

Example 1: Production of dsRNA from linear DNA templates with different ITS variants using *in vitro* transcription (IVT) reactions

GS1, a 601-base pair dsRNA, was produced in *in vitro* transcription (IVT) reactions in buffer using linear DNA templates comprising expression cassettes comprising a promoter operably linked to different ITS variants (as shown in Table 3) placed immediately upstream of the sequences-of-interest (GS1 sense and GS1 antisense strands) to be expressed. Each GS1 strand synthesized in the IVT reactions comprises a 5' single-stranded r-ITS overhang corresponding to the different ITS variants. DNA templates lacking a specific ITS (ITS_none) were used as a control in the experiment. Note that, for each ITS variant and the control, two DNA templates were utilized, one of which comprised a sequence of interest (SOI) encoding the sense strand of GS1 and the other of which comprised a SOI encoding the antisense strand of GS1. FIG. 2 shows the general architecture of the linear DNA templates with expression cassettes and the expected dsRNA product (with 5' single stranded r-ITS overhangs).

Table 3: ITS variants for use in Example 1

	ITS sequence	Promoter* + ITS sequence
ITS_None	None	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TA (SEQ ID NO: 31)
ITS_6	GGGAGA (SEQ ID NO: 8)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGA (SEQ ID NO: 32)
pT7-g10	GGGAGACCACAACGT (SEQ ID NO: 5)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGACCACAACGT (SEQ ID NO: 33)
pT7-g5	GGGAGACCGGAATT (SEQ ID NO: 6)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGACCGGAATT (SEQ ID NO: 34)
GL-Hybrid_A9	GGGAGACCAGGAATT (SEQ ID NO: 1)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGACCAGGAATT (SEQ ID NO: 35)

*The promoter operably linked to each ITS in these templates was the 47 bp extended T7 promoter (i.e. SEQ ID NO: 8), comprising the 17 bp minimal class III T7 promoter with 30

ITS_6	GGGAGA (SEQ ID NO: 8)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGA (SEQ ID NO: 49)
GL-Hybrid_A9	GGGAGACCAGGAATT (SEQ ID NO: 1)	TCGATTTCGAACTTCTGATAGACT TCGAAATTAATACGACTCACTA TAGGGAGACCAGGAATT (SEQ ID NO: 35)

As shown in FIG. 15, the GL-Hybrid_A9 ITS resulted in a 1.9 fold improvement in dsRNA synthesis over the consensus ITS_6 naturally found in the T7 bacteriophage genome as well as other shorter ITSes.

Example 9. ITSes comprising an A-start produce capped RNA at high titer and purity using co-transcriptional capping reagents

A pair of mRNA molecules were designed and produced in cell-free reactions. Both molecules consisted of a similar sequence architecture, incorporating the 5' ITS GL-hybrid_A9 (A start) (AGGAGACCAGGAATT (SEQ ID NO: 38)). mRNA sequences, were encoded on a pUC-19 derived plasmid template along with a T7 promoter at the 5' end and a restriction endonuclease recognition site. Plasmids were propagated in *E. coli* strain DH10b, purified by Plasmid Giga Kits (Qiagen), linearized by digestion with Esp3I restriction endonucleases (New England Biolabs), and further purified by phenol-chloroform extraction. RNA synthesis reactions were performed using the cell-free production platform as described in PCT/US2020/025824. Reactions producing capped RNAs also included CleanCap AG reagent (TriLink Biotechnologies). Template DNA was removed by treatment with DNase I, then RNA was recovered by lithium chloride precipitation. Recovered RNA was quantified by UV absorbance at 260nm and analyzed for size and quality using a 2100 BioAnalyzer instrument (Agilent Technologies).

As shown in FIG. 16, cell-free reactions producing capped RNA using CleanCap AG and an AG ITS (GL-hybrid A9 (A Start), SEQ ID NO: 38) achieved similar titers as reactions producing uncapped RNA using the GG ITS (GL-hybrid_A9, SEQ ID NO: 1) (FIG. 16A). Analysis by BioAnalyzer demonstrated RNA products from both reactions were of the expected size and similar purity (FIG. 16B).

Example 10. ITSes in mRNAs encoding different proteins results in consistent production titers and molecule quality