In Vitro Elongation Using Tailed Template

(Jess and Bret version)

NOTE 1: Tailed template allows for promoter-less transcription. All you actually need is RNA polymerase II (optimised using TAP purified RNApII, via Rpb9-TAP). Works extremely well once the calmodulin elutions have been pooled and concentrated 3-6 fold. Use 6-8μl of this concentrated protein per 60μl reaction. Future experiments will add recombinant or TAP purified elongation factors.

NOTE 2: This protocol is written for a single 60μl reaction. *** Need to scale up reaction volumes if taking time points or doing multiple conditions. Remove 60μl for each time point of each reaction condition.

1. Aliquot appropriate volume of salt (1M KOAc) and **DEPC-H_2O** (see **Note 4**) to each reaction tube taking into account the protein volume to be added in **Step # 3** (total volume 25μl / 60μl reaction).

   NOTE 3: Want final salt concentration to be 100mM. Calculate contribution from protein buffers and complete up to 100mM with 1M KOAc. If proteins are in a buffer with a different salt (like KCl), use that in place of KOAc. Prefer all proteins in same buffer with same salt.

   NOTE 4: Water treated with DEPC is used for work with RNA; the DEPC should kill any contaminating ribonucleases. Add 1ml of fresh DEPC to 1L of H_2O to make a final concentration of 0.1% (v/v) and shake to disperse DEPC thoroughly. Incubate at 37°C for at least 12 hours, then autoclave at 15psi on liquid cycle for 20min to inactivate the remaining DEPC.

2. Add 30μl of Master Mix / 60μl reaction to each tube and pipette to mix.
3. Add appropriate volume of RNA Pol II (and other proteins) / 60μl reaction.
4. Let tubes stand for 5 min at RT°.
5. During incubation, make up NTP mix and add α^{32}P-CTP immediately prior to adding NTP mix to reactions.
6. Add 5μl of NTP mix for each 60μl reaction. Pipette gently to mix.
7. Incubate at RT° for 30min.
8. Remove 60μl aliquot for 0 min time point/pulse control and place in new tube. For t=0min, skip to **step # 11**.
9. Add 1μl Chase NTP / 60μl reaction to tube.
10. Incubate at RT° for 30min (or appropriate time points).
11. Add 60μl of Stop Buffer to 60μl reaction aliquot.
12. Add 1.2μl 20% SDS, and 2μl of 10mg/mL Proteinase K to reaction tube.
13. Incubate for 15min at RT°.

14. Add 2μl of tRNA (10mg/mL) and 40μl 10M NH_4OAc.
15. Add 400μl of P:C:I (25:24:1, Acid PhOH (buffered with ddH_2O): $CHCl_3$: Isoamyl Alcohol). Vortex and spin at max for 4 min.
16. Remove aqueous layer and add 400μl $CHCl_3$. Vortex and spin at max for 4 min.
17. Remove aqueous layer and transfer to new tube. Add 850μl 100% EtOH (-20ºC) to precipitate overnight at -20°C or 40 min at -80°C (recommend O/N ppt).
18. Spin tubes at max for 15min. Remove EtOH with 1ml pipetman.

NOTE 5: Be VERY CAREFUL removing the EtOH. The pellet will be small, glassy and sometimes very loose. You do not want to suck it up accidentally.

19. Wash pellets with 400μl 70% EtOH (do not vortex!) and spin at max 5 min at RTº.
20. Remove EtOH with pipetman and let dry for 15 min.

NOTE 6: I use a 10μL pipetman to remove the last drops of EtOH.

21. Dissolve pellets in 10-14μL loading buffer (100% formamide, 1mM EDTA, bromophenol blue, xylene cyanol). Heat 95°C for 1-2min to dissolve pellet.
22. Run on 5-7μl on thin, ChIP-sized urea-polyacrylamide denaturing gel (I use 8% acryl, 7M urea). Pre-run gel at 160V for 30min and then run gel at 400V for 3-4hr at RT.

NOTE 7: I usually run the gels until the xylene cyanol is about 1 inch from the bottom of the gel. Should give you a nice ladder of RNA transcripts up to 200-600nt or greater depending on elongation time/conditions.

23. Dry gel for 1hr at 80°C.
24. Expose gel to phosphoimager screen for 1hr up to O/N, depending on strength of signal.

Solutions and Reagents	
MgOAc	1 M
Tris-HCl pH 7.9	1 M
DTT	0.5 M
10X Salts (no KOAc)	30 mM HEPES-KOH pH 7.9 30% Glycerol 5 mM EDTA pH 8.0
Glycerol	10%
BSA	10 mg/mL
RNAsin	40 u/μL
KOAc	1M

Pulse Solution	1.5 mM ATP 1.5 mM GTP 60 μM CTP
Chase Solution	61 μM UTP
STOP solution	10 mM Tris HCl pH 7.5 0.5 mM EDTA 300 mM NaCl
SDS	20%
Proteinase K	10 mg/ml
tRNA	10 mg/ml
NH_4OAc	10 M
Phenol / $CHCl_3$ / IAA	25:24:1 buffered in dH_2O
$CHCl_3$	
EtOH	100%
EtOH	70%
DNA template	*see attached protocol for preparation*

Use the following table to calculate volumes for scaled up reactions

Master Mix	**Stock**	**Final**	**μL/ 60μL rxn**	**# of rxns**
MgOAc	1M	8 mM	0.48	
TrisHCl pH 7.9	1M	20 mM	1.2	
DTT	500mM	1 mM	0.12	
10x Salts	10x	1x	6	
Glycerol	10%	3%	18	
BSA	10 mg/ml	0.5 mg/ml	3	
RNAsin	40 u/μl	8u	0.2	
Tailed template	100ng/μl	100ng/rxn	1	
			30μL/rxn	
Proteins	**Date made:**		**μL / 60μL rxn**	
Pol II		vary		
KOAc	1M	100mM total		
DEPC H_2O	**---**	Que to 25μL		
			25μL/rxn	
NTP Mix	**Stock**		**μL / 60μL rxn**	
Pulse Solution	ATP, GTP, CTP	2μL mix		
α^{32}P-CTP	---	1μL (~10μCi)		
DEPC H_2O	---	2μL		
			5μL/rxn	

Supplementary Protocol: Preparation of Tailed Template

NOTE 7: Using a 3' oligo(dC)-tailed template in an *in vitro* elongation assay allows for RNApII transcription to occur in the absence of a promoter and general transcription factors (TBP, TFIIB, etc). One can directly observe a factor's elongation activity independent of other transcription factors by adding it to the reaction with RNApII.

1. Digest pADGR220 **(pCpGR220;** Rice *et al* (1991) *PNAS* **88:**4245**)** with SmaI.

NOTE 8: pADGR220 plasmid (full sequence on next page) appears to be toxic to *E. coli*. Therefore, always perform a new transformation before starting cultures for plasmid isolation.

2. Run on agarose gel and cut out linear plasmid.
3. Isolate DNA with preferred method.
4. Determine concentration of isolated linear DNA.
5. Perform test tailing reactions with 1μg DNA per reaction and varying concentrations of dCTP (5μl NEB #4, 5μL 2.5mM $CoCl_2$, 1μL 10, 5, 2.5, and 1.25mM dCTP, 0.5μL TdT, total vol. 50μL). Be sure to include a control with no dCTP. Incubate 37°C for 30min.
6. Stop reaction by incubation at 70°C for 20min.
7. Ethanol ppt reaction and resuspend in 10μl TE. It is necessary to EtOH ppt as KpnI is inhibited by components of the tailing reaction.
8. Digest with KpnI to check tail length on agarose gel. The length from SmaI to KpnI is 240nt (see below for sequence). Look for increase over the no dCTP control. I found that using 1μl of 1.25mM dCTP gave a tail length of 0-50nt, or an average of 25nt. I chose this for scaling up.
9. Repeat above steps at determined [dCTP] and at chosen scale, omitting KpnI digestion. Digest instead with BamHI to remove downstream tail. Heat inactivate BamHI.

pADGR220 Sequence:

(BamHI, SmaI, KpnI **bolded**; U-less cassette underlined; major pause sites highlighted):

ggaaattgtaaacgttaatattttgttaaaattcgcgttaaatttttgttaaatcagctcatttttttaaccaataggccgaaatcggcaaaatcccttataaatcaaaagaatagaccgagatagggttgagtgttgttccagtttggaacaagagtccactattaaagaacgtggactccaacgtcaaaggcgaaaaaccgtctatcagggcgatggcccactacgtgaaccatcaccctaatcaagttttttggggtcgaggtgccgtaaagcactaaatcggaaccctaaagggagcccccgatttagagcttgacggggaaagccggcgaacgtggcgagaaaggaagggaagaaagcgaaaggagcgggcgctagggcgctggcaagtgtagcggtcacgctgcgcgtaaccaccacacccgccgcgcttaatgcgccgctacagggcgcgtcgcgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagtgaattgtaatacgactcactatagggcgaattggagctccaccgcggtggcggccgctctagaactagt**ggatcc**ggggctataaaaggggtgggggcgcgttcgtcctcaca**cccggg**aagaaaaagcaaaccgcagagaggaagccaaccccagaagagaggaaccagaagcggcaagcccaggagcgagacgcaacaggcgacgaggcacccgcgggcacgacaagcacaacaccagcgagcaaggcgTTTcggggaagaaaaagcaaaccgcagagggaagccaaccccagaagagaggaaccagaagcgccaagcacaaacggcaagatcgaggggggcccc**ggtacc**cagcttttgttccctttagtgagggttaattccgagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgttttccataggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagtactcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttccttttcaatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaataggggttccgcgcacatttccccgaaaagtgccacctg