In vitro transcriptions assays

Protocol for multiround transcription assays

The final volume of the reaction is 25µl – 24µl of cocktail and the reaction is started by the addition of 1µl polymerase.

i) Cocktail: for 15 x 24 µl where RNAP (1 µl of each in 1 x transcription buffer) is added individually to each reaction:

36 µl	10 x transcription buffer (Tris/acetate buffer)	2.4y
3.6 µl	10 mg/ml BSA	0.24y
15 µl	20 ng/µl pSR/lacUV5(short)	y
15 µl	5 mM GTP	y
15 µl	5 mM ATP	y
15 µl	5 mM CTP	y
15 µl	0.25 mM UTP	y
3.75 µl	[α^{32}P]-UTP (2.5 µCi per reaction)	0.25y
241.65 µl	sterile, RNase-free water	
360 µl		

iv) Prepare RNAP dilutions - dilute RNAPs in 1 x transcription buffer + 100 µg/ml BSA:

Usually use three different concentrations for each polymerase – 4ng/ml, 2ng/ml + 1ng/ml

Reaction number	Cocktail	RNAP dilution
1	24µl	1 µl 1ng/ml
2	24µl	1 µl 2ng/ml
3	24µl	1 µl 4ng/ml
etc		

iv) Add 1 µl of RNAP solution to each reaction, each one staggered by 30 seconds, mix by pipetting up and down and moving the tip around, incubate for 15 minutes at 30°C.

v) Adde25 µl of stop solution - mix by gentle pipetting.

vi) Load 15 µl of each reaction onto the denaturing gel.

vii) Run gel for ~ 1 hour at 224 V (~ 15V/cm) then dry without fixing.

viii) Cover gel with cling film and exposed to phosphorimager.

ix) Analyse the results, measurimg the amount of RNA I and the lacUV5 transcripts produced in each lane.

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Notes

Transcription buffer:

40 mM Tris acetate pH 8
100 mM KCl
10 mM $MgCl_2$
1 mM DTT

DNA Template:

PSR is approx. 4000bp ∴ 20ng per reaction is a final conc. Of approx. 0.3 nM.

Protein Concentration:

The DNA concentration is approx. 0.3nM and there are more than 1 promoter per plasmid. If using the polymerase concentrations of 4μg/ml, 2μg/ml and 1μg/ml these equate to 89μM, 44μM and 22μM. This means that the final concentrations in the reactions are 3.52nM, 1.76nM and 0.88nM respectively.