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Troubleshooting Guide for *in vitro* Transcription

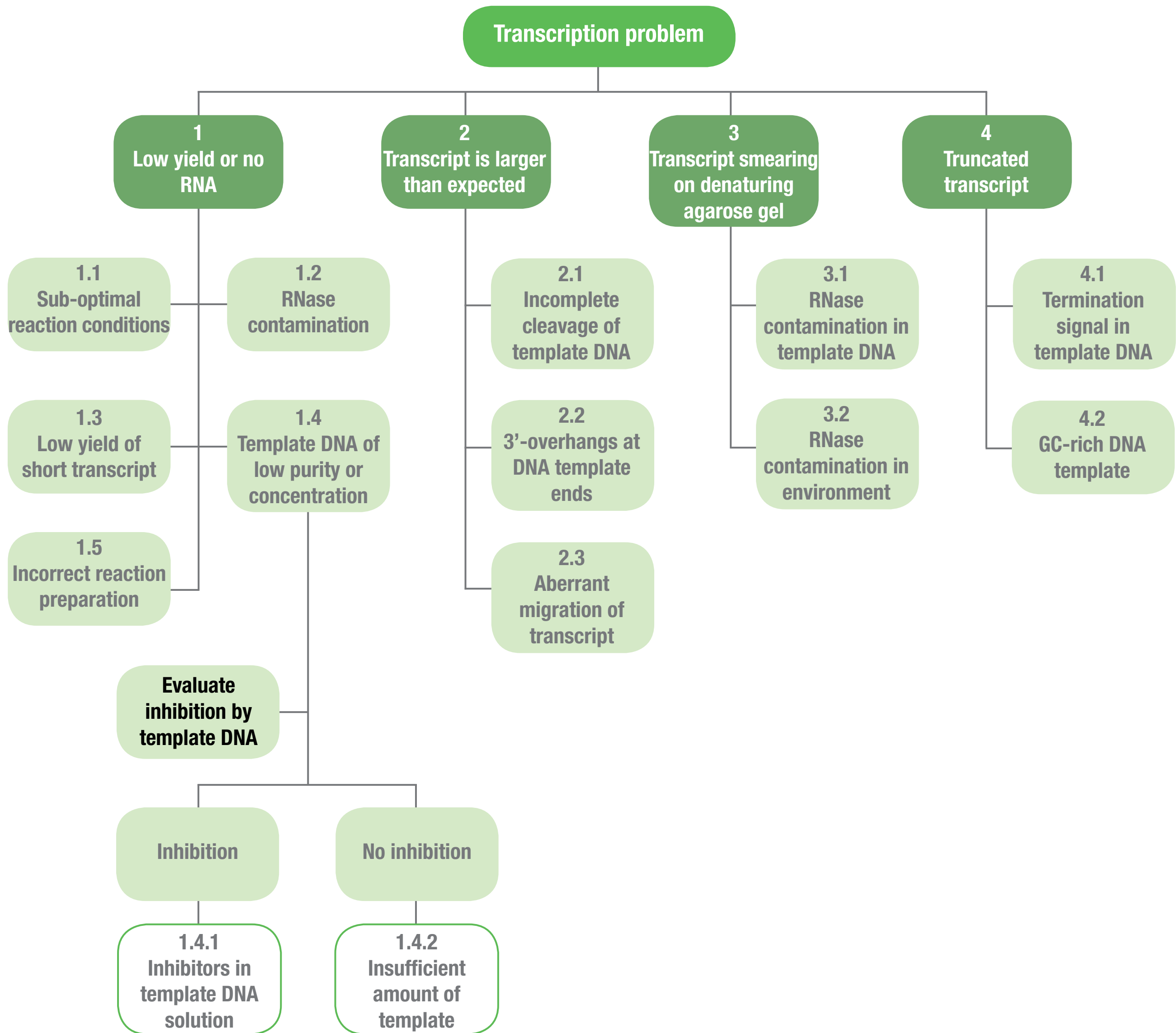

Table 6.1. Troubleshooting Guide for *in vitro* Transcription.

Problem	Possible cause and recommended solution
1. Low yield or no RNA transcript	**1.1. Non-optimal reaction conditions.** Conventional *in vitro* transcription reaction, using stand alone RNA polymerases or the T7 Transcription Kit (#K0411) should produce at least 10 µg of RNA transcript from 1 µg of template. For higher RNA yields (up to 200 µg), the TranscriptAid™ T7 High Yield Transcription Kit (#K0441) should be used. Addition of Pyrophosphatase, Inorganic (#EF0221) at 0.02-0.1 u (0.2-1 µl) per 20 µl of reaction volume may increase the yield of RNA by reducing the effect of reaction inhibition by pyrophosphates.
	1.2. RNase contamination. Working environment, DNA template, reagents or electrophoresis systems may be contaminated with RNases. • Follow general recommendations for working with RNA (p.366). • Use RNase-free enzymes, nucleotides and DEPC-treated water (#R0601). • Use RiboLock™ RNase Inhibitor (#EO0381) to protect synthesized RNA from RNases. ***Note*** RiboLock™ RNase Inhibitor inhibits the activity of RNases A, B and C. It does not inhibit the following RNases: I, T1, T2, H, U1, U2 and CL3. Do not use electrophoresis tanks which have been previously used for plasmid DNA miniprep analysis as they may be contaminated with RNases A or T1.
	1.3. Insufficient yield of short transcript. High yields of short transcripts (≤100 bases) can be achieved by increasing the amount of template and extending the incubation time. Use 2 µg of template and prolong the reaction time to 4-8 hours.
	1.4. DNA template of low purity or concentration. Evaluate your template in conjunction with a control template to determine if contaminants are inhibiting the reaction. If your template generates considerably lower RNA yields compared to the control template, modify the transcription reaction described in p.367 by mixing equal amounts of experimental template to the control template and adjusting the volume of DEPC-treated water (#R0601). Evaluate the transcript on agarose gel as described in p.369: C S C/S1 C/S2 **Fig. 6.5. Evaluation of mixing experiment results** **C** – control template **S** – sample template **C/S1** – mixture of **C** and **S**: control reaction inhibited by sample template solution **C/S2** – mixture of **C** and **S**: control reaction not inhibited by sample template ***If control reaction was inhibited by sample template (see Fig.6.5. C/S1), this indicates:*** **1.4.1. Reaction inhibitors in template DNA solution.** Template DNA may contain residual SDS, EDTA, proteins, salts* and RNases. Repurify the template by phenol/chloroform extraction and ethanol precipitation. An $A_{260}/_{280}$ ratio of 1.8-2.0 should be observed. To remove EDTA and salts, wash the pellet with 70% cold ethanol. * T7 and SP6 RNA Polymerases are inhibited ~50% by NaCl or KCl at concentrations above 150 mM (T3 RNA Polymerase at above 250 mM). Greater than 50% inhibition of the polymerases is observed with ammonium sulphate. ***If control reaction is not inhibited by sample template (see Fig.6.5. C/S2), but low RNA yields are observed, this indicates:*** **1.4.2. Insufficient amount of template.** Low amounts of template produce significantly lower RNA yields. The presence of RNA and chromosomal DNA in the DNA template preparation may interfere with UV absorbance readings and lead to misinterpretation of template DNA concentration. To accurately determine the concentration, size and integrity of the template, analyze the DNA concentration by UV absorbance and gel electrophoresis.
	1.5. Incorrect reaction preparation. If the reaction is prepared on ice or in the incorrect order, the DNA may precipitate in the presence of spermidine in the reaction buffer. Water should always be added to the transcription reaction first.

(continued on next page)

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2. Transcript is larger than expected	**2.1. Incomplete cleavage of template plasmid DNA.** Even small amounts of undigested circular DNA can produce large amounts of long transcripts. Check the template for complete digestion and, if required, additionally digest with the appropriate restriction enzyme (*see* Troubleshooting Guide for DNA Digestion on p.204). For faster and more efficient plasmid cleavage, use FastDigest™ enzymes. If complete digestion is unachievable, gel-purify the digested template using DNA Gel Extraction Kit (#K0513).
	2.2. 3'-overhangs at DNA template ends. Avoid plasmid linearization with restriction enzymes that generate 3'-overhangs. Alternatively, blunt 3'-overhangs with T4 DNA Polymerase (#EP0061) before use in transcription. *See* protocol on p.336 .
	2.3. Aberrant migration of transcript. Due to secondary structures, RNA may run aberrantly on a native gel. On a denaturing gel, these transcripts normally migrate as single bands at the expected size.
3. Transcript smearing on denaturing agarose gel	**3.1. RNase contamination in template DNA.** During preparation, plasmid DNA templates are often contaminated with RNases. This can affect the length and yield of synthesized RNA, and is seen as a smear below the expected transcript length If using a commercial kit, such as the GeneJET™ Plasmid Miniprep Kit (#K0502), omit the RNase A from plasmid preparation solutions and use DEPC-treated water (#R0601) for plasmid elution. If RNase A is premixed in the purification buffers, perform phenol/chloroform extraction after plasmid DNA linearization, then ethanol precipitate the DNA and dissolve in DEPC-treated water (#R0601) (*see* Plasmid Templates on p.366).
	3.2. RNase contamination in working environment. • Follow general recommendations for working with RNA (p.367). • Use RNase-free enzymes, nucleotides and water. • Use RiboLock™ RNase Inhibitor (#EO0381) to protect synthesized RNA from RNases. ***Note*** RiboLock™ RNase Inhibitor inhibits the activity of RNases A, B and C. It does not inhibit the following RNases: I, T1, T2, H, U1, U2 and CL3. Do not use electrophoresis tanks which have been previously used for plasmid DNA miniprep analysis as they may be contaminated with RNases A or T1.
4. Truncated transcript	**4.1. RNA polymerase recognizes a termination signal in template DNA sequence.** Try another RNA polymerase system or perform the transcription reaction at a lower temperature (e.g. 30°C). This may increase the length of transcript. However, RNA yield may be decreased at lower temperatures.
	4.2. GC-rich DNA template (or template with high secondary structure). For templates with secondary structure, incubating at 42°C or using a single-stranded binding (SSB) protein has been reported to improve yield and transcript length (2).

References

1. Schenborn, E.T and Mierendorf, R.C., Nucl. Acids Res.,13, 6223-6236, 1985.
2. Aziz, R.B. and Soreq, H., Nucl. Acids Res., 18, 3418, 1990.