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# Stable Expression of T7 RNA Polymerase Mediated by Lentivirus in MDBK Cells

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**Abstract.** The low efficiency of transcripts *in vitro* mediated virus rescue seriously restricts the development and application of reverse genetics technology. T7 RNA polymerase (RNAP), a member of a family of single-subunit RNAPs that includes the phage RNAPs, transcribes the single or double-strand DNA at the downstream of T7 promoter and subsequently synthesizes RNA which complements with its corresponding DNA template, and is extensively applied to viruses rescue *in vivo*. In order to establish a novel and efficient system for bovine viral diarrhea virus (BVDV) rescue, we successfully established a Madin-Darby Bovine Kidney (MDBK) -T7 cell line which stably expresses T7 RNAP. In this study, we amplified the coding gene of T7 RNAP from *Escherichia coli (E. coli)* DE3 (BL21) genome. After cloning into a prokaryotic expression vector pET-32a, the protein of T7 RNAP was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Meanwhile, lentivirus was packaged with the recombinant lentiviral vector pLEX-T7 RNAP and its corresponding helper plasmids. After lentivirus infection and puromycin selection, the drug-resistant cells were obtained. To identify the validity of T7 RNAP, pET-32a-IERS-EGFP plasmid was transfected into MDBK-T7 cells and the expression of enhanced green fluorescent protein (EGFP) was monitored by Western blotting. The results showed T7 RNAP has been stably integrated into MDBK-T7 cells and has complete transcription activity, which provides an efficient tool for BVDV rescue.

**Keywords:** MDBK-T7 cells, T7 RNA polymerase, lentivirus, stable expression, puromycin selection.

## INTRODUCTION

During bacteriophage T7 infection, T7 RNAP, a phage-encoded DNA-dependent RNA polymerase, transcribes most 80% bacteriophage T7 genome (Tabor and Richardson, 1985). T7 RNAP is a member of the family of single-subunit RNA polymerases that includes the phage RNAPs (T3, K11, SP6, N4, and others) as well as the mitochondrial RNAPs (McAllister, 1993; McAllister and Raskin, 1993). T7 RNAP consists of a single polypeptide of 883 amino acids (98-kDa) (Moffatt *et al.*, 1984). Meanwhile, T7 RNAP exhibits extremely high affinity and specificity for the sequences of T7 promoters, class II and class III T7 promoters, a conserved 23-base pair (bp) sequence from -17 to +6 (Gunderson *et al.*, 1987). Comparing with other RNA polymerases in bacteria, the sequence recognized by T7 RNAP is extremely high specificity and the transcription efficiency of T7 RNAP is higher. Transcription *in vitro* by T7 RNAP is used widely to produce RNA for biophysical, biochemical and molecular biological experiments (Doudna *et al.*, 1993; Milligan *et al.*, 1987; Sampson and Uhlenbeck, 1988; Xiong and Lommel, 1991). Simultaneous intracellular expression of genetically marked full-length rabies virus (RV) antigenome-like T7 RNAP transcripts and RV N, P and L proteins from transfected plasmids result in formation of transcriptionally active nucleocapsids and subsequent assembly and budding of infectious rabies virions (Schnell *et al.*, 1994). The plasmids encoded bovine respiratory syncytial virus (BRSV) N, P, M2, and L proteins are co-transfected into BSR T7/5, a BHK-derived cell line stably expressing T7 RNAP, and recombinant BRSV is reproducibly recovered from cDNA constructs after T7 RNAP-driven expression of antigenome sense RNA (Buchholz *et al.*, 1999). Rift

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Valley fever virus (RVFV) rescues are based on the expression of positive-sense copies of the three viral RNA segments by bacteriophage T7 RNAP.

To establish a conventional reverse genetics system for bovine viral diarrhea virus (BVDV) rescue, MDBK cells stably expressed T7 RNAP named MDBK-T7 cells were established. The coding gene of T7 RNAP was cloned and integrated into lentiviral vector pLEX-MCS to construct the recombinant lentiviral vector pLEX-T7 RNAP which was applied to package lentivirus, following by infecting MDBK cells. After puromycin selection, the cells were identified using real-time PCR and Western blotting. The results showed T7 RNAP has been integrated into MDBK-T7 cell line and stably expressed at high level, which provides an efficient tool for BVDV rescue.

## MATERIALS AND METHODS

*Cells and plasmids*

The HEK-293T and MDBK cells were purchased from the Cell Bank of Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified minimal essential medium (DMEM) respectively supplemented with 10% fetal bovine serum (FBS) and horse serum (HS) at 37 °C with 5% $CO_2$. All of those medium and nutritional supplements were from Thermo Scientific (Logan, UT, USA). The plasmids of lentiviral packing system (pLEX-MCS, pSPAX2 and pMD2.G) were kindly provided by Dr. Mingjun Liu (Liu *et al.*, 2012).

*Construction of plasmids*

The genomic DNA of *E. coli* DE3 (BL21) (Tiangen, China) was extracted using a TIANamp Bacteria DNA Kit (Tiangen, China) according to the product's description. The coding gene of T7 RNAP was amplified from the genomic DNA of *E. coli* DE3 by Plus Taq DNA polymerase (TaKaRa, China). The primers for T7 RNAP gene (GenBank accession number M38308) were designed using Primer Premier 5.0 software (Primer, Canada) and listed as following:

forward:
5'CGAGGATCCCACCATGAACACGATTAACATCG 3'
reverse:
5'TACCTCGAGCGCGAACGCGAAGTAAGACTC 3'

Subsequently, T7 RNAP gene was cloned into the BamH I and Xho I restriction sites of lentiviral vector pLEX-MCS and prokaryotic expression vector pET-32a (+) (Novagen, USA) to yield the pLEX-T7 RNAP and pET-32a-T7 RNAP recombinant plasmids. Meanwhile, the coding gene of enhanced green fluorescent protein (EGFP) (GenBank accession number U55762) was cloned into the Spe I and Xho I restriction sites of pLEX-MCS to constructe pLEX-EGFP as positive control.

*Prokaryotic expression of T7 RNAP*

pET-32a-T7 RNAP plasmid was transformed into *E. coli* DE3 (BL21). Expression was then induced by addition of 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sangon, China), following by growth for an additional 8 h at 37°C. The cells were collected at different time intervals (0, 2, 4 and 8 h) and incubated for 10 minutes in a boiled water bath, following by separating on 12% SDS-PAGE. The coomassie brilliant blue (CBB) staining method was used to determine the expression of T7 RNAP gene.

*Lentivirus-mediated T7 RNAP transcation*

pLEX-MCS, pLEX-T7 RNAP and pLEX-EGFP plasmids were prepared using a Endofree Maxi Plasmid Kit (Tiangen, China) and co-transfected with the helper plasmids (pSPAX2 and pMD2.G) into HEK-293T cells by using Lipofectamine$^{TM}$ 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol, respectively. At 48 h post-transfection, the supernatants and suspensions containing infectious lentiviral particles were harvested and concentrated with 100-kDa ultrafiltration tubes (Millipore, USA) at 10,000g for 1 h at 4°C. The viral titer was measured using the limited dilution method based on the expression level of EGFP (Sastry *et al.*, 2002). In this study, lentivirus which was respectively packaged with pLEX-MCS, pLEX-T7 RNAP and pLEX-EGFP was named negative-lentivirus, T7 RNAP-lentivirus and EGFP-lentivirus.

Three strains of lentiviruses (MOI = 5) were respectively used to infect monolayer MDBK cells (50-60% confluence) in the presence of 8 μg/ml polybrene (Sigma, USA). At 36 h post-infection (pi), cells were fed with fresh medium plus 5% HS and 4 ug/ml puromycin every 2 days. The drug-resistant cells derived from T7 RNAP-lentivirus, EGFP-lentivirus and negative-lentivirus was named as MDBK-T7, MDBK-EGFP and MDBK-negative, respectively.

*Identification of drug-resistant cells*

About $10^5$ MDBK-T7 and MDBK-EGFP cells were respectively harvested and subjected to extract total RNA using a total RNA extraction kit (Tiangen, China). First-strand cDNA was synthesized from 1 ug of total RNA using a reverse transcription kit (Tiangen, China) and applied into real-time PCR to identify the drug-resistant cells. The primers for T7 RNAP, EGFP and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as following:

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T7 RNAP-F: 5' GTAACTCCTGATGGTTTCCCTGTG 3'
T7 RNAP-R: 5' GAGCGATACCAGACTCCTGTTTGTG 3'
EGFP-F: 5' CTACGTCCAGGAGCGCACCATCT 3'
EGFP-R: 5' TTCTTCTGCTTGTCGGCCATGATAT 3'
GAPDH-F: 5' TGAACCACGAGAAGTATAACAACACCC 3'
GAPDH-R: 5' TAAGTCCCTCCACGATGCCAAAG 3'
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The mRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to the endogenous levels of GAPDH.

pET-32a-IERS-EGFP plasmid was constructed. Briefly, the coding gene sequences of internal ribosome entry site (IRES) of BVDV NADL (GenBank accession number NC_001461) and EGFP (GenBank accession number U55762) were cloned into a prokaryotic expression vector pET-32a (+) to yield the recombinant plasmids pET-32a-IERS-EGFP. About 5 μg of pET-32a-IERS-EGFP plasmid was respectively electroporated into MDBK-T7 and MDBK-negative using a Bio-Rad Gene Pulser Xcell (Gene Pulser® Electroporation Buffer, 340 V, 5 ms, 4 mm cuvettes, one pulse). At 24 h post-transfection, the localization of EGFP protein was observed under a fluorescence microscope (TE200; Nikon, Tokyo, Japan). Meanwhile, about $10^6$ cells were harvested and lysed with Cell Lysis Buffer (Beyotime, China). The protein concentration was determined using a BCA protein quantification assay kit (Tiangen, China). The equal amounts of protein were subjected to 15% SDS-PAGE and electrophoretically transferred onto a 0.45-μm nitrocellulose membrane (Bioworld, USA). After blocking with 5% non-fat milk, the membranes were incubated with a rabbit anti-GFP tag antibody (BS6507, Bioworld, USA), following by incubating with a goat anti-rabbit IgG antibody (BS10350, Bioworld, USA) labeled with horseradish peroxidase (HRP). A rabbit anti-β-actin antibody (Bioworld, USA) was used for β-actin served as the internal control. Western blots were developed with using a DAB HRP color development kit (Tiangen, China).

*Identification of stable integration*

To identify the stability of MDBK-T7 cell line, the cells were passaged 40 times were harvested and identified with real-time PCR and Western blot analysis as described above.

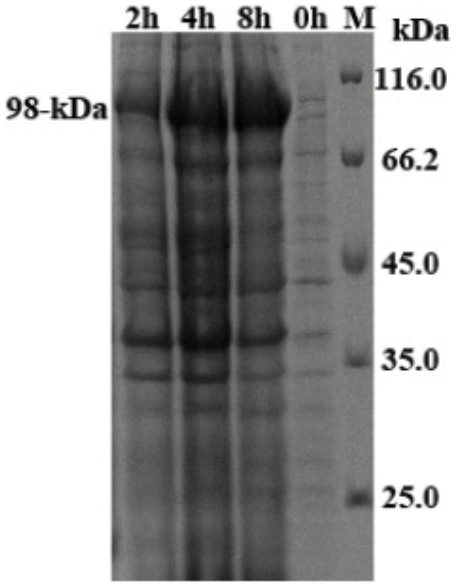


Fig. 1. Prokaryotic expression of T7 RNAP. The coding gene of T7 RNAP was cloned into a pET-32a vector and the recombinant plasmid pET-32a-T7 RNAP was transformed into *E. coli* DE3 (BL21). After IPTG induction, SDS-PAGE was performed to examine the protein of T7 RNAP. A 98-kDa protein highly expressed at 8 h after induction. n = 2.

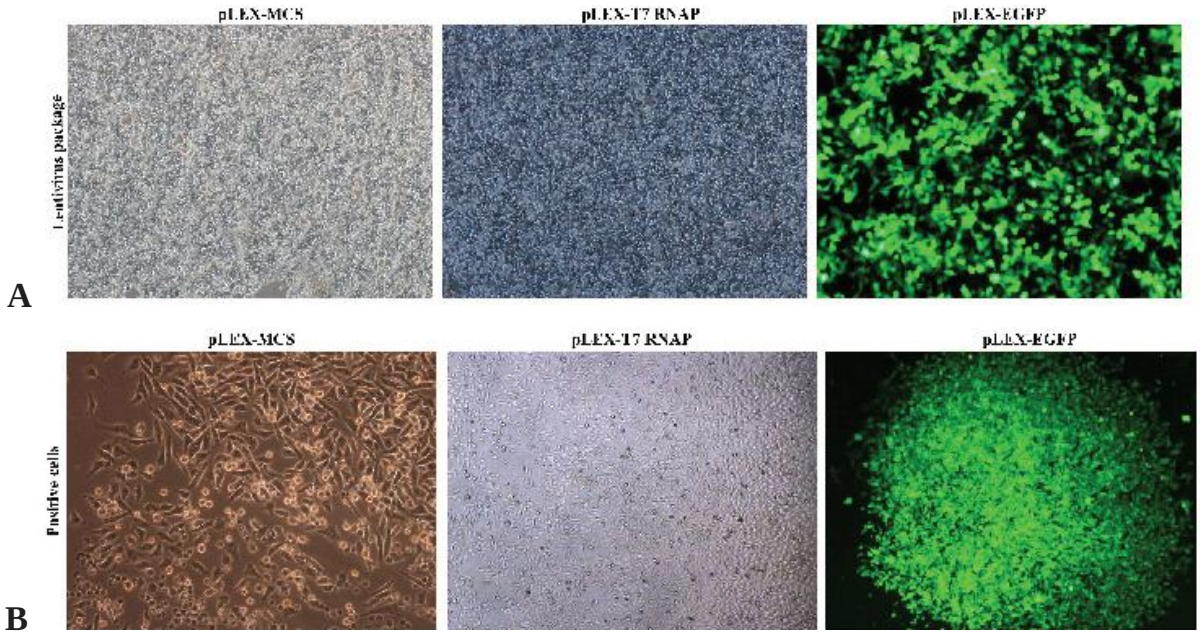


Fig. 2. Lentivirus packaged and positive cells selection. (A) T7 RNAP gene was cloned lentiviral expression vector pLEX-MCS and transfected into HEK-293T cells. At 48 h post-transfection, lentiviruses were observed under a fluorescence microscope. (B) MDBK cells were infected with lentivirus and puromycin selection was introduced to select the positive cells. After puromycin selection, the drug-resistant cells were observed under a fluorescence microscope.

*Statistical analyses*

Statistical analyses were performed using SPSS 17.0 for Window (SPSS Inc. Chicago, IL, USA). Values of *P < 0.05 and **P < 0.01 were considered to be significant and highly significant, respectively. The results are shown as the mean ± standard deviation (SD), and error bars represent the SD.

## RESULTS

*Prokaryotic expression of T7 RNAP*

To identify the protein molecular weight of T7 RNAP, T7 RNAP gene was cloned into a prokaryotic expression vector pET-32a (+) and SDS-PAGE was performed to examine the protein of T7 RNAP. As shown in Figure 1, translation of T7 RNAP generated a single protein of approximately 98-kDa on SDS-PAGE.

*Lentiviral-mediated T7 RNAP intergration*

Lentivirus was introduced to integrate T7 RNAP into MDBK cells. T7 RNAP gene was cloned into lentiviral vector pLEX-MCS and co-transfected into HEK-293T cells with its corresponding helper plasmids. At 48 h post-transfection, the lentiviral particles were harvested and concentrated (Fig. 2A). Meanwhile, pLEX-MCS and pLEX-EGFP were respectively packaged and served as negative control and positive control. Three strains of lentiviruses were respectively used to infect monolayer MDBK cells. After puromycin selection, the drug-resistant cells were observed under a fluorescence microscope and subjected to identification (Fig. 2B).

*Identification of drug-resistant cells*

Real-time PCR was introduced to identify the drug-resistant cells. As shown in Fig. 3A, the $2^{-\Delta\Delta Ct}$ value of T7 RNAP and EGFP expression levels were expected to be close to 1, suggesting that the positive rate of MDBK-T7 and MDBK-EGFP cells were almost near to 100%. Meanwhile, in order to further examine the transcription activity of T7 RNAP, pET-32a-IERS-EGFP plasmid was

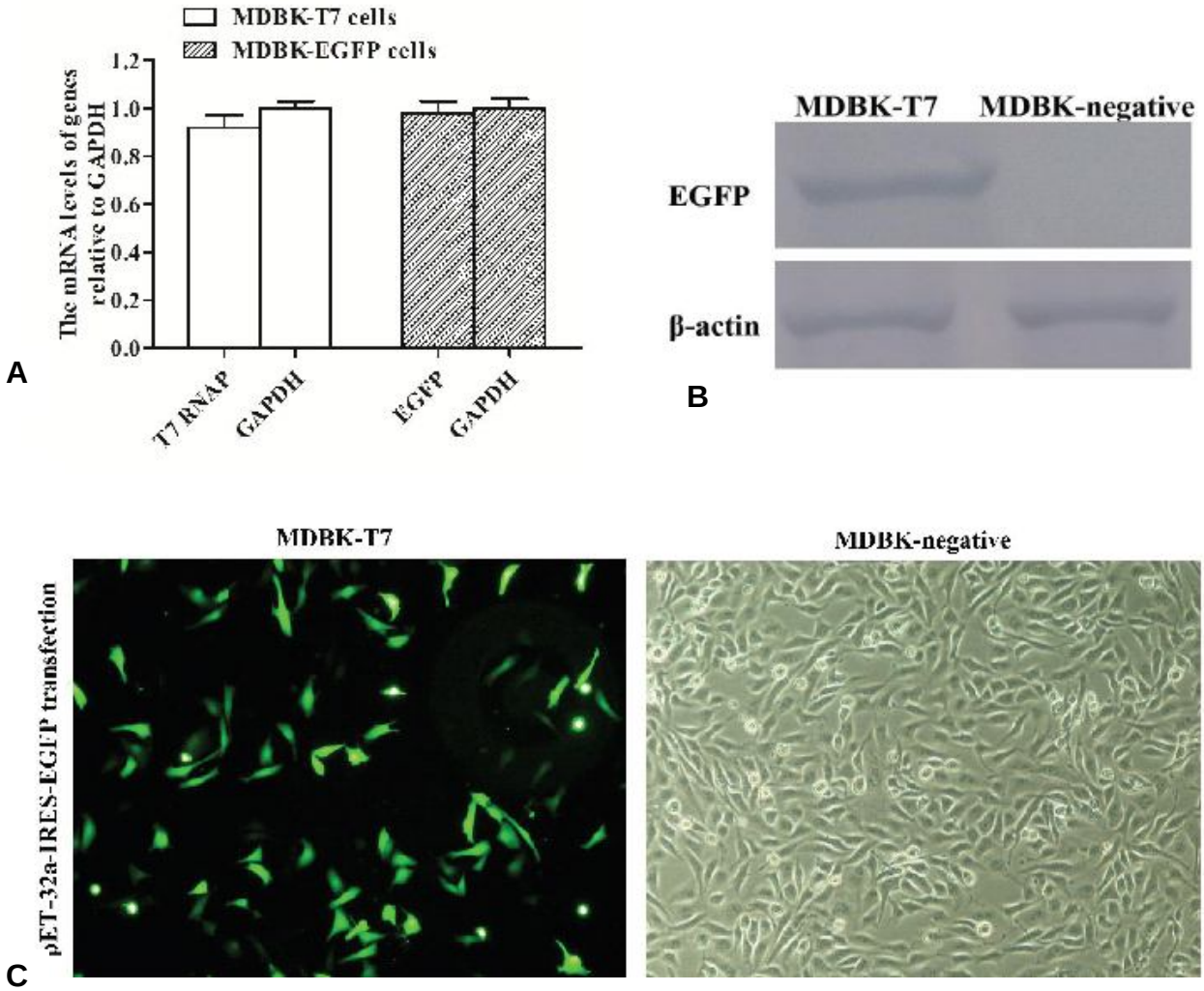


Fig. 3. Identification of drug-resistant cells. (A) Real-time PCR was performed to identify the drug-resistant cells. The $2^{-\Delta\Delta Ct}$ value of T7 RNAP and EGFP expression levels in MDBK-T7 and MDBK-EGFP cells were expected to be close to 1. Meanwhile, pET-32a-IERS-EGFP plasmid was respectively electroporated into the MDBK-T7 and MDBK-MCS cell lines. At 24 h post-transfection, fluorescence microscopy and Western blotting were used to detect the expression levels of EGFP. (B) Under a fluorescence microscope, numberous green fluorogenic cells were observed in MDBK-T7 cells. (C) The expression of EGFP protein was detected in MDBK-T7 cells, suggesting that T7 RNAP exhibited transcription activity and triggered the expression of EGFP.

constructed and electroporated into MDBK-T7 and MDBK-MCS, respectively. At 24 h post-transfection, fluorescence microscopy and Western blotting were used to detect the expression levels of EGFP. The results showed that numberous green fluorogenic cells were observed under a fluorescence microscope in MDBK-T7 cells (Fig. 3B). Meanwhile, the results of Western blot assays showed that T7 RNAP exhibited the transcription activity and triggered the expression of EGFP (Fig.3C).

*Identification of stable integration*

To examine stable expression of T7 RNAP in MDBK-T7 cells, the cells were collected at 40th passage for identifying using real-time PCR and Western blotting. As shown in Figure 4, the results of real-time PCR and Western blotting were similar

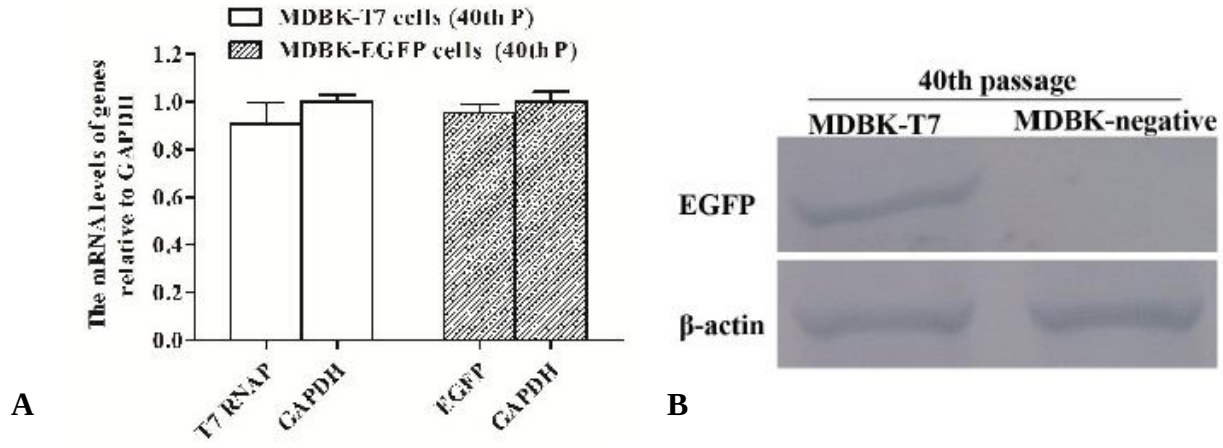


Fig. 4. T7 RNAP stably expressed in MDBK-T7 cells. The cells were collected at 40th passage for identifying using real-time PCR and Western blotting. (A) and (B) The results of real-time PCR and Western blotting were similar with the results in Fig. 3A and B, suggesting that T7 RNAP stably expressed in MDBK-T7 cells.

with the results in Figure 3, suggesting that T7 RNAP stably expressed in MDBK-T7 cells.

## DISCUSSION

Reverse genetics system is an efficient approach to investigate the function of a gene by analyzing the phenotypic effects of specific gene sequences obtained by DNA sequencing. Meanwhile, reverse genetics technology is extensively applied to investigate the structure and function of genes of RNA virus through directly operating at the DNA molecular level. In recent years, reverse genetics systems for recombinant virus rescue have proven to be of great value for viruses research and vaccines development (de Wit *et al.*, 2007). However, the low efficiency of virus rescue with transcription *in vitro* is a bottle-neck restricting the development and application of reverse genetics technology (Boyer and Haenni, 1994). Therefore, the methods for rescuing virus *in vivo* with high efficiency and no need for RNA preparation *in vitro* are urgently established.

T7 RNAP, one of single-subunit RNA polymerases, is widely employed to generate high specific activity labeled RNA probes, RNA for *in vitro* translation, biologically active mRNA, and preparative quantities of defined length RNA by the run off transcription method (Schenborn and Mierendorf, 1985). Since T7 RNAP has strict promoter and terminator specificities and makes unprocessed RNAs, the cell line stably expressing T7 RNAP can be used to assess the requirements for stability and translation of mRNA (Elroy-Stein and Moss, 1990). When CV-1 monkey kidney cells were infected with the recombinant vaccinia virus which was constructed with T7 RNAP ligating to a vaccinia virus transcriptional promoter and integrating within the vaccinia virus genome and transfected with plasmids containing the target genes, the latter were expressed at high levels (Fuerst *et al.*, 1986).

In our study, we successfully established MDBK-T7 cell line by lentivirus-mediated T7 RNAP gene integration. Meanwhile, we demonstrated T7 RNAP stably expresses in MDBK-T7 cells and has complete transcription activity.

## ACKNOWLEDGEMENTS

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## REFERENCES


BOYER, J.C. AND HAENNI, A.L., 1994. Infectious transcripts and cDNA clones of RNA viruses. *Virology*, **198:** 415-426.

BUCHHOLZ, U.J., FINKE, S. AND CONZELMANN, K.K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J. Virol.*, **73:** 251-259.

DE WIT, E., SPRONKEN, M.I., VERVAET, G., RIMMELZWAAN, G.F., OSTERHAUS, A.D. AND FOUCHIER, R.A., 2007. A reverse-genetics system for Influenza A virus using T7 RNA polymerase. *J. Gen. Virol.*, **88:** 1281-1287.

DOUDNA, J.A., GROSSHANS, C., GOODING, A. AND KUNDROT, C.E., 1993. Crystallization of ribozymes and small RNA motifs by a sparse matrix approach. *Proc. nat. Acad. Sci. USA*, **90:** 7829-7833.

ELROY-STEIN, O. AND MOSS, B., 1990. Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells. *Proc. Nat. Acad. Sci. USA*, **87:** 6743-6747.

FUERST, T.R., NILES, E.G., STUDIER, F.W. AND MOSS, B., 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. nat. Acad. Sci. USA*, **83:** 8122-8126.

GUNDERSON, S.I., CHAPMAN, K.A. AND BURGESS, R.R., 1987. Interactions of T7 RNA polymerase with T7 late promoters measured by footprinting with methidiumpropyl-EDTA-iron(II). *Biochemistry*, **26:** 1539-1546.

LIU, C., LI, W., ZHANG, X., ZHANG, N., HE, S. AND HUANG, J., 2012. The critical role of myostatin in differentiation of sheep myoblasts. *Biochem. biophys. Res. Commun.*, **422:** 381-386.

LIVAK, K.J. AND SCHMITTGEN, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, **25:** 402-408.

MCALLISTER, W.T., 1993. Structure and function of the bacteriophage T7 RNA polymerase (or, the virtues of simplicity). *Cell. mol. Biol. Res.*, **39:** 385-391.

MCALLISTER, W.T. AND RASKIN, C.A., 1993. The phage RNA polymerases are related to DNA polymerases and reverse transcriptases. *Mol. Microbiol.*, **10:** 1-6.

MILLIGAN, J.F., GROEBE, D.R., WITHERELL, G.W. AND UHLENBECK, O.C., 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucl. Acids Res.*, **15:** 8783-8798.

MOFFATT, B.A., DUNN, J.J. AND STUDIER, F.W., 1984. Nucleotide sequence of the gene for bacteriophage T7 RNA polymerase. *J. mol. Biol.*, **173:** 265-269.

SAMPSON, J.R. AND UHLENBECK, O.C., 1988. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*. *Proc. nat. Acad. Sci. USA*, **85:** 1033-1037.

SASTRY, L., JOHNSON, T., HOBSON, M.J., SMUCKER, B. AND CORNETTA, K., 2002. Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. *Gene Ther.*, **9:** 1155-1162.

SCHENBORN, E.T. AND MIERENDORF, R.C. JR., 1985. A novel transcription property of SP6 and T7 RNA polymerases: dependence on template structure. *Nucl. Acid Res.*, **13:** 6223-6236.

SCHNELL, M.J., MEBATSION, T. AND CONZELMANN, K.K., 1994. Infectious rabies viruses from cloned cDNA. *EMBO. J.*, **13:** 4195-4203.

TABOR, S. AND RICHARDSON, C.C., 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. nat. Acad. Sci. USA*, **82:** 1074-1078.

XIONG, Z.G. AND LOMMEL, S.A., 1991. Red clover necrotic mosaic virus infectious transcripts synthesized *in vitro*. *Virology*, **182:** 388-392.


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