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CONSTRUCTION OF IRES-INCORPORATED pMG36e LACTOCOCCAL VECTOR AND ITS SEGREGATIONAL INSTABILITY IN *Escherichia coli*

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SUMMARY

The increasing demand of *Lactococcus lactis* (*L. lactis*) beyond application in food industry raises the need to develop lactococcal bicistronic vector with target gene expression in a eukaryotic system for vaccination purposes. Despite the importance of functional studies of the expression vector in eukaryotic system, the stable and efficient host for vector propagation should be tackled. Therefore, the objectives of this study were to construct a pMG36e lactococcal vector containing modified eukaryotic expression cassette, with internal ribosome entry site (IRES) inserted between CMV promoter and Poly A signal to allow transcription and translation of two proteins in a single cassette and, study its stability in *Escherichia coli* (*E. coli*) TOP 10 as a propagation host. IRES sequence was PCR-amplified from pRetroX-IRES-ZsGreen1 vector, digested with *Eco*RV and *Xho*I, and ligated into eukaryotic expression cassette from pcDNA 3.1 HisA plasmid/vector. Constructed vector was transformed into *E. coli* TOP 10 and the orientation of IRES fragment in the modified eukaryotic expression cassette was confirmed by DNA sequencing. The modified eukaryotic expression cassette was PCR-amplified from pcDNA3.1HisA/IRES before sub-cloning into lactococcal vector pMG36e and transformed into *E. coli* TOP 10. pMG36e harbouring modified eukaryotic expression cassette was successfully constructed and transformed into *E. coli* TOP 10. However, the segregational instability of constructed vector in *E. coli* Top10 host was detected, which may have been caused by production of single-stranded intermediates and high-molecular weight DNA due to rolling-cycle replication of the pMG36e vector.

Keywords: E. coli Top10, eukaryotic expression cassette, IRES, Lactococcus lactis, pMG36e

INTRODUCTION

Lactococcus is a Gram-positive cocci from the lactic acid bacteria group with novel properties for application in the preservation and flavor production of food. Due to their contribution in the food industry, *Lactococcus* has been designated as generally recognized as safe (GRAS) organism (Pontes *et al.*, 2011). *Lactococcus* has become a suitable candidate for both production of live delivery of vaccines and therapeutics including in non-dairy applications by synthesizing lactococcal plasmid with heterologous proteins through plasmid-borne gene expression systems (Glenting *et al*., 2002). The non-pathogenic characteristic coupled with the ability to express heterologous proteins rendered *Lactococcus* the ideal choice for safe production of antigens and medically important proteins (De Ruyter *et al.*, 1996). Traditionally, co-transfection of two different vectors; one carrying reporter protein and another carrying protein of interest are needed for most delivery of vectors into mammalian cells. The transfection of the protein of interest which is presumed to be successful with the successful transfection of the reporter protein is unreliable and unpredictable (Hunt, 2005). Within the past decades, expression of multigenes within a single plasmid has been successfully performed by inserting internal ribosome entry sites(IRES). IRES is a translational enhancer naturally present in a series of mRNAs, mediating internal initiation of translation when present between the genes of interest (Renaud-Gabardos *et al.,* 2015). IRES elements are able to internally initiate the translation of RNA and facilitate the expression of two or more proteins. When modified into expression vectors, these bicistronic expression vectors are able to translate the first gene in a cap-dependent manner and the second in IRES dependent manner (Vagner *et al.*, 2001; Mutalib *et al*., 2014). This technique offers several benefits which mostly occur in prokaryotes and need a strict co-expression of transgenes in vitro, cell culture and transgenic animals and plants (Hennecke *et al*., 2001). Meanwhile, Wang *et al*. (2005) reported the good genetic stability of expression plasmid vector pMG36e in *Escherichia coli (E. coli)* JM109 and *Lactococcus lactis (L. lactis)* MG1363/36e with 120 and 100 generations representatively. Therefore, the objectives of this study were to construct IRES incorporated-lactococcal vector pMG36e containing eukaryotic expression cassette denoted as pMG: IRES/Hyg and study its stability in *E. coli* Top 10 hosts.

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MATERIALS AND METHODS

Bacteria Strains Culture Conditions and Plasmids

Escherichia coli TOP 10 was used as a propagation host for constructed vectors and was grown at 37˚C in Luria Bertani (LB) agar or broth with agitation at 200 rpm. *Lactococcus lactis* NZ9000 was used as cloning host for pMG36e and was grown at 30˚C in M17 broth

supplemented with 0.5% (w/v) glucose without aeration. Ampicillin (100 µg/mL) and erythromycin (5 µg/mL) were added for the recombinant *E. coli Top10* and *L. lactis* cultures respectively for selection purposes. The IRES fragment was isolated from pRetroX-IRES-ZsGreen1 vector (Clontech, USA) and cloned into eukaryotic expression cassette from pcDNA 3.1 HisA (Invitrogen, USA). The eukaryotic expression cassette containing IRES sequence was isolated and cloned into lactococcal vector pMG36e (obtained from Prof. C.J. Leenhouts from University of Gronigen, Holland).

Plasmid Construction

pRetroX-IRES-ZsGreen1 and pcDNA 3.1 HisA plasmids were extracted from *E. coli Top10* culture using alkaline lysis method with minor modifications (Sambrook *et al.*, 1989). Subsequently, pMG36e plasmid was extracted from *L. lactis* using EasyPure Plasmid MiniPrep Kit (TransGene, Beijing). IRES sequence initially was amplified from pRetroX-IRES-ZsGreen1 polymerase chain reaction (PCR) using specific primer set in Table 1.

The PCR product was purified using GeneJET PCR Purification Kit (Thermo Scientific, USA) and were subjected to restriction enzyme (RE) digestion of specific RE as shown in Table 2. The RE digestion mixture was incubated at 37˚C for three hours, deactivated at 65˚C for 20 min before gel purification. The purified fragments of IRES was then ligated into pcDNA 3.1 HisA through overnight incubation at 4˚C and deactivated at 65˚C for 10 min. The ligation product; recombinant plasmid denoted as pcDNA3.1HisA/IRES were then transformed into *E. coli Top10* using heat shock method and the transformation mixture was spread on LB plates supplemented with 100 µg/mL ampicillin. The white colonies grown on the plates after overnight incubation were then subjected to plasmid extraction using alkaline lysis method before subjected to double digestion with *Sac*I and *Xho*I to verify the correct orientation of the inserted IRES. Once verified, pcDNA3.1HisA/IRES and pMG36e were double digested with *Sal*I and *Afe*I (Table 2) to obtain CMV-IRES-PolyA fragments and a cut pMG36e plasmid. Both CMV-IRES-PolyA fragments and the cut of pMG36e plasmid were ligated using DNA ligation mixture containing 1x T4 ligase buffer, 50% PEG 4000 solution, vector: insert ratio of 1:3, 2 units of T4 DNA ligase (Thermo Scientific, USA) and distilled water. The ligation mixture was incubated overnight at 4˚C and deactivated at 65˚C for 10 min. The ligated DNA products; denoted as pMG: IRES/Hyg was transformed into *E. coli Top10* using electroporation heat shock method and check for its stability. The colony of the transformants were observed and verified via DNA sequencing.

Table 1. Primer design for PCR amplification of IRES and CMV-IRES-PolyA fragment

Primer name	Sequence	Annealing temperature (Ta)	RE site	Expected size of the amplicons (bp)
F_IRES	5'- CCG GAT ATC ATT AAT GTG CAC GCC CCT CTC CCT CCC-3'	68.3˚C	*Eco*RV	600[a]
R_IRES	5'-CCG CTC GAG CCC GGG TGA TCA TAT TAT CAT CGT GTT TTT-3'	66.4˚C	*Xho*I	
F_pcmv	5'-GCG TCG ACA GGC AGC TGA CTT CAC ATT GTT GAG ATC AGC TGC CTC GCG TTG ACA T-3'	72.1˚C	*Sal*I	1750[b]
R_PolyA	5'-GCA GCG CTA ACC ATA GAG CCC ACC GCA TCC CCA GC-3'	72.0˚C	*Afe*I	

[a] IRES fragment (Set of F_IRES and R_IRES primers)
[b] CMV-IRES-PolyA fragments (Set of F_pcmv and R_PolyA primers)

Table 2. The role and characteristics of restriction enzymes used

Restriction Enzymes	Buffer	Role
*Eco*RV and *Xho*I	2X Tango	Cloning of IRES fragment into pcDNA 3.1 HisA
*Sac*I and *Xho*I	2X Tango	Verification of IRES orientation
*Sal*I and *Afe*I	1X Buffer O	Cloning of CMV-IRES-PolyA fragment into pMG36e
*Sal*I	1X Buffer O	Verification of the identity of pMG36e

RESULTS

pcDNA3.1 HisA/IRES Construction

IRES fragment was PCR-amplified from pRetroX-IRES-ZsGreen1 while the eukaryotic expression cassette containing CMV enhancer, CMV promoter and Poly A signal was obtained from pcDNA 3.1 HisA. The IRES sequence flanked by *Eco*RV and *Xho*I was designed to be inserted between CMV promoter and Poly A signals to construct the CMV-IRES-PolyA. PCR amplification of IRES sequence successfully generated a single amplicon of approximately 600 bp. Figure 1 (A and B) show the amplified IRES fragment and extracted pcDNA 3.1 HisA, with their respective RE-digested and gel purified product. The sizes of digested and undigested IRES fragment differ by 23 bp, hence the differences in size could not be determined on agarose gel. The result for the digestion of pcDNA 3.1 HisA showed an approximately 5500 bp band, denoting complete digestion of the vector. After ligation process, the ligation products were then transformed into *E. coli* TOP 10 and were cultured on LB agar supplemented with ampicillin. Colony PCR was employed to screen for putative positive transformants harboring pcDNA/IRES vector. F_pcmv which binds to the vector and R_IRES which binds to the IRES fragment was used as primers resulted on 2 out of 15 (C5 and C14) colonies screened yield an approximately 1500 bp PCR band, suggesting that these positive transformants may harbor the pcDNA/IRES vector (Figure 2). Double digestion of the pcDNA 3.1 HisA/IRES using *Eco*RV and *Xho*I restriction enzymes yielded two fragments; the ~5500 bp pcDNA 3.1 HisA vector and the ~600 bp IRES fragment indicating that those plasmids carried IRES fragment (Figure 3A). Restriction enzymes *Sac*I and *Xho*I were successfully used to verify the orientation of the IRES fragment by fragmenting it into two bands with ~5300 bp and ~800 bp (Figure 3B). PCR was carried out using another two sets of primers; the first set which consists of F_pcmv and R_IRES that will amplify a product with an expected size of 1500 bp while the second set which consists of F_IRES and R_IRES that will amplify the 600 bp IRES fragment before subjected for sequencing (Figure 4). The sequencing result confirmed the insertion of IRES fragment into the pcDNA 3.1 HisA vector in correct orientation and location with alignment score is 1143 with a similarity of 0.9982578. Figure 5 summarise the pcDNA 3.1 HisA/IRES construction while Figure 6 shows map and sequence position of pcDNA3.1HisA/IRES vector created using Snapgene Viewer Version 2.7(GSL Biotech LLC; http://www.snapgene.com/)

pMG: IRES/Hyg Construction

The cloning strategy of CMV-IRES-PolyA fragment into pMG36e was similar to cloning strategy of IRES fragment into pcDNA 3.1 HisA. The CMV-IRES-PolyA fragment flanked by *Afe*I and *Sal*I RE site was isolated from pcDNA3.1HisA/IRES vector constructed previously. The CMV-IRES-PolyA fragment (~1750 bp) and pMG36e vector (~3000 bp) were successfully digested using *Afe*I and *Sal*I prior to ligation and transformation

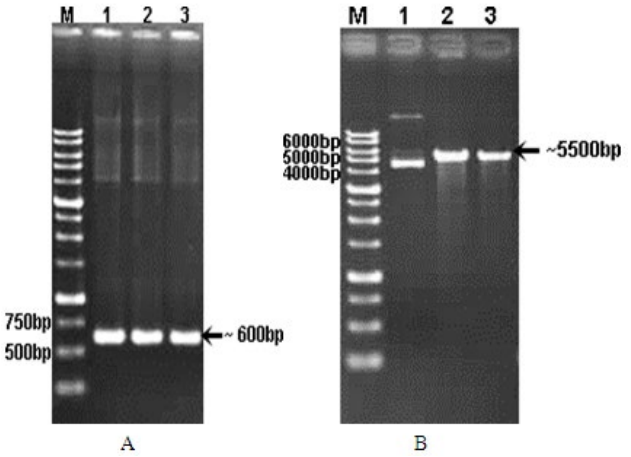

Figure 1. RE digestion and gel purification of IRES insert and pcDNA 3.1 HisA vector. A) M: GeneDirex 1kb DNA ladder RTU. Lane 1: Undigested IRES PCR product. Lane 2: Digested IRES. Lane 3: Digested IRES after gel purification. B) M: GeneDirex 1kb DNA ladder. Lane 1: Undigested pcDNA 3.1 HisA. Lane 2: Digested pcDNA 3.1 HisA. Lane 3: Digested pcDNA 3.1 HisA after gel purification

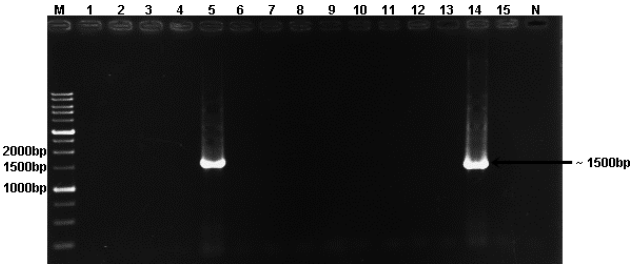

Figure 2. Colony PCR screening for colonies formed from antibiotic selection LB agar supplemented with ampicillin. M: GeneDirex 1kb DNA ladder RTU. Lanes 1 to 15: PCR product amplification from Colonies 1 to 15 respectively. N: negative control without DNA template

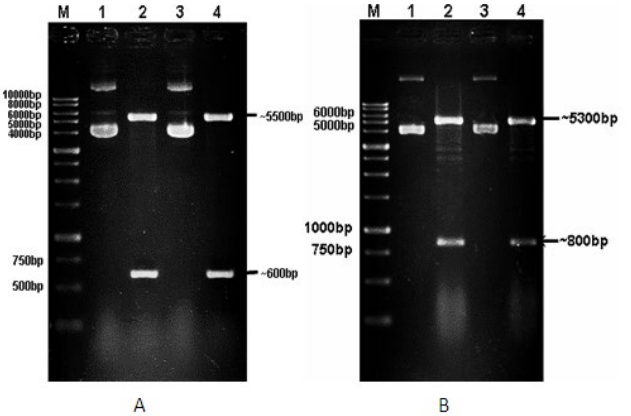

Figure 3. Verification of positive transformant harboring pcDNA3.1HisA/IRES vector (A) and orientation of IRES fragment (B) using double digestion. (A and B) M: GeneDirex 1kb DNA ladder RTU. Lane 1: extracted plasmid from C5. Lane 2: digested C5 plasmid. Lane 3: extracted plasmid from C14. Lane 4: digested C14 plasmid

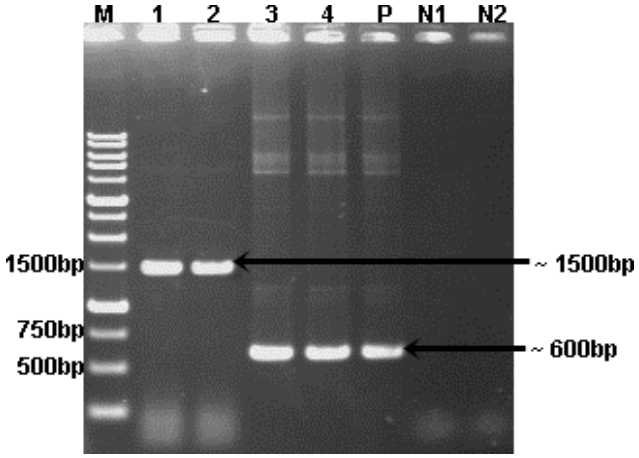

Figure 4 (on the left). Verification of positive transformant harbouring pcDNA3.1HisA/IRES using PCR. M: GeneDirex 1kb DNA ladder RTU. Lanes 1 and 2: PCR product amplified from C5 and C14 plasmid using F_pcmv and R_PolyA. Lane 3: PCR product amplified from C5 and C14 plasmid using F_IRES and R_IRES. P: positive control, IRES sequence amplified from pRetroX-IRES-ZsGreen using F_IRES and R_IRES. N1: negative control, pcDNA 3.1 HisA amplified with F_pcmv and R_PolyA. N2: negative control without template

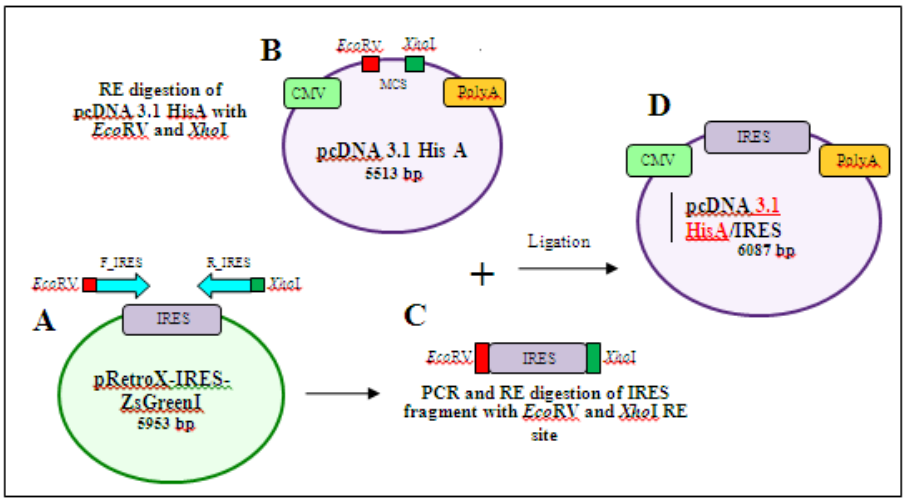

Figure 5 (on the left). Construction of CMV-IRES-PolyA fragments. (A) pRetroX-IRES-ZsGreen1, template for IRES sequence. (B) pcDNA 3.1 HisA harboring eukaryotic expression cassette. (C) IRES insert flanked by EcoRV and XhoI RE site. (D) pcDNA 3.1 HisA harboring constructed CMV-IRES-PolyA fragment is renamed as pcDNA 3.1 His A/IRES

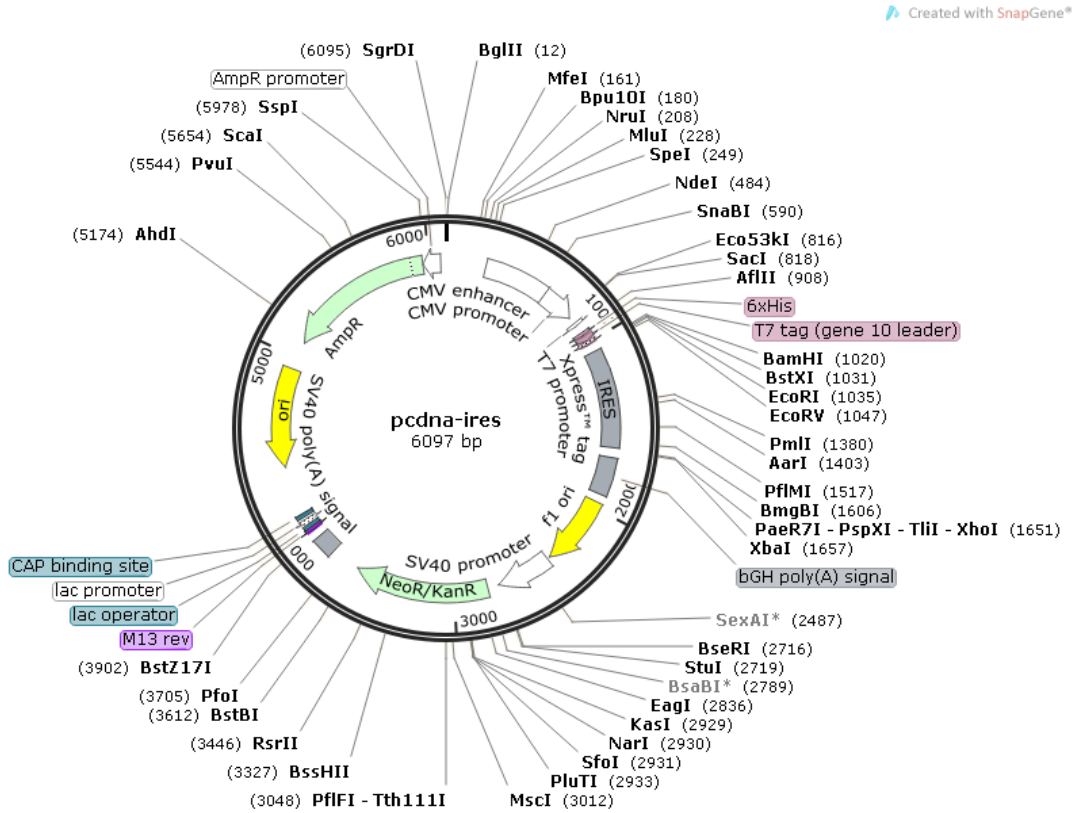

Figure 6. A map and sequence position of pcDNA3.1HisA/IRES vector

into *E. coli* Top10. The positive transformants were then screened using 5 µg/mL of erythromycin. The result showed that four out of eight colonies grown may harbor the putative vector as PCR product of approximately 1500 bp was successfully amplified when F_pcmv and R_IRES primers were used (Figure 7).Two colonies, C5 and C8 were further subcultured for their plasmid stability. It was observed the intensity of plasmid DNA band decreases with each subsequent subculture (Figure 8) suggested segregational plasmid instability.

DISCUSSION

The objective of this study was to construct a lactococcal vector containing modified eukaryotic expression cassette, with internal ribosome entry site (IRES) inserted between CMV promoter and Poly A signal to allow transcription and translation of two proteins in a single cassette. This concept has been applied in recombinant vaccine development and a study of plasmid containing the CMV promoter and bovine growth hormone termination signal has been reported to work well in avian vaccination (Tupperwar *et al*., 2010; Suarez and Schultz-Cherry, 2000). CMV promoter exhibited as a strong promoter and has been used by many workers for high level protein expression and enable transcription to occur in eukaryotic system (Mutalib *et al*., 2014). Internal initiation of translation will occur in the presence of IRES element which enables expression of two or more unrelated reading frames inserted within a single vector (Hennecke *et al*., 2001). Renaud-Gabardos *et al*. (2015) stated that IRES is a RNA constituent with 5' untranslated regions of a few mRNAs that play as a powerful tool to co-express several genes. To enable the cloning of the eukaryotic cassette containing CMVpromoter, multiple cloning sites (MCS) and IRES in between the MCS, a suitable restriction enzyme was chosen. In this study, *Eco*RV and *Xho*I RE site was chosen because both enzyme sites were present in between CMV promoter and Poly A signal, and is not present in the IRES sequence. The enzymes present along the IRES sequence should differ with the restriction enzyme chosen for digestion of the eukaryotic promoter and Poly A signal as cloning can become complicated when genes contain internal restriction sites that are also present in the MCS (Celie *et al*., 2016) and if not the IRES sequence will be digested causing incomplete IRES sequence inserted in the cassette. The IRES sequence flanked by *Eco*RV and *Xho*I was designed to be inserted

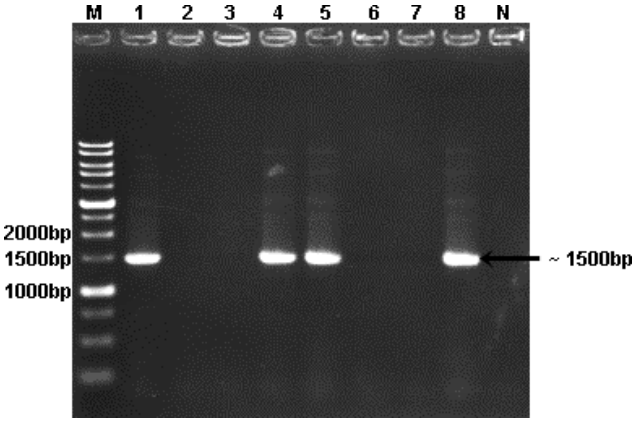

Figure 7 (on the left). Colony PCR screening for colonies formed from antibiotic selection LB agar supplemented with erythromycin. M: GeneDirex 1kb DNA ladder RTU. Lanes 1 to 8: PCR product amplified from Colonies 1 to 8 respectively. N: negative control without DNA template.

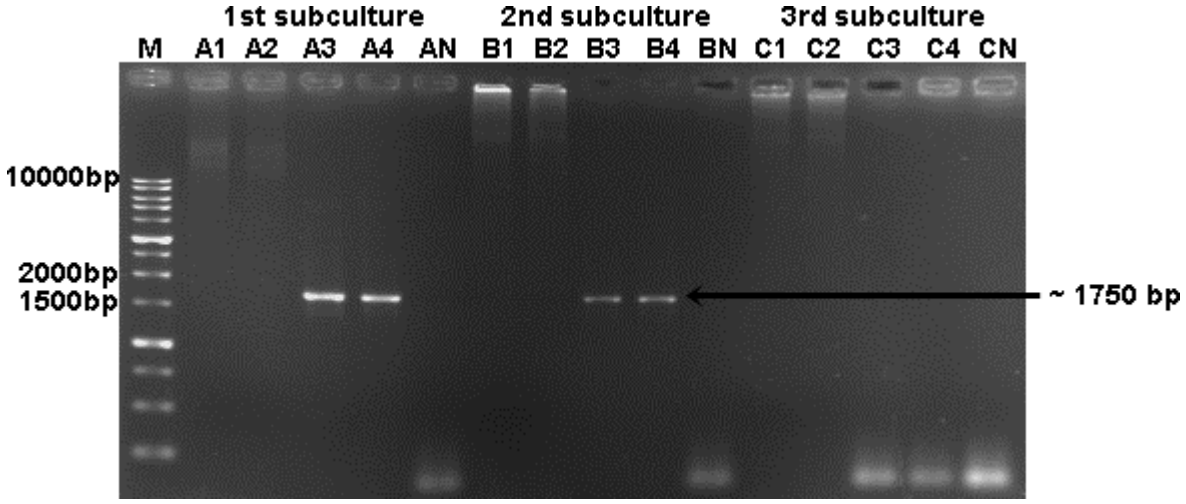

Figure 8. Putative pMG36e/C5 and pMG36e/C8 extracted from three subcultures of *E. coli Top10* and their PCR products amplified using F_pcmv and R_PolyA. M: GeneDirex 1kb DNA ladder RTU. Lanes 1 and 2: Extracted putative pMG36e/C5 and pMG36e/C8. Lanes 3 and 4: PCR products amplified from putative pMG36e/C5 and pMG36e/C8. Lane N: Negative control without any DNA template. First, second and third subcultures were represented by A, B and C respectively.

between CMV promoter and Poly A signal to construct the CMV-IRES-PolyA fragment. Double RE digestion using *Eco*RV and *Xho*I was carried out to the amplified IRES fragment and pcDNA 3.1 HisA vector before subjected to ligation. The ligation product (pcDNA 3.1 HisA/IRES) was transformed into *E. coli* TOP 10 and positive transformants were screened on LB plate supplemented with ampicillin. RE digestion, colony PCR and DNA sequencing showed positive results where IRES gene was successfully inserted between the CMV and Poly A signal with correct orientation. The agarose gel of extracted pMG36e from *L. lactis* showed differences in the profile compared to the extracted pcDNA 3.1 HisA/IRES from *E. coli* Top10. The positive control pcDNA 3.1 HisA/IRES was observed as two bands while the extracted pMG36e was observed as smearing with faint bands. Spectrophotometer readings for OD260/280 ratio and DNA concentration of pMG36e demonstrated the presence of high concentration of nucleic acid in the sample. The results indicated that the smear on the lane may be contributed by high molecular weight (HMW) plasmid DNA multimers and single-stranded DNA (ssDNA), which were produced due to the mode of replication of pMG36e, a rolling-cycle (RC) plasmid. ssDNA are the intermediate formed during RC plasmid replication while HMW plasmid DNA was proposed to be a product of impaired termination of RC replication which occurs occasionally in wild-type RC plasmid (Gruss and Ehrlich, 1988) and pMG36e might not be stable.

The CMV-IRES-PolyA fragments from pcDNA3.1 HisA/IRES then were sub clone into pMG36e; denoted as pMG: IRES/Hyg before subjected to *E. coli* Top10 transformation using heat shock method, positive colonies were obtained, indicating that the ligation mixture was prepared correctly and ligation of CMV-IRES-PolyA fragment into pMG36e occurred. Generally, ligated DNA electrotransformation efficiency is lower than covalently closed circular (CCC) plasmid DNA due to greater resistance conferred by conformation while increasing plasmid size will also reduce transformation efficiency. Two colonies of *E. coli* Top10 cells (Colony 5 and 8) were further subcultured and it was observed that the putative vector was not stable in *E. coli* Top10. Intensity of plasmid DNA band decreases with each subsequent subculture, in parallel with estimated DNA concentration measured using Nanodrop$^{(R)}$ spectrophotometer. The smearing of the high molecular weight plasmid bands was decreased. In the third subculture, the concentration of putative pMG36e/Colony could not be determined using spectrophotometer and the plasmid DNA band failed to be visualized using gel electrophoresis. No PCR product amplification was obtained when extracted putative pMG36e/Colony from the third generation was used as template, indicating the loss of the plasmid. Since the concentration of the putative pMG36e/Colony was too low, sequencing of the plasmid DNA samples were not possible and the identity could not be confirmed. The instability of the plasmid may be caused by large DNA insert and the RC type replication of pMG36e. Segregational instability of RC type plasmid was shown to be drastically higher and the copy number was reduced with increased size of DNA inserts. CMV-IRES-PolyA fragment (1744 bp) was half the size of the digested pMG36e (3009 bp), thus contributed to the plasmid instability. Recombinant RC plasmid with large inserts produced ssDNA intermediates and HMW plasmid multimers, which have been implicated to cause both structural and segregational instability of the plasmid (Kiewet *et al*., 1993). In addition, research done by Darbet *et al*. (1992) concluded that the nucleotide composition of the insert may also cause HMW formation. This correlate with the result in this study as the IRES sequence in the inserted CMV-IRES-PolyA, believed to exist in secondary form may have contributed to the formation of the HMW DNA. Increased amount of HMW DNA were paralleled by decreased numbers of circular plasmid forms and also shown to reduce cell viability of certain *E. coli* Top10 strain (Kusano *et al*., 1989). It was observed that the growth of *E. coli* Top10 culture harboring the putative pMG36e/Colony was slower, thus *E. coli* Top10 may not be a suitable host for the transformation of the ligated product due to plasmid instability and reduced cell viability. However, Hongying *et al*. (2014) reported a 97% stability of pMG36e-hp0410 with erythromycin and 90% without erythromycin (control) which also successfully constructed a stable recombinant *L. acidophilus* vaccine containing pMG36e expression vectors. Nanyan et al. (2011) was also reported the plasmid pMG36e was found to be 100% stable in *L. lactis* M4 where all of the 100 colonies were able to grow on the medium containing erythromycin even after 120 generations or ~72 h of cultivation. Thus, finding the suitable host might also play the critical role to improve the plasmid stability.

CONCLUSION

The modified eukaryotic expression cassette of ~1750 bp, designated as CMV-IRES-PolyA fragment was successfully amplified from pcDNA3.1HisA/IRES and sub-cloned into pMG36e lactococcal vector which later was denoted as pMG: IRES/Hyg.The transformation of the constructed pMG: IRES/Hyg into *E. coli* Top10 propagation host was successful but shows segregational instability mostly due to rolling-cycle replication of the pMG36e which produces single-stranded intermediates and high-molecular weight DNA.

CONFLICT OF INTEREST

None of the authors of this paper has any financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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