive NTPs was described in this paper, however fluorescent nucleotides can also be employed<sup>21</sup>. Quality of the DNA template is critical for the success of transcription assay. Sufficient spacing between the promoter/pause/terminator must be included, allowing the various transcription products to be properly separated and examined. The sequencing gel must be properly poured and relatively fresh TBE buffer must be used for electrophoresis to achieve highest possible resolution of RNA transcripts. If degradation of transcription product is noticed, high quality RNase inhibitor must be incorporated in the transcription reaction mixture. Tubes and pipette tips must be double autoclaved and gloves must be worn at all times when performing the assay, in order to minimize introduction of RNase.

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#### **DISCLOSURES:**

The authors have nothing to disclose.

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