

# *in vitro* Transcription on DNA Templates Immobilized to Streptavidin MagneSphere® Paramagnetic Particles



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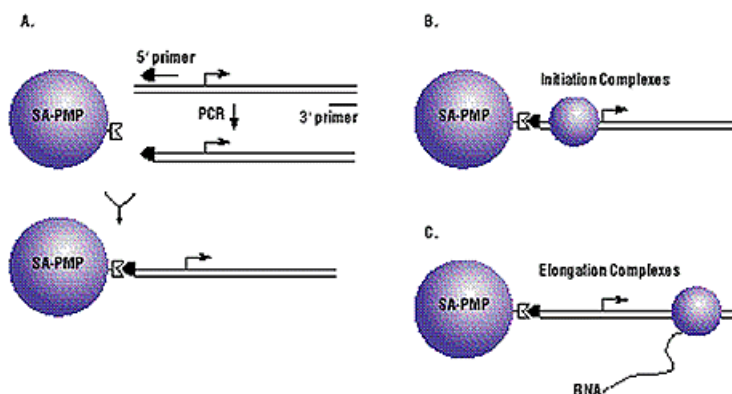
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The immobilization of molecules has successfully aided research in many areas, including purification of proteins and the study of protein-protein and protein-nucleic acid interactions. We have used Promega's Streptavidin MagneSphere® Paramagnetic Particles (Cat.# Z5481) to immobilize DNA templates for use in the study of RNA polymerase II complex formation and transcription *in vitro*. The ability to rapidly isolate transcription complexes formed on the immobilized templates and the ease with which reaction conditions can be manipulated are particularly helpful for dissecting the complex interactions inherent in transcription.

## INTRODUCTION

A detailed *in vitro* study of the mechanistic details of factors involved in transcription by RNA polymerase II requires a completely defined and reconstituted *in vitro* transcription system. However, the complex nature of eukaryotic transcription systems makes purification of the numerous components present in crude nuclear extracts a daunting task. Fortunately, this significant problem is partially circumvented by using immobilized components in the transcription reaction. Immobilization of components enables quick isolation and subsequent manipulations such as transcription factor 'add-back' procedures. Immobilized proteins or DNA templates have been used widely for the identification and characterization of transcription factors, as well as for a variety of structural studies (1-3).

The immobilization of template DNA, to which the protein complex can assemble itself, instead of direct immobilization of protein factors, helps to maintain the activity of the transcriptional components allowing for the study of initiation (4,5), elongation (6,7) and termination (2,8) of transcription by RNA polymerase II. In a typical experiment, unfractionated nuclear extract is incubated with the immobilized promoter-containing DNA template to form preinitiation complexes. Transcription by RNA polymerase II is then initiated by adding one radiolabeled ribonucleotide triphosphate (rNTP) and the remaining three cold rNTPs to the reaction. Elongation complexes that contain nascent transcripts are isolated for further manipulation or "chased" to yield longer transcripts with the addition of excess unlabeled rNTPs. The transcription complex can be stalled during transcription by addition of EDTA to chelate magnesium. Once halted, RNA polymerase II complexes can be washed, reaction conditions altered and the complexes then allowed to reenter active transcription through the addition of required components. Using this strategy, the properties of the transcription complexes or the effects of protein factors on complexes during different stages of transcription can readily be studied (Figure 1), unless they are magnesium-insensitive. In this report, we describe one procedure for using Promega's Streptavidin MagneSphere® Paramagnetic Particles (Cat.# Z5481), or SA-PMPs, to generate immobilized templates for use in studying RNA polymerase II transcription. To demonstrate this technology, we used unfractionated nuclear extracts in transcription studies of the human immunodeficiency virus long terminal repeat (HIV-LTR) and the *Drosophila actin 5C* (*Act5C*) promoter *in vitro*.



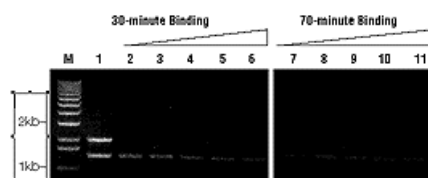
**Figure 1. Schematic diagram of the SA-PMPs, biotinylated PCR product and formation of initiation complexes on immobilized template DNA. Panel A:** The generation of immobilized template by PCR. The biotinylated 5' primer and a standard 3' primer are used in PCR amplification to generate the DNA fragment containing the promoter of interest. The biotinylated template DNA then is immobilized on Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs). Although drawn as spheres in the diagram, the SA-PMPs are irregularly shaped. **Panel B:** RNA polymerase II initiation complexes can form on the immobilized template. **Panel C:** With the addition of nucleotides, the RNA polymerase II early elongation complexes can form on the immobilized template.

## GENERATION OF IMMOBILIZED DNA TEMPLATE

Promega's Streptavidin MagneSphere<sup>®</sup> Paramagnetic Particles bind biotinylated DNA templates through one of the strongest known biological interactions ( $K_d = 10^{-15}$ M). Since the SA-PMPs are paramagnetic they can be attracted by magnets. Magnetics capture technology can be used to isolate the immobilized template rapidly and efficiently.

Immobilized templates used in these experiments were generated by PCR using a linearized pGL2-Basic Vector<sup>(a)</sup> (Cat.# E1641) DNA template containing the promoter region (475 to +76 relative to the transcription start site) of HIV-LTR. This fragment included the TAR RNA-encoding region that is required for transactivation of Tat (9). Our PCR amplification approach consisted of generating a product slightly larger than the insert, which we were then able to restrict at various sites in the 3'-end of the fragment downstream of the transcription start site. Therefore, the 3' primer was designed to hybridize to the pGL2-Basic Vector and function with any insert. This 3' primer and a biotinylated, 5' primer were used to generate an initial 3.5kb product. The PCR product then was restricted approximately 700bp 3' to the transcription start site. We have determined that the immobilization site can be as close as 50bp upstream of the TATA box or the upstream activator sequences of RNA polymerase II promoters without significant loss of transcription or activation (data not shown). The PCR product was then separated from unincorporated biotinylated primers by size-exclusion (Sephadex<sup>®</sup> G-200 column, Pharmacia) chromatography. The recovered promoter-containing DNA was subjected to digestion with *EcoR* I to cleave the template 692bp downstream of the transcription start site. The digestion produced a biotinylated, 2.1kb fragment and a nonbiotinylated 1.4kb fragment. Sodium chloride was added, to a final concentration of 0.5M, to the reaction. The SA-PMPs were washed five times with TE buffer (pH 8.0) in 0.5M NaCl and incubated with the digestion reaction mix with agitation for 30 minutes to allow binding (Figure 2). The SA-PMPs were isolated by magnetics capture and the supernatant was assayed on a 1% agarose gel. The particles were washed four times in TE buffer before being resuspended in TE buffer and stored at 4°C where they were stable for several months.

<sup>(a)</sup>The method of recombinant expression of Coleoptera luciferase is covered by U.S. Pat. No. 5,583,024 assigned to The Regents of the University of California.



**Figure 2. The binding of template DNA to Streptavidin MagneSphere<sup>®</sup> Paramagnetic Particles after 30 and 70 minutes.** Lane M, DNA markers (Life Technologies). Lane 1, 2µl of template DNA before binding. All other lanes, 2µl of supernatant from the binding reactions after 30 or 70 minutes. The binding conditions are described in the text. Each binding reaction contained 0.2mg of SA-PMPs and the indicated increasing amounts of template DNA (lanes 2 and 7, 5µg; lanes 3 and 8, 10µg; lanes 4 and 9, 12µg; lanes 5 and 10, 15µg; lanes 6 and 11, 20 µg).

As shown in Figure 2, binding of the 2.1kb biotinylated DNA template to the SA-PMPs is completed before 30 minutes. The binding capacity estimated from the gel and quantitated by UV spectrophotometry was approximately 30µg of 2kb DNA per milligram of SA-PMPs. The binding capacity of the DNA template was only 2.5-5%, relative to the reported binding capacity of biotinylated oligo(dT). The extent of binding of biotinylated DNA to SA-PMPs is inversely proportional to the length of the DNA, presumably due to charge repulsion between DNA molecules. This effect appears to be a universal characteristic and is independent of the properties of the SA-PMPs. Performing the binding reaction in the presence of elevated salt concentrations increases binding efficiency of biotinylated templates, but does not compensate completely for a decrease in their length. Therefore, we recommend selecting the shortest DNA sufficient for the reaction or complex formation to increase efficiency of binding. We observed no significant binding of nonbiotinylated, 1.4kb-long template DNA to the SA-PMPs.

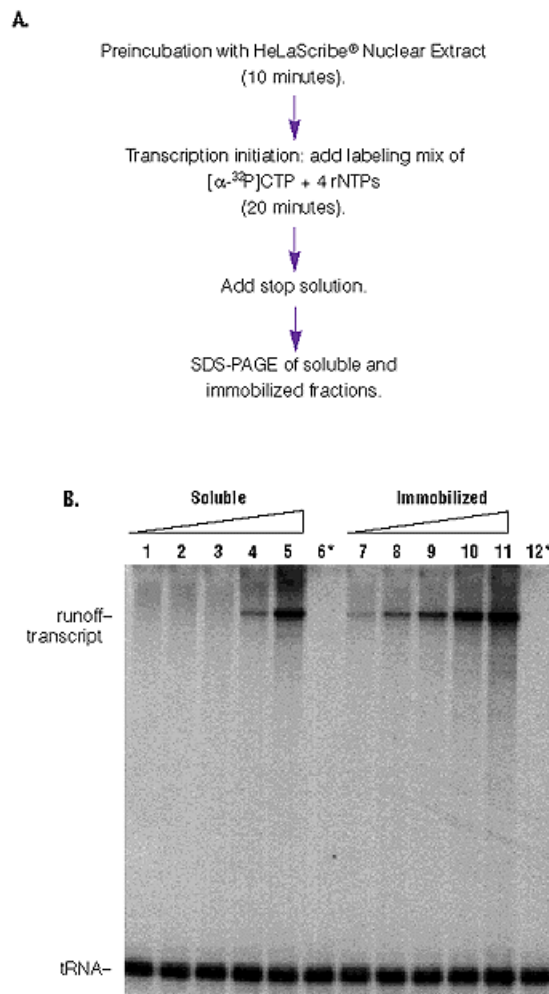
In addition to the PCR method described above, an end-fill protocol was used to generate immobilized templates. A plasmid containing a promoter of interest was linearized upstream of the promoter with *EcoR* I to generate a 5'-overhang. The Klenow fragment of DNA polymerase I in the presence of biotinylated dATP was used to fill in the overhang. The linear, biotinylated template was purified and immobilized as described for the PCR-generated templates. Biotinylated templates generated using the end-fill technique displayed similar binding and transcriptional properties to those synthesized using the PCR approach (data not shown).

## TRANSCRIPTION FROM AN IMMOBILIZED TEMPLATE

The immobilized DNA template containing the HIV-LTR was tested in an *in vitro* transcription reaction using Promega's HeLaScribe<sup>®</sup> Nuclear Extract (*in vitro* Transcription Grade, Cat.# E3091). The continuous labeling protocol used in the following experiments (Figure 3) is as follows. Increasing amounts of soluble and immobilized HIV-LTR template were incubated separately at 30°C for 10 minutes in a 10µl reaction mixture that contained 5 units of the HeLaScribe<sup>®</sup> extract, 20mM HEPES (pH 7.6), 7mM MgCl<sub>2</sub> and 65mM KCl, to allow RNA polymerase II preinitiation complexes to form on the promoter. Transcription was initiated by addition of 2µl of a labeling mix that contained 5µCi [alpha-<sup>32</sup>P]CTP and gave a final concentration of 30µM unlabeled CTP and 600µM GTP, ATP and

UTP for 20 minutes at 30°C. The reaction was stopped by the addition of 200µl stop solution (1% Sarkosyl, 100mM NaCl, 100mM Tris [pH 8.0], 10mM EDTA and 100µg/ml tRNA). The transcripts were recovered and analyzed by 6% denaturing polyacrylamide gel electrophoresis (10).

RNA polymerase II runoff transcripts generated from the HIV-LTR template (Figure 3) were quantitated using a Packard InstantImager<sup>TM</sup>. An accumulation of runoff transcripts could be detected using 5µg/ml of immobilized DNA template (Figure 3). When template concentration was raised to 10µg/ml, the transcript signal increased 1.4-fold; when raised to 20µg/ml, the signal increased 2.3-fold. The soluble template generated little runoff transcript at 5µg/ml concentration, but the transcript signal increased 2.9-fold using 10µg/ml and 8.4-fold when using template at 20µg/ml. At a concentration of 40µg/ml, the immobilized template gave approximately the same signal as did 20µg/ml of the soluble template, a concentration that routinely gives a distinct and reasonable transcription signal in the *in vitro* transcription systems. These results highlight that the easy isolation and manipulation of the paramagnetic particles does not significantly compromise the *in vitro* transcription reaction or transcript yield.



**Figure 3. Transcription with continuous labeling of the immobilized template.** The continuous labeling transcription protocol is described in the text. To ensure accurate quantitation, more particles than necessary for complete binding of biotinylated template DNA were used in the generation of immobilized template in this experiment. Increasing amounts of DNA were used in the transcription reactions and the generated transcripts were analyzed on a 6% polyacrylamide gel. Alpha-amanitin was added during preincubation to a final concentration of 1µM for the highest concentrations of template DNA tested; these lanes are indicated with an asterisk (\*). The tRNA bands were due to transfer of [alpha-<sup>32</sup>P]CTP to the 3'-end by nucleotidyltransferase activity in the nuclear extract. Concentration of DNA in soluble fractions: lane 1, 1.25ng/µl; lane 2, 2.5ng/µl; lane 3, 5ng/µl; lane 4, 10ng/µl; lane 5, 20ng/µl; lane 6, 20ng/µl + alpha-amanitin. Concentration of DNA in immobilized fractions: lane 7, 2.5ng/µl; lane 8, 5ng/µl; lane 9, 10ng/µl; lane 10, 20ng/µl; lane 11, 40ng/µl; lane 12, 20ng/µl + alpha-amanitin.

## TERMINATION ASSAYS

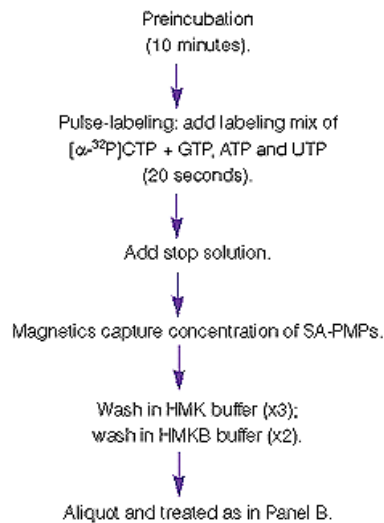
The mechanistic study of a *Drosophila* transcription termination factor (factor 2) is easier with immobilized templates in the transcription reaction. Factor 2 causes the release of transcripts from RNA polymerase II early elongation complexes (EECs) in an ATP-dependent manner (8). To demonstrate the effect of factor 2 on stalled EECs, we first generated an immobilized template containing the *Drosophila Act5C* promoter using the PCR approach already described. The template was restricted with *Hpa* II to generate a 780nt runoff transcript. Binding was performed under conditions identical to those described above. The binding capacity of SA-PMPs to the

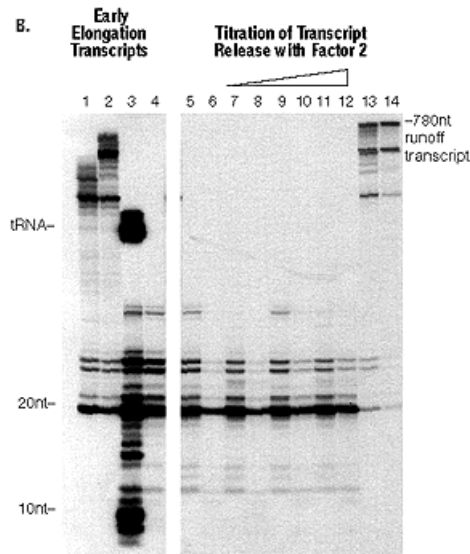
980bp template was estimated by Kodak 1D<sup>TM</sup> software analysis and UV spectrophotometry as approximately 33µg of 1kb-long DNA per milligram of SA-PMPs (data not shown).

For the termination assay, immobilized *Act5C* template (approximately 25µg SA-PMPs per reaction) were incubated with *Drosophila* nuclear extract from the K<sub>c</sub> cell line, prepared as described in reference 9, for 10 minutes at room temperature in a reaction mixture of 20mM HEPES (pH 7.6), 5mM MgCl<sub>2</sub> and 60mM KCl, to allow preinitiation complexes to assemble. Transcription was initiated by the addition of 5µCi [alpha-<sup>32</sup>P]CTP and 600µM of GTP, ATP and UTP to each reaction. This pulse labeling was stopped at 20 seconds by addition of 20mM HEPES, 60mM KCl and 50mM EDTA, for a final EDTA concentration of 10mM. (Note that while these conditions are designed to halt transcription by chelating magnesium, a required cofactor for transcription, a fraction of the transcription complexes will remain transcriptionally engaged and competent for further elongation.) After halting the pulse reaction, the SA-PMPs were concentrated using magnetic capture and washed three times in 1M HMK buffer (20mM HEPES [pH 7.6], 5mM MgCl<sub>2</sub> and 1M KCl) followed by two washes with 60mM HMKB buffer (20mM HEPES [pH 7.6], 5mM MgCl<sub>2</sub>, 60mM KCl and 200µg/ml BSA). After washing, the SA-PMPs were aliquoted into new tubes, and ATP only or ATP and factor 2 were added as indicated in [Figure 4](#). With the exception of chase solutions performed in 250mM KCl, all of the reactions had a final volume of 12µl containing 20mM HEPES (pH 7.6), 5mM MgCl<sub>2</sub>, 60mM KCl and 600µM of each rNTP as indicated. After a 5-minute incubation (or as indicated for the chase reactions in [Figure 4](#)), the reactions were stopped by the addition of an equal volume of 1M HGKE buffer (20mM HEPES [pH 7.6], 15% glycerol 1M KCl and 20mM EDTA). The SA-PMP fractions and supernatant fractions were separated by magnetic capture and 200µl of Sarkosyl stop solution was added to all reactions. The transcripts were recovered and analyzed on 15% denaturing polyacrylamide gels.

[Figure 4](#) shows the recovery and properties of the EECs formed on the immobilized *Act5C* template. Because the reaction loaded in lane 1 was stopped immediately after pulse labeling, the labeled tRNA band is still present in the lane, as well as some extremely short RNA transcripts (~5-8nt long). The salt wash removed the soluble tRNAs and very short (<10nt), aborted initiation products to levels below detection. As quantitated by the InstantImager<sup>TM</sup>, about 44% of the bands between ~19-35nt were recovered after a total of five salt washes. About 59% of these EECs were "chased" up to >35nt region in a 1-minute chase (lane 1) and 67% in a 3-minute chase (lane 2). About 78% of the EECs were "chased" up after a 10-minute chase and the runoff transcripts were all released into the supernatant fraction indicating little nonspecific binding of long transcripts to the beads under these conditions ([Figure 4](#)).

A.





**Figure 4. Pulse-chase experiments of early elongation complexes (EECs) and titration of transcript release from the immobilized *Drosophila Act5C* template.** EECs were generated as described in the text. EECs were washed 3 times with 1M KCl and twice with 60mM KCl. EECs then were "chased" in 250mM KCl for 1 (lane 1) or 3 (lane 2) minutes. Potassium chloride (250mM) suppresses the effect of negative transcription elongation factors of RNA polymerase II and promotes productive elongation (7). Reactions before (lane 3) and after (lane 4) washing are shown. Titration of the transcript release activity of factor 2 was assessed by washing the EECs followed by incubation with ATP only (lanes 5 and 6) or ATP + factor 2 (lanes 7-12). Purified factor 2 was tested at 6, 12 or 24ng. The reactions were stopped, and the particles were separated from the supernatants as described in the text. The transcripts remained bound to the immobilized template (lanes 5, 7, 9 and 11), and fractions of those released into the supernatant (lanes 6, 8, 10 and 12) were assessed. EECs that were chased in 250mM KCl for 10 minutes were separated into bead (lane 13) and supernatant (lane 14) fractions. The 780nt-long runoff transcript is indicated to the right of the gel. The transcripts were assayed on a 15% polyacrylamide gel and quantitated using a Packard InstantImager™.

When the EECs were incubated with ATP only, most of the transcripts were retained in the bead fraction (lane 5 versus lane 6). However, when increasing amounts of factor 2 along with ATP were added to the reactions, increasing amounts of transcripts were released into the supernatant indicating the transcript release activity of factor 2. At the highest factor 2 amount used, about twice as much transcript was released into the supernatant after a 5-minute incubation in the presence of ATP compared to the lowest amount of added factor 2. Factor 2 was added to elongation complexes for 3 minutes prior to a 5-minute incubation with ATP (Figure 4).

## SUMMARY

In this report, we tested immobilized templates made with Promega's Streptavidin MagneSphere® Paramagnetic Particles in our *in vitro* transcription assays and found the binding capacity of the particles for a 2kb fragment to be 30µg of DNA per milligram of particles. The *in vitro* transcription reaction showed the immobilized template gave a 50-70% transcription signal compared to the same concentration of soluble template. We obtained good recovery of isolated early elongation complexes following washes in salt solution, and successfully demonstrated the termination activity of *Drosophila* factor 2. The quick magnetic separation and easy manipulation make Promega's Streptavidin MagneSphere® Paramagnetic Particles a reliable tool for studies on *in vitro* transcription.

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### Ordering Information

Product	Size	Cat.#
Streptavidin MagneSphere® Paramagnetic Particles	(15 x 0.6ml) 9ml	Z5481
	25ml	Z5482
Streptavidin MagneSphere® Paramagnetic Particles Plus M13 Oligo		Z5392
MagneSphere® Technology Magnetic Separation Stand (two-hole)	0.5ml	Z5331
	1.5ml	Z5332
	12 x 75mm	Z5333
MagneSphere® Technology Magnetic Separation Stand (twelve-hole)	0.5ml	Z5341
	1.5ml	Z5342
	12 x 75mm	Z5343
MagneSphere® Technology Magnetic Separation Stand, 24 well		Z5441
MagneSphere® Technology Magnetic Separation Stand, 96 well		Z5431
Biotinylated Oligo(dT) Probe	35µl	Z5261
HeLaScribe® Nuclear Extract <i>in vitro</i> Transcription Grade	40 reactions	E3091
	160 reactions	E3092

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