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Asymmetric PCR provides alternatives for preparation of (GT)₅ tailed duplex DNA promoter for promoter trapping

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Abstract

Synthesis of (GT)₅ tailed duplex DNA promoter is an important first step for purifying transcription complexes by promoter trapping purification. In our earlier publication we have shown that the purification of the c-jun promoter using lambda exonuclease digestion of PCR produced DNA with single stranded tails. Asymmetric polymerase chain reaction (PCR) can also produce tailed single strands which can be annealed to yield the desired promoter. An effective method utilizes asymmetric PCR and double digestion. After PCR, first a restriction enzyme, in this case *SacII*, cuts duplex strands remaining after asymmetric PCR, leaving 5'-phosphoryl ends susceptible to a second digestion with lambda exonuclease to effectively degrade any duplex. The resulting single strands are then annealed to produce a duplex DNA with a single stranded (GT)₅ tail at the 3' end of each strand of the duplex. Unlike the previously described method, this novel procedure produces the desired tailed promoter devoid of any untailed duplex.

Keywords

transcription; transcription factors; Southwestern blotting; affinity chromatography

Introduction

In promoter trapping chromatography (Fig. 1), an intact, transcriptionally active promoter is used to isolate the components of the transcription complex [1]. The promoter, at nanomolar concentrations, is mixed with nuclear extract to allow the transcription complex to form. The protein-DNA complex is then applied to a column where single stranded ends engineered into the promoter DNA anneals with its complement sequence immobilized on the stationary phase. After washing to remove proteins not involved in transcription of this promoter, the transcription complex proteins can be eluted for further characterization. Trapping on the column requires that the promoter DNA contain a single stranded tail, typically (GT)₅, which anneals with a column containing an (AC)₅ DNA. Production of (GT)₅ tailed duplex DNA promoter is an important first step. Previously, we used a single 5' phosphoryl primer and lambda exonuclease digestion to produce single strands by symmetric PCR, that is by using equal concentrations of the forward and reverse primers [1]. By performing two such reactions, the upper and lower strands are purified and annealed to yield the final tailed promoter. This relies on the fact that lambda exonuclease only degrades strands containing a

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5' phosphoryl end, allowing us to choose which strand will be digested and which strand be retained undigested. One problem with this approach is that if the phosphorylation of the primers is incomplete, the resulting DNA will contain some that is untailed, which will diminish yield during subsequent trapping.

PCR can also be performed asymmetrically, using an excess of one primer to predominantly produce that primer's strand in excess. Here, we report that asymmetric PCR can provide the single strands which easily separate by agarose gel purification. Alternatively, since digestion with a restriction endonuclease only digests duplex DNA and leaves 5'-phosphoryl ends, restriction alone or combined with lambda exonuclease digestion can be used to effectively remove any remaining duplex DNA from the prepared single strands.

2. Materials and Methods

2.1. Materials

All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). Lambda exonuclease, Taq DNA polymerase, and *SacII* restriction endonuclease enzymes were from New England BioLabs (Ipswich, MA, USA). RNasin and rNTPs were from Promega (Madison, WI, USA). Creatine phosphate kinase, phosphocreatine and actinomycin D were from Sigma Chemical Co. (St. Louis, MO, USA). Poly (2'-deoxyinosinic-2'-deoxycytidylic acid) (poly dI:dC) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase were from Thermo Scientific (Waltham, MA, USA). (AC)₅-Sephacrose was prepared as previously described [1].

2.2 PCR

All PCR reactions were 50 µl which contained primers as specified, 0.2 mM of each dNTP, 5 mM MgCl₂, 20 ng pUC19-cjun, and 0.5 units of Taq polymerase in the reaction buffer supplied with the enzyme. As the template, the pUC19-c-jun plasmid described previously [1], containing c-jun core promoter DNA sequence from -200 to +81, was used. This was heated to 95°C for 5 min, thermocycled 95°C 1 min, 55°C 1 min and 72°C 2 min for 35 cycles, and extension at 72°C for another 10 min. When needed, multiple identical reactions were pooled to obtain the desired amount of DNA. Excess primers and mononucleotides were removed from PCR products using the GenCatch Advanced PCR Extraction kit (Epoch Biolabs Inc., Missouri City, TX).

2.3 Preparation of c-jun promoter without a (GT)₅ tail by symmetric PCR

Symmetric PCR was performed to produce the c-jun core promoter without a GT tail. PCR reactions contained equal amounts, 1 pmol of FP and RP (Table 1). This c-jun promoter DNA was used for Southwestern blotting and primer extension assays.

2.4 Preparation of c-jun promoter with a single (GT)₅ tails by symmetric PCR and lambda exonuclease digestion

To produce the DNA with single-stranded 3'-(GT)₅ tails for promoter trapping experiment, two different PCR reaction were performed. The first reaction utilized 1 pmol each of FP and (AC)₅RP primers, while the second reaction utilized RP and (AC)₅FP primers (Table 1). In each case, the primer containing the (AC)₅ is phosphorylated and susceptible to subsequent exonuclease digestion. For each reaction, multiple identical tubes were combined to obtain 250 µg DNA product which was then digested with 100 units of lambda exonuclease for 2h at 37°C, using the buffers and protocol provided by the manufacturer. The phosphorylated strand is thus removed by digestion leaving complementary single strands in the two reactions; the single strands with a 3'-(GT)₅ tails remained after lambda exonuclease digestion. The resulting two (GT)₅ tailed single strands were then mixed in

equal proportion and annealed to yield a final duplex c-jun promoter with 3'-(GT)₅ single stranded ends.

2.5 Preparation of c-jun promoter with (GT)₅ tails by asymmetric PCR and enzyme digestion

Asymmetric PCR uses different concentration of primers to produce one strand predominantly in PCR. Again, two reactions are performed to produce the two desired strands. Reaction 1 utilized 50 pmol FP2 and 1 pmol of (AC)₅RP2, a 50 fold excess. Reaction 2 utilized 50 pmol RP2 and 1 pmol (AC)₅FP2. Notice in this case none of the primers are phosphorylated. For asymmetric PCR, we found it necessary, after the last cycle to add 0.5 μ l of 0.5 M EDTA to each reaction to prevent degradation of the produced single stranded DNA.

Restriction can be used to cut any remaining duplex and can aid in gel purification. The c-jun promoter sequence has a *Sac II* restriction site (CCG/GG) at position 32. Since restriction also yields a 5'-phosphoryl at the cleavage site, these fragments are then susceptible to lambda exonuclease digestion. In the end a single stranded (GT)₅ tailed DNA was produced. After PCR (50 μ l) and GenCatch Advanced PCR Extraction, *SacII* digestion buffer and 15 unit of *SacII* was added in 50 μ l. The reaction was incubated for 90 min at 37°C and then adding 1.5 unit of lambda exonuclease continuing the incubation for another 30 min.

2.6 Annealing

Once prepared, single strands were ethanol precipitated and mixed in equal proportions in TE0.1 buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl). The mixture was heated to 95°C and either allowed to cool slowly to room temperature or cooled in a thermocycler over 1 h to 4°C.

2.7 Gel Purification

After annealing, the DNA was separated on, a 2% agarose gel by electrophoresis. The duplex band was excised and then gel extraction was performed using the Gel extraction kit (Epoch Biolabs Inc.), using the supplied protocol.

2.8 Purification of large amount of (GT)₅ tailed c-jun promoter by DNA affinity chromatography

Equal amounts of the two single stranded DNAs were annealed. The annealed product was applied to a 500 μ l (AC)₅-Sephacrose column at 4°C. The column was washed thoroughly with TE 0.1 buffer (10mM Tris-HCl, pH 7.5, 10 mM EDTA and 0.1 M NaCl) at 4°C. Then the column was eluted with TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) at 60°C. Fractions containing the purified duplex tailed c-jun promoter were collected and the purity determined by 2% agarose gel electrophoresis. It was store at -20°C before to use.

2.9 Preparation of nuclear extract

Nuclear extract was prepared from HEK293 cells as previously described [2] and stored as 3–5 mg/ml protein as determined by the Bradford protein assay [3].

2.10 Promoter trapping chromatography

500 μ g of nuclear extract was diluted to a final volume of 1ml with binding buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 0.1 % Tween-20, 50 μ M ZnSO₄ and 25 μ g/ml poly dI:dC) and incubated for 10 min on ice. The (GT)₅ tailed c-jun promoter DNA was then added to a final concentration of 60 nM and the mixture gently

mixed on a wheel rotator at 4°C for additional 30 min before passing over a 250 µl of (AC) 5-Sepharose column. The protein was eluted with TE0.5 (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl). Fractions were concentrated and desalted using a centrifugal desalting column. The protein was immediately used for 2DGE-Southwestern (2DGE-SW).

2.11 2DGE-Southwestern blotting (2DGE-SW)

For two-dimensional gel electrophoresis and Southwestern blotting, the first dimension of electrophoresis consisted of nuclear extract or proteins from promoter trapping rehydrated onto a pH 3–10 IPG strip and isoelectric focusing (IEF) was performed using 250 v for 15 min followed by 4 000 V for a total of 20 000 V/h. For second dimension of electrophoresis, the strips were transferred to 10% SDS-PAGE gel and electrophoresed for 2 hours at 100 volts. The gels were then either silver stained or blotted to PVDF membranes. For Southwestern blot, the membrane was blocked in binding buffer containing 5% milk for overnight at 4°C. Then the blot was probed with 2 nM ³²P 5' end labeled c-jun promoter (Section 2.3).

2.12 Primer Extension Assay

The primer extension assay as described [4] was followed with the following minor modifications. Briefly, 200 µg of nuclear extraction was diluted to 200 µl in promoter trapping binding buffer with or without 30 nM of c-jun promoter without a GT tail (Section 2.3). The sample was incubated on ice for 30 min before beginning transcription. For transcription, 12 µl of 10 mM rNTPs, 0.625 µl of 40 unit/µl RNasin, 1.5 µl of 1 M DTT, 1.5 µl of 2 unit/µl creatine phosphate kinase and 2 µl of 1.2 M phosphocreatine were to each sample. The samples were incubated at 30°C for 60 min to synthesize mRNA. RNA was phenol/chloroform extracted and ethanol precipitated. The dried mRNA was dissolved in 10 µl annealing buffer (5 mM Tris-HCl, pH 8.3, 1 mM EDTA, and 75 mM KCl) containing 0.1 pmol ³²P labeled RP3 (Table 1) and annealed at 50°C for 45 min. Primer extension then was performed by adding 20 µl of annealing buffer containing additions to make the final 30 µl mixture 15 mM DTT, 4.5 mM MgCl₂, 0.5 mM dNTP, 1.5 µg actinomycin D, 25 units RNasin, and 200 units M-MLV at 37 °C for 60 min. The product was resolved by 8 M urea in 6% polyacrylamide gel electrophoresis and detected by autoradiography.

3. Results and Discussion

3.1 Restriction endonuclease *SacII* can be used to digest double-stranded DNA to facilitate the production of single stranded DNA by asymmetric PCR

Promoter trapping (Fig. 1) is used to purify the components of a transcription complex. A necessary first step is to prepare the duplex promoter with the necessary single stranded ends used in recovery of the transcription complex. To prepare a duplex promoter containing single stranded tails for promoter trapping, there are essentially two approaches that could be used: 1. The duplex DNA could be produced and subsequently the tails ligated to the ends. This approach was explored and discarded because it was not found to yield sufficient tailed DNA, devoid of untailed DNA, for our purposes (data not shown). 2. The individual single strands can be prepare and later annealed to yield the desired product. This approach is further explored here.

Restriction endonucleases only cleave duplex DNA and can be used to effectively deplete it from single stranded DNA, which is not digested. The 281 base pair c-jun promoter DNA has a site of *SacII* digestion at position 32. Lane 1 (Fig. 2) shows the symmetric PCR product, the 281 bp duplex DNA c-jun promoter sequence. Digestion with *SacII* endonuclease produces the expected size products. Asymmetric PCR (lane 3) produces both the duplex band (ds) and a light band between 200 and 100 bp presumed to be the single

stranded product (ss). Since duplex DNA stains better with ethidium bromide than does single strands, the proportion is not clear. Restriction with *SacII* effectively digests the duplex band while leaving the single strand intact and can be used to effectively remove duplex.

3.2 Preparation of a single stranded c-jun promoter DNA with a single (GT)₅ tail by symmetric PCR and Lambda exonuclease digestion

In our earlier publication [1], we have produced (GT)₅ single stranded tailed duplex c-jun promoter DNA by symmetric PCR and lambda endonuclease digestion. This approach is shown in Fig. 3A. Briefly, two reactions with phospho (AC)₅FP and RP (not phosphorylated, lane 1) or phospho (AC)₅RP with FP (not phosphorylated, lane 3) are performed. Lambda endonuclease only digests strands containing a 5' phosphorylation and is then used to remove one strand from each reaction to yield the desired single strands in lanes 2 and 4. Lanes 2 and 4 show that after lambda exonuclease some duplex DNA always remains. Even further digestion with more lambda endonuclease and for longer times did not change the outcome (data not shown) indicating that incomplete digestion is not at fault. More likely, phosphorylation during synthesis of the oligonucleotides is incomplete. Since the transcription complex being purified will also bind this remaining duplex and not be subsequently trapped, it will diminish the yield of promoter trapping. To prevent this, we sought an alternative approach.

3.3 Preparation of tailed promoter by asymmetric PCR and dual digestion with *SacII* and lambda endonuclease

In Fig. 3B is shown an effective approach to solve this problem of remaining duplex. Asymmetric PCR yields both duplex and single stranded DNA (lane 1). Digestion with *SacII* restriction endonuclease (lane 2) cleaves this duplex leaving a 5' phosphoryl on either side of the cut site. *SacII* digestion effectively removes the duplex and further digestion with lambda exonuclease (lane 3) removes the *SacII* fragments leaving the desired single strand. Using the other asymmetric PCR primer pair yields similar results (data not shown).

3.4 The Overall Yield of the Two Methods is Similar

Table 2 shows a balance sheet for the preparation of (GT)₅ single tailed c-jun promoter by the asymmetric and symmetric PCR methods. The absorbance 260 nm reveals that both reactions by either method yield approximately the same amount of DNA. In these experiments, the absorbance of the added primers, which together with mononucleotides should have been removed by the PCR extraction purification (section 2.2), is only about 3% of the absorbance measured showing that the measurement is primarily recording the amount of DNA synthesized during PCR. After digestion, annealing of the two strands, and gel purification of the duplex, symmetric PCR results in about 50% more DNA, however, as shown in Fig. 3A, it may be contaminated with untail duplex promoter. The extent of this contamination was next investigated.

3.5 Preparation of (GT)₅ single stranded tailed c-jun promoter by affinity chromatography

Once the single strands are prepared, they are mixed in equal amounts and annealed in TE0.1 buffer. The heating also denatures any remaining enzymes used and the buffer here is important since all DNases require divalent cations and the EDTA prevents any digestion by any enzymes activity that remains.

Fig. 4 shows the outcome of purifying the (GT)₅ tailed duplex c-jun promoter DNA by using (AC)₅-Sepharose column and also reveals the amount that would bind in a promoter trapping experiment. A duplex DNA was produced using the asymmetric PCR approach

using *SacII* and Lambda exonuclease and the two single strands annealed (Fig. 4A, input). The (GT)₅ tail on the promoter anneals at 4°C to the (AC)₅ sequence on the column and no detectable DNA appears in the flow through (unretained, FT) fraction from the column; the same volume of flow through (FT) as input was loaded on the gel. After thorough washing (W), the column was heated to 60°C and eluted with TE buffer. Under these conditions, (GT)₅ does not anneal with (AC)₅, and the tailed duplex promoter elutes (E) and little elutes in subsequent fractions (data not shown). Fraction E, containing the (GT)₅ tailed duplex c-jun promoter DNA, was retained and concentrated by ethanol precipitation.

When the tailed duplex promoter was prepared by symmetric PCR and lambda exonuclease digestion and the duplex gel purified, the results of affinity chromatography are notably different (Fig. 4B). In this case, the unretained fraction (FT) contains a significant amount of DNA. This is presumably untailed promoter DNA which is an undesirable side product of preparing the promoter DNA by this method. Some does elute in E1 and E2. Densitometry of the gels shown in panels A and B reveals the difference. The asymmetric PCR method (panel A) results in 84% of the applied DNA (input) eluting (fraction E), while the symmetric PCR method only yields 34.7% eluting (E1+E2). Thus, the contaminating untailed duplex promoter in the symmetric method would result in significantly lower yield in promoter trapping if not removed while the asymmetric method does not suffer from this problem. Thus, losses upon affinity chromatography (Fig. 4) negate the modest increase in the symmetric yield seen in Table 2.

3.6 The produced promoter DNA is transcriptionally active

Unless the prepared promoter is capable of driving transcription, it would not be useful. This was next demonstrated using a primer extension assay. The prepared promoter binds a transcriptionally active complex as shown in Fig. 5. In this primer extension assay, the produced mRNA should be 81 nt in length. The RNA is then reverse transcribed using a primer (RP3, Table I) complementary to the 3' end of the RNA and yields an 81 nt cDNA (Fig. 5) only when the promoter DNA (+) was added. When other primers from the upstream region of the c-jun promoter were used, no cDNA was produced (data not shown). Thus, promoter trapping with this DNA has the potential to purify the components necessary for transcription.

3.7 (GT)₅ tailed promoter trapping is applicable to the purification of transcription factors

In Fig. 6, we performed a 2DGE silver stain and Southwestern blot of nuclear extract (Fig. 6 A and B, respectively) and after c-jun promoter trapping (Fig. 6 C and D). The silver staining of nuclear extract (A) shows many spots in 2DGE. It is not easy to identify each spot on the gel by subsequent methods such as mass spectroscopy, although in rare cases this has been possible [5]. When the nuclear extract gel blot is subjected to southwestern blotting, using the entire 281 bp c-jun promoter DNA (2 nM) as probe (panel B), over 30 spots are revealed to be high affinity DNA-binding proteins, or alternatively, high abundance, lower affinity proteins. In panel C, is shown the silver staining resulting after promoter trapping. Now there are far fewer spots which are well resolved and could be further analyzed, suggesting that promoter trapping has purified a much simpler group of proteins. Since we had previously shown that promoter trapping yields a transcriptionally active complex capable of transcribing RNA [1], we assume these proteins are components of the transcription complex. When these proteins are probed with the 281 bp c-jun promoter DNA, we count only 10 spots representing high affinity DNA-binding proteins. This region of the c-jun promoter is known to have two AP1 sites, a single Sp1, CTF, MEF2, NF-jun and SMAD3/4 site, and the Ku-jun site we recently identified [6], a total of 8 transcription factor binding sites. Since some of these are heterodimers, 10 is a reasonable number for the

expected DNA-binding transcription factors. Thus likely, these high affinity DNA-binding spots are the transcription factors in this transcription complex.

More recently, we have begun to apply these techniques both to the study of the 281-mer c-jun promoter but also the human telomerase (hTERT) 317-mer core promoter (data not shown). Preliminary results with hTERT also strongly suggest successful isolation of a transcription complex for hTERT.

Purification of (GT)₅ tailed promoter DNA (c-jun or hTERT) is a first step for producing promoter duplex DNA with (GT)₅ tails at each 3' end. Here, we have shown that asymmetric PCR might provide a useful alternative (Figs.2–4) approach which effectively produces the desired, tailed duplex promoter without any remaining untail duplex and should give higher yield in promoter trapping. Promoter trapping successfully purified the desired, transcriptionally active complex.

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Fig. 1.

A schematic representation of the promoter trapping technique. The promoter trapping technique was described previously [1]. A duplex promoter is prepared that contains single stranded ends used for recovery. This promoter, typically at 50 nM concentration, is mixed with nuclear extract on ice and a transcription complex assembles on the promoter. The protein-DNA complex is then recovered by annealing at 4°C the single stranded tails (here (GT)₅) which binds to Sepharose beads containing the immobilized, complementary single stranded ((AC)₅) in a column. The column is then thoroughly washed to remove proteins not bound in the complex, and then eluted with a 0.5 M salt containing buffer, which weakens protein-DNA interactions, allowing the transcription complex proteins to be recovered while the DNA remains on the column.

Fig. 2.

SacII digested only the double stranded 281 bp core c-jun promoter DNA resulting from symmetric PCR (lane 1 and 2), which uses an equal amount of forward (FP) and reverse (RP) primers and asymmetric PCR (lane 3 and 4) using a 50 fold excess of RP. Primer sequences are given in Table 1. Lane 1 and 3 without *SacII* enzyme digestion (–) as shown; lane 2 and 4 with *SacII* restriction endonuclease digestion (+).

Fig. 3.

Preparation of a single (GT)₅ tailed promoter DNA by using 5'-phosphorylated primers and lambda exonuclease. **A**, Symmetric PCR was performed by using a pair of (AC)₅ FP and RP (lane 1) or (AC)₅ RP and FP (lane 3). The primers containing the (AC)₅ tail are also phosphorylated (Table 1) during synthesis. In lanes 2 and 4, the DNAs from lanes 1 and 3 are digested with lambda exonuclease at 37°C for 30 min. This yields the two single strands which can then be annealed to produce the desired tailed duplex DNA. **B**, Asymmetric PCR was performed by utilizing 50 pmol FP2 and 1 pmol of (AC)₅RP2, a 50 fold excess. Lane 1 shows the product from this asymmetric PCR reaction. The duplex band is efficiently restricted by *SacII* digestion (lane 2) and subsequent lambda endonuclease digestion leave the single strand in lane 3 with no remaining duplex. In the figure, an asterisk (*) is placed next to the double stranded DNA, a double asterisk (**) next to the *SacII* fragment, and "ss" denotes the single stranded DNA.

Fig. 4. Purification of (GT)₅ tailed c-jun promoter by DNA affinity chromatography. **A.** Duplex DNA prepared by asymmetric PCR, *SacII* endonuclease and lambda exonuclease digestion, annealing of the two strands and gel purification. The annealed DNA contains the duplex c-jun core promoter with a 3'-(GT)₅ single stranded tail on each strand. The annealed tailed duplex DNA (input) was loaded to a (AC)₅-Sephrose column as described in methods. FT, flow through, W, wash, and eluted (E) fractions. **B.** Symmetric PCR and lambda exonuclease digestion, followed by annealing and gel purification of the duplex was performed and the DNA loaded onto an (AC)₅-Sephrose column. The column was loaded, washed and eluted as in panel A.

Fig. 5.

Nuclear extract was used to show that the produced promoter binds an active transcription complex. Nuclear extract was incubated with (+) or without (–) c-jun promoter DNA and transcription was demonstrated using a primer extension assay. The product, was resolved by 8M urea, 6% polyacrylamide gel electrophoresis and detected by autoradiography. The product is an 81 nt cDNA, the expected size.

Fig. 6. 2DGE-SW analysis of nuclear extract and of purification by c-jun promoter trapping. 100 μ g of HEK293 cell nuclear extract was separated by IEF (pH range 3 to 10), followed by a second dimension of SDS-PAGE on a 10% gel. After electroblotting, one gel was stained with silver nitrate (A), while another gel was transferred to PVDF membrane for SW using radiolabeled 281-mer c-jun promoter DNA as probe (B). In panel C and D, the samples were also separated by 2DGE after c-jun promoter trapping, showing silver staining (C) and SW (D). For all SW, the probe was 2 nM c-jun promoter labeled with [γ - 32 P]-ATP.

Table 1

Oligonucleotide sequences

c-jun promoter	
Name	Primer sequence
FP	5'-cgggatcccagcgggagcattacatc-3'
RP	5'-cgggaattcgtggctgtgtctgtctgc-3'
(AC) ₅ FP*	5'-Phos/acacacacggatcccagcgggagcattacc-3'
(AC) ₅ RP*	5'-Phos/acacacacgaattcgtggctgtgtctgtctgc-3'
FP2	5'-cagcgggagcattacatc-3'
RP2	5'-ctggctgtgtctgtctgtct-3'
(AC) ₅ FP2	5'-acacacacaccagcgggagcattacatc-3'
(AC) ₅ RP2	5'-acacacacactggctgtgtctgtctgtct-3'
For Primer extension	
Name	Primer sequence
RP3	5'-gctggctgtgtctgtctgtct-3'

* "Phos/" in a sequence denotes a 5' phosphate added during synthesis

Table 2**Balance sheet of Tailed Promoter Synthesis**

Asymmetric PCR (n=4)		
Rxn1	Rxn2	After digestion, annealing, and gel purification
$6.98 \pm 1.13^{**}$	6.90 ± 0.55	2.07 ± 0.27
Symmetric PCR (n=4)		
6.53 ± 0.38	6.22 ± 0.51	3.16 ± 0.40

* For asymmetric PCR, digestion is with Sac II, followed by lambda exonuclease, followed by annealing and gel purification. Symmetric PCR is the same except without Sac II digestion.

** Shown is the mean of the absorbance at 260 nm $\pm$ standard deviation