

A novel method for assessment of *in vitro* transcription for a bacterial promoter

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In vitro transcription (IVT) assay is a useful tool to monitor the transcriptional activity of a specific promoter under defined conditions *in vitro*. Conventional IVT assay involves use of radiolabeled probes which makes it tedious to perform and limits its utility for large scale applications. Here, a reverse-transcription-PCR (RT-PCR) based transcript detection method has been developed for bacterial *in vitro* transcription assay. Unlike conventional radiolabeling approach, this method is simple, fast, needs smaller reaction volumes, does not require special infrastructure and could be potentially used for any large-scale screening applications. The present study demonstrates the feasibility of this method by showing Cyclic AMP Receptor Protein (CRP) mediated activation of a CRP dependent *E. coli* promoter.

Keywords: CyclicAMP Receptor Protein (CRP), IVT assay

In vitro transcription (IVT) assays are carried out to assess the ability of bacterial RNA polymerase to transcribe from a specific promoter or to probe differential transcription from a given promoter under different conditions e.g. in presence of a transcription factor. The components of a typical bacterial IVT assay include a ds-DNA template containing a promoter, ribonucleotide triphosphates (rNTPs), and bacterial RNA polymerase (RNAP) in appropriate buffer conditions. The conventional assays use radioactive probe to label the nascent transcript which necessitates appropriate infrastructure for safe storage, usage and disposal of radioactivity. To add to these problems are limited half-life of commonly used radiolabels, use of tedious denaturing PAGE gels for resolving the transcripts, longer exposure periods required for X-ray films or requirement of expensive instruments such as phosphorimager.

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In recent studies¹⁻³, non-radioactive methods of labeling have been developed where detection is done by direct fluorescent staining of the newly synthesized transcript. However, these would also detect any non-specific nucleic acid present in the reaction. Alternative non-radioactive methods involving incorporation of bulky modified rNTP does not seem to be promising as bacterial RNA polymerases do not incorporate these modified nucleotides efficiently (e.g. Dig-UTP or biotin-UTP by *E. coli* RNAP)¹. Further, the necessity of purification during different stages of assay makes it tedious and vulnerable to losses. Moreover, in all the above approaches, the final handling material is RNA which is highly prone to degradation by RNases. RT-PCR is currently among the most sensitive methods of RNA detection and is widely used for semi-quantitation of RNA levels⁴⁻⁷. Here, we demonstrate a novel and better method for *in vitro* transcription (IVT) assays which uses Reverse transcription (RT)-PCR as an alternative to radiolabeling for IVT. The feasibility of this method has been demonstrated by evaluation of transcription activation of a Cyclic AMP Receptor Protein (CRP) dependent *E. coli* gene promoter in presence of purified CRP.

Materials and Methods

Template preparation for *in vitro* transcription assay

Table.1 shows the list of primer sequences used in this study. CC (-41.5) promoter, a CRP-dependent promoter with consensus CRP-binding site centered at position -41.5 upstream of the *melR* gene, is fused to *lac* operon in pRW50 (a *lac* expression vector: tet^R)⁸. CC (-41.5) promoter DNA fragment (~470 bp), comprising of 80 bp upstream and 266 bp downstream of *melR* gene fused to *lac* operon along with some plasmidic region, was PCR amplified using

SU123F	AGAGCCATCCATGAATACAGATACGTTTAT
SU125F*	ACCCGGGGATCAGGTAAA----- TGGATCCCCCTCACTCCT
SU126R*	AGGAGTGAGGGGGGATCCA----- TTTACCTGATCCCCGGGT
SU128F	TTTCCAACGCGAGCCATTCGCGAGA
SU129R	GCAGGTCGTTGAACTGAGCCTGAAATTCA

*position of CRP target site deletion is shown by dashed lines

pRW50 as a template and plasmid specific forward (SU128F) and reverse (SU129R) primers (as depicted in Fig. 1A). CC ($\Delta 15$) (~455 bp) was synthesized using overlap extension PCR method as described in previously⁹. The flanking primers (SU128F and SU129R) and the overlapping primers (SU125F and SU126R), carrying deletion of 15 bases spanning CRP target site, were used with pRW50 plasmid DNA as template (Fig. 1A). Final PCR products were purified using High pure PCR purification kit (Roche Molecular Biochemicals, Germany).

Electrophoretic mobility shift assays (EMSA)

EMSAs were carried out as described previously⁹. Recombinant CRP protein was purified as described previously¹⁰. Briefly, the DNA samples (PCR products) were mixed with 200 nM purified His-tagged CRP protein in 10 μ L of 1X binding buffer (100 mM KCl, 40 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1 mM dithiothreitol [DTT], 200 μ M cAMP, 100 μ g of bovine serum albumin/mL) and incubated at 37°C for 10 min. Samples were mixed with loading dye (bromophenol blue, 0.25%; xylene cyanol FF, 0.25%; Ficoll 400, 15%) and loaded on to a 10% non-denaturing polyacrylamide gel containing 200 μ M

cAMP (Sigma Aldrich, USA). The gel was electrophoresed at constant voltage (15 V/cm), stained in 1X SYBR green I nucleic acid gel stain (Roche Molecular Biochemicals, Germany), and visualized under UV light using gel documentation system (Syngene, UK).

In vitro transcription reaction coupled with RT-PCR

In vitro transcription

IVT reactions were set up in 10 μ L with appropriate DNA template at ~0.1 nM in a buffer containing 100 mM KCl, 40 mM Tris-HCl pH 8.0, 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 2 mM spermidin, 50 μ g/mL bovine serum albumin (BSA), 200 μ M cAMP, without or with 0.5 nM CRP. The reaction mixture was pre-incubated with 1 U of *E. coli* RNA polymerase Holoenzyme (USB, USA), 16 units of RNase inhibitor (Protector RNase Inhibitor, Roche Molecular Biochemicals, Germany) and CRP (if present) at 37°C for 10 min followed by addition of NTPs (ATP, CTP, GTP, and UTP; 200 μ M) (Roche Molecular Biochemicals, Germany) and further incubation at 37°C for 15 min. The buffer conditions and presence of additional supplements may vary for specific promoters.

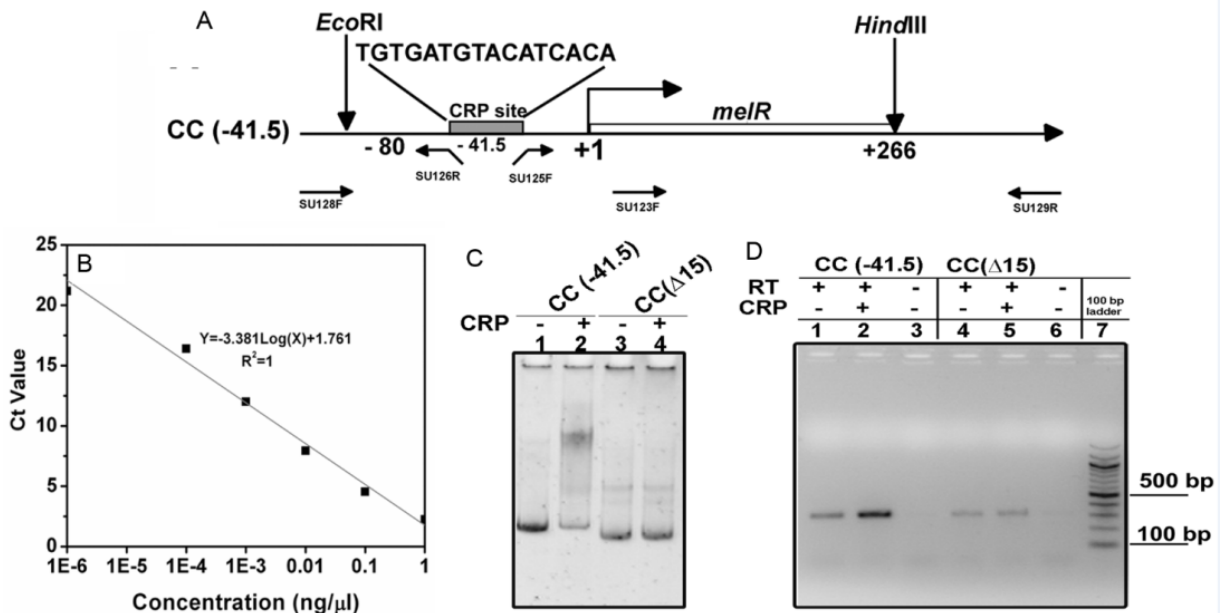


Fig. 1 — (A) A line diagram depicting the *EcoRI*-*HindIII* CC (-41.5) synthetic *melR* fragment (-80 to +266 bp) fused upstream of *lac* operon in pRW50. The position of all the primers is shown by an arrow. TSS (+1) used for $P_{CC(-41.5)}$ transcription is marked; (B) The plot of Ct value as a function of concentration (log axis) of CC (-41.5) PCR product (+13 to +266 using SU123F/SU129R primers) (0.000001 ng/ μ L to 1 ng/ μ L) for calculation of PCR efficiency. The trend line equation and R^2 value are displayed; (C) Electrophoretic mobility shift assays (EMSA) showing CRP binding to CC (-41.5) and CC ($\Delta 15$) promoters; and (D) *In vitro* transcription assays using Reverse transcription (RT)-PCR: Ethidium bromide stained agarose gel showing PCR products after *in vitro* transcription (IVT), and RT-PCR for CC (-41.5) (lane 1-3) and CC ($\Delta 15$) (lane 4-6) promoters in presence (lane 2, 5) and absence of CRP (lane 1,4), PCR products from No RT reactions for CC (-41.5) (lane 3) and CC ($\Delta 15$) (lane 6) are also included.

Reverse transcription

As the RNA transcribed during IVT is finally detected by RT-PCR, any template DNA present may interfere and give rise to false positives during PCR. Therefore, it is necessary to remove the template DNA before proceeding for RT using DNaseI treatment of the IVT sample. Template DNA was removed by treating 10 μ L of IVT reaction with 10 units of DNaseI (Roche Molecular Biochemicals, Germany) (final volume 20 μ L), incubation at 37°C for 1 h followed by heat inactivation of DNaseI at 65°C for 15 min. It should be noted here that no purification step or buffer exchange was performed preceding either DNaseI treatment or reverse transcription thereby minimizing the purification losses. Moreover, the efficiency of DNase treatment or downstream reverse transcription reaction was reasonably good in spite of the carryover from the IVT buffer.

Following removal of the template DNA contamination, the RNA synthesized *in vitro* (2.5 μ L) was converted to cDNA (reaction volume 10 μ L) using a reverse primer (SU129R) complementary to downstream sequence present in the transcript and SuperScript II reverse transcriptase (Invitrogen, USA) as per the manufacturer's instructions. A parallel reaction without RT was also set-up as a control to rule out any template DNA amplification (if there is inefficient DNasing) during PCR.

PCR/Real Time PCR

The Conventional PCR was performed using 1 μ L of cDNA product (final volume 25 μ L) in presence of both forward (SU123F) and reverse primers (SU129R) (Fig. 1A) using Paq5000 DNA polymerase (Agilent technologies, USA) with 25 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 30 s). The same Reverse primer (SU129R) was used for template amplification, RT and PCR. The PCR products were run on a 1.5% ethidium bromide stained agarose gel. Image of the gel was captured under UV light using a gel documentation system (Syngene, UK). Intensity of DNA bands was quantified using ImageJ software (<http://rsb.info.nih.gov/ij>). Intensity of the PCR product was taken as a measure of the net transcript synthesized during IVT. The fold activation was expressed as a ratio of the intensity values in the presence and absence of CRP. The experiment was repeated at least three times and the results were found to be consistent.

Real Time PCR reaction with 0.5X Syber Green was run in Real-Time PCR cycler (Rotor-Gene Q of Qiagen, Germany). Ct value (Threshold Cycle) is defined as the cycle number at which the PCR product crosses a threshold of detection where the threshold line was automatically set by the software algorithm of the instrument. A standard curve analysis was performed with PCR amplification using varying concentration (0.000001 ng/ μ L to 1 ng/ μ L) of CC (-41.5) DNA (+13 to +266) as template and SU123F/SU129R primers for estimation of the PCR efficiency (Fig. 1B). Standard curve was generated manually by plotting Ct values as a function of respective template DNA concentration (log axis) and then by fitting a logarithmic regression trend line. The PCR efficiency, as calculated from the slope of the trend line using following formula, was found to be 1.998 ($R^2=1$)¹¹ (Fig. 1B):

$$E=10^{[-1/\text{slope}]}$$

Subsequently, the Ct values obtained for individual RT-PCR samples were analyzed by REST analysis¹¹ to find out the fold regulation (R) using the following formula (Table 2):

$$R = \frac{(E_{cc})^{\Delta CP_{cc}}}{(E_{\Delta 15})^{\Delta CP_{\Delta 15}}}$$

where, CC is CC (-41.5) and $\Delta 15$ is CC ($\Delta 15$), E is efficiency (same for both CC and $\Delta 15$) and ΔCP is the difference between the Ct values for the sample with no CRP and the sample with CRP.

Results and Discussion

The feasibility of *in vitro* transcription assay, coupled with RT-PCR was demonstrated by evaluation of transcription activation of a Cyclic AMP Receptor Protein (CRP) dependent *E. coli* gene promoter in presence of purified CRP. CRP, in presence of cAMP, regulates gene transcription by binding to a 22 base pair consensus sequence present in the upstream region of some of the *E. coli* promoters¹². In this study, CC (-41.5) promoter, synthetic derivative of *E. coli melR* promoter with consensus CRP target site located at -41.5 with respect to the transcription start⁸, was used to measure

Table 2 — Results from Real Time PCR using REST analysis¹¹

Promoter DNA	Mean Ct values (CRP ⁻)	Mean Ct values (CRP ⁺)	Expression ratio (Based on Rest analysis)
CC (-41.5)	23.515±0.235	21.215±0.115	4.362±0.94204
CC ($\Delta 15$)	21.810±0.140	21.635±0.095	

activation by CRP (Fig. 1A). CRP has been shown to bind and activate transcription from CC (-41.5) promoter both *in vivo* and *in vitro*⁸. CC (Δ 15), with CRP target site (15 bp) deleted from CC (-41.5), was also used to reaffirm that the CRP target site is necessary for the activation. Purified PCR fragments CC (-41.5) and CC (Δ 15), were used as template for *in vitro* transcription. Binding of CRP to the template fragments was checked using electrophoretic mobility shift assays (EMSA) (Fig. 1C). As expected, CC (-41.5), having CRP target site, showed binding to CRP (Fig. 1C, Lane 1-2) while CC (Δ 15), with CRP target site deleted, did not show any binding (Lanes 3 & 4).

IVT reaction products were subjected to reverse transcription (RT) after DNase treatment to get rid of template DNA contamination. RT products were evaluated by both conventional PCR and real time Syber green based PCR method. Here, the level of the PCR product was an indicative measure of the transcript produced during *in vitro* transcription. In line with the previous results based on conventional radiolabeling used for IVT⁸, both CC (-41.5) and CC (Δ 15) promoters exhibited a basal level (in absence of CRP) of transcription as seen by the presence of a faint PCR product with RT (Fig. 1D, lane 1, 4). Absence of a PCR product in no RT sample (lanes 3 & 6) ruled out any template DNA contamination indicating that the DNaseI treatment was successful and the PCR product in the RT samples (lane 1, 2, 4, 5) is originating from the cDNA only. The RT-PCR product from the CC (-41.5) promoter showed an increase (~ 4 fold) in presence of CRP (lane 2) as compared to the levels in the absence of CRP (lane 1) indicating that CRP activates CC (-41.5) transcription. However, CC (Δ 15) lacking the CRP target site, did not show any change in the RT-PCR product upon CRP addition (lanes 4 & 5) reaffirming that the CRP mediated activation for CC (-41.5) is mediated through binding of CRP to the target site. On the same lines, results from Real Time PCR showed a similar increase (4.36 ± 0.94 fold) for CC (-41.5) promoter in presence of CRP (Table 2) while CC (Δ 15) did not show any increase. Hence, the results from IVT-RT-PCR based assay were in line with already published results for CRP mediated CC (-41.5) activation using conventional radiolabeling⁸ and reaffirmed that CRP activates transcription from CC (-41.5) promoter by binding to the CRP target site. Though we have demonstrated the use of this method only for an *E. coli* promoter, it could be potentially used for any

bacterial promoter¹³⁻¹⁶. Use of Real Time PCR makes this method amenable to scale-up and automation which have the potential to be used for large scale drug screening assays e.g. for discovering new class of bacterial RNA polymerase inhibitors¹⁷⁻²¹.

Conclusion

The present study is a proof of concept to show that RT-PCR based *in vitro* transcription assay could be used for qualitative assessment of the basal level of transcript as well as differential transcription from a bacterial promoter *in vitro*. To summarize, the major advantages of this method over conventional radiolabeling are that (i) it is less time consuming, simple and easy to perform; (ii) being non-radioactive, it does not need special infrastructure; (iii) cDNA being less prone to degradation than RNA, makes the handling easier; (iv) can be performed in very small volumes thereby reducing the reagent costs; (v) absence of any purification step minimizes the losses; and (vi) use of Real Time PCR makes this method amenable to scale-up and automation.

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Conflict of interest

The authors declare no conflict of interest regarding this publication.

References

- 1 Kuhlman P, Duff HL & Galant A, A fluorescence-based assay for multisubunit DNA-dependent RNA polymerases. *Anal Biochem*, 324 (2004) 183.
- 2 Sim J, Byun JY & Shin YB, Transcription immunoassay: light-up RNA aptamer-based immunoassay using *in vitro* transcription. *Chem Commun*, 55 (2019) 3618.
- 3 Cook A, Hari-Gupta Y & Toseland CP, Application of the SSB biosensor to study *in vitro* transcription. *Biochem Biophys Res Commun*, 496 (2018) 820.
- 4 Joyce C, Quantitative RT-PCR. A review of current methodologies. *Methods Mol Biol*, 193 (2002) 83.
- 5 Gupta S, Dongre A, Saxena J & Jyoti A, Computation and *in silico* validation of a Real-Time PCR array for quantitative detection of pathogens isolated from blood sample in sepsis patients. *Indian J Biotechnol*, 16 (2017) 505.
- 6 Gupta S, Singh SV, Gururaj K, Chaubey KK, Singh M, Lahiri B, Agarwal P, Kumar A, Misri J, Hemanti Z & Bhatia AK, Comparison of IS900 PCR with 'Taqman probe PCR' and 'SYBR green Real Time PCR' assays in patients suffering with thyroid disorder and sero-positive for

- Mycobacterium avium* subspecies *paratuberculosis*. *Indian J Biotechnol*, 16 (2017) 228.
- 7 Gurjar MS, Aggarwal R, Jogawat A, Sharma S, Kulshreshtha D, Gupta A, Gogoi R, Thirumalaisamy PP & Saini A, Development of Real Time PCR assay for detection and quantification of teliospores of *Tilletia indica* in soil. *Indian J Exp Biol*, 55 (2017) 549.
 - 8 Gaston K, Bell A, Kolb A, Buc H & Busby S, Stringent spacing requirements for transcription activation by CRP. *Cell*, 62 (1990) 733.
 - 9 Uppal S, Maurya SR, Hire R & Jawali N, Cyclic AMP Receptor Protein (CRP) regulates *cspE*, an early cold-inducible gene, in *Escherichia coli*. *J Bacteriol*, 193 (2011) 6142.
 - 10 Uppal S & Jawali N, Cyclic AMP Receptor Protein (CRP) regulates *cspA*, *cspB*, *cspG* and *cspI*, members of *cspA* family, in *Escherichia coli*. *Arch Microbiol*, 197 (2015) 497.
 - 11 Pfaffl MW, Horgan GW & Dempfle L, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in Real-Time PCR. *Nucl Acids Res*, 30 (2002) e36.
 - 12 Busby S & Ebright RH, Transcription activation by Catabolite Activator Protein (CAP). *J Mol Biol*, 293 (1999) 199.
 - 13 Morita T, Nishino R & Aiba H, Role of the terminator hairpin in the biogenesis of functional Hfq-binding sRNAs. *RNA*, 23 (2017) 1419.
 - 14 Miura C, Komatsu K, Maejima K, Nijo T, Kitazawa Y, Tomomitsu T, Yusa A, Himeno M, Oshima K & Namba S, Functional characterization of the principal sigma factor RpoD of phytoplasmas via an *in vitro* transcription assay. *Sci Rep*, 5,(2015) 1.
 - 15 Gupta M, Mukherjee S, Dutta A, Mukhopadhyay J, Bhattacharyya D & Mandal S, Identification of a suitable promoter for the sigma factor of *Mycobacterium tuberculosis*. *Mol Biosyst*, 13 (2017) 2370.
 - 16 Plessis DJ, Cloete R, Burchell L, Sarkar P, Warren RM, Christoffels A, Wigneshweraraj S & Sampson SL, Exploring the potential of T7 bacteriophage protein Gp2 as a novel inhibitor of mycobacterial RNA polymerase. *Tuberculosis (Edinb)*, 106 (2017) 82.
 - 17 Singh SN, Anand R, Pasha ST, Gupta S, Basir SF, Khare S & Lal S, Molecular characterization of mutation associated with rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. *Indian J Exp Biol*, 44 (2006) 547.
 - 18 Debasmita D, Rath S, Sahu MC, Debata NK, Padhy RN & Rabindra N, Antimicrobials of plant origin against TB and other infections and Economics of plant drugs – Introspection. *Indian J Tradit Knowl*, 11 (2012) 225.
 - 19 Dutt M & Khuller GK, Poly (DL-lactide-co-glycolide) microparticles as carriers for antimycobacterial drug rifampicin. *Indian J Exp Biol*, 38 (2000) 887.
 - 20 Sharma A, Magotra A, Bhatt S, Dogra A, Wazir P, Satti P, Naresh K, Singh G, Bhusari S & Nandi U, Potential herb-drug interaction of a flavone glycoside from *Cuminum cyminum*: Possible pathway for bioenhancement of rifampicin. *Indian J Tradit Knowl*, 17 (2018) 776.
 - 21 Solankee A, Tailor R & Kishor K, *In vitro* antimycobacterial and antimicrobial activity of some new pyrazoline, isoxazole and benzodiazepine derivatives containing 1,3,5-triazine nucleus via chalcone series. *Indian J Chem Sec B*, 55 (2016) 1277.