

## Cloning and expression of Human Papilloma virus type 16 L1 capsid protein in bacteria

DOI: [dx.doi.org/10.22435/hsji.v10i2.2442](https://doi.org/10.22435/hsji.v10i2.2442)

Silvia Tri Widyaningtyas<sup>1</sup>, Sofy Meilany<sup>1</sup>, and Budiman Bela<sup>1,2</sup>

<sup>1</sup>Research Center for Virology and Pathobiology Cancer, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital (PRVKP FKUI-RSCM)

<sup>2</sup>Department of Microbiology, Faculty of Medicine, Universitas Indonesia

Corresponding author: Silvia Tri Widyaningtyas

Email: [silvia.widyaningtyas@ui.ac.id](mailto:silvia.widyaningtyas@ui.ac.id)/[silvia\\_3w@yahoo.com](mailto:silvia_3w@yahoo.com)

Received: September 18, 2019; Revised: October 25, 2019; Accepted: December 11, 2019

### Abstrak

**Latar belakang:** Secara alamiah protein kapsid L1 Human Papillomavirus (HPV) tipe 16 dapat mengalami auto assembly untuk membentuk Viral like particle (VLP). Terkait dengan penelitian vaksin HPV, VLP dapat digunakan untuk berbagai keperluan seperti vaksin, pseudovirion atau SpyTag-Spycatcher. Penelitian ini ditujukan untuk mendapatkan plasmid rekombinan yang digunakan untuk produksi protein L1 HPV 16.

**Metode:** Gen penyandi protein L1 HPV 16 diklonasi ke dalam vector pQE80L, suatu plasmid yang mengandung sistem ekspresi untuk prokariota. DNA penyandi HPV 16 L1 disisipkan pada situs restriksi BamHI dan Hind III plasmid pQE80L. Plasmid rekombinan yang mengandung gen L1 HPV 16 dikonfirmasi menggunakan PCR dan analisis enzim restriksi. Lebih lanjut untuk memastikan bahwa gen rekombinan L1 HPV 16 dapat diekspresikan dalam prokariota, plasmid rekombinan ditransformasikan ke bakteri *Escherichia coli* BL21 (DE3). Bakteri diinduksi dengan Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) dengan berbagai konsentrasi dan berbagai waktu inkubasi.

**Hasil:** protein rekombinan L1, berat 56 kDa, telah berhasil diekspresikan dalam sistem prokariota. Protein rekombinan L1 dapat dimurnikan menggunakan Talon<sup>R</sup> dalam kondisi denaturasi.

**Kesimpulan:** gen L1 HPV 16 telah dikloning ke dalam pQE80L dan berhasil diekspresikan dalam sistem prokariota. (*Health Science Journal of Indonesia* 2019;10(2):82-9)

**Kata kunci:** L1, HPV 16, cervical cancer

### Abstract

**Background:** Naturally Human Papillomavirus (HPV) type 16 L1 capsid protein can auto assemble to form Viral like particles (VLP). Concerning to vaccine development for HPV, VLP can be used for a variety of needs such as a vaccine, pseudovirion or SpyTag-Spycatcher. In this study, to obtain a vector expression that can be used in the production of HPV L1 protein, we cloned gene coding HPV 16 L1 protein into pQE80L a plasmid contains an expression system for prokaryote.

**Methods:** The DNA coding HPV 16 L1 was inserted at BamHI and Hind III restriction sites of pQE80L plasmid. The recombinant plasmid containing the HPV L1 gene was confirmed using PCR colony and enzyme restriction. Further to ensure the recombinant HPV 16 L1 gene could be expressed in a prokaryote, the recombinant plasmid was transformed into bacteria *Escherichia coli* BL21 (DE3). The bacteria were induced with IPTG with various concentrations and various incubation time.

**Result:** L1 recombinant protein, 56 kDa in weight, has successfully been expressed in prokaryote system. L1 recombinant protein can be purified using Talon<sup>R</sup> under denaturing conditions.

**Conclusion:** L1 HPV 16 gene has been cloned into pQE80L and successfully expressed in prokaryote system. (*Health Science Journal of Indonesia* 2019;10(2):82-9)

**Keywords:** L1, HPV 16, cervical cancer

Human papillomavirus (HPV) is the major cause of cervical cancer that causes death of 311,000 women in 2018.<sup>1</sup> Based on their capability in inducing cancer, the HPV viruses are divided into 2 groups, the high risk and the low-risk group.<sup>2</sup> Consisted of at least 9 HPV subtypes, those are HPV 6,11,16,18,31,32, 45,52, and 58, the high-risk group is associated with 90% of cervical cancer and 90% of genital warts.<sup>3</sup> Approximately 67% of cervical cancer globally was caused by HPV16 and 18.<sup>4</sup> The main strategy to prevent HPV infection recommended by WHO is by vaccination. Nowadays there are three kinds of commercial prophylactic HPV vaccines, that are Gardasil, a quadrivalent vaccine containing HPV 6,11,16,18 antigens, Gardasil 9, a 9-valent vaccine containing HPV 6,11,16,18,31,33,45,52,58 antigens and a bivalent vaccine against HPV16 and 18 antigens.<sup>3</sup> The WHO recommends that the commercial HPV vaccine must cover at least two kinds of HPV subtypes, HPV 16 and HPV18 antigens.<sup>5</sup>

HPV is an unenveloped virus that has a circular double-stranded DNA genome of approximately 8 kb in size.<sup>6</sup> HPV has a capsid that formed by HPV major and minor capsid protein, which are Late (L) 1 and L2 proteins respectively. Naturally, L1 without the presence of other HPV structural and non-structural proteins has the capability to self-assemble to form virus-like particles (VLP).<sup>7</sup> Capsid proteins contain conformational epitopes that induce neutralizing antibodies.<sup>8</sup> Capsid proteins contain positively charged amino acids causing DNA that has negative charge can be assembled into VLP.<sup>9</sup> Based on its natural unique properties, many studies have explored the potentiality of L1 protein to be developed as vaccine, pseudovirion, and vehicle to deliver immunomodulator, adjuvant, DNA and proteins.<sup>7,8,9</sup>

Infection of HPV will be followed by serologic immune responses that mainly directed against conformational epitopes of viral capsid proteins, and this response will persist for many years.<sup>9</sup> Immunization of VLP will induce immune responses that resembling HPV infection because VLP presenting neutralizing antibody epitopes that are similar to those presented on the virus.<sup>8</sup> Thus, unlike a virus, VLP does not contain viral genome so the expression of proteins inducing cancers such as E6 or E7 can be avoided. Nowadays, in vaccine industries, there are 2 technologies used to produce L1 HPV vaccine, which is a yeast-expression (*Saccharomyces cerevisiae*) and Baculovirus-expression system.<sup>8</sup> By using these techniques, the mass of production of HPV vaccine becomes possible. The other promising expression

system that could be developed as a production host of L1 protein is prokaryote.<sup>11,3</sup>

Due to the limitation in providing a susceptible cell culture that can be infected by HPV, the capability of the antibody to prevent HPV infection was measured by using pseudovirion. Pseudovirion is L1 VLP that assemble DNA coding a certain reporter protein, such as green fluorescent protein (GFP). The production of pseudovirion is conducted by co-transfecting plasmid coding L1 and L2 and plasmid coding reporter genes in 293 TT cell culture. L1 and L2 protein will be produced and assembled into VLP, and during the formation of VLP the DNA coding reporter gene will incorporate to VLP. The incorporation of DNA to VLP is through the interaction of negative charge of the DNA with positive charges of the L1 amino acid. Like in HPV infection, pseudovirion enters cells via the interaction of VLP with HPV receptor expressed on 293TT cell, Heparan sulfate.<sup>12</sup> In neutralization assay, in the absence of neutralizing antibody, pseudovirion can enter cell culture, 293TT, and the reporter protein will be expressed. On the other hand, in the presence of neutralizing antibodies, the pseudovirion will be neutralized and cannot enter the cell causing reporter protein will not be expressed.

Beside beneficial in medicine, VLP also useful in nanotechnology.<sup>13</sup> SpyTag-Spy catcher technology or protein in nanotechnology, VLP can be decorated with a different antigen or protein. Decorating VLPs with target-antigens by genetic fusion of chemical modification is time-consuming and often leads to L1 protein miss folding or miss assembly. Some studies have been conducted to establish a platform for irreversibly decorating VLP simply by mixing with a protein of interest. One of the technologies used to develop irreversible decoration of VLP is Sphycatcher and SpyTag. Sphycatcher is a genetically-encoded protein designed to spontaneously form a covalent bond to its peptide partner.<sup>13</sup> Generally, a protein that has the capability to form a highly organized supramolecular structure with unique biological properties is chosen to be a Spy catcher.<sup>14</sup> The protein of interest tagged with peptide SpyTag form an irreversible covalent bond to the spy catcher protein via a spontaneous isopeptide linkage to create a peptide interaction that resists force and harsh conditions.<sup>15</sup> Even though the usage of L1 HPV as Spycatcher has been reported yet, L1 HPV is potential to be developed as Spy catcher. L1 could assemble to form big, stable molecules. A peptide that mediates spy catcher and spy tag interaction can be inserted in one of 5 major loops of L1 protein.

In this study, we construct the plasmid containing gene coding L1 HPV subtype 16. Plasmid will be used in the production of HPV 16 L1 protein. The HPV 16 L1 protein will be used in many studies, such as in the development of spy taq-spy catheter nanotechnology or producing specific antibodies that can be used for diagnostic or other serological assays for HPV 16. More over producing HPV 16 L1 will give valuable experiences for developing vaccine or pseudovirion for other high risk HPV. Technically, producing recombinant protein in a prokaryote is simpler than in yeast or Baculovirus. The production of recombinant protein in bacteria does not need the expensive facility.<sup>1,23,24,25,26</sup>

## METHODS

**L1 coding gene.** L1 used in this study a sequence is obtained from a back translation of the consensus sequence of L1 protein using DNA2 and has been codon optimized in accordance with prokaryote expression system. The L1 consensus sequence was generated by picking up the most frequent amino acid present at each position in a L1 protein alignment. The sequence of L1 full length proteins were collected from Genbank with accession number : AD33259.1, AIQ82831.1, ACN91168.1, AIQ82846.1, AIQ82845.1, AIQ82844.1, AIQ82843.1, AIQ82842.1, AIQ82841.1, AIQ82840.1, AIQ82839.1, AIQ82838.1, AIQ82837.1, AIQ82836.1, AIQ82835.1, AIQ82834.1, AIQ82833.1, AIQ82832.1, AIQ82829.1, AIQ82830.1. The L1 gene containing an open reading frame (ORF) of L1 protein with length 1515 bp was synthesized in IDT Malaysia via local supplier. The gene was cloned into universal cloning vector pUC 19, and plasmid containing L1 gene was name pUCL1col. The sequence of L1 gene and protein is confidential because a patent is currently being filed.

**Subcloned gen coding HPV 16 L1 protein into pQE80L.** Gene coding HPV 16 L1 was subcloned from pUCL1col (obtained from PRVKP FKUI RSCM) to pQE80L (Qiagen) inserted in BamHI and HindIII fragment. Vector and insert used in the subcloned reaction were prepared by restricted 10 µg plasmid of each pQE80L and pUCL1col. The restriction was performed by adding 10 µg in a tube containing 1x Neb2 buffer, 1xBSA (NEB) and 40.000 Unit BamHI (NEB) and DNase/RNase free water (Ambion) to volume 100µl. The mixture

was incubated for 4 hours at 37°C. After that, the DNA was desalted using QiaecII gel extraction kit (Qiagen) following the procedure described by the manufacturer. Further, DNA was restricted by HindIII. DNA that has been cut with BamHI was added to a tube containing 1x Neb4 buffer, 1xBSA, and 40.000 Unit HindIII (NEB) and DNase/RNase free water (Ambion) to volume 100µl. After incubated overnight at 37°C, DNA was run on 0.8% LMA containing crystal violet (Invitrogen). DNA was purified from LMA by using S.N.A.P UV-free DNA isolation kit (Invitrogen). Ligation was performed by using vector: insert comparison = 1:3. Ligation reaction is 80 ng vector, 80 ng insert, 1x ligation buffer (NEB), 2.5 Unit T4 Ligase (NEB) and water to volume 20 µl. reaction ligation was incubated at 16°C for overnight. Ligation was transformed into chemically competent *Escherichia coli* Top10<sup>16</sup> by the heat shock method.

## Modeling Ribo Nucleic acid (RNA) secondary structure

The secondary structure of messenger RNA is generated from the HPV 16 L1 sequence (the sequence is unpublished) using the dynamic programming algorithm described in MaxExpect program.<sup>17</sup> The software can be accessed freely at [Mathews Lab Home – University of Rochester](https://rna.urmc.rochester.edu) (<https://rna.urmc.rochester.edu>)

## Selection of recombinant cloned

Recombinant bacteria containing recombinant plasmid coding HPV 16 L1 was screened using PCR colonies. Primers pQEF (GTATCACGAGGCCCTTTCGTCT) and pQER (CATTACTGGATCTATCAACAGGAG) that recognizing specific sites in pQE80L was used to amplify the DNA inserted in multiple cloning sites. The PCR reaction was performed using DreamTaq DNA polymerase (Thermoscientific) following the manufacturer's instruction (Thermoscientific). Bacterial colonies were picked up using sterile toothpick strikes on replica plates and put into PCR tubes as PCR template. The colonies producing expected DNA amplicons were grown in 4 ml LB broth containing 100 µg/ml ampicillin, incubated at 37°C overnight. Plasmids were isolated using Miniprep (Qiagen) and characterized using enzyme restriction.<sup>16</sup> Further plasmid was sent to IDT Malaysia via local supplier for sequencing by using Sanger Method to verify the gene sequence. The shipment of DNA was accompanied by Material transfer agreement (MTA) signed by sender and receiver. MTA stated that the receiver was allowed to use the DNA only for method stated in research protocol.

**Protein expression**

The verified plasmid was transformed into bacteria *Escherichia coli* BL21 (DE3) chemically competence using heat shock transformation.<sup>23</sup> Three colonies were picked up and cultured in 4 ml LB broth containing 100 µg/ml ampicillin, incubated overnight at 37°C. One part of overnight culture was grown in 20 part of Terrific broth (Gliserol 1%, Trypton 1,2%, Yeast extract 1,2%, KH<sub>2</sub>PO<sub>4</sub> 0,34%, K<sub>2</sub>HPO<sub>4</sub> (1,1%) containing 100 µg/ml ampicillin, after incubated for 1.5 hours, the cultures were incubated on ice for 15 minutes, and IPTG with various final concentration (0,1; 0,2 dan 1 mM ) was added, and 1 ml cultures were collected after 3, 6 and overnight after induction. The incubation temperature of induction was 37°C.

**Protein purification**

Bacteria were lysed under native and denature conditions. In native condition, bacteria were lysed using buffer A (500mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 20%Sucrose, 1mg/ml Lyzosome. Under denature condition, bacteria were lysed using denature buffer (50mM Sodium Phosphate, 6 M Guanidine HCl, 300mM

NaCl (pH8)). After incubated for 1 hour 20 minutes on rocking incubator 60 rpm (BioRad) at room temperature. After incubates, samples were centrifuged at 12000 rpm for 5 minutes at 4°C. Protein was purified using TALON® (Clontech) and the purification was conducted following the manufacturing instruction. Wash buffer used here was 45 mM Sodium Fosfat, 300mM NaCl, 5,4 M Urea, 10 mM imidazole (pH 8,0). Recombinant protein was eluted using 45 mM Sodium Fosfat, dan 300mM NaCl, 5,4 M Urea, 150/200 mM imidazole (pH 8,0). The purified recombinant protein was analyzed by run it on 12% SDS PAGE.

**RESULTS**

Linearized vector and L1 gene insert were successfully isolated from LMA, 4800 and 1515 bp respectively (Figure 1). After ligated recombinant plasmids were transformed into *E.coli* Top10, the growing colonies were subjected to PCR colony. The colonies produced 1840 bp amplicon indicated those colonies possibly contain the interested DNA. The pQE80L Wild type (WT) produced 289 bp DNA that closely migrated to 300 bp marker (Figure 2).

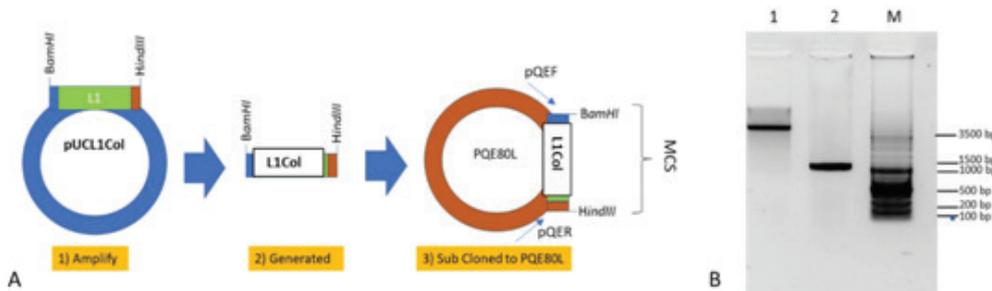


Figure 1. Schema of Subcloning plan (A) and vector (line 1) and insert (line 2) (B)

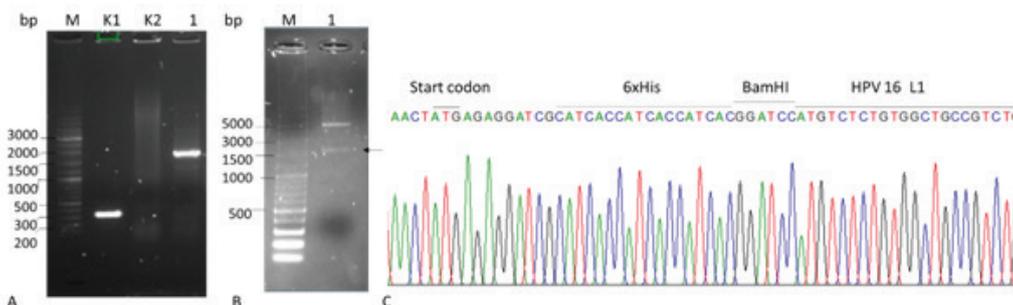


Figure 2. Screening of Recombinant plasmid. (A) PCR colony. M : Marker; K1 pQE80L WT, K2: Negative control, 1 pQE80L L1. (B) Restriction enzyme analysis pQE80L L1 with BamHI and HindIII, the arrow showed the fragment of L1 gene. (C). Sequencing result showed the position of L1 gene that was in frame with 6xHis.

Plasmids were isolated from PCR confirmed colonies. Once run on 0.8% agarose gel, recombinant plasmids migrated slower than pQE80L WT, indicating the size of those plasmids due to the insertion of L1 gene became larger than pQE80L WT (data was not shown). The recombinant plasmids were further analyzed using restriction enzyme. The recombinant plasmids produced DNA fragments 4800 and 1515 bp in length (Figure 2). The sequencing showed the inserted-gene sequences were not mutated and can be expressed in frame with 6xHis Tag (the full sequence of L1 was not shown).

The verified recombinant plasmid was transformed in bacteria hopes that used for recombinant protein production, BL21 (DE3). After inducing with IPTG

with various concentrations showed there was overexpression of protein that migrates between marker 55 and 70 kDa, indicating L1 recombinant proteins were successfully expressed. Based on SDS page analysis, the 0.1 mM IPTG could induce the recombinant L1 as efficient as other IPTG concentrations. The recombinant bacteria could be expressed 3 hours after the addition of IPTG. Prolonged induction time to overnight also induced unspecific host protein (Figure 4). Analysis of messenger RNA secondary structure showed the RBS (AGGAG) in a single-stranded structure, and the last base (G24) of start codon formed external closing pair with T91, however that interaction did not infer protein expression.

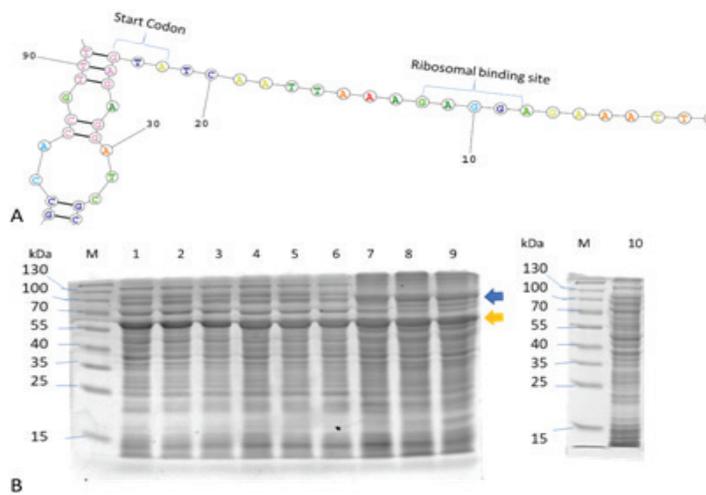


Figure 3. (A) The secondary structure modeling of 5'UTR L1-mRNA covering the ribosomal binding site and start codon generated by Maxexpect program. (B) The expression of L1 recombinant protein in E.coli BL21 (DE3) induced with various concentrations of IPTG and length of induction time. M: marker; 1-3 bacteria were induced for 3 hours at various concentrations of IPTG, no 4-6 were induced for 6 hours at various concentrations of IPTG, no 7-9 were induced for overnight at various concentrations of IPTG. The concentrations of IPTG: 0.1, 0.25 and 1 mM respectively. No 10: wild type BL21 (DE3). UTR: untranslated region.

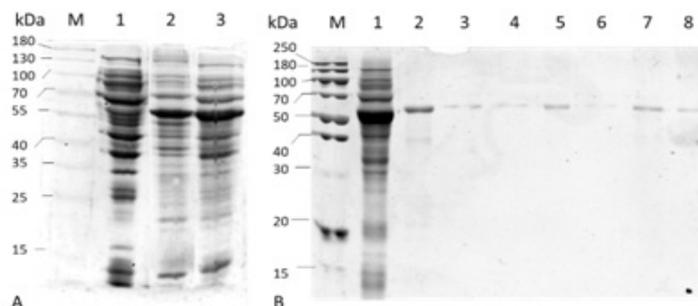


Figure 4. (A) Lysed bacteria M: Marker, 1: Supernatant 2: pellet 3: BL21 (DE3) expressing L1. (B) Purification of L1 recombinant using Talon, M: Marker, 1 BL21 (DE3) expressing L1, 2-6: the elution of L1 recombinant, 7-8: washed.

After lysed using native buffer, the recombinant L1 predominantly found in the pellet (Figure 4), indicating the protein retains in inclusion bodies. L1 recombinant protein fused with 6xHistidine Tag was successfully purified under denaturing condition using Talon<sup>R</sup>. However, the protein also could be found in washing filtrate. Protein started to elute when incubated with elution buffer containing 150 mM Imidazole (Figure 4).

## DISCUSSIONS

Bacteria containing plasmid coding gene of interest can be selected by combining some methods, such as PCR colonies, restriction profile of enzyme restriction of plasmid, a specific selectable marker, and sequencing. In this study, we used PCR colony as the 1<sup>st</sup> method to screen the bacteria bearing interested recombinant plasmid. In this study primer pair, pQEF and pQER, that recognized a specific region that flanks open reading frame of pQE80L was used in PCR colony. The amplification using pQEF and pQER causes the addition of 289 bp to the insert length (figure 2A) and the amplification of 289 pb in wild type plasmid (figure 2A). Specific primers that recognize gene of interest also can be used, but the possibility of the altering of primer annealing temperature after that gene was cloned into a vector must be taken into consideration. The second method to screen plasmid is based on the migration of plasmid on an agarose gel. The insertion of a gene into a plasmid causes the addition of plasmid size. Once run on agarose gel the recombinant plasmid will run slower than wild type. Further, the recombinant plasmid can be confirmed by DNA restriction pattern. Enzyme restriction has a specific target on DNA either plasmid or insert. In this study, the recombinant plasmid was cut with BamHI and HindIII. Those enzymes locate at 5' and 3' terminal of pQE80L cloning sites. The restriction of recombinant plasmid using BamHI and HindIII causes the separation of vector, 4800 bp, and insert 1515 bp (figure 2B). The sequence of interesting genes was confirmed using sequencing. By using proper software, the amino acid sequence of the inserted gene can be analyzed whether it in frame with 6xHistidine Tag or not. In this study, the L1 HPV 16 gen was cloned in frame with 6x His (figure 2C). L1 HPV16 recombinant can be expressed in prokaryote system that showed by the overexpression of 56 kDa protein band after induction with IPTG,

whereas that band could not be found in wild type. The L1 gene used here was codon optimized in accordance to prokaryote to minimize the hindering of protein translation due to codon bias [11]. Based on mRNA modelling, the ribosomal binding site was in a single-stranded or un structure. Modelling was used to predict the translation probability of a mRNA in host cells.<sup>18,19</sup> Protein production in *E.coli* depends on the capability of 16sRNA to bind to a specific segment of mRNA called Shine Dalgano or ribosomal binding site [20] and the interaction only occurs when the SD/RBS in a single-stranded or unstructured state.<sup>21</sup> The interaction of SD/RBS with 16sRNA will be followed by the interaction of ribosome-bound initiator tMet with start codon (ATG). Based on RNA modelling, the last base of HPV 16 L1 ATG interacted with T91. However, this interaction does not hinder protein translation. Predicted RNA structure that generated based on the full sequence of mRNA probable difference to those generated in living system.<sup>22</sup> During transcription and translation process in vivo the elongation of mRNA chain occurs simultaneously with protein synthesis. Ribosome complex has occupied SD and ATG before the mRNA elongation reaches certain base that pairing with ATG.<sup>18</sup> By using Talon (Clontech), the recombinant HPV 16 L1 could be purified under denature condition, because under native condition, the protein retained in pellet. Previously been reported, L1 protein expressed in *E.coli* retained in inclusion bodies.<sup>23,24</sup> High level protein expression, high temperature during expression, high concentration of IPTG, the usage of strong promoter, partially folded or misfolded protein often results in aggregation of the recombinant protein into inclusion bodies.<sup>25</sup> By using chaotropic agents such as Guanidine HCl and Urea, the recombinant protein in inclusion bodies can be solubilized.<sup>26</sup> On the other hand, the presence of chaotropic agents can denature the protein. The denatured recombinant protein can renature by dialysis or diluting in native buffer.<sup>26,27</sup> Therefore, modification of L1 protein at N-terminal has been reported could solubilize L1, and such modification did not affect the capability of L1 to self-assembly.<sup>3</sup>

The L1 protein of HPV 16 can be produced in various expression system, and the currently HPV prophylaxis vaccines were produced in mammalian system, that are Baculovirus and Yeast.<sup>11,24,28,29,30,31,32</sup> Compared to those mammalian expression systems, *E.coli* has some advantages such as fast growth, easy gene manipulation, low production cost and

easy to scale up.<sup>28</sup> Thus this system is suitable for low-income countries where cervical cancer results in higher mortality, and this expression system has been used to produce a low-cost HPV vaccine by Xiamen Innovax Biotech, China (Huang et al, 2017).

The sources of L1 gene used to developed VLP are varying. The gene could be amplified from keratinocyte cell line W12 (28), synthesized based on L1 gene downloaded from Genbank<sup>11</sup>, extracted from patients<sup>30</sup>, or ordered from ATTC company.<sup>33</sup> L1 gene used in this research is synthetic gene that codes a consensus L1 protein that represents the majority amino acid presented in different L1 HPV 16 proteins. Consensus sequence can accommodate the diversity of L1 HPV 16.<sup>34</sup> Vaccine based on consensus sequence expectantly could induce strong and broad immune response.<sup>34</sup>

In conclusion, gene coding HPV 16 L1 has been successfully cloned into pQE 80L, a prokaryote expression system. The cloned gene can be expressed *E. coli* in BL21(DE3) and can be purified using Talon<sup>R</sup>.

#### Acknowledgement

This study was supported by grants from Insentif Riset Sistem Inovasi Nasional (Insinas) Ministry of Research, Technology and Higher Education (Proposal code: RT-2016-0583).

#### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

#### REFERENCES

1. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global cancer observatory: cancer today. Lyon, France: International Agency for Research on Cancer. 2018 Available from: <https://gco.iarc.fr/today>
2. Doorbar J, Egawa N, Griffin H, Kranjec C, Murakami I. Human papillomavirus molecular biology and disease association. Review in Medical. Virology. 2016; 25: 2–23.
3. Wei M, Wang D, Li Z, Song S, Kong X, Mo X, et al. N-terminal truncations on L1 proteins of human papillomaviruses promote their soluble expression in *Escherichia coli* and self-assembly in vitro. Emerg Microbes Infect. 2018 Sep 26;7(1):160. doi: 10.1038/s41426-018-0158-2. PMID: 30254257; PMCID: PMC6156512. 9-6
4. Bruni L, Barrionuevo-Rosas L, Albero G, Aldea M, Serrano B, Valencia S, et al. Barcelona: ICO information centre on HPV and cancer; Human papillomavirus and related diseases in Senegal. Summary report 2016-02-26. Available from: <http://www.hpvcentre.net/statistics/reports/SEN.pdf>
5. WHO/BS/2018.2348. Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines. Geneva, 12 to 16 October 2015. Available from: [https://www.who.int/biologicals/BS.2018.2348.Recommendations\\_HEP\\_E\\_vaccines.pdf?ua=1](https://www.who.int/biologicals/BS.2018.2348.Recommendations_HEP_E_vaccines.pdf?ua=1)
6. Pinidis P, Tsikouras P, Iatrakis G, Zervoudis S, Koukouli Z, Bothou A, et al. Human Papilloma virus life cycle and carcinogenesis. Maedica (Buchar). 2016 Mar;11(1):48-54. PMID: 28465751; PMCID: PMC5394500.
7. Buck CB, Day PM, Trus BL. The papillomavirus major capsid protein L1. Virology. 2013;445(1-2),169–174. doi:10.1016/j.virol.2013.05.038
8. Wu X., Ma X., Li Y, Xu Y, Zheng N, Xu S, et al. Induction of neutralizing antibodies by human papillomavirus vaccine generated in mammalian cells. Antibody Therapeutics. 2019;2(2). Pages 45–53, doi.org/10.1093/abt/tbz004
9. Bousarghin L. Positively charged sequences of human papillomavirus type 16 capsid proteins are sufficient to mediate gene transfer into target cells via the heparan sulfate receptor. Journal of General Virology. 2003;84(1)157–64. doi:10.1099/vir.0.18789-0
10. Wu WH, Gersch E, Kwak K, Jagu S, Karanam B, et al. Capsomer vaccines protect mice from vaginal challenge with Human Papillomavirus. PLoSONE 2011 6(1): e27141. doi:10.1371/journal.pone.0027141
11. Bang HB, Lee TH, Lee YJ, Jeong KJ. High-level production of Human Papillomavirus (HPV) type 16 L1 in *Escherichia coli*. J. Microbiol. Biotechnol. 2016;26(2), 356–363. <http://dx.doi.org/10.4014/jmb.1511.11010>
12. Barzegar H, Sharifi H, Langroudi L, Azadmanesh K, Arashkia A. Human papillomavirus genotype 16 pseudovirus production and purification in HEK-293FT cells. Vaccine Research. 2017;4(3 and 4):46-50.
13. Brune, KD. Plug-and-Display: decoration of virus-like particles via isopeptide bonds for modular immunization. Sci. Rep. 6, 19234; doi: 10.1038/srep19234 (2016).
14. Diaz D, Care A, Sunna A. Bioengineering strategies for protein-based nanoparticles. Genes (Basel). 2018 Jul 23;9(7):370. doi: 10.3390/genes9070370. PMID: 30041491; PMCID: PMC6071185
15. LLi L, Fierer JO, Rapoport TA, Howarth M. Structural analysis and optimization of the covalent association between SpyCatcher and a Peptide Tag. Journal of Molecular Biolog. 2014;426(2)309–17. doi:10.1016/j.jmb.2013.10.021.
16. Ausubel M, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology volumes 1 and 2. John Wiley & Sons, Inc., Media, PA, 1988. <https://doi.org/10.1002/mrd.1080010210>.

17. Lu ZJ, Gloor JW, Mathews DH. Improved RNA secondary structure prediction by maximizing expected pair accuracy. *RNA*. 2009 Oct;15(10):1805-13. doi: 10.1261/rna.1643609. Epub 2009 Aug 24. PMID: 19703939; PMCID: PMC2743040.
18. Lai D, Proctor JR, Meyer IM. On the importance of cotranscriptional RNA structure formation *RNA* 2013 19: 1461-73.
19. Mathews DH. How to benchmark RNA secondary structure prediction accuracy. *Methods*. 2019 Jun 1;162-163:60-67. doi: 10.1016/j.ymeth.2019.04.003.
20. Sterk M, Romilly C, Wagner EGH. Unstructured 5'-tails act through ribosome standby to override inhibitory structure at ribosome binding sites. *Nucleic Acids Res*. 2018 May 4; 46(8): 4188-4199. doi: 10.1093/nar/gky073.
21. Wagner EJ, Burch BD, Godfrey AC, Salzler HR, Duronio R.J, Marzluff WF. A genome-wide RNA interference screen reveals that variant histones are necessary for replication-dependent histone pre-mRNA processing. *Mol Cell*. 2007 Nov 30;28(4):692-9.
22. Leamy KA, Assmann SM, Mathews DH, Bevilacqua PC. Bridging the gap between in vitro and in vivo RNA folding. *Q Rev Biophys*. 2016 Jan;49:e10. doi: 10.1017/S003358351600007X. Epub 2016 Jun 24. PMID: 27658939; PMCID: PMC5269127.
23. Lai WB, Middelberg. The production of human papillomavirus type 16 L1 vaccine product from *Escherichia coli* inclusion bodies. *Bioprocess and Biosystems Engineering*. 2002;25(2)121-8. doi:10.1007/s00449-002-24. Chen Y, Liu Y, Zhang G, Wang A, Dong Z, Qi Y, et al. Human papillomavirus L1 protein expressed in *Escherichia coli* self-assembles into virus-like particles that are highly immunogenic. *Virus Res*. 2016 Jul 15;220:97-103. doi: 10.1016/j.virusres.2016.04.017.
25. Singh A, Upadhyay V, Upadhyay AK, Singh SM, Panda AK. Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process. *Microb Cell Fact*. 2015 Mar 25;14:41. doi: 10.1186/s12934-015-0222-8. PMID: 25889252; PMCID: PMC4379949.
26. Singh A, Upadhyay V, Panda AK. Solubilization and refolding of inclusion body proteins. *Insoluble Proteins, Microb Cell Fact*. 2015 Mar 25;14:41. doi: 10.1186/s12934-015-0222-8.
27. Hoffmann D, Ebrahimi M, Gerlach D, Salzig D, Czermak P. Reassessment of inclusion body-based production as a versatile opportunity for difficult-to-express recombinant proteins. *Critical Reviews in Biotechnology*. 2017;38(5)729-44. doi:10.1080/07388551.2017.1398134.
28. Zhang W, Carmichael J, Ferguson J, Inglis S, Ashrafian H, Stanley M. Expression of Human Papillomavirus type 16 L1 protein in *Escherichia coli*: Denaturation, Renaturation, and Self-assembly of virus-like particles in vitro. *Virology* 1998;243(2)423-31. doi:10.1006/viro.1998.9050.
29. Coimbra, E. C., Gomes, F. B., Campos, J. F., D'arc, M., Carvalho, J. C., Mariz, F. C., Freitas, A. C. Production of L1 protein from different types of HPV in *Pichia pastoris* using an integrative vector. *Brazilian Journal of Medical and Biological Research* 2011 44(12), 1209-1214. doi:10.1590/s0100-879x2011007500141.
30. Combata AL, Touzé A, Bousarghin L, Christensen ND, Coursaget P. Identification of two cross-neutralizing linear epitopes within the L1 major capsid protein of human papillomaviruses. *J Virol*. 2002;76(13):6480-6. doi:10.1128/jvi.76.13.6480-6486.2002.
31. Seo PS, Heo SY, Han EJ, Seo JW, Ghim SJ, Kim CH. Bacterial expression and purification of human papillomavirus type 18 L1. *Biotechnol. Bioprocess Eng*. 2009;14:168-74.
32. Huang X, Wang X, Zhang J, Xia N, Zhao Q. *Escherichia coli*-derived virus-like particles in vaccine development. *Npj Vaccines* 2017 2(1). doi:10.1038/s41541-017-0006-8.
33. Akuzum B, Kim S, Nguyen TT, Hong J, Lee S, Kim E, et al. L1 recombinant proteins of HPV tested for antibody forming using sera of HPV quadrivalent vaccine. *Immune Netw*. 2018 Jun;18(3):e19. <https://doi.org/10.4110/in.2018.18.e19>.
34. Wheatley AK, Kent SJ. Prospects for antibody-based universal influenza vaccines in the context of widespread pre-existing immunity. *Expert Review of Vaccines*. ;14(9):1227-39. doi:10.1586/14760584.2015.1068125.