

## PRODUCTION OF DSRNAS FOR INDUCTION OF POSTTRANSCRIPTIONAL GENE SILENCING AGAINST COXSACKIE VIRUS B1 INFECTION

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### ABSTRACT

RNA interference is a highly conserved and sequence-specific mechanism for silencing the transcriptional product of an activated gene. We use 2 virus-based systems for cost-effective efficient production of dsRNA targeting specific regions of Coxsackie viruses. In vitro dsRNA production system utilizes the combination T7 RNA polymerase and RNA-dependent RNA polymerase of bacteriophage  $\phi 6$ . The in vivo dsRNA production system is set up in *Pseudomonas syringae* cells that constitutively express the  $\phi 6$  polymerase complex from plasmids pLM1086 and pLM991, and a plasmid with the target sequence to produce large amounts of dsRNA of up to 4.0 kb. dsRNA is Dicer-digested with recombinant endoribonuclease producing siRNA fragments.

**Key words:** *Coxsackie B1 virus, VP1, dsRNAs, siRNAs*

### INTRODUCTION

RNA interference (RNAi) is a highly conserved process of post-transcriptional gene silencing (PTGS) by which double stranded RNA (dsRNA), when introduced into a cell, causes sequence-specific degradation of homologous mRNA sequences. RNAi and related silencing mechanisms have been used to target expression of several cancer- and disease-related genes and to alleviate viral infections in cell cultures and *in vivo* models (Uprichard 2005; van Rij and Andino 2006). Gene silencing experiments have been successfully carried out using sequence-specific siRNAs or short hairpin RNAs that have been either synthesized chemically or transcribed from template DNA using DNA-dependent RNA polymerases (Amarzguioui et al. 2005). However, since several factors affect the efficacy of a siRNA (Reynolds et al. 2004; Heale et al. 2005), identifying a functional sequence often is laborious and expensive. One way to circumvent this trial-and-error approach is to digest long dsRNAs into siRNA pools containing molecules of multiple sequences (Myers et al. 2003; Seyhan et al. 2005). Current methods for producing long dsRNAs rely on annealing two single-stranded RNA (ssRNA) strands. This may result in poor-quality dsRNA. That's why we used two novel flexible virus-based systems for the high quality production of dsRNA: (1) an *in vitro* system utilizing the combination of T7 RNA polymerase and RNA-dependent RNA polymerase (RdRp) of bacteriophage  $\phi 6$  (Makeyev and Bamford 2000a,b) to generate dsRNA molecules, and (2) an *in vivo* RNA replication system based on carrier state bacterial cells containing the  $\phi 6$  polymerase complex (Onodera et al. 1992; Sun et al. 2004) to produce virtually unlimited amounts of dsRNA.

### MATERIALS AND METHODS

#### *In vitro* dsRNA production system

The dsRNA is synthesized by combined *in vitro* transcription and replication from a DNA template. The DNA template for the dsRNA synthesis is produced by PCR using Phusion High-Fidelity DNA polymerase. The PCR primers are designed so that the resulting PCR product contains the target sequence (VP1) flanked by T7 promoter sequence at the 5' end and  $\phi 6$  qRdRP promoter sequence at the 3' end. The PCR product is purified and transcribed into single-stranded RNA (ssRNA) by T7 RNA polymerase. This ssRNA is replicated into double stranded RNA (dsRNA) by

$\phi 6$  qRdRP in the same incubation step (Fig. 1). The target sequence is of 800 bp from the CBV1 genome which includes VP1 gene (Fig.2).

### ***In vivo* dsRNA production system**

The system utilizes the T7 RNA polymerase and the polymerase complex of bacteriophage  $\phi 6$  to produce high amounts of target-specific dsRNA. The *in vivo* system is set up in *Pseudomonas syringae* cells that constitutively express the T7 RNA polymerase (from plasmid pLM1086). Plasmid pLM991 carries a cDNA copy of the  $\phi 6$  L<sub>kan</sub> – segment, which contains all the components required to form the polymerase complex (PC), as well as a kanamycin resistance gene. The other plasmid contains the required  $\phi 6$  S-segment 5'packaging (\*) and 3'-replication (†) signals, between which the gene of interest (VP1). Upon transformation into the host cells, the T7 RNA polymerase transiently synthesizes ssRNA from the cDNA plasmids. The (+)ssRNA of the  $\phi 6$  L<sub>kan</sub> – segment directs the translation of viral proteins, which yield empty PCs. Packaging begins with the S-segment specific ssRNA, followed by L-segment specific ssRNA. Upon packaging, an exact complementary strand is synthesized inside the PC particle by the viral RdRP.

### **Generating small interfering RNA pools by Dicer-digestion**

For gene silencing, dsRNA must be Dicer-digested for applications in mammalian systems, yielding a pool of small interfering RNAs (siRNAs). Since every siRNA has its own off-targets, pooling is thought to reduce the individual nonspecific contributions of each sequence, revealing the “true” RNAi-phenotypes. However, dsRNA molecules can be used as such to induce RNA silencing in plants, fungi and most invertebrates.

The PowerCut Dicer is a recombinant endoribonuclease from *Giardia intestinalis*. It cleaves dsRNA efficiently, producing siRNA fragments with a length of 25-27 nucleotides. (Fig.4)

## **RESULTS AND DISCUSSION**

We produce dsRNA targeting VP1 region of the Coxsackie virus B1 to stop viral replication (Fig.3).

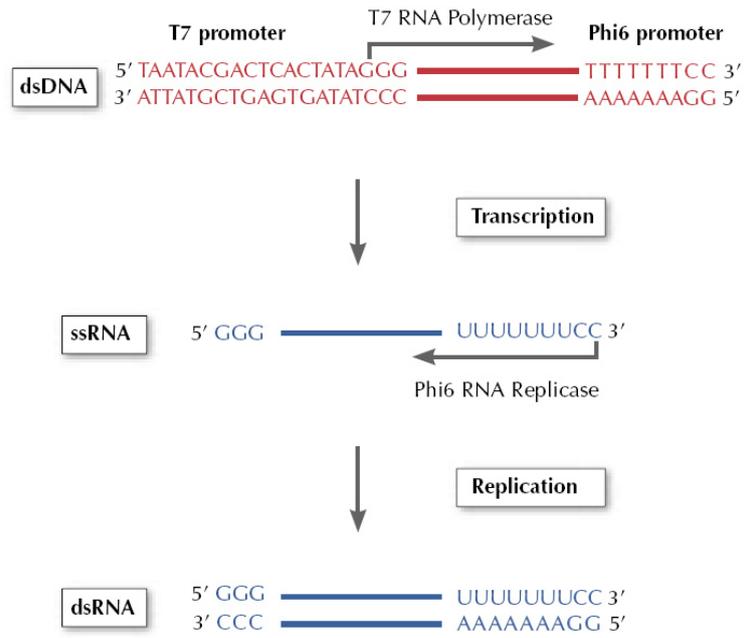
*In vitro* dsRNA production method combines the use of T7 RNA polymerase to synthesize ssRNA templates of Coxsackie virus cDNA..  $\phi 6$  RdRP performs de novo initiation and produces full-length dsRNAs from given template ssRNAs.

Selection of stable carrier state cells is achieved by inserting a kanamycin resistance (kan) gene in the 39 untranslated region of the L segment. Plasmids containing a T7 promoter and any heterologous cDNA, flanked by the viral packaging and replication signals, are transcribed and packaged into viral PC particles, followed by synthesis of the complementary RNA strand.

Medium and large quantities of dsRNA of a given sequence may be generated by *in vitro* or *in vivo* recombinant  $\phi 6$  RdRP-based dsRNA production systems. The *in vitro* system offers an efficient and flexible method to produce high-quality dsRNA from an ssRNA molecule of practically unlimited length.

The *in vivo* system allows dsRNA to be produced in very large quantities for industrial-scale implementation. This approach requires some 300 bp of  $\phi 6$ -specific sequence at both ends of the desired gene.

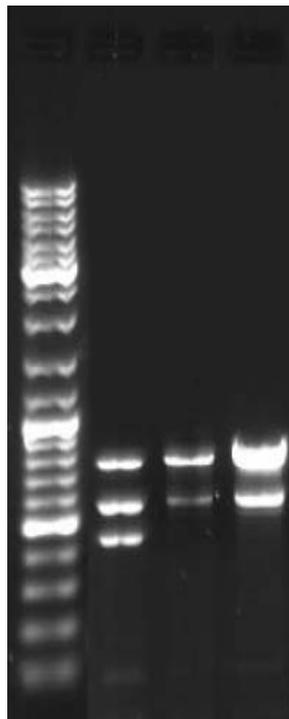
The novel methods described here provide an example of how large amounts of dsRNA required for efficient therapy could be synthesized cost effectively. When mucosal membranes are the route of infection, as is the case with influenza, HIV, and the common cold, delivery of siRNAs is feasible. The use of long target sites would reduce the generation of escape mutants, which are often detected when using single siRNAs (Westerhout et al. 2005; Wilson and Richardson 2005). Furthermore, interferon induction due to the controlled presence of long dsRNA molecules in combination with siRNAs might constitute a future direction of research.



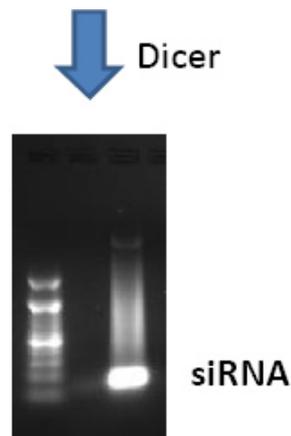
**Fig. 1** *In vitro* dsRNA production system (Finnzymes, Finland)



**Fig. 2** CBV1 genome



**Fig. 3** dsRNA targeting VP1 region of Coxsackie virus B1 (800 bp)



**Fig. 4** siRNAs from produced dsRNA of VP1 of Coxsackie virus B1

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