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Construction of a Minigenome Rescue System for Measles Virus, *AIK-c* Strain

Mostafa Ghaderi [1]; Farzaneh Sabahi [1,*]; Majid Sadeghizadeh [1]; Zahra Khanlari [1]; Azam Jamaati [1]; Seyed Dawood Mousavi Nasab [1]; Nasrin Majidi Garenaz [1]

[1]Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, I.R. IRAN

[*]*Corresponding author*: Farzaneh Sabahi, Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, I.R. IRAN. Tel: +98-2182883590, E-mail: sabahi_f@modares.ac.ir

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Background: In the recent decade, the reverse genetics method has been broadly used for rescue of negative-stranded RNA viruses from cDNA or viral minigenomes. This technique has been applied to study different steps in virus replication and virus-host interactions. Reverse genetics could also be implemented for design of new vaccines. The T7 RNA polymerase activity as well as virus (nucleocapsid protein) N, (phosphoprotein) P and (Large) L proteins are necessary to rescue the virus or viral minigenome. Measles virus is a negative-stranded non-segmented RNA virus. There are useful vaccine strains to prevent measles disease.

Objectives: Here, we describe the construction of a new helper cell line for rescue of measles virus minigenome. The helper cell line stably expresses T7 RNA polymerase as well as measles virus N and P proteins by tricistronic mRNA.

Materials and Methods: For rescue of measles virus minigenome a stable helper cell line by using tricistronic expression vector was developed which expressed T7 RNA polymerase as well as measles virus N and P proteins. To construct the tricistronic expression vector, T7 RNA polymerase gene was cloned after cytomegalovirus (CMV) promoter and measles virus N and P proteins were under control of IRES (internal ribosome entry site) sequences.

Results: Our results indicated that measles virus minigenome could be rescued in this constructed helper cell line.

Conclusions: Through this system, the measles virus minigenome was rescued. Further studies are necessary to improve the rescue efficiency. This may be possible by replacing the CMV promoter with the T7 promoter.

*Keywords:*Measles Virus; Reverse Genetics; Minigenome.

1. Background

Taxonomically, measles virus belongs to the genus *Morbilivirus* of the family *Paramyxoviridae*. Measles virus contains a negative-stranded non-segmented RNA as a genome (1). The genome of measles virus strongly binds to nucleocapsid (N) proteins, called ribonucleoprotein (RNP). RNP is associated with viral polymerase complex, consisting of the phospho- (P) and large (L) proteins (1, 2).

Measles virus genomic RNA contains six transcription units that encode the nucleocapsid protein (N), phosphoprotein, V and C protein (P/V/C), matrix protein (M), fusion protein (F), hemagglutinin protein (H) and large protein (L). The genomic termini of measles virus contain non-coding regions (NCR), which are necessary for genome replication, genome packaging and mRNA synthesis. The NCR sequences located on the 3'-end and 5'-end of the genome are called leader and trailer sequences, respectively. The leader sequence has a role in mRNA production and antigenome synthesis. The trailer sequence after production of an antigenome, plays a role in genomic RNA synthesis (3).

Reverse genetics is an efficient method to study different aspects of RNA viruses replication and interaction with the host (4). By reverse genetics, RNA viruses could be rescued from cDNA (5-10). Reverse genetics has also been used to design new vaccines and to produce oncolytic viruses (11-16). Traditionally, construction of a minigenome instead of the full genome cDNA has been applied in many studies to appraise the efficiency of the virus rescue system and virus replication (17-21).

The minigenome constructs consist of non-coding termini (leader and trailer) of the virus genome, which are located on both sides of a reporter gene such as e*GFP* or *CAT* (22). To transcribe the viral minigenome RNA with accuracy, 3'-and 5'-ends, T7 promoter, hepatitis delta virus ribozyme (R.D) and T7 terminator sequences were used. The T7 promoter starts RNA transcription from the first nucleotide of the 3'-end minigenome. The hepatitis delta virus ribozyme cuts the last nucleotide of the 5'-end of the minigenome from itself (5).

Here, we describe the design of a successful minigenome system for the *AIK-c* strain of measles virus.

Implication for health policy/practice/research/medical education:
This project evaluates the effectiveness of reverse genetics system for rescue of measles virus by tricistronic expression vector in the helper cell line.

Figure 1. Schematic Representation of e*GFP* Expression as a Reporter Gene Included in Measles Virus Minigenome. After transfection of the minigenome into HEK-T7N1P5, negative sense RNA (vRNA) is transcribed by T7 RNA Polymerase. Then, the minigenome vRNA establishes vRNP that could be detected by viral polymerase complex (P-L). Finally, the viral polymerase complex starts to transcribe e*GFP* mRNA by using non-coding region of vRNA (leader).

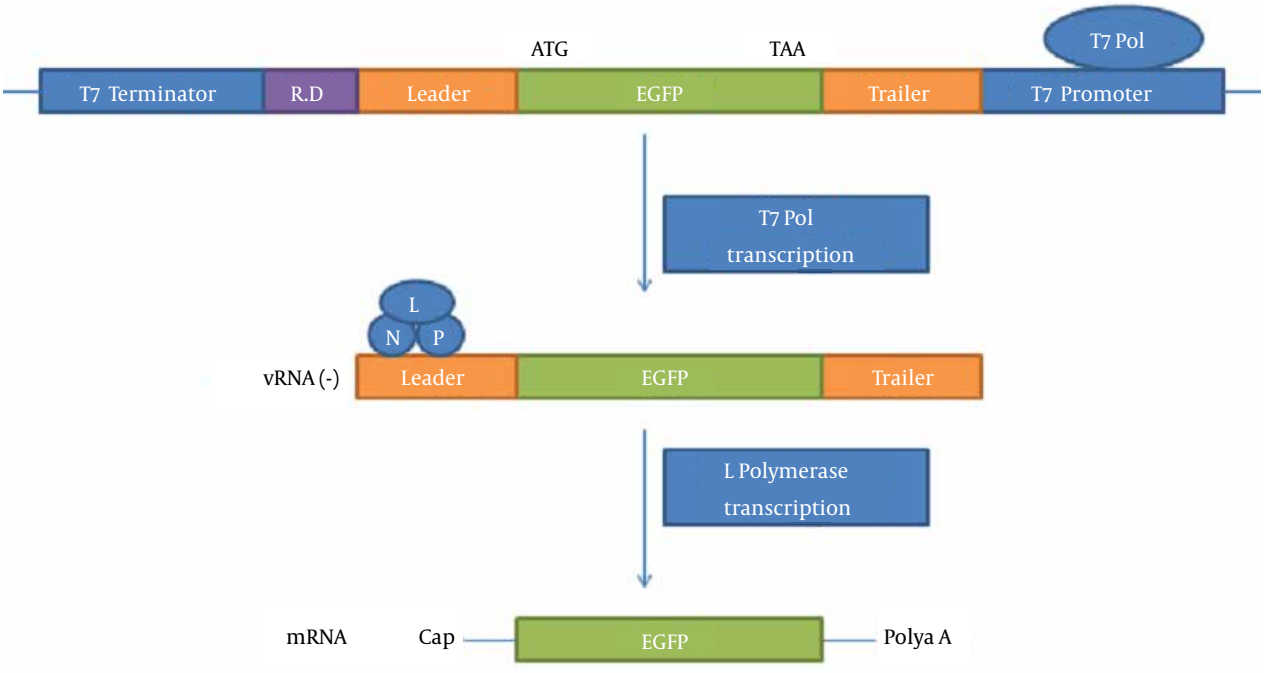

Figure 2. Structure of Tricistronic Expression Plasmid Used for Expression of T7 RNA Polymerase, and Measles Virus N and P Proteins

In this study, to rescue the minigenome from cDNA, a helper cell line was constructed which stably expressed measles virus N and P proteins as well as T7 RNA polymerase (Figure 1). A recombinant tricistronic expression vector was constructed in which T7 RNA polymerase as well as measles virus N and P genes were inserted. Previous studies have used tricistronic expression systems to simultaneously produce three proteins (23, 24). The T7 RNA polymerase in this tricistronic vector was cloned into multiple cloning sites (MCS) of the plasmid downstream to the CMV promoter, and measles virus N and P genes were separately inserted after IRES sequences (Figure 2).

2. Objectives

The objective of the study was to construct a rescue system for the vaccine strain of the measles virus.

3. Materials and Methods

3.1. Cell Line

HEK-293 cells were grown in high glucose Dulbecco's Modified Eagle's Minimal medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), the antibiotics penicillin (100 U.ml $^{-1}$) and streptomycin (0.1 mg.ml $^{-1}$).

3.2. Construction of Helper Plasmids

The full genome cDNA of *AIK-c* strain of measles virus along with accessory elements consisting of T7 promoter, hammer-head, ribozyme delta and T7 terminator was synthesized. The cDNA of measles virus was cloned into the pUC57 plasmid (Genscript Company, Hong Kong).

3.2.1. PCR Amplification

PCR amplification of measles virus N and P genes was carried out using specific primers and the Platinum-pfx

Table 1. Primers for Amplification of Measles Virus N and P Genes as Well as T7 RNA Polymerase Gene

Gene	Primer Sequence
T7, Forward primer	5'CTAGCTAGCCCACCATGAACACGATTAACATCGCTAAGAACGAC3'
T7, Reverse primer	5'CCGCTCGAGTTACGCGAACGCGAAGTCCGACTC3'
N, Forward primer	5'AACCATGGCCACACTTTTAAGGAGCTTAGCA3'
N, Reverse primer	5'ATAAGAATGCGGCCGCCTAGTCTAGAAGATTTCTG3'
P, Forward primer	5'AACCATGGCAGAAGAGCAGGCACGC3'
P, Reverse primer	5'ATAAGAATGCGGCCGCCTACTTCATTATTATCTTCATCAGCA3'

kit (Invitrogen). All primers for PCR amplification are listed in Table 1.In these reactions, we used the full genome cDNA of measles virus as a template.

The bacteriophage T7 RNA polymerase gene was amplified using BL-21 genome lysate as a template. Forward and reverse primers for T7 RNA polymerase amplification are listed in Table 1.

3.2.2. Cloning Steps

The PCR products for each gene were purified using the Qiagen purification kit. The T7 RNA polymerase gene was digested with NheI and XhoI. Then, after purification with a gel extraction kit (Qiagen), the DNA fragment of T7 RNA polymerase (in length of 2600Kb) was cloned into the pIRES2-EGFP plasmid (Clontech) and the recombinant vector was called pIRES-T7.

The cloning process for N and P genes was similar. The PCR products for each gene was purified and digested with NotI. The NotI site was at the 5'-end of reverse primers, but there was no restriction enzyme site in the forward primers. The forward primers contained akozak consensus ribosome binding site (AACC) and ATG initiation codon. The pIRES2-EGFP plasmid was digested in a step by step process. First, pIRES2-EGFP was digested with BstxI and then, the digestion product of the plasmid was treated with Klenow to produce a blunt end. Finally, pIRES2-EGFP was digested with NotI. The DNA fragments of N and P genes were cloned into the pIRES2-EGFP and recombinant vectors were named pIRES-N and pIRES-P, respectively.

To produce a tricistronic expression vector containing the T7 RNA polymerase and N and P genes, T7 RNA polymerase gene after digestion with NheI and XhoI was cloned into pIRES-P and the new recombinant vector was named pT7P5. Next, the N gene along with the IRES sequences of pIRES-N, after digestion with XhoI and HpaI, was cloned into pT7P5 at the site of XhoI and SmaI. The final tricistronic construct was named pT7N1P5 (Figure 2).

To analyze the functionality of the T7 RNA polymerase, a plasmid was constructed expressing the e*GFP* reporter gene under control of the T7 promoter and IRES sequences. This plasmid was called pFT7A. The e*GFP*, IRES and polyadenylation signal in pFT7A was produced by PCR amplification from pIRES2-EGFP as a template.

To generate pAIKc-mini as a measles virus minigenome, the essential sequences for rescue of the reporter gene consisting of the T7 promoter, measles virus leader and trailer elements, e*GFP* gene, ribozyme delta and T7 terminator were designed by the Bioedit software. In this plasmid, the sequence of e*GFP* was located in the negative sense direction. After designing, the whole sequence was synthesized (Genscript Company).

3.3. Transfection

To generate a stable cell line expressing the T7 RNA polymerase as well as measles virus N and P proteins, the pT7N1P5 was linearized by digestion with SspI. Then, the linearized plasmid was purified for transfection into HEK cells. Cells were grown in six-well tissue culture plates to 80% confluence and transfected with 5 μg plasmids via Lipofectamine 2000 (Invitrogen). Cells were incubated for 16 hours at 37 ℃, and then washed once with phosphate buffer saline (PBS) and maintained in DMEM containing 10% FBS. After two days, the cells of six-well plates were split into 25 cm^2 flasks and selected by 1 $mg.mL^{-1}$ G418. The medium containing 1 mgmL G418 was changed every second day. After 12 days, 20 colonies were cultured into 24 well tissue culture plates. After expansion of cells to six-well plates, they were prepared for analysis of each gene. The T7 RNA polymerase activity was assayed by transfection of G418 resistance cells of each colony with the pFT7A plasmid. The expression of measles virus N and P proteins by the tricistronic expression vector was evaluated by western blotting using the total lysate of the confluent 25 cm^2 cell culture flask. For protein detection, we used a monoclonal antibody to measles virus N and P proteins. In each step of analysis, including T7 RNA polymerase activity assay and western blotting for detection of N and P proteins, we used HEK-293 cells as a negative control. In order to assess whether recombinant HEK-293 helper cell line (HEK-T7N1P5) could rescue the measles virus minigenome, co-transfection of pAIKc-mini with

pEMC-La (a gift from Hussein Y.Naim), containing the measles virus L polymerase was, carried out.

4. Results

4.1. Construction of Tricistronic Expression Vector

In order to gain a higher expression of T7 RNA polymerase, the cloning site of the gene was designed in MCS of the pIRES2-EGFP plasmid, under control of the CMV promoter. The bacteria genome, containing the bacteriophage T7 RNA polymerase, was isolated from BL-21, and sequences of the T7 RNA polymerase gene was amplified by PCR (Figure 3).

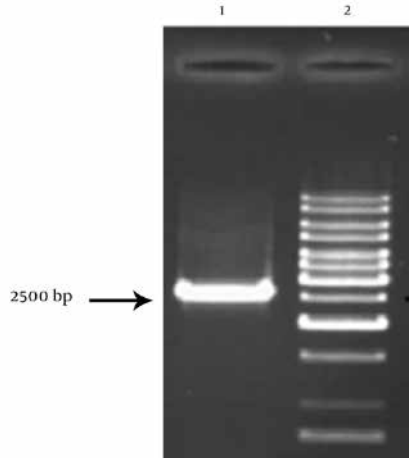

Figure 3. PCR Amplification of T7 RNA Polymerase Gene. 2600 bp Fragment in lane 1 shows amplification of the T7 RNA polymerase gene and lane 2 is related to the 1 kb DNA ladder (Fermentas, Germany).

Then, the PCR product of T7 RNA polymerase was inserted into MCS of the pIRES2-EGFP plasmid (Figure 4).

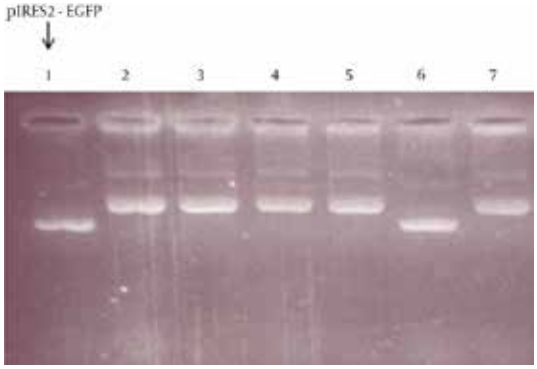

Figure 4. Lane 1 shows the pIRES2-EGFP Plasmid as a control and bands in lanes 2-5 and 7 are miniprep products of the pIRES-T7 vector; lane 6 is the miniprep product of non-ligated T7 RNA polymerase gene in the pIRES2-EGFP plasmid.

For simultaneous expression of the three genes including T7 RNA polymerase and measles virus N and P genes; firstly, the last two genes after PCR amplification, were cloned into the pIRES2-EGFP plasmid directly after IRES sequences resulting in the pIRES-N and pIRES-P vectors (Figure 5 and 6); then, subcloning processes to fuse the three genes for production of pT7P5 and pT7N1P5 vectors were carried out (Figure 7). Digestion with the SsPI enzyme to confirm the size of pT7N1P5 (10200 bp) was done (Figure 8). The correctness of positioning of the resulting tricistronic pT7N1P5 plasmid was confirmed by DNA sequencing.

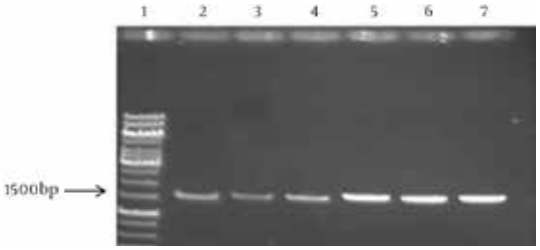

Figure 5. Lane 1 is related to the 1 Kb ladder (Fermentas, Germany) Lanes 2-4 and 5-7 are related to PCR products of measles virus N and P genes, respectively.

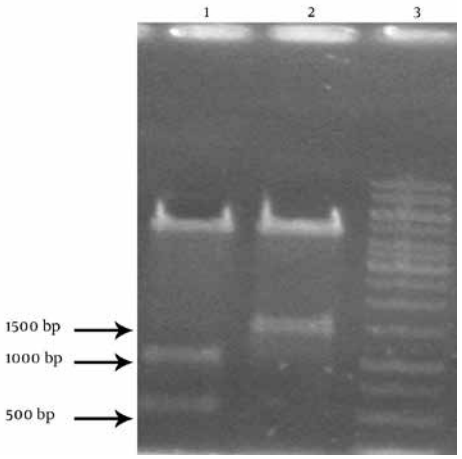

Figure 6. Lanes 1 and 2 digested PIRES_P and PIRES-N for confirmation of measl virus P and N genes, cloned PIRES-EGFP, respectively. Lane 3 1 Kb ladder.

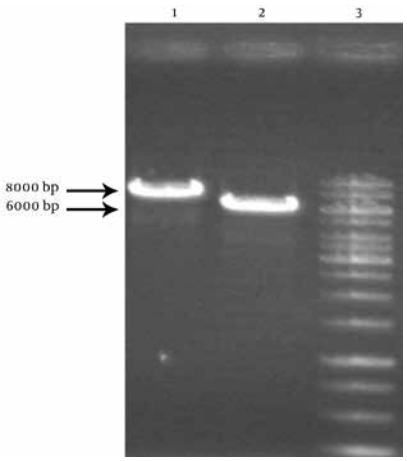

Figure 7. Confirmation of T7 RNA Polymerase Subcloning into pIRES-P. Lane 1 is positive for the pT7P5 vector (8700 bp) after digestion with the NheI enzyme, Lane 2 is related to the pIRES-P (as a control plasmid) and Lane 3 shows the 1 Kb ladder.

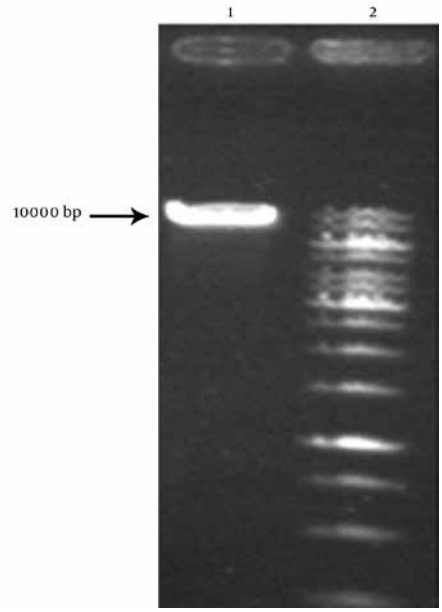

Figure 8. Subcloning Results of Measles Virus N Gene Along With IRES Sequence into pT7P5. lane 1 is positive for pT7N1P5 (10200bp) and lane 2 represents the 1Kb ladder.

4.2. Construction of a Stable Cell Line

For stable expression of genes by pT7NP5 as a tricistronic vector, the linearized plasmid was transfected into the HEK-293 cell line. In order to evaluate stable construction of the cell line by integration of plasmid into cell chromosomes, the linearized pIRES2-EGFP plasmid was used with the same digestion enzyme used for pT7N1P5 digestion. After third and tenth passage of the recombinant HEK-T7N1P5, the T7 RNA polymerase activity, indicated by use of the pFT7A plasmid from the four colonies (assigned as a, b, c, and d), was positive (Figure 9). pFT7A plasmid contains the e*GFP* gene under control of the T7 promoter and IRES sequences. The e*GFP* gene included in the pFT7A plasmid could be expressed by T7 RNA polymerase activity in recombinant HEK-T7N1P5. The e*GFP* fluorescence was also verified in transfected HEK-293 cells by pIRES2-EGFP at third and tenth passage.

There was successful expression of measles virus N and P proteins in stably HEK-T7N1P5 cells. Although there were positive results for expression of N and P proteins from 'a', 'b' and 'd' colonies, there was no detection of these proteins expression from the 'c' colony. Among these colonies, it seems that colony 'a' was the best selected colony (Figure 10).

4.3. Assay of Measles Virus Minigenome in Recombinant HEK-T7N1P5

According to the results of gene expression, colony 'a' of HEK-T7N1P5 was used for rescue of the minigenome. To visualize e*GFP* fluorescence as a reporter gene by the minigenome, not only the activity of T7 RNA polymerase is necessary to transcribe the negative sense of the minigenome RNA, but also the measles virus L polymerase is important to synthesize positive sense of the minigenome RNA and e*GFP* mRNA.

Therefore, co-transfection of the minigenome (pAIKc-mini) along with the pEMC-La plasmid containing the measles virus L polymerase was performed. At 72 hours post-transfection, e*GFP* fluorescence was observed in a few cells (Figure 11).

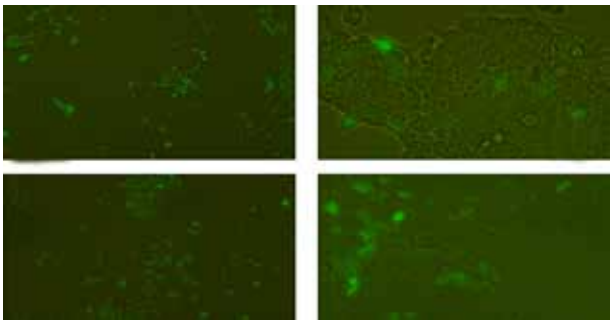

Figure 9. Assay of T7 RNA Polymerase Activity by Using the pFT7A Plasmid. e*GFP* fluorescence from the four colonies was positive.

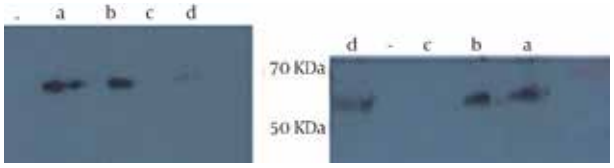

Figure 10. Western Blotting Assay for Examination of Measles Virus N and P Expression, the (-) is Related to Control Negative from HEK-293 Cell Lysate and Lanes 'a', 'b , 'c' and 'd' Show Measles Virus P (70kDa) and N (60kDa) Bands in Left and Right Figure, Respectively.

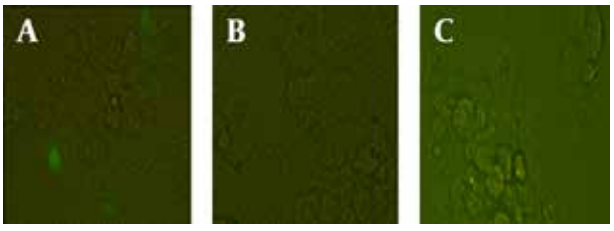

Figure 11. e*GFP* Expression Assay in Recombinant HEK-T7N1P5 by Transfecting With Measles Virus Minigenome. A) shows the negative control of HEK-T7N1P5 which was not transfected with any plasmid; B) indicates transfection of pAIKc-mini alone into HEK-T7N1P5; and C) represents the e*GFP* fluorescence after co-transfection of pAIKc-mini and pEMC-La into HEK-T7N1P5.

5. Discussion

In reverse genetics for the order *Mononegaviral*, T7 RNA polymerase activity as well as virus nucleoprotein and phospho-protein are necessary to rescue the virus or minigenome (5). For this reason, we designed a tricistronic

expression vector, in which all of the genes were under control of one CMV promoter. The T7 RNA polymerase gene was placed at the MCS and next, measles virus N and P proteins were inserted directly after the IRES sequence. This strategy gives rise to transfection of one recombinant vector instead of three vectors for each gene.

In step one, stable expression of e*GFP* in transfected HEK-293 with linearized pIRES2-EGFP plasmid indicated that the linearized plasmid consisting of pIRES2-EGFP and pT7N1P5 had been successfully integrated into cell chromosomes. In the next step, stable expression of eGFP gene in the pFT7A, which was expressed by the T7 RNA polymerase and translated with the IRES sequences, showed that integration of pT7N1P5 was successful and there was enzymatic activity of T7 RNA polymerase in recombinant HEK-T7N1P5 cells. The results of western blotting for expression of measles virus N and P proteins also confirmed stable expression of these genes by pT7N1P5 in recombinant HEK-T7N1P5 cells.

Finally, although there was e*GFP* fluorescence via the e*GFP* gene under control of measles virus minigenome, the number of recombinant HEK-T7N1P5, which showed e*GFP* expression was low. It can be speculated that the reason for low e*GFP* expression by the minigenome was using the CMV promoter for transcription of tricistronic mRNA from pT7N1P5. The transcription of mRNA by the CMV promoter is suppressed in some cells after many passages (25, 26). Use of the T7 promoter and IRES sequences instead of the CMV promoter in tricistronic expression vector could be effective in increasing the rate and stability of gene expression. However, *eGFP* expression by our minigenome indicated that pT7N1P5 could be used to rescue the measles virus minigenome after co-transfection of pT7N1P5, measles virus L gene and minigenome into HEK-293 cells. On the other hand, successful rescue the measles virus minigenome by this system could be effective for rescue the measles virus full genome.

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Author's Contribution

Mostafa Ghaderi performed this project as part of his PhD, Farzaneh Sabahi designed and managed the project, and all the other authors were involved in the experiments, technical support and writing of the manuscript.

Financial Disclosure

There is no conflict of interest.

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