A comprehensive analyses of dsRNA by-products formed during *in vitro* transcription.

Monica Z. Wu, Haruichi Asahara, George Tzertzinis, and Bijoyita Roy* (*broy@neb.com)

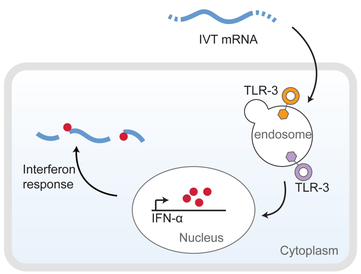
RNA and Genome Editing, New England Biolabs, Ipswich, MA 01938

Introduction

The use of synthetic RNA for therapeutics requires that the *in vitro* synthesis process be robust and efficient. The technology used for the synthesis of these *in vitro*-transcribed (IVT) mRNAs using phage RNA polymerase (RNAP) is well established. Introduction of these IVT RNAs into *in vivo* models results in an immune response, which is undesirable for some therapeutic applications, and previous studies have identified double-stranded RNA (dsRNA)—a major by-product of the *in vitro* transcription process—as a trigger of cellular immune responses. To avoid these unwanted immune responses, it is critical to either eliminate these dsRNA by-products from the mRNA preparations or minimize their formation. Therefore, it is pivotal to understand the mechanistic origins and the sequence dependencies of these contaminants.

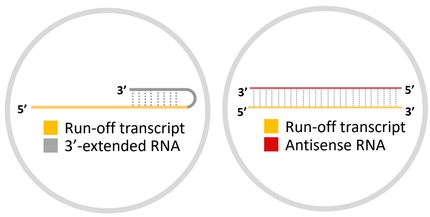
dsRNA is a common by-product formed during IVT

A. IVT mRNA triggers an immune response



*Based on Kariko *et al.*, 2004. J. Biol. Chem.

B. Two distinct mechanisms for dsRNA by-product formation during IVT

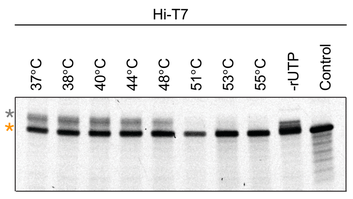


How can we remove both types of dsRNA by-products?

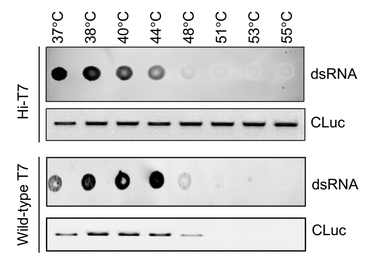
Reduction of 3’-extended dsRNA by-products

Effect of temperature on 3’-extended dsRNA formation

A. Denaturing gel

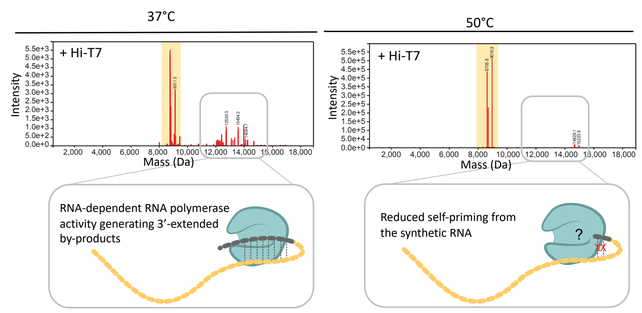


B. dsRNA immunoblot



- Validated with both thermostable RNA polymerases.
- 3’-extended dsRNA by-products are reduced in high-temperature IVT reactions.

Assessing the RNA-dependent RNA polymerase activity at high temperature

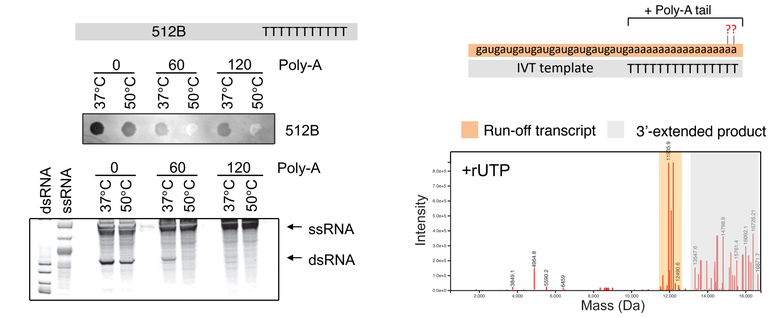


High-temperature IVT prevents the self-extension of the run-off product.

Effect of poly-T sequence on dsRNA by-products

A. Antisense dsRNA by-products

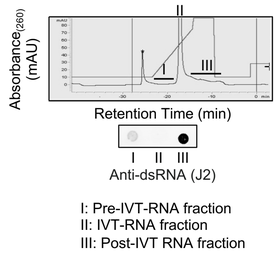
B. 3’-extended dsRNA by-products



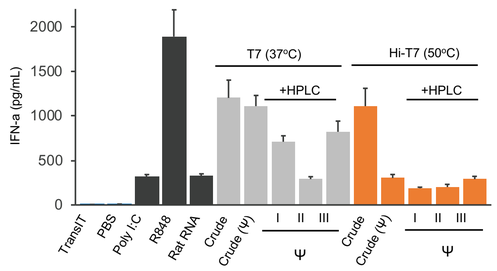
- Addition of poly-T sequence reduces antisense dsRNA
- Poly-A tail has no effect on 3’-extended dsRNA by-product formation

Immune response from high-temperature IVT

A. HPLC purification of mRNA



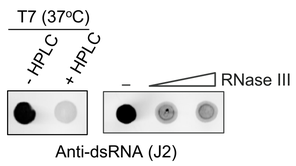
B. Interferon secretion in dendritic cells in response to IVT RNA



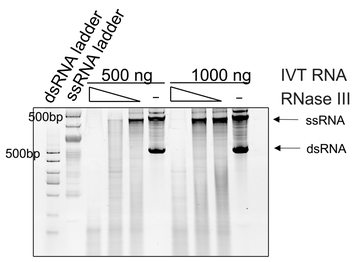
HT-IVT mRNA is functional and demonstrates a reduced immune response *in vivo*.

Methods

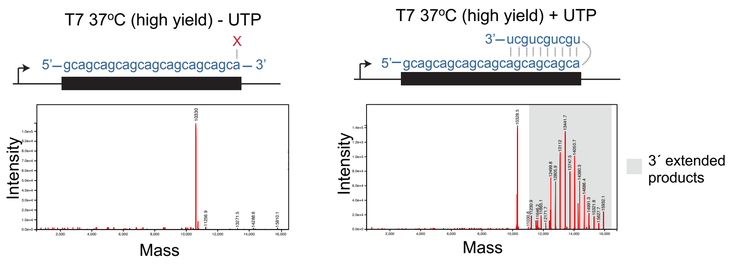
A. dsRNA immunoblot for long mRNA



B. Gel-based assays for short/mid-sized RNA



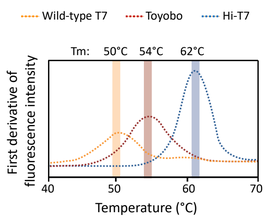
C. MS-based assays for short RNA



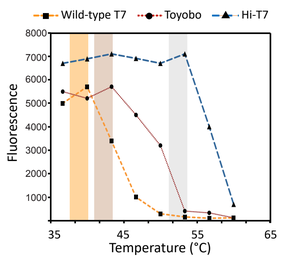
dsRNA by-products can be detected in IVT reactions using three independent approaches.

Characterization of thermostable RNA polymerases

A. Protein melting temperature



B. Transcription activity at higher temperatures



Thermostable RNAP variants are more active at higher temperatures.

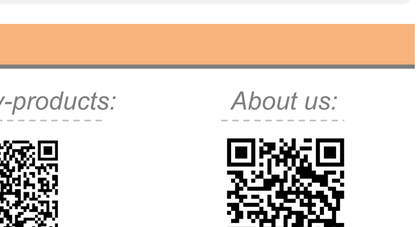
*Commercial thermostable RNA polymerases: Toyobo Thermostable T7 RNA polymerase; NEB Hi-T7® RNA polymerase

Reduction of antisense dsRNA by-products

Effect of high-temperature IVT on antisense dsRNA

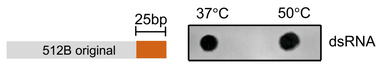
A. dsRNA immunoblot

B. Native gel

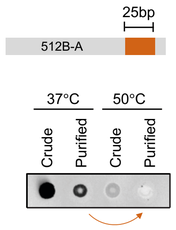


High-temperature IVT does not affect formation of antisense RNA by-products.

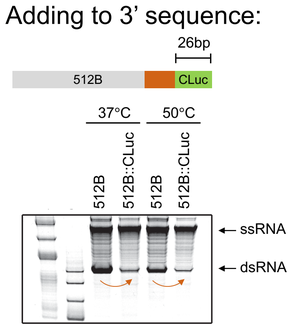
Assessing antisense dsRNA from modified 512B templates



A. Moving 3’ sequence:



B. Adding to 3’ sequence:



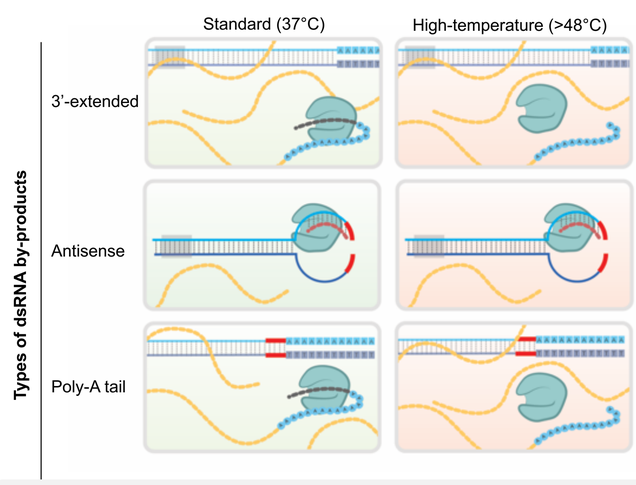
Antisense dsRNA formation is sequence-dependent.

More information

Learn more about dsRNA by-products:

About us:

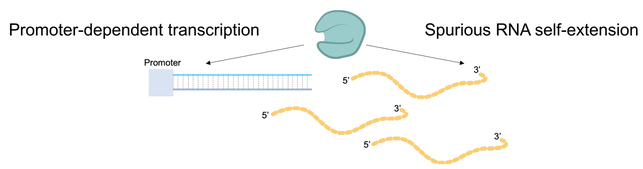
Model



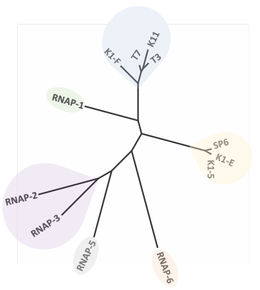
A combination of high temperature and poly-A tail reduces both types of dsRNA.

Future Directions

Mechanism of 3-extended dsRNA by-product formation and effect of high-temperature IVT



dsRNA by-products in other RNAPs



Quantitation of dsRNA by-products