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High-salt transcription of DNA cotethered with T7 RNA polymerase to beads generates increased yields of highly pure RNA

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High yields of RNA are routinely prepared following the two-step approach of high-yield *in vitro* transcription using T7 RNA polymerase followed by extensive purification using gel separation or chromatographic methods. We recently demonstrated that in high-yield transcription reactions, as RNA accumulates in solution, T7 RNA polymerase rebinds and extends the encoded RNA (using the RNA as a template), resulting in a product pool contaminated with longer-than-desired, (partially) double-stranded impurities. Current purification methods often fail to fully eliminate these impurities, which, if present in therapeutics, can stimulate the innate immune response with potentially fatal consequences. In this work, we introduce a novel *in vitro* transcription method that generates high yields of encoded RNA without double-stranded impurities, reducing the need for further purification. Transcription is carried out at high-salt conditions to eliminate RNA product rebinding, while promoter DNA and T7 RNA polymerase are cotethered in close proximity on magnetic beads to drive promoter binding and transcription initiation, resulting in an increase in overall yield and purity of only the encoded RNA. A more complete elimination of double-stranded RNA during synthesis will not only reduce overall production costs, but also should ultimately enable therapies and technologies that are currently being hampered by those impurities.

Our understanding of RNA's central role in biology continues to expand and be exploited. Researchers across a wide swath of basic science and applied technologies require high yields of pure RNA. Solid-phase chemical synthesis can, in principle, generate RNAs up to 50 to 100 nt in length, but both yield and purity decrease with increasing lengths (1–3). Enzymatic synthesis *in vitro* by T7 RNA polymerase is widely used to synthesize high yields of RNA of all lengths for structural studies, basic RNA biology (splicing, riboswitches, CRISPR, lncRNA), therapeutics applications (mRNA vaccines and therapies, siRNA, gRNA for CRISPR), and nanotechnology (4–7).

T7 RNA polymerase binds its consensus promoter sequence with near nanomolar affinity *in vitro* (8, 9), initiates transcription at a unique site in the DNA, transitions to stable elongation, and runs off the end of a linear DNA template to synthesize the encoded RNA (4, 6, 10). It has long been known that in addition to the full-length encoded RNA, T7 RNA polymerase produces short abortive RNAs 2 to 7 nucleotides in length (6, 11) and RNAs longer than the encoded length. These longer products have been proposed to be generated through templated and nontemplated additions (6, 12–14), *cis* or *trans* primed extension of RNA (15–22), or strand jumping (23). Our lab recently demonstrated that in high-yield transcription reactions, the most significant contribution to the longer, undesired RNA products is through the *cis* self-primed extension mechanism (16, 24). As high yields of encoded RNA accumulate in solution, mass action drives the polymerase to rebind the accumulated RNA at its 3' end and self-extend *via* a nonpromoter-dependent mechanism. The process is heterogeneous and distributive, leading to a diverse pool of products often abundant in (partially) double-stranded RNAs longer than the encoded length.

In RNA therapeutics applications, dsRNA contamination from *in vitro* synthesized RNA can invoke the innate immune response, as dsRNA is classified as a potent pathogen-associated molecular pattern in the body. This can happen by way of activating natural sensors such as retinoic acid inducible gene (RIG-I) (25, 26), Toll-like receptor 3 (TLR-3) (27–29), and protein melanoma-differentiation-associated antigen 5 (MDA5) (25). It can cause the production of type I interferon, which can inhibit translation through the activation and upregulation of protein kinase R (PKR) (30). It can also cause cellular mRNA degradation by activating the 2'-5' oligoadenylate synthetase (OAS) enzyme family (31). Therefore, therapeutics researchers must follow up *in vitro* T7 RNA polymerase transcription with often very extensive purification methods (32–36). Gel or chromatic (HPLC) purification methods are time-consuming, result in a loss of yield, and are imprecise, as the encoded product may not always be readily identified. At long RNA lengths, the resolution in these separations becomes progressively worse, making the purification of the precisely encoded RNA unattainable. A recently developed method removes dsRNA impurities from the *in vitro* transcription pool

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by their selective binding to cellulose in an ethanol-based buffer (37). However, the effectiveness of this method is expected to depend on the relative lengths of double-stranded regions and may remove desired RNAs with natively structured regions.

Although researchers have long focused on improving the overall yield of *in vitro* transcription reactions, we have confirmed with RNA-Seq that the very conditions of high-yield synthesis often drive the correct product into primer extended, double-stranded impurities (24). This not only impacts the overall purity, but also the yield of the encoded RNA.

In this research, we present a novel method of *in vitro* transcription that allows promoter-directed transcription while preventing primer extension activity, thereby dramatically reducing double-stranded impurities. In brief, by increasing salt concentrations in solution, we reduce all protein–nucleic acid interactions. To selectively restore promoter binding, we tether both T7 RNA polymerase and promoter DNA to a solid support (beads). This drives their association even at high salt concentrations. Near elimination of RNA rebinding and extension not only results in a dramatic reduction in longer, primer-extended products, but also nets a dramatic increase in the yield of encoded RNA.

Results

The goal of this study is to eliminate RNA product rebinding and subsequent extension activities of T7 RNA polymerase, while retaining promoter-directed transcription. Like almost all protein–nucleic acid interactions, both initial binding of T7 RNA polymerase to its promoter and rebinding of product RNA are stabilized in part by electrostatic interactions between positively charged residues on the RNA polymerase surface and the negatively charged phosphate backbone of the DNA or RNA (38–41). As a result, increasing salt concentrations should destabilize both promoter DNA binding and product RNA rebinding. We have previously shown that covalently cross-linking an engineered cysteine (A94C) in the N-terminal domain of T7 RNA polymerase to a 3' thiol-modified template DNA creates a locally high concentration of the promoter near its binding site, allowing promoter binding, even at high salt (42). Initiation proceeds well and at least some of these complexes transition to the stable elongation phase (42). Elongation by T7 RNA polymerase is stabilized by the topological locking of the RNA around the template DNA in the enzyme active site (10) and elongation has been shown to be resistant to added salt concentrations up to at least 0.2 M NaCl (40, 43).

By tethering T7 RNA polymerase to its DNA promoter and carrying out transcription at elevated salt concentrations, we can achieve promoter-initiated transcription while preventing product RNA rebinding that otherwise would lead to *cis* primed extension activity. We expect this approach to reduce substantially the production of longer, double-stranded RNA impurities.

Design of a tethered *in vitro* transcription system

In order to tether the polymerase to the promoter DNA and still allow functional initiation and substantial transition to

elongation, we bound each, independently to Strep-TactinXT magnetic beads. The N-terminal domain of T7 RNA polymerase (together with a hairpin loop from the C-terminal domain) forms the promoter binding platform (44, 45), and many N-terminal fusions of T7 RNA polymerase function well in promoter-directed transcription (46–48). Thus, we fused the Strep-tag II peptide (WSHPQFEK), followed by a short and flexible peptide linker (GGS), to the N-terminus of recombinant T7 RNA polymerase (49). Herein, we will call this Strep-tagged variant Strep-T7 RNA polymerase. The Strep-tag II peptide has nanomolar binding affinity to specifically engineered Strep-TactinXT-coated magnetic beads (50). We also used 5'-biotinylated nontemplate DNA to independently bind the promoter DNA to the Strep-TactinXT beads. Biotin is reported to have picomolar binding affinity to Strep-TactinXT-coated magnetic beads (50).

To confirm that the Strep-tag II peptide addition at the N-terminus of T7 RNA polymerase does not affect transcription activity, we performed *in vitro* transcription reactions using Strep-T7 RNA polymerase and promoter DNA (without a biotin tag) encoding a 24 base RNA (RNA-24) under high-yield transcription conditions. The gel analysis in Fig. S2 demonstrates identical transcription profiles using T7 RNA polymerase and Strep-T7 RNA polymerase. This confirms that the addition of the Strep-tag II peptide has no adverse effect on the activity of T7 RNA polymerase. Similarly, biotinylating the upstream end of the promoter has no effect on promoter function, as also shown in Fig. S2. Finally, we tested these constructs for transcription activity at 0.4 M added NaCl and, as expected for the uncoupled species, observed essentially complete inhibition of transcription in all constructs.

Tethered system favors promoter-directed transcription at high salt

Having demonstrated that the DNA and protein modifications do not perturb promoter binding and transcription, we proceeded to test the cotethered system for function. Given that at low salt the dissociation constant for duplex promoter binding by T7 RNA polymerase is ≈ 4 nM (9) we first pre-incubated the 5'-biotinylated nontemplate strand, template strand encoding a 24 base RNA and Strep-T7 RNA polymerase at final equimolar concentrations, as described in the Methods section. We then incubated the assembled promoter complex with tetrameric Strep-TactinXT-coated magnetic beads to form the tethered *in vitro* transcription system illustrated in Figure 1.

While elevated salt concentrations weaken both promoter binding and RNA rebinding activities of T7 RNA polymerase, indirectly tethering the polymerase to the promoter (as demonstrated in Fig. 1) should restore promoter binding by increasing the local concentration of promoter DNA compared with free RNA in solution, as observed previously using a direct tethering approach (42). To test the hypothesis with our tethered *in vitro* transcription system, we performed a comparative analysis of transcription between tethered and untethered systems as a function of increasing concentrations of added NaCl. In order to

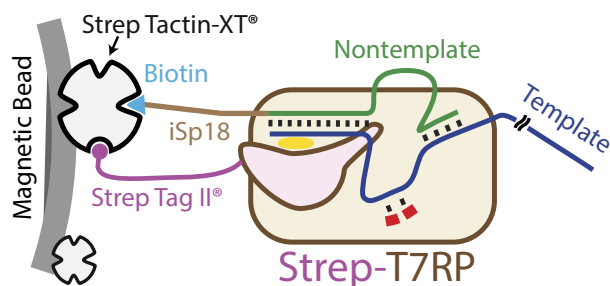


Figure 1. Cross-linked transcription complex. T7 RNA polymerase containing an N-terminal Strep-tag II peptide and duplex DNA labeled with biotin at the 5' end of the nontemplate strand are bound to (tetraivalent) Strep-TactinXT coated magnetic beads.

see the direct effect on *cis* primed extension activity, we selected a template strand that encodes a 24 base RNA (RNA-24) known to serve effectively in 3' self-extension (24). The gel analysis presented in Figure 2A shows that as added NaCl concentration is increased from 0 M to 0.4 M in 0.1 M intervals, all transcription activity decreases for the untethered system and is negligible at 0.4 M added NaCl. In the tethered system, promoter binding (and transcription) is relatively resistant to increasing concentrations of added salt, as expected. The data also confirm that product RNA rebinding to polymerase is inhibited, as there is a dramatic reduction in the formation of primer extension products. At 0.3 M added NaCl, most of the primer extension activity is inhibited, leading directly to an increase in the encoded RNA yield. At 0.4 M added NaCl, the overall yield is decreased somewhat relative to that at 0.3 M, but the purity of the encoded RNA is at its highest (and the concentration of the encoded RNA is substantially higher than in the untethered control). Overall, the tethered transcription system under high salt produces significantly improved purity and increased yield of the correct length product.

Synthesis of encoded RNA is not impaired by tethering

Not all encoded RNAs participate in 3' self-extension (24). To confirm that the described tethering does not have an effect

on the fundamental efficiency of promoter-directed transcription, we repeated the above comparative analysis with a template strand encoding another 24 base RNA (RNA-24Alt) that is known not to participate substantially in 3' self-extension (24). For the untethered system, gel analysis of the products presented in Figure 3A (and quantified in 3B) shows an overall loss of yield in RNA transcription with increasing added NaCl concentration, as expected (more subtly, there is an initial increase in 24 base RNA at 0.1 M added NaCl, which then decreases to barely detectable levels by 0.4 M added NaCl).

In contrast, Figure 3, A and B show that the tethered system, while also initially showing an increase in the yield of 24 base RNA with increasing NaCl, continues to transcribe well up to at least 0.3 M added NaCl and produces more 24 base RNA at 0.4 M than at 0 M added NaCl. Close inspection of the gel lanes in Figure 3A suggests that the RNA-24Alt sequence is in fact producing primer extended products at 0 M added NaCl, as evidenced by a broad smear above the 24 base RNA band that decreases with increasing NaCl. Quantification of the extended products is shown in gray at 10× magnification in Figure 3B to better depict this observation. Thus, even for this construct, the intensity of the 24 base RNA band underestimates the total RNA produced. According to the model, inhibition of the primer extension that produces the broad smear would result in higher net amounts of the initially synthesized 24 base RNA, as observed.

Generality of the system

The results presented in Figures 2 and 3 use DNAs encoding 24 base RNAs with different sequences. To further test the generality of this system, we took the RNA-24 template sequence introduced in Figure 2 and inserted ten bases at position +8 to yield DNA that encodes a 34 base RNA (RNA-34), as shown in Fig. S1. Paralleling the experiment of Figure 2, we compare in Figure 4 untethered and tethered transcription of RNA-34 at low (0 M) and high salt (0.4 M) added NaCl concentrations. As predicted by the general model, the

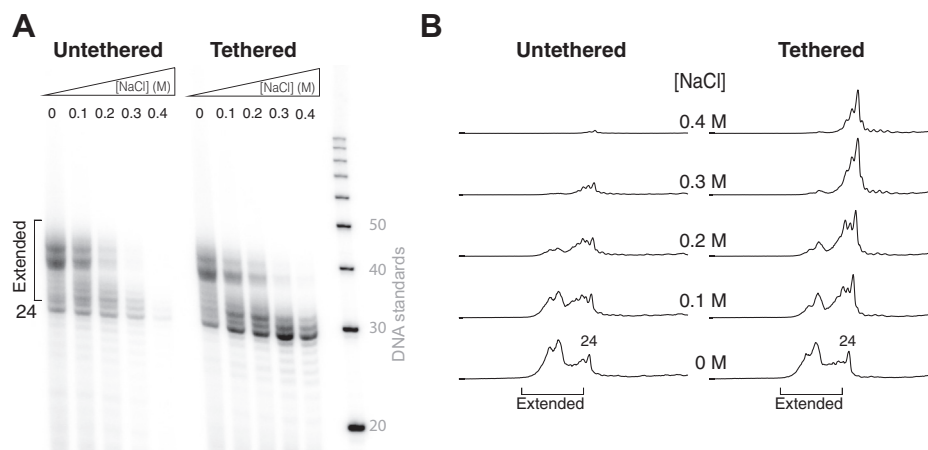


Figure 2. Tethered, high-salt transcription dramatically reduces primer extension. A, salt dependence of transcription profiles for untethered and tethered complexes analyzed by 20%, 7 M urea denaturing gel electrophoresis, labeled via incorporation of [α - 32 P]ATP. The final concentrations of NaCl added to the standard reaction mixture are shown. B, quantification of individual gel lanes in A.

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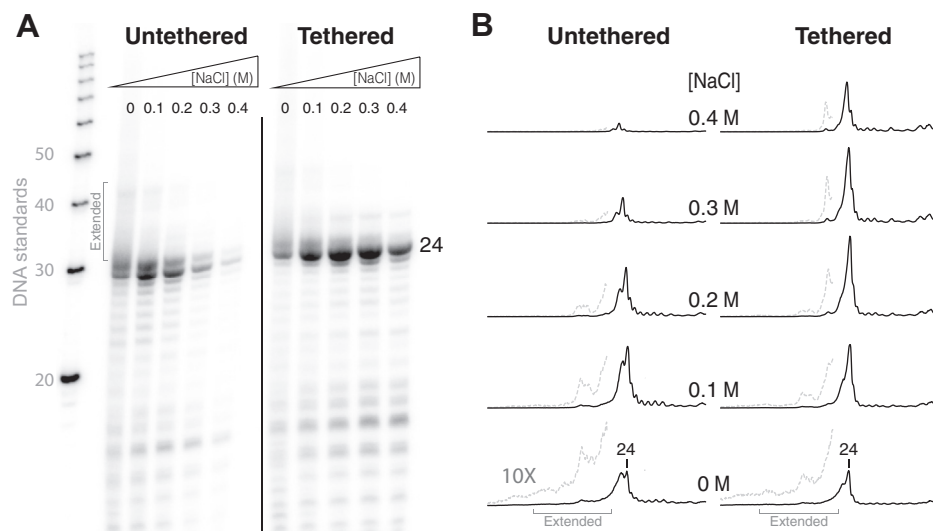


Figure 3. Transcription by tethered complexes is salt-resistant. *A*, salt dependence of transcription profiles for untethered and tethered complexes encoding RNA-24Alt, analyzed as in [Figure 2](#). *B*, quantification of individual lanes in *A*. Extended products shown in *gray* at 10x scale.

tethered *in vitro* transcription system produces primarily the encoded 34 base RNA at high salt. This result confirms that the system can be used for RNA of longer lengths to generate high yields of encoded RNA while preventing the formation of self-extended longer RNA impurities.

Biotinylated DNA and Strep-T7 RNA polymerase bind at nearby tetrameric sites

To encourage both enzyme and DNA to attach to the same Strep-TactinXT tetramer in the above experiments, we pre-incubated (at low salt) Strep-T7 RNA polymerase and biotinylated promoter DNA before adding the Strep-TactinXT magnetic beads. Following assembly, a high salt wash was used to remove components (free polymerase and DNA) not strongly bound to the beads. As controls, we prepared tethered transcription complexes using DNA and T7 RNA polymerase with only one or neither of the two modifications. The

resulting *in vitro* transcription reactions with DNA encoding RNA-24 under high-yield conditions, shown in [Fig. S3](#), confirm that the absence of one or both of the modifications destroys RNA synthesis, both at low and high salt concentrations, as expected.

Despite the high affinity of T7 RNA polymerase for its promoter, and the strategic preforming of the promoter complex before tethering to the beads, we expected that some enzyme might couple to beads lacking nearby DNA or vice versa. Without a partner, on well-washed beads, these species should be inactive in transcription. To test for enzyme immobilized without a promoter DNA partner, we challenged an assembled and washed system encoding RNA-24Alt by introducing in solution unmodified promoter DNA-34Alt that encodes RNA-34Alt. At low salt, RNA polymerase without a locally (and functionally) tethered 24-Alt DNA partner should bind the free DNA-34Alt and synthesize a 34 base RNA. The results in [Fig. S4](#) demonstrate this hypothesis to be correct. While at low salt, both 24 base and 34 base RNAs are produced at levels similar to that of untethered transcription, at high salt, only the 24 base RNA from the tethered DNA template is observed.

To further confirm that (RNA-34-encoding) DNA-34 is binding to and reacting with enzyme lacking a DNA partner, we increased the ratio of labeled 24 base encoding DNA-24 to protein from 1:1 to 2:1, followed by a high-salt wash to remove all unbound DNA. The results shown in [Fig. S5](#) reveal an approximately twofold increase in the overall RNA-24Alt production for the 2:1 prep compared with the 1:1 prep, while RNA-34Alt production decreases only slightly. This suggests that under these conditions, a significant portion of enzyme bound to beads may not have a functionally tethered DNA nearby. Alternatively, biotinylated DNA-24 could be binding to an empty binding site in the tetramer, further increasing the DNA concentration near tethered polymerase. Future development in this system will focus on optimizing binding capacity.

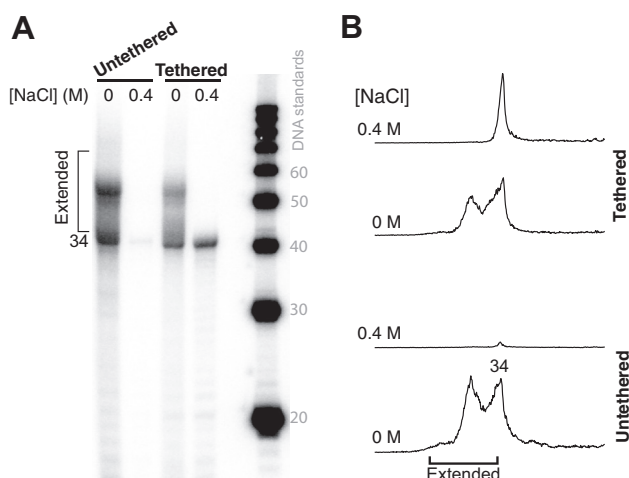


Figure 4. Improvements are independent of RNA length. *A*, low- and high-salt transcription profiles for tethered and untethered complexes analyzed as in [Figure 2](#). *B*, gel quantification of *A*.

Generally speaking, since promoter DNA and product RNA are in competition for binding to the polymerase active site, excess RNA polymerase (relative to the DNA) should always be avoided, as there will necessarily be free enzyme available to bind and extend RNA in solution. While one might argue that excess DNA is to be preferred, DNA can be resource limiting. In any case, a tethered 1:1 complex should provide the highest efficiency (relative to either DNA or polymerase), while at the same time proximity tethering of the DNA helps it compete with RNA for rebinding to the enzyme.

The system is stable and reusable

In this system, Strep-T7 RNA polymerase and promoter nontemplate DNA are immobilized on Strep-TactinXT magnetic beads. This allows that this bead-immobilized transcription complex could be reutilized for multiple rounds of transcription. To test this, we carried out three rounds of transcription using the above bead tethered transcription complex, as described in further detail in the Methods section and illustrated as a scheme in Figure 5A. Results in Figure 5B show that there is a significant loss in overall transcription after each round. We hypothesized that this could be due to washing off of the template DNA with each round, as template DNA is only bound to the tethered system *via* the strong promoter contacts. Although promoter binding is tight, off rates of the enzyme are fast (51–53). This suggests that with each round of transcription, it is not unreasonable to expect some template DNA to wash off of the tethered system if promoter contacts are lost during the off states.

With the observation that there is significant loss in overall transcription at each round, we hypothesized that strengthening promoter contacts might allow greater persistence of the complex on washing. In the two domain model of the 17-base pair consensus T7 promoter, the duplex recognition element (responsible for binding) stretches from position –17 upstream to position –5, while the AT rich melting region (required for optimal catalysis) is situated between positions –4 and –1 (54).

Part of the binding energy from the strong duplex binding interactions upstream of (and including) position –5 is used to institute conformational change in the downstream DNA, essentially predicted to melt bases from position –4 to about +3 (9, 55). As a result, the enzyme binds a partially single-stranded promoter DNA that has no bases downstream of position –5 in the nontemplate strand (referred to here as pss[–5]) at least four times tighter than it binds double-stranded (or pss[+2], as used here) promoter (9, 55). With significantly slower off rates (51–53) and strengthened promoter binding, use of the pss[–5] promoter in the tethered system should reduce loss by washing and result in greater overall retention of transcription activity after each round of transcription.

Comparison of the results in Figure 5C with those in Figure 5B confirms that use of the pss[–5] construct significantly increases the reusability of the tethered system, at both low and high salt concentrations. The gel analysis compares the products from each reaction cycle, using the template encoding RNA-24, under both low (0 M) and high (0.4 M) added salt conditions, and nontemplate DNA pss[+2] and pss[–5] respectively. The overall transcription yield decreases with each cycle using pss[+2] and is nearly lost by round 3. In contrast, using pss[–5] DNA, the overall transcription yield at each added salt concentration remains reasonably constant through three rounds of washing and reuse. The losses associated with the pss[+2] reuse point out that yields will likely depend on the extent of washing of the system. The pss[–5] system appears more resistant to such losses and so can be used at least three times, with essentially no loss in yield, by simply removing the product and adding transcription buffer with fresh NTPs.

Discussion

Transcription *in vitro* by T7 RNA polymerase is a long-established method to synthesize RNAs of diverse lengths and sequences, due to the promoter specificity and robust

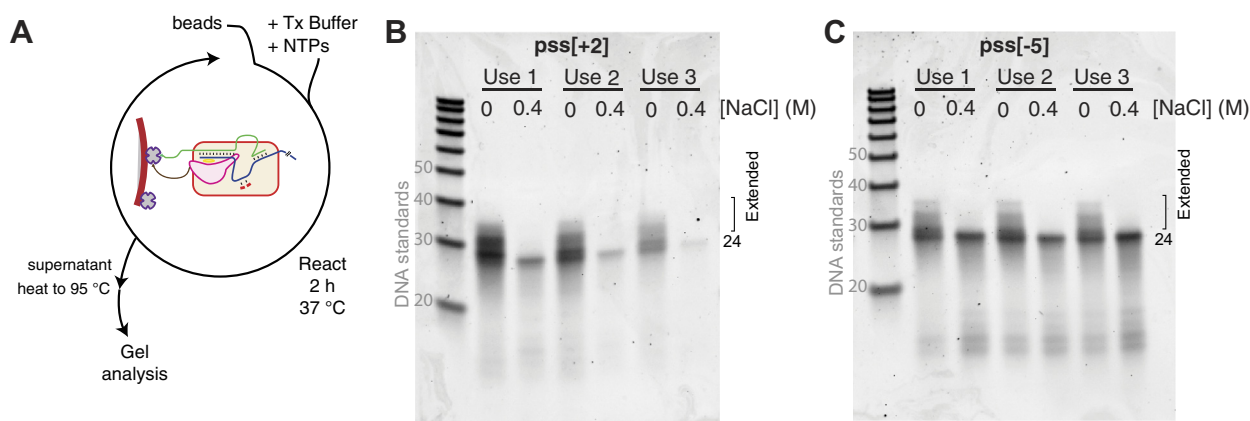


Figure 5. The tethered system can be reused. A, reusability experimental order scheme. After 2 h of “Use 1,” the reaction solution was removed, and a new substrate NTP solution was added to initiate a 2 h “Use two” reaction (and repeated to initiate a 2 h “Use three” reaction). B, fifteen percent polyacrylamide denaturing urea gel stained with SYBR Gold Nucleic Acid Gel Stain. Transcription profiles at low (0 M) and high (0.4 M) added NaCl for tethered transcription of RNA-24 using pss[+2], analyzed as in Figure 2. C, fifteen percent polyacrylamide denaturing urea gel, stained with SYBR Gold Nucleic Acid Gel stain. Transcription profiles at low (0 M) and high (0.4 M) added NaCl for tethered transcription of RNA-24 using pss[–5].

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nature of this system (6). Over the past 30 years, researchers have noted nonpromoter-driven activities of this enzyme that contaminate the product pool with other than encoded RNA products, often termed incorrectly as nontemplated additions (6, 12–14). The quest for high-yield RNA synthesis only exacerbates the production of undesired, longer products (24). As high concentrations of encoded RNA accumulate, T7 RNA polymerase binds RNA at its 3' end and self-extends *via cis* primed extension, now independent of the promoter.

High-yield RNA synthesis efforts to date have focused on a two-step approach: 1) high-yield transcription using T7 RNA polymerase, followed by 2) (sometimes extensive) purification of the encoded RNA using gel or chromatographic purification methods. High-yield conditions drive primer extension, resulting in the correct length RNA product being converted into heterogeneous, longer than desired double-stranded RNA contaminants. The very nature of high-yield reactions leads directly to longer, double-stranded impurities and reduces the encoded RNA yield. This process is sequence-dependent, and in cases where the encoded RNA product does not significantly participate in *cis* primed self-extension, gel electrophoretic or chromatographic purification methods may be adequate to address the purity problem. However, each purification step generally reduces overall product yield. Moreover, electrophoretic or chromatographic purification approaches have highest success for relatively short RNAs. Preparative purification of longer RNA (*e.g.*, separating an encoded 300 base RNA from products extended by 20–30 bases) is often difficult, if not impossible. With increased emphasis on mRNAs many kilobases in length, the problem becomes increasingly difficult.

The goal then is to prevent the rebinding of the synthesized RNA from the outset, to then prevent the RNA-primed self-extension that leads to longer impurities. Noting that high ionic strength inhibits *all* polymerase–nucleic acid interactions, we introduce here a novel *in vitro* transcription method, transcribing at high salt to limit RNA product rebinding. To restore promoter binding and initiation of transcription, we cotether promoter DNA and T7 RNA polymerase to beads. Specifically, we cotether Strep-T7 RNA polymerase and biotinylated promoter DNA to Strep-TactinXT beads, bringing the enzyme in close proximity to its promoter to restore promoter binding, even at high ionic strength. This results in overall higher yields of the desired RNA at greater initial purity, reducing the need for subsequent (and low yield) purification steps.

Tethering plus salt leads to improved transcription purity and yield

Increasing salt concentrations leads to complete inhibition of all enzymatic activity in the untethered system, as shown in Figures 2–4. In the tethered system, high salt inhibits only the undesired *cis*-primed extension activity of T7 RNA polymerase, while allowing promoter-directed transcription. This reflects as a dramatic decrease of the longer, double-stranded impurities. Since production of the longer double-stranded

products derives from and therefore consumes encoded-length RNAs, tethered system recovers higher yields of the desired (directly encoded) product. As expected, at sufficiently high concentrations of added salt, promoter-driven transcription begins to be inhibited, even for the tethered system. For these constructs and these conditions, a practical optimum of about 0.3 M added NaCl provides a good trade-off of purity *versus* yield.

To characterize the salt dependence of promoter-driven transcription in the (relative) absence of primer extension, we used a sequence, RNA-24Alt, previously observed to yield less of the resolvable primer extended products (24). The results in Figure 3 show that while in the untethered system, overall transcription is inhibited progressively with increasing added NaCl, the tethered system continues to generate high yields of RNA-24Alt at moderate salt concentrations. Note that at 0.1 M added NaCl, encoded RNA yield increases somewhat for both untethered and tethered transcription systems, when compared to 0 M added NaCl. As discussed earlier, this observation suggests that this RNA sequence is in fact subject to primer extension, but to a lower extent and in a more dispersed heterogeneous (and therefore less readily visualized in a gel) manner. Thus, even in an untethered system, low salt concentrations may reduce primer extension with limited effect on promoter binding and initiation.

As the titration approaches 0.3 M added NaCl, the tethered system reaches maximal yield of encoded length RNA, while the untethered system is inhibited significantly. At 0.4 M added NaCl, the overall yield in the tethered system starts to drop, as the effect of high salt on inhibiting even promoter-driven transcription becomes significant. This behavior parallels the results observed for RNA-24 in Figure 2. For practical consideration, users can determine the optimal concentration of added salt, depending on their RNA sequence, targeted degree of purity, and desired yield.

Generality of the system

The characterization experiments shown in Figures 2 and 3 have been carried out on relatively short RNAs 24 bases in length. To test the generality of the system, we used a DNA that encodes a 34 base RNA (RNA-34) and carried out transcription using the tethered system. The results in Figure 4 exhibited similar characteristics in terms of transcription profile, salt sensitivity, and overall transcription yield. The similarity in transcription trends in 24 base and 34 base encoding tethered systems suggests these characteristics to be generalizable for other RNA as well. The synthesis here of relatively short 24 and 34 base RNAs allows precise analytical characterization of both yield and impurities. We fully anticipate that the cotethering concept can be extended to much longer RNAs; however, preliminary results suggest that the 6% agarose-coated Streptactin-XT beads used here may not bind >120 base DNA duplexes efficiently. We anticipate that with appropriate immobilization of the DNA, this system should find ready utility in the synthesis of CRISPR guide RNAs, long noncoding RNAs, and mRNA.

Stability of the system

In RNA therapeutics applications, high yields of pure RNA are needed for research and drug production purposes. In order to achieve high yields, researchers routinely carry out *in vitro* transcription using T7 RNA polymerase for long hours under high RNA generating conditions (5, 6, 56, 57). Production under those conditions results in the generation of longer, double-stranded impurities, which then requires extensive purification to recover only the encoded RNA (33–35, 58–60). At long RNA lengths, purification methods do not have the resolution to eliminate all impurities (for example, separating a 2000 base transcript from a 2040 base impurity) and purifications lead to a loss in yield. Moreover, since longer, double-stranded impurities are generated *via* the extension of correct length, encoded RNA, the synthesis itself leads to a loss in yield of the intended length product. The approach outlined here improves yield in both respects.

As the needed overall yield increases, as in therapeutic production, economic considerations play a significant role in the design of synthesis approaches. Earlier studies have described approaches to reuse DNA in transcription reactions. In one such approach, Davis and colleagues tethered synthetic DNA to a solid surface and claimed 15 rounds of transcription using the same DNA, with the ability to store the system between rounds (61). In that system, however, RNA polymerase must be added at each round; but more importantly, the polymerase is untethered and, as in solution reactions, as RNA product accumulates, it will rebind the polymerase, driving primer extension, lowering purity and target RNA yield. Thus, while template DNA is reused, that approach is not expected to improve the profile of the RNA generated in high yields.

The system described here is fundamentally different, in that it tethers both promoter DNA and T7 RNA polymerase, in proximity, to magnetic beads and carries out transcription at high salt, so that *cis*-primed extension activities are inhibited, resulting in an increase in overall yield and purity (desired RNA is not chased to longer impurities) of the encoded RNA. To further improve the yield of this system, we reused the beads for multiple rounds of transcription, using the same DNA and enzyme, but additionally strengthening promoter binding by use of a partially single-stranded promoter construct. Comparing the results in Figure 5, B and C, strengthening promoter contacts using pss[–5] indeed helps retain template DNA during washing and allows at least three repetitions of 2 h reactions. When compared with the routine (untethered) transcription reaction profile seen in Figure 2A, the overall transcription yield of the encoded RNA is improved dramatically. This new system offers dramatic improvements in encoded RNA yield and purity.

In summary, the approach described here yields substantial improvements in RNA synthesis compared with the typical approaches of the past. The near elimination of primer extended products that typically occur in high-yield transcription reactions improves purity dramatically, while eliminating the extension of correct length RNA increases the overall yield of the desired RNA product. This approach also

allows easy separation of product from RNA polymerase and from the templating DNA, while allowing for reuse of these components.

Experimental procedures

Reagents

DNA oligonucleotides used in transcription reactions were purchased from Integrated DNA Technologies (IDT). Sequences with modifications are shown in Fig. S1. Unless otherwise noted, all transcription reactions were performed using partially single-stranded DNA constructs where the nontemplate DNA extends to the +2 position downstream of the promoter sequence (referred to here as pss[+2]). This promoter DNA construct is known to have the same functionality as fully double-stranded promoter DNA (6), and we have found in these studies that it behaves identically to fully double-stranded DNA with respect to salt. The following buffers were optimized in house and used where indicated. High-yield transcription buffer contained final concentrations of 30 mM HEPES, 40 mM magnesium acetate, 25 mM potassium glutamate, 0.25 mM EDTA, 0.05% Tween20, pH 7.8, 10 units/ml pyrophosphatase (New England Biolabs). Wash buffer contained 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA. Storage buffer contained 30 mM HEPES, 15 mM magnesium acetate, 25 mM potassium glutamate, 0.25 mM EDTA, 0.05% Tween20, pH 7.8. Nucleoside triphosphates (New England Biolabs) were added to transcription reactions at final concentrations of 7.5 mM each.

T7 RNA polymerase

His-tagged T7 RNA polymerase was prepared from *Escherichia coli* strain BL21 carrying the plasmid pBH161, purified and characterized as previously described (62).

Strep-T7 RNA polymerase

An ATG start codon, the Strep-tag II sequence (WSHPQFEK) and a linker sequence (GGG) (followed by the T7 RNA polymerase gene) were inserted between the Nco I and Hind III restriction sites in the plasmid vector pBAD HisA. Strep tagged T7 RNA polymerase was expressed under the inducible control of the L(+) arabinose promoter. Dry cell pellet containing the induced protein was then resuspended in 100 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT, and lysed using a sonicator (sample maintained at 4 °C). A gravity column packed with 5 ml of Strep-Tactin-Sepharose (IBA-LS) was equilibrated with wash buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The lysate supernatant was then loaded onto the column by gravity. The column was then washed with five times the bed volume of the column (25 ml). The recombinant protein was later eluted using three bed volumes of elution buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin). Fractions of 0.5 ml were collected and 20 µl of each fraction was analyzed by SDS-PAGE, purified fractions were then pooled together and dialyzed against storage buffer

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(20 mM potassium phosphate, pH 7.8, 50% glycerol, 100 mM NaCl, and 1 mM EDTA) and stored at -80°C .

Untethered transcription reactions

All reactions were performed with high-yield transcription buffer in the presence of $0.8\ \mu\text{M}$ (each) of nontemplate and template DNA, $0.8\ \mu\text{M}$ T7 RNA polymerase, and $7.5\ \text{mM}$ of each NTP, in an overall 10 or 20 μl reaction volume at 37°C for 4 h. Transcription was stopped by heat denaturation at 95°C for 5 min.

Tethered transcription reactions

To assemble the promoter complex, equimolar ($0.8\ \mu\text{M}$) concentrations of biotinylated nontemplate DNA, template DNA, and Strep-T7 RNA polymerase were incubated at 4°C for 30 min. A 5% slurry of Strep-TactinXT 6% agarose-coated beads (MagStrep “type 3” XT, iba Life Sciences) were washed with storage buffer three times and then incubated with the above enzyme-promoter complex at 4°C overnight to form the tethered transcription system. Tethered transcription reactions contained the equivalent of $0.13\ \mu\text{l}$ dry beads per $10\ \mu\text{l}$ reaction ($0.26\ \mu\text{l}$ for $20\ \mu\text{l}$ reactions). Before each transcription reaction, the tethered transcription system was washed twice with wash buffer and once with storage buffer. Transcription was initiated by the addition of high-yield transcription buffer (containing additional NaCl where indicated) with $7.5\ \text{mM}$ of each NTP, in a final reaction volume of 10 or $20\ \mu\text{l}$ at 37°C for 4 h. After 4 h, the supernatant was separated from the beads and heat denatured at 95°C for 5 min.

Reuse of the tethered complex for transcription reactions

For the reuse reactions of [Figure 5](#), transcription was carried out at 37°C for 2 h. As indicated below, partially single-stranded promoter DNA with a nontemplate strand that extends only to +2 position downstream (pss[+2]) or a partially single-stranded promoter DNA with a nontemplate strand that extends only to -5 position downstream (pss[-5]) was used for [Figure 5](#), B and C, respectively. After the first reaction was complete, the supernatant carrying the transcription products was separated from the beads and processed for gel quantification. The second reaction was then initiated as above, with the addition of fresh high-yield transcription buffer and NTPs and carried out at 37°C for 2 h. This process was repeated for a third time.

Gel electrophoretic analyses

All transcription reactions, except the reusability reactions shown in [Figure 5](#), were labeled with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (PerkinElmer). The gel showing reusability reactions in [Figure 5](#) was stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen). All reactions were analyzed by 20% polyacrylamide (19:1 acrylamide:bisacrylamide) denaturing (7 M) urea gel electrophoresis. Gels labeled with radioactivity were dried and then imaged with a GE Typhoon FLA 9500 Phosphorimager. Gels labeled with SYBR Gold stain were imaged directly with a Bio-Rad Gel Doc Go Imaging System Blue Tray. Quantifications of

gel lanes were performed using Fiji ([63](#)) software on raw TIF output.

Data availability

All data are contained within the manuscript and accompanying supporting information.

Supporting information—This article contains [supporting information](#).

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Conflict of interest—E. C. and C. T. M. are listed as inventors on a patent application related to the described technologies.

Abbreviations—The abbreviations used are: MDA5, melanoma-differentiation-associated antigen 5; OAS, oligoadenylate synthetase; RIG-I, retinoic acid inducible gene; TLR-3, Toll-like receptor 3.

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