Construction of a Novel Recombinant Adenovirus (Ad5-p53-DsRed Monomer N1)

Asita Elengoe, Salehhuddin Hamdan*

Department of Biological Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
asitaelengoe@yahoo.com, saleh65@utm.my*

Abstract: Oncolytic adenoviruses are a class of promising anti-cancer agents, which are engineered to infect, replicate within, and lyses cancer cells without damaging normal cells. In this study, we reported for first time the construction of adenovirus serotype 5 (Ad5) by cloning p53 gene into defective recombinant adenovirus vector containing red fluorescence protein (Ad5-p53-DsRed Monomer N1). This recombinant virus can be a potential anti-cancer agent in cancer treatment. Firstly, human tumor protein p53 gene (Entrez ID: 7157) was successfully cloned into pDsRed Monomer N1 vector. The size of the recombinant plasmid (p53-DsRed Monomer N1) obtained was approximately 5.9 kb. The p53-DsRed Monomer N1 construct was then used to insert into entry clone (pENTR3C^{TM}). The p53 gene was successfully cloned into the pAd/CMV/V5-DEST^{TM} vector through the entry clone (pENTR3C^{TM}). The size of PCR product of Ad5-p53-DsRed Monomer N1 was about 10.3 kb. Finally, Ad5-p53-DsRed Monomer N1 was transfected in Vero cells to determine expression of p53. The transfected cells presented diffuse red fluorescence from the DsRed Monomer N1 that appeared to be present throughout the cytoplasm and nucleus. The presence of DsRed Monomer N1 in cells was a strong indicator of transfection of the recombinant construct and thus possible p53 expression. This study reveals future perspective to develop a promising treatment for cancer patients.
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1. Introduction

Gene therapy is a new therapeutic approach for cancer. It specifically targets the tumor cells including metastatic cells in the body (Abaan and Criss, 2002). It has been shown to be effective with different types of diseases (Rubanyi, 2001). Therefore, it may be applicable for the treatment of cancer patients. Recombinant adenoviruses are a class of promising anti-cancer agents, which are engineered to infect, replicate within, and lyses cancer cells without damaging normal cells (Yamamoto and Curiel, 2009).

Adenovirus (Ad) is a non-enveloped and double-stranded DNA virus. Although over forty different serotypes of adenoviruses have been discovered but adenovirus serotype 2 and 5 were the most extensively used virus in developing oncolytic adenoviruses. Most primarily, these viruses infect the epithelial tissue lining the respiratory tract. The most common symptoms are nasal congestion and rhinorrhoea (runny nose), conjunctivitis and cough. Adenovirus serotype 2 (Ad2) and 5 (Ad5) infection occurs via high affinity binding of the adenoviral fiber to cellular receptors such as the coxsackie adenovirus receptor or $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Mei and Wedell, 1996). Once bound, the virus is internalized through receptor-mediated endocytosis; it escapes from the endosomal compartment to the cytosol and brought to the nucleus. Then, the viral capsid is disassembled during transport; therefore allowing the viral DNA to enter via the nuclear pores. Viral transcription and replication begins once the viral genome enters the nucleus.

One of the widely used vectors in clinical trials is adenovirus when compared to other vectors such as adeno-associated viral (AAV) or hybrid adenoviral, retroviral, lentiviral, herpes simplex viral (HSV) and non-viral vector. It is because broad host range, very high transducing efficacy, no chromosomal integration, high vector yield ($>10^{11}$) and host cell proliferation not required. Furthermore, it is more safe and suitable than other vectors to deliver the therapeutic gene.

p53 is a labile protein, comprising folded and unstructured regions that function in a synergistic manner (Bell et al., 2002). It acts as a tumor suppressor in many tumor types. It induces growth arrest or apoptosis depending on the physiological circumstances and cell type. It also involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process.

The human p53 gene will be tagged with *Discoscoma* red fluorescence (DsRed). The advantage of using a fluorescence marker (DsRed Monomer N1) is that there is no need for addition of any antibodies, co-markers or substrates (cells with p53 are easily detected according to their direct fluorescence). Moreover, only a few cancer studies were conducted

using DsRed Monomer N1 protein when compared with green fluorescence protein (EGFP) to study specific proteins in cells.

In this study, we reported for first time the construction of Ad5-p53-DsRed Monomer N1. This recombinant virus can be a potential anti-cancer agent in cancer treatment.

2. Material and Methods

2.1 Cells

The African green monkey kidney (Vero) cell line was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 100 U/ml penicillin and 10% fetal calf serum (FCS). Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

2.2 Vectors

pDsRed-Monomer-N1 (Clontech): vector which encodes for DsRed-Monomer N1 whose expression is under control of cytomeagalovirus (CMV) promoter. DsRed Monomer N1 is a monomeric mutant of the *Discosoma* sp. This vector was used to construct a p53 fusion protein expression plasmid.

pENTR3C™ (Invitrogen): vector that allows restriction cloning of the gene of interest (p53-DsRed Monomer N1) for entry into the pAd/CMV/V5-DEST™ vector to generate the expression of clone. The gene of interest was replaced with ccdB gene which located between the two attL sites. Besides that, it contains *Escherichia coli* (*E. coli*) ribosome binding site for efficient initiation of translation in prokaryotic cells.

pAd/CMV/V5-DEST™ (Invitrogen): vector that contains human adenovirus type 5 sequences (Ad 1-458 and 3513-35935) encoding genes and elements (e.g. Left and Right Inverted Terminal Repeats (ITRs), encapsidation signal sequence, late genes) required for proper packaging and production of adenovirus (Hitt et al., 1999; Russell, 2000). The expression of recombinant protein of interest (p53-DsRed Monomer N1) is under control of the CMV promoter. It consists of two recombination sites, attR1 and attR2 for recombinational cloning of the protein of interest from an entry clone (pENTR3C™). In addition, it also contains C-terminal V5 epitope for detection of recombinant protein using the Anti-V5 antibodies.

2.3 Bacterial strains

Bacterial strain *Escherichia coli* (*E.coli*) *DH5α: dlacZ Delta M15 Delta (lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1* was used as host for cell transformation which carries plasmids which size up to 40 kb.

2.4 p53 protein

Homo sapiens tumor protein p53 (Li-Fraumeni syndrome) (Entrez ID: 7157) was purchased from Invitrogen.

2.5 Construction of p53-DsRed Monomer N1

2.5.1Determination of plasmid DNA concentration

The plasmid DNA concentration of pDsRed Monomer N1, p53 and pENTR3C™ was determined using Nanodrop 1000 spectrophotometer machine (*Thermo* Scientific). The OD_{260}: OD_{280} ratio of purified plasmid DNA was expected to be in the range of 1.8 and 2.0.

2.5.2 Design primers of p53 gene

Complete DNA sequence of human IRAU p53 gene (Entrez Gene ID: 7157) was retrieved from the CCSB ORFeome Human Collection database (Human ORFeome version 5.1) and used to identify the coding sequence of p53 gene using open reading frame (ORF) server. Specific forward and reverse primers were designed based on parameters such as length, melting temperature, GC content, hair-pin formation, self-complementary and self-annealing sites. These parameters were evaluated through OligoAnalyzer version 3.1 and OligoCalc: Oligo Properties Calculator. BLAST was carried out to find significance of the primers via E-value and identity value. Finally, the predicted primers were validated using Sequence Extractor program and the size of PCR product was obtained.

2.5.3 Cloning of p53 into pDsRed Monomer N1

Complete DNA sequence of p53 gene was amplified by Polymerase Chain Reaction (PCR) using the specific forward (*Sal*I) and reverse (*Bam*HI) primers (refer Table 1). Then, pDsRed Monomer N1 was digested with restriction endonucleases *Sal*I and *Bam*HI and ligated with the PCR products. The ligation mixture was used for transformation of competent *E.coli* DH5α. After that, the recombinant bacteria were screened using LB agar medium containing 30 µg/ml kanamycin. The presence of the insert was confirmed by PCR and double digestion of *Sal*I and *Bam*HI. Finally, the cloned sequence was confirmed by DNA sequencing.

2.5.4 Construction of Ad5-p53-DsRed Monomer N1

pENTR3C™ entry clone and p53-DsRed Monomer N1 were digested with *ECo*RI and *Not*I restriction enzymes. pENTR3C™ entry clone was ligated with p53-DsRed Monomer N1 and used for transformation of competent *E.coli* DH5α. The successful transformants were selected by plating cell suspension on 30 µg/ml kanamycin LB agar. The presence of the insert was confirmed by PCR. Lastly, the PCR product was sent for DNA sequencing. pAd/CMV/V5-DEST™ adenovirus expression vector was purchased from Invitrogen. The LR

recombination reaction was carried out between the entry clone, pENTR3C™ containing gene of interest (p53-DsRed Monomer N1) and destination vector (pAd/CMV/V5-DEST™) according to the manufacturer's protocols (Invitrogen). The plasmid resulting from the insertion of p53 tagged with DsRed Monomer N1 into the pAd/CMV/V5-DEST™ was designated as Ad5-p53-DsRed Monomer N1. Ad5-p53-DsRed Monomer N1 was transfected by Lipofectamine™ 2000 reagent (Invitrogen) into Vero cells.

3 Results and Discussion

3.1 Construction of p53-DsRed Monomer N1

To clone and express p53 in p53-negative cells, the production of an appropriate expression vector is necessary. This study involves the production of full length p53 and cloning into suitable vector (pDsRed Monomer N1) both for sequencing. The vector backbone contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen, a pUC origin of replication for propagation in *E. coli*, and a f1 origin for single-stranded DNA production. The multiple cloning site (MCS) in pDsRed-Monomer-N1 is positioned between the immediate early promoter of CMV (PCMV IE) and the DsRed-Monomer N1 coding sequence. The complete coding sequence of human p53 cloned into the MCS was expressed by *Discoscoma* red fluorescence (DsRed). The p53-DsRed Monomer N1 construct was then used to insert into entry clone (pENTR3C™).

3.1.1 Isolation of plasmid DNA

Verification of isolated plasmid plays an important role in validate the presence of correct plasmid before subsequently being used for any research. pDsRed Monomer N1, p53 and pENTR3C™ plasmid DNA were isolated through Mini-Prep Isolation method (Qiagen) and analyzed through agarose gel electrophoresis (Figure 1). The size of pDsRed Monomer N1, p53 and pENTR3C™ plasmid DNA was approximately at 4.7 kb, 2.5 kb and 3.8 kb respectively. p53 was further for PCR amplification.

3.1.2 Determination of plasmid DNA concentration and purity

Concentration and purity of plasmid DNA is also another essential factor in molecular cloning. Plasmid DNA should be of high quality or minimal contaminants of primer dimers, nucleotides and salts to obtain good results in PCR amplification. Good quality of plasmid DNA would have an A_{260}/A_{280} ratio of 1.8-2.0. Plasmid DNA concentration and purity was determined using Nanodrop1000 Spectrophotometer (*Thermo* Scientific). Results were shown in Table 1.

Table 1. Plasmid DNA concentration and purity

Plasmid DNA	Reading 1	Reading 2	Reading 3	Average
pDsRed Monomer N1				
Concentration (ng/µl)	300.80	300.50	327.50	309.50
Ratio	1.88	1.87	1.89	1.88
p53				
Concentration (ng/µl)	123.36	121.66	131.32	125.45
Ratio	1.87	1.88	1.88	1.88
pENTR3C™				
Concentration (ng/µl)	85.80	85.10	83.40	84.77
Ratio	1.86	1.85	1.87	1.86

3.1.3 Primer design of p53

Primer design is essential to obtain specificity and efficiency of PCR amplification. Therefore, the primers which follow the general rule of PCR primer design such as length (18-27 bases), melting temperature (T_m=50-65°C), GC content (45-60%), no hairpin formation, no self-complementary and no self-annealing sites will produce good PCR result (Dieffenbench et al., 1993). Table 2 shows forward and reverse primers were chosen for PCR amplification based on the criteria explained above.

Table 2. Specific forward and reverse primers designed for PCR amplification

Primer	Forward	Reverse
Sequence	5' ACT GCC TTC CGG TCG ACT 3'	5' CAG CCT GGG GAT CCT TGA GTT 3'
Restriction enzyme	*Sal*I	*Bam*HI
Length	18	21
Tm	58.9	59.2
GC (%)	61.1	57.1
Hairpin formation	NO	NO
Complementarity	NO	NO
Self-annealing sites	NO	NO
Identity	100%	100%
E-value	0.005	0.005

3.1.4 PCR amplification of p53

Template (p53) was mixed with forward and reverse primers, dNTPs, *Pfx* amplification buffer, *Pfx* enhancer buffer, $MgSO_4$ and Platinum® *Pfx* DNA Polymerase and subjected to 30 cycles on a DNA thermocycler (Bio-Rad Thermocycler). Nuclease free-water was used in place of DNA in the negative control. The negative control was also known as non-template control (NTC). Upon completion, 6X loading dye was added to PCR reaction mixture before

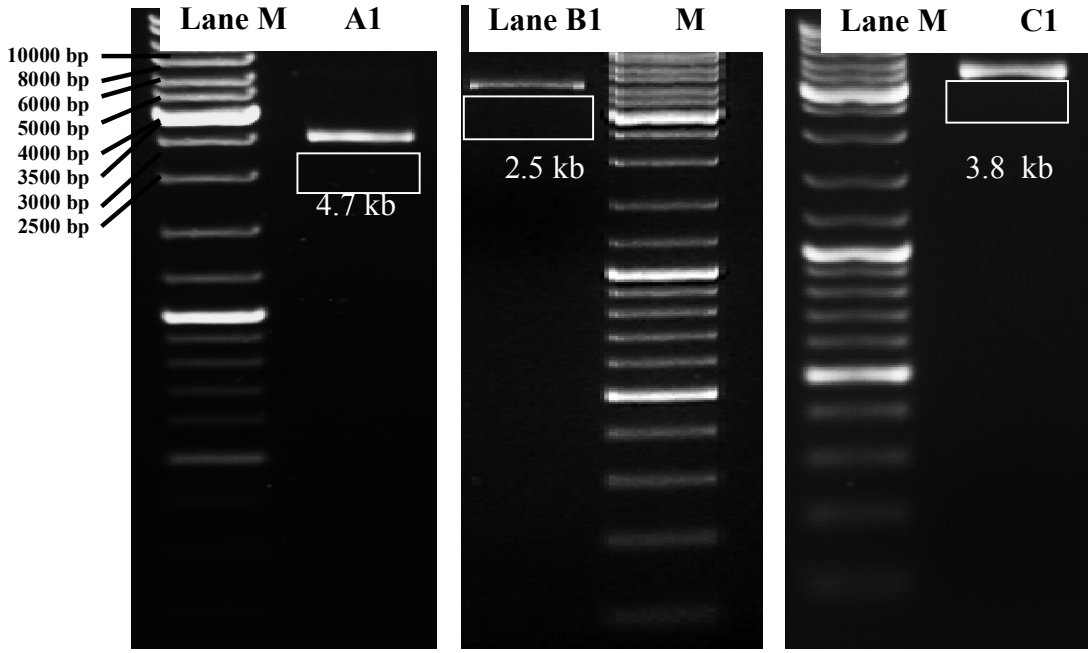
analysis by 1.2% agarose gel electrophoresis. DNA bands were detected by EtBr staining under UV illumination. The full length p53 was isolated successfully, as shown by the production of an approximately 1.2 kb band in the positive control sample, indicating the correct sized fragment for p53, with no products detected in the negative control (NTC) sample (Figure 2). This implied that water nuclease free water, primers and Platinum® *Pfx* DNA Polymerase in good quality and no contamination from any other sources such as pipette tips or PCR tubes.

3.1.5 Restriction Endonuclease (RE) Digestion of pDsRed Monomer N1 vector

pDsRed Monomer N1 vector was digested with *Sal*I and *Bam*HI restriction enzymes *Sal*I and *Bam*HI were added separately into two different PCR tubes at a total volume of 20 µl for single digestion whereas double digestion was performed by adding *Sal*I and *Bam*HI together in one PCR tube at a total volume of 30 µl. The RE digested plasmid DNA was separated by 1.2% agarose gel electrophoresis, stained with EtBr and viewed under UV illumination.

An approximately 4.7 kb band was produced from both single and double digestion of pDsRed Monomer N1 vector (Figure 3). There was no distinct band for the double digestion of pDsRed Monomer N1 vector. This was due to the fact that only about 20 bases were cut by *Sal*I and *Bam*HI restriction endonucleases (Figure 4). The double digestion of pDsRed Monomer N1 with both sticky ends of *Sal*I and *Bam*HI sites were extracted from gel and further performed for ligation process.

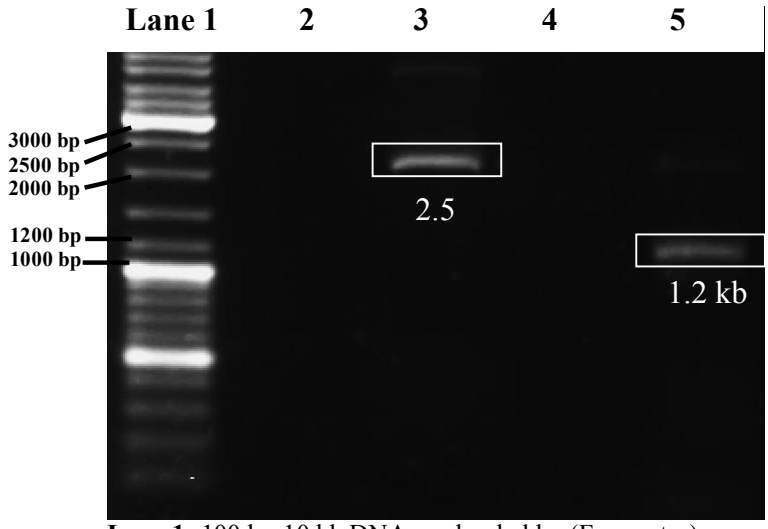
3.1.6 Analyze transformants (p53-DsRed Monomer N1) using PCR amplification and RE digestion



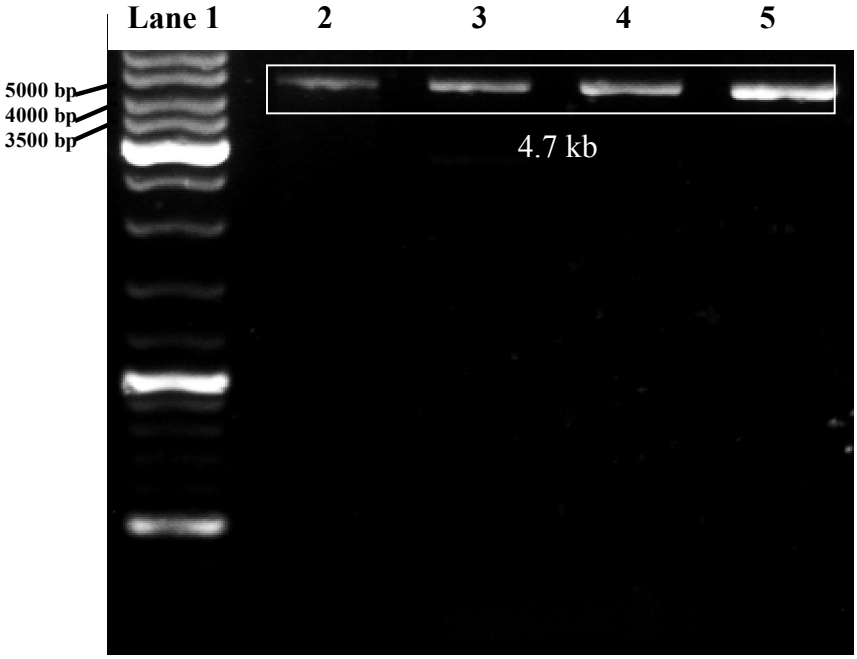
Lane M: 100 bp-10 kb DNA marker ladder (Fermentas)
Lane A1: p53
Lane B1: pDsRed Monomer N1
Lane C1: pENTR3C™

Figure 1. Electrophoretic analysis of pDsRed Monomer N1, p53 and pENTR3C™ plasmid DNA amplification

After the ligation mixture was incubated at 4°C overnight, it was mixed with *E.coli* competent cells for transformation and selection of successful transformants (p53-DsRed Monomer N1) was achieved by plating cell suspension on 30 µg/ml kanamycin LB-Agar. Then, the transformants was amplified by PCR. Finally, the PCR products were separated by electrophoresis on a 1.2% agarose gel and detected by EtBr staining and UV illumination.



Lane 1: 100 bp-10 kb DNA marker ladder (Fermentas)
Lane 3: Undigested p53
Lane 4: Negative control (NTC, Non-Template Control)
Lane 5: PCR amplification of p53
Figure 2. Electrophoretic analysis of p53 PCR amplification



Lane 1: 100 bp-10 kb DNA marker ladder (Fermentas)
Lane 2: Undigested pDsRed Monomer N1 vector
Lane 3: Single digestion of pDsRed Monomer N1vector with *Sal*I
Lane 4: Single digestion of pDsRed Monomer N1 vector with *Bam*HI
Lane 5: Double digestion of pDsRed Monomer N1 vector with *Sal*I and *Bam*HI
Figure 3. Electrophoretic analysis of single and double digestion of pDsRed Monomer N1 vector

Figure 5 shows band in lane 4 and 6 obtained same size of DNA in positive control (lane 2) which was 1.2 kb. This revealed that out of three colonies selected, two of the colonies carried p53 fragment or recombinant p53-DsRed Monomer N1. In addition, colony 1 had fainted band in comparison with colony 3 and positive template. Yet, no band was observed in lane 5. This was due to colony 2 did not carry either p53 fragment or recombinant p53-DsRed Monomer N1. A volume of 20 µl of PCR product of colony 3 was sent to 1st Base Sdn. Bhd for sequencing.

The selected colony 3 was cultured overnight at 37°C in an orbital shaker at 180 rpm. After plasmid DNA of recombinant p53-DsRed Monomer N1 was isolated using a mini preparation protocol, restriction endonuclease analysis was performed using *Sal*I and *Bam*HI restriction enzymes to confirm the presence of the correct insert. The plasmid digest products were separated by 1% agarose gel electrophoresis and visualized by UV illumination. As shown in Figure 6, the insert gave a band (lower band) of approximately 1.2 kb, corresponding to the p53 fragment while the other band (upper band) implies DsRed Monomer N1(vector) which at 4.7 kb size for double digestion with *Sal*I and *Bam*HI. The size of undigested p53-DsRed Monomer N1 was lower than the single and double digestion of plasmid DNA. This was because the undigested p53-DsRed Monomer N1 in high concentration and supercoiled form when ran for agarose gel electrophoresis. James et al. (2004) demonstrated that supercoiled DNAs which are compact and have small effective volume migrate more rapidly than relaxed circular DNAs of equal mass. However, single digestion of p53-DsRed Monomer N1 by *Sal*I and *Bam*HI had a band at size approximately 5.9 kb. This was because the enzyme cleaved at one site only. Thus, it generated one -strand cut only.

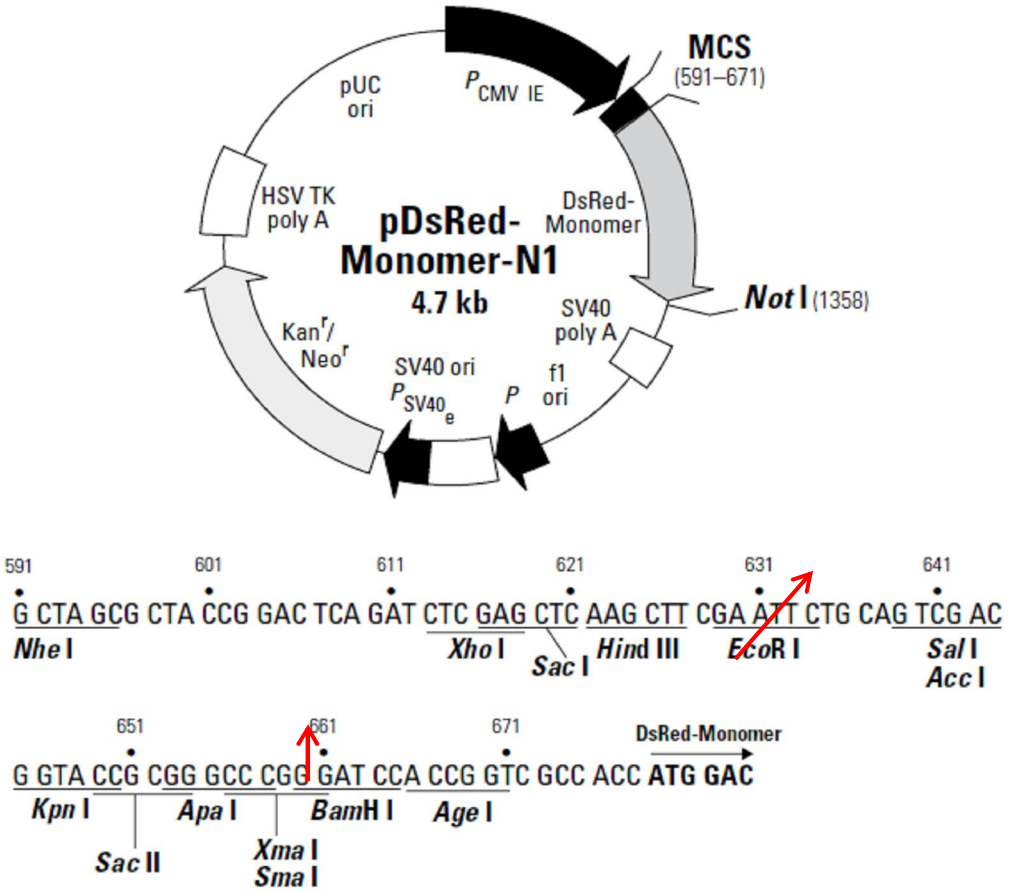
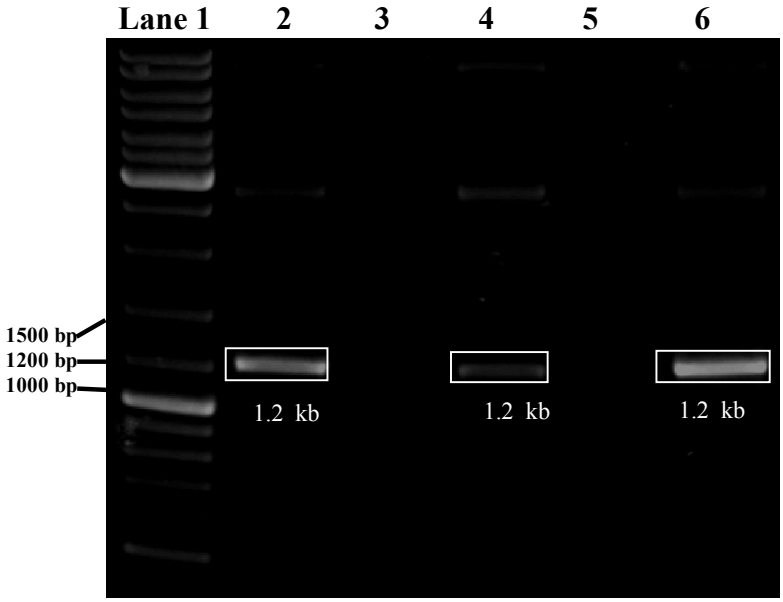
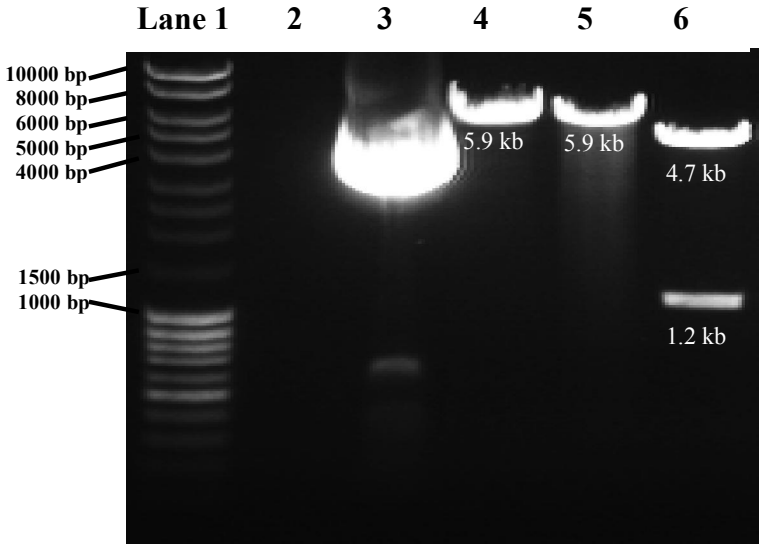


Figure 4. A schematic representation of restriction map and Multiple Cloning Site (MCS) of pDsRed Monomer N1 vector (Adapted from Clontech TAKARA BIO Company, 2006)



Lane 1: 100 bp-10 kb DNA marker ladder (Fermentas)
Lane 2: Positive control (p53 PCR product)
Lane 3: Negative control (NTC, Non-Template Control)
Lane 4: Colony 1
Lane 5: Colony 2
Lane 6: Colony 3
Figure 5. Screening of the insert (p53) from selective transformant colonies using amplification of PCR



Lane 1: 250 bp-10 kb DNA marker ladder (Promega)
Lane 3: Undigested p53-DsRed Monomer N1 recombinant
Lane 4: Single digestion of p53-DsRed Monomer N1 recombinant with *Sal*I
Lane 5: Single digestion of p53-DsRed Monomer N1 recombinant with *Bam*HI
Lane 6: Double digestion of pDsRed Monomer N1-p53 recombinant with *Sal*I and *Bam*HI
Figure 6. Electrophoretic analysis of single and double digestion of p53-DsRed Monomer N1 recombinant

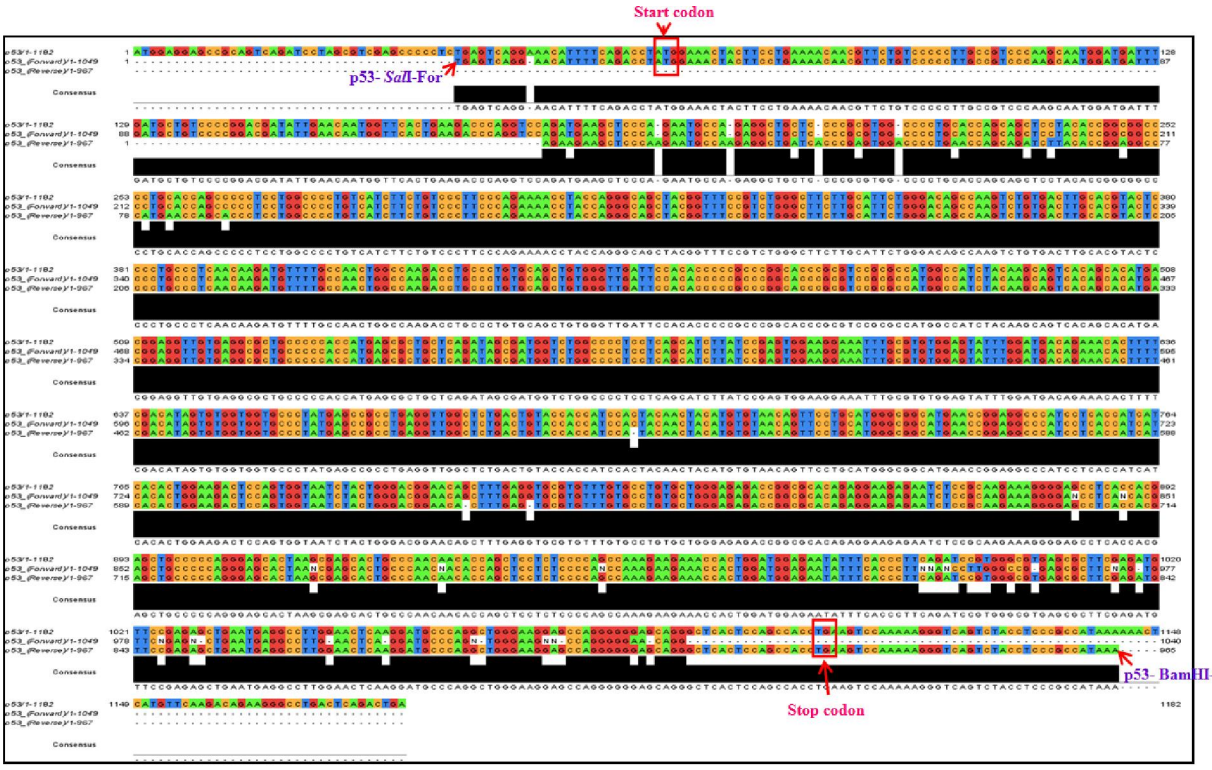


Figure 7. Alignment of forward and reverse sequencing of nucleic acid of the p53 PCR product (p53-DsRed Monomer N1) and *Homo sapiens* tumor protein p53 gene (Entrez Gene ID: 7157)

3.1.7 DNA sequencing analysis of p53-DsRed Monomer N1

3.1.7.1 Sequencing of PCR product of p53

DNA sequencing was performed using the specific forward and reverse primers (Table 1) to confirm the presence of the correct sequence of the p53 within the pDsRed Monomer N1 vector. The DNA sequence data obtained from 1st Base Sdn. Bhd was then verified by aligning with the sequence of the human tumor protein p53 gene (Entrez Gene ID: 7157) from the CCSB ORFeome Human Collection database (Human ORFeome version 5.1) using NCBI BLAST analysis.

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size (Dennis et al., 2006). It decreases exponentially as the Score (S) of the match increases. This describes that E-value is inversely proportional to the Score value. Essentially, the E-value describes the random background noise. The lower the E-value, or the closer it is to zero, the more "significant" the match is (James et al., 2006). Score or bit score is a value calculated from the number of gaps and substitutions associated with each aligned sequence (David et al., 2006). As the Score is higher, the more significant alignment will be obtained. Each Score links to the corresponding pairwise alignment between query sequence and hit sequence (subject sequence). Results from NCBI BLAST show that the DNA sequence of p53 PCR product as similar with human tumor protein p53 gene (Entrez Gene ID: 7157).

Furthermore, E-value was 0 for forward and reverse sequences which was considered low. Therefore, they were statistically significant. Both sequences were homologous as the E-value was lower than 10^{-5}. In addition, the identity value for forward sequence obtained was 96% whereas for reverse sequence was 98%. This revealed that the forward and reverse sequences were 96% and 98% respectively identical towards query. Moreover, 0% gaps were found in reverse sequence. This indicates that no insertion and deletion occurred. However, there were 2% gaps observed in forward sequence. The insertion and deletion was happened within the forward sequence. The Score value of the corresponding pairwise alignment between query sequence (human tumor protein p53 gene) and hit sequence (forward) was 1725 bits (934) while reverse was 1696 bits (918). Figure 7 shows a summary of a representation of a three-way sequence alignment with the *Homo sapiens* tumor protein p53 gene (5' to 3') which displayed using Jalview program. The location of the start and stop codons for p53 were also indicated.

In conclusion, human tumor protein p53 gene was successfully cloned into pDsRed Monomer N1

vector. The size of the recombinant plasmid (p53-DsRed Monomer N1) was approximately 5.9 kb.

3.2 Construction of Ad5-p53-DsRed Monomer N1

This section describes about cloning of p53-DsRed Monomer N1 into the pAd/CMV/V5-DEST™ vector through the entry clone (pENTR3C™). The vector that contains human adenovirus type 5 sequences (Ad 1-458 and 3513-35935) encoding genes and elements (e.g. Left and Right Inverted Terminal Repeats (ITRs), encapsidation signal sequence, late genes) required for proper packaging and production of adenovirus (Hitt et al., 1999; Russell, 2000). The expression of recombinant protein of interest (p53-DsRed Monomer N1) is under control of the CMV promoter. Human CMV is an immediate early promoter for high level constitutive expression of the gene interest in a wide range of mammalian cells (Andersson et al., 1989; Boshart et al., 1985; Nelson et al., 1987). It also contains a pUC origin for high-copy replication and maintenance of the plasmid in *E.coli*. Besides that, it consists of two recombination sites, attR1 and attR2 for recombinational cloning of the protein of interest (p53-DsRed Monomer N1) from an entry clone (pENTR3C™). The Ad5-p53-DsRed Monomer N1 construct was then used to determine the presence of p53 tagged with DsRed Monomer N1 in transfected Vero cells.

3.2.1 RE digestion of pENTR3C™

pENTR3C™ entry clone was digested with *ECo*RI and *Not*I restriction enzymes. For double digestion, *ECo*RI and *Not*I were added together in one PCR tube at a total volume of 30 µl. The RE digested plasmid DNA was separated by 1.2% agarose gel electrophoresis, stained with EtBr and viewed under UV illumination.

Figure 8 shows that the size of undigested pENTR3C™ entry clone was lower than the actual size of plasmid DNA (3.8 kb). This was because the undigested pENTR3C™ in supercoiled form when ran for agarose gel electrophoresis. Double digestion of pENTR3C™ by *ECo*RI and *Not*I obtained two bands at size about 2.3 kb (upper band) and 1.5 kb (lower band). The enzyme cleaved at two sites. Therefore, it generated two strands cut. The upper band of double digestion of pENTR3C™ with both sticky ends of *ECo*RI and *Not*I sites were extracted from gel and further performed for ligation process.

3.2.2 RE digestion of p53-DsRed Monomer N1

Restriction endonucleases digestion of p53-DsRed Monomer N1 was performed using *ECo*RI and *Not*I restriction enzymes. *ECo*RI and *Not*I were added separately into two different PCR tubes at a total volume of 20 µl for single digestion while double digestion was performed by adding *ECo*RI and *Not*I together in one PCR tube at a total volume of 30 µl. The recombinant plasmid DNA was digested by RE. After that, the DNA bands were separated by 1.2% agarose gel electrophoresis, stained with EtBr and viewed under UV illumination.

From the analysis of the gel electrophoresis (Figure 9), it is evident that the lower band of approximately 1.2 kb in lane 5 corresponded to the p53 fragment, with the higher band (4.7 kb) corresponding to pDsRed Monomer N1 for double digestion with *ECo*RI and *Not*I. In addition, the size of undigested recombinant plasmid DNA of p53-DsRed Monomer N1 in lane 3-4 was at approximately 5.9 kb. The lower band of p53-DsRed Monomer N1 in double digestion was extracted from gel and further proceeded for ligation process.

3.2.3 Analyzes transformants using PCR amplification

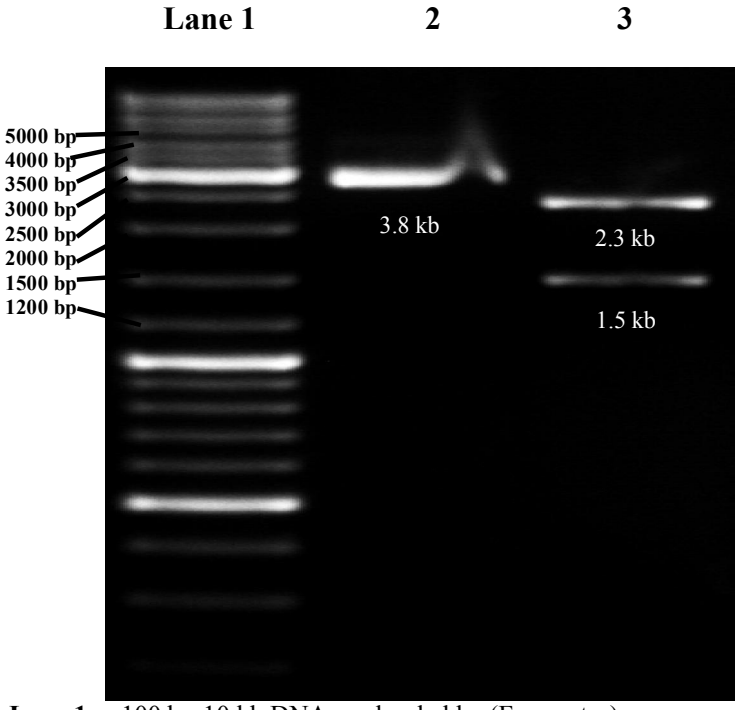
After the ligation mixture was incubated at 4°C overnight, it was mixed with *E.coli* DH5α competent cells for transformation and successful transformants (pENTR3C™ that carries p53-DsRed Monomer N1 gene) were selected by plating cell suspension on 30 µg/ml kanamycin LB agar. Pink color colonies were observed. Then, PCR amplification was performed for transformants. Upon completion, 6X loading dye was added to reaction mixtures before 1.2% agarose gel electrophoresis analysis. The DNA bands were detected by EtBr staining under UV illumination.

Figure 10 shows that the PCR products gave expected insert of p53-DsRed Monomer N1 approximately 1.2 kb in lane 2, 3 and 4. This revealed that p53-DsRed Monomer N1 was inserted within the pENTR3C™. Nevertheless, no band was observed in lane 1. This was because colony 1 did not carry p53-DsRed Monomer N1 DNA fragment within the pENTR3C™. A volume of 20 µl of PCR product of colony 2 was sent to 1st Base Sdn. Bhd for sequencing.

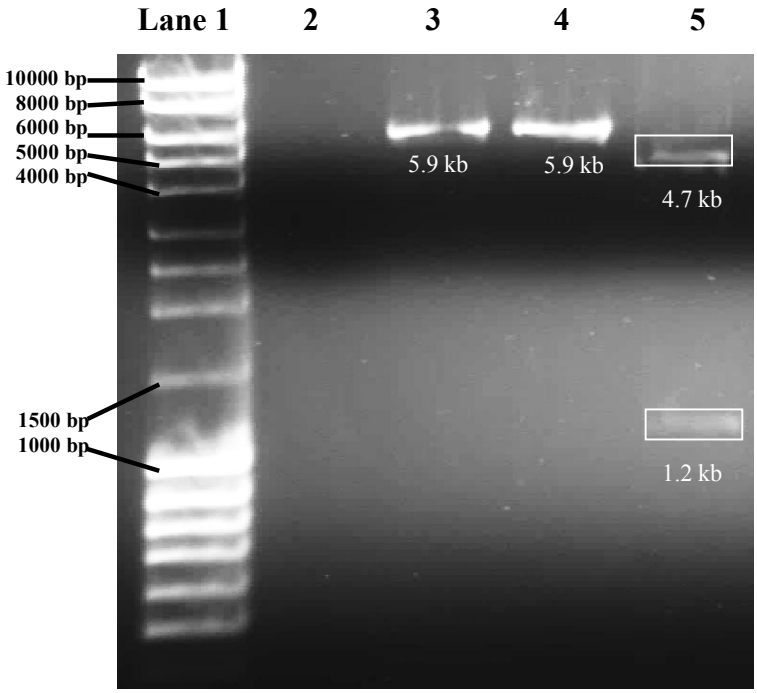
3.2.4 DNA sequencing analysis of transformants of pENTR3C™-p53-DsRed Monomer N1

DNA sequencing was performed using the pENTR3C™ forward and reverse primers to confirm the presence of the correct sequence of the p53-DsRed Monomer N1 fragment within the pENTR3C™ entry clone. The DNA sequence data obtained from 1st Base Sdn. Bhd was then verified by aligning with the sequence of the human tumor protein p53 gene (Entrez Gene ID: 7157) from the CCSB ORFeome Human Collection database (Human ORFeome version 5.1) using NCBI BLAST analysis.

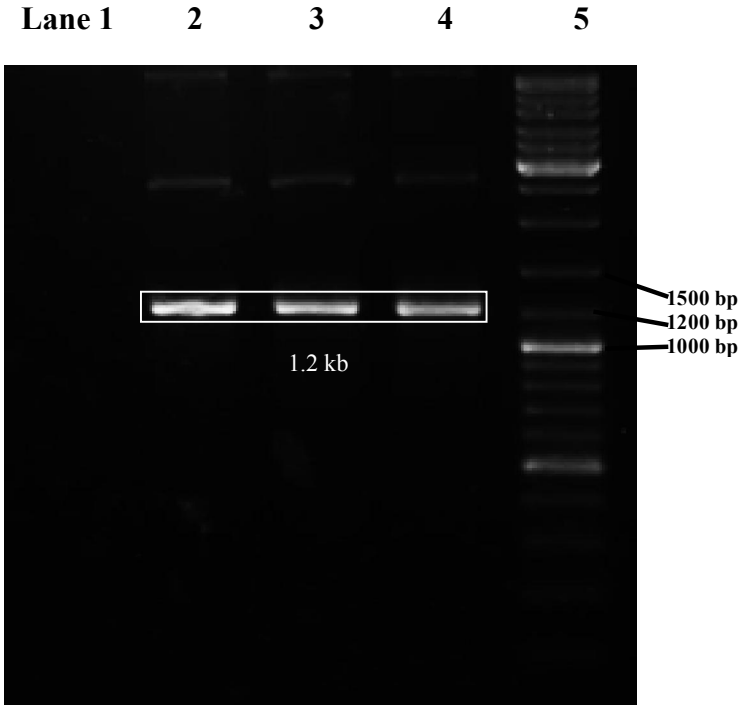
NCBI BLAST analysis shows that the forward and reverse sequences were corresponded 98% and 99% respectively similarity to the human tumor protein p53 gene (Entrez Gene ID: 7157).



Lane 1 : 100 bp-10 kb DNA marker ladder (Fermentas)
Lane 2 : Undigested pENTR3C™
Lane 3 : Double digestion of pENTR3C™ with *ECo*RI and *Not*I
Figure 8. Electrophoretic analysis of double digestion of pENTR3C™



Lane 1 : 250 bp-10 kb DNA marker ladder (Promega)
Lane 3-4 : Undigested p53-DsRed Monomer N1 recombinant
Lane 5 : Double digestion of p53-DsRed Monomer N1 with *ECo*RI and *Not*I
Figure 9. Electrophoretic analysis of double digestion of p53-DsRed Monomer N1 recombinant



Lane 1: Colony 1
Lane 2: Colony 2
Lane 3: Colony 3
Lane 4: Colony 4
Lane 5: 100 bp-10 kb DNA marker ladder (Fermentas)

Figure 10. Screening of the insert (p53) from selective transformant colonies using amplification of PCR

Moreover, the scoring hit was 1892 bits (1024) and 1759 bits (952) for forward and reverse sequences respectively. Each score links to the corresponding pairwise alignment between query sequence and hit sequence (subject sequence). The E-value obtained from this analysis was 0 for both sequences. Thus, they were statistically significant. They were homologous as the E-value was lower than 10^{-5}. In addition, the identities value for forward and reverse sequence obtained was 98% and 99% respectively. This infers that the forward and reverse sequence was 98% and 99% identical with the query sequence. Moreover, 0% gaps were found in both sequences. This indicates that no insertion and deletion occurred. Figure 11 shows a summary of a representation of a three-way sequence alignment with the *Homo sapiens* tumor protein p53 gene (5' to 3') which displayed using Jalview program. The location of the start, stop codons for p53 and the positions of the *ECo*RI and *Not*I restriction sites were also indicated.

As a conclusion, p53-DsRed Monomer N1 was successfully cloned into the entry clone (pENTR3C™).

3.2.5 Ligation of p53-DsRed Monomer N1 with pAd/CMV/V5-DEST™ vector through the entry clone (pENTR3C™)

LR recombination reaction between pAd/CMV/V5-DEST™ vector and entry clone, pENTR3C™ containing gene of interest (p53-DsRed Monomer N1) was performed in a final volume of 10 µl. After ligation and transformation of the DNA mixture into *E.coli* DH5α cells, recombinant colonies containing Ad5-p53-DsRed Monomer N1 were obtained as clear crystal on 50 µg/ml ampicillin LB-agar. PCR amplification was carried out for transformants. The PCR products were then separated by electrophoresis on a 0.8% agarose gel and detected by EtBr staining and UV illumination. Samples were run alongside a 1 kb extension DNA marker ladder to calibrate the gel. As shown in Figure 12, the size of PCR product of Ad5-p53-DsRed Monomer N1 was approximately 10.3 kb while no band was observed for p53. Yet, the 1 kb DNA extension ladder can detect linear double-stranded DNA fragments from 500 bp-40 kb only. Therefore, the size of p53 might be lower than 500 bp. A volume of 20 µl of PCR product of Ad5-p53-DsRed Monomer N1 was sent to 1st Base Sdn. Bhd for p53 sequencing and further proceeded for transfection.

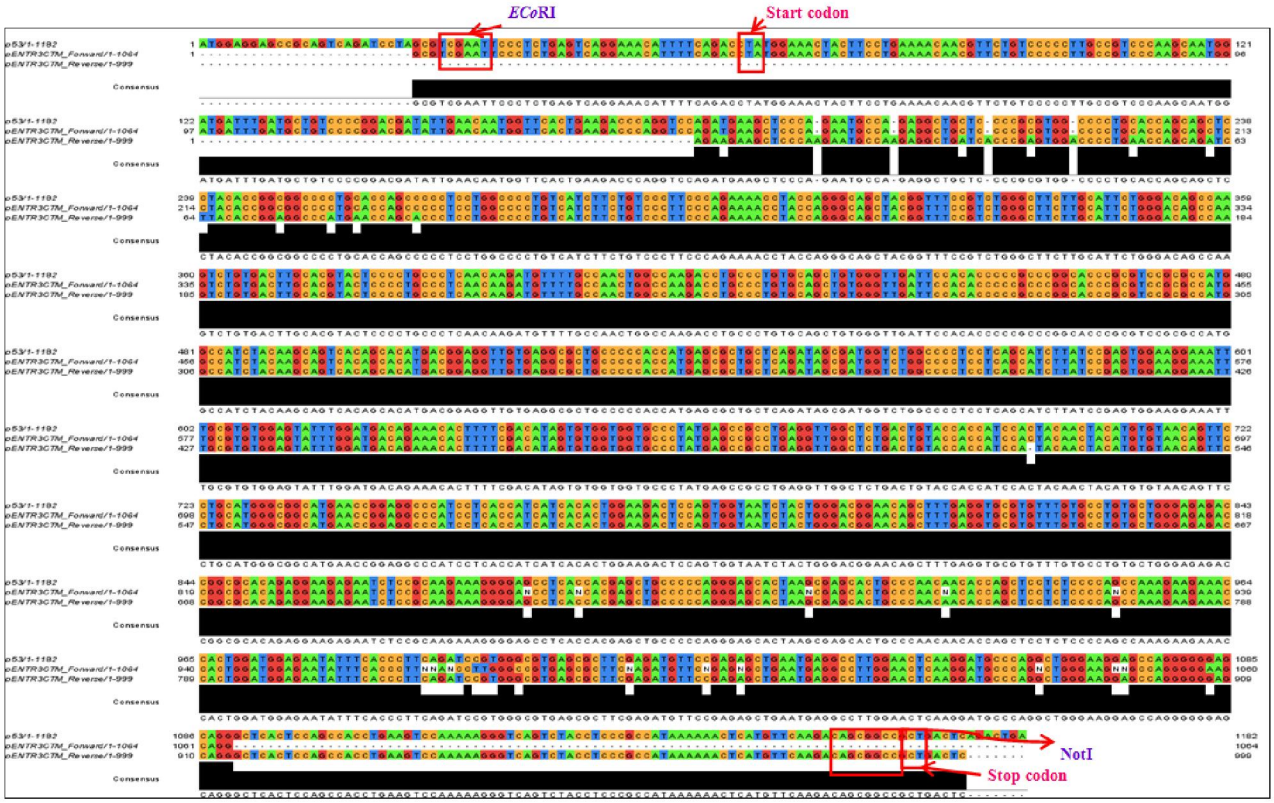
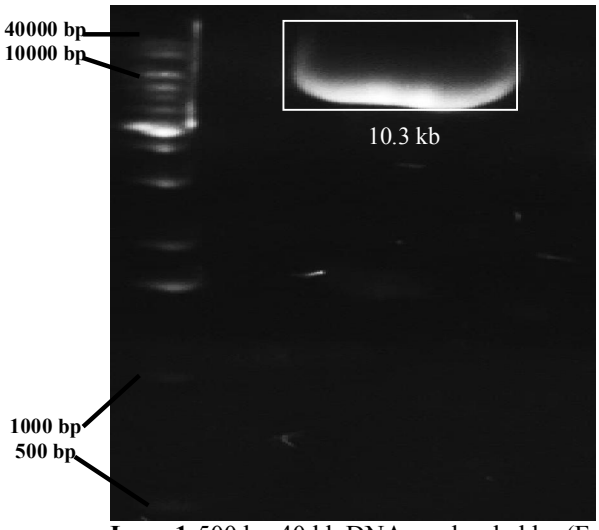


Figure 11. Alignment of forward and reverse sequencing of nucleic acid of the p53 (pENTR3CTM-p53-DsRed Monomer N1) PCR product and human tumor protein p53 gene (Entrez Gene ID: 7157)



Lane 1:500 bp-40 kb DNA marker ladder (Fermentas)
Lane 2: p53 PCR product
Lane 3-4: Ad5-p53-DsRed Monomer N1 PCR product

Figure 12. Electrophoresis analysis of p53 and Ad5-p53-DsRed Monomer N1 PCR amplification

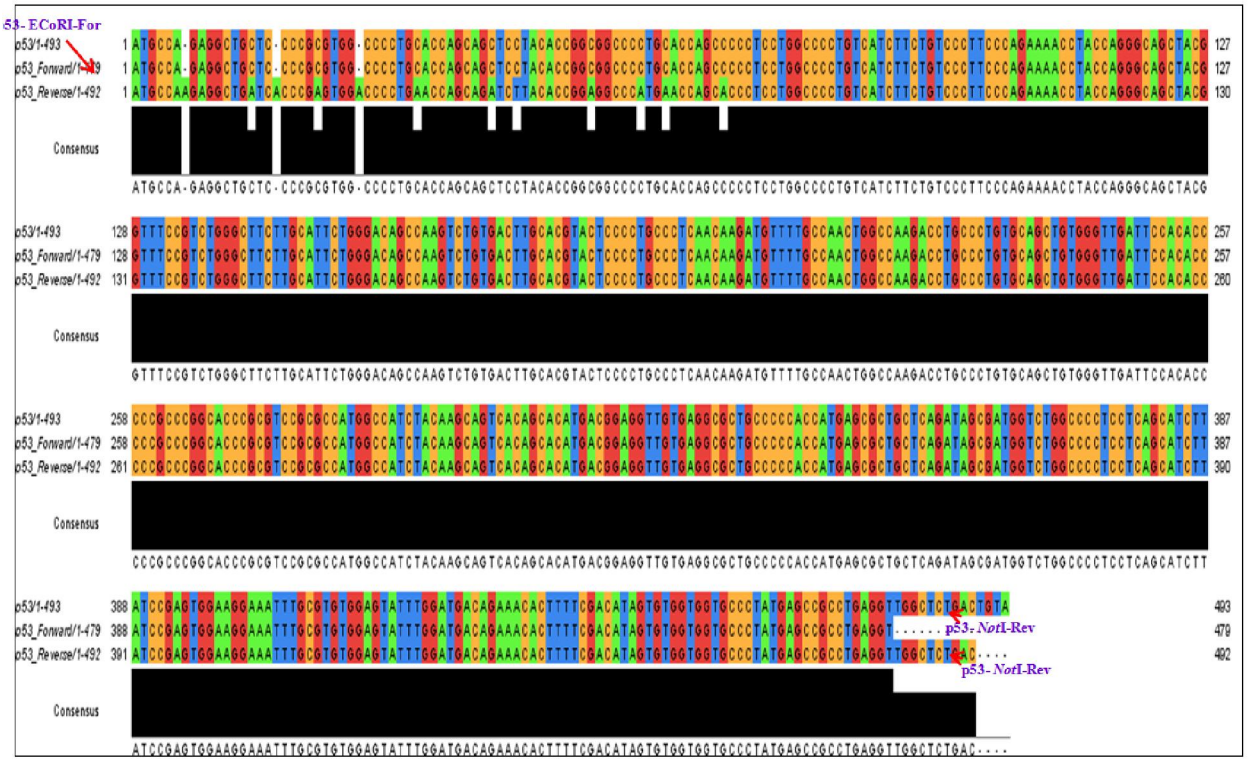


Figure 13. Alignment of forward and reverse sequencing of nucleic acid of the p53 (Ad5-p53-DsRed Monomer N1) PCR product and *Homo sapiens* tumor protein p53 gene (Entrez Gene ID: 7157)

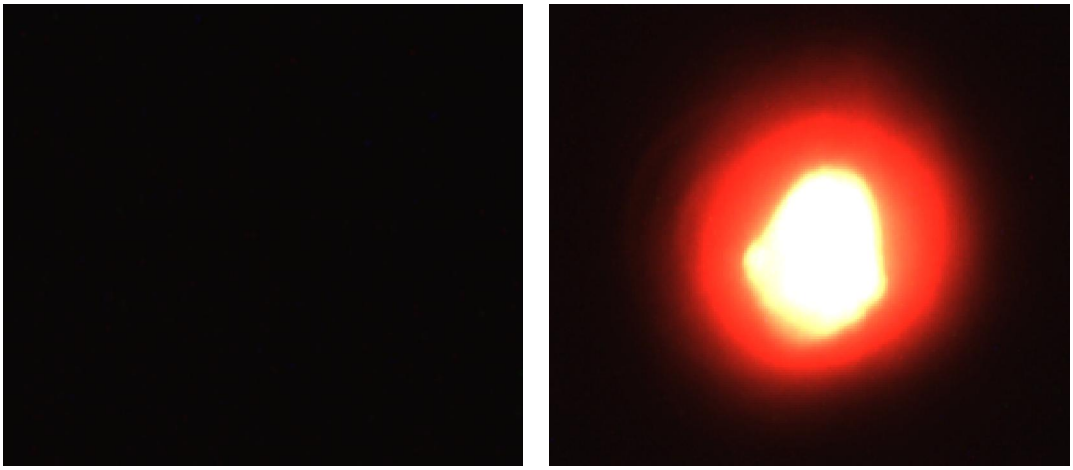
3.2.6 DNA sequencing analysis of transformants of Ad5-p53-DsRed Monomer N1

The p53 sequence was obtained by aligning the DNA sequence data (analyzed from 1st Base Sdn. Bhd) and human tumor protein p53 gene (Entrez Gene ID: 7157) using NCBI BLAST analysis. The forward and reverse sequences were corresponded 100% and 98% similarity to the human tumor protein p53 gene (Entrez Gene ID: 7157). For forward and reverse sequences, the scoring hit was 885 bits (479) and 839 bits (454) respectively. Each score links to the corresponding pairwise alignment between query sequence (Entrez Gene ID: 7157) and hit sequence (subject sequence). Furthermore, the E-value was 0 for both sequences. Therefore, they were statistically significant. They were homologous too as the E-value was lower than 10^{-5}. Moreover, the identities value for forward and reverse sequence obtained was 100% and 98% respectively. This implies that the forward and reverse sequence was 100% and 98% identical towards the query. In addition, 0% gaps were found in both sequences. This explains that there was no insertion and deletion occurred. Figure 13 shows a summary of a representation of a three-way sequence alignment with the *Homo sapiens* tumor protein p53 gene (5' to 3') which visualized using Jalview program.

In conclusion, human tumor protein p53 gene was successfully cloned into the pAd/CMV/V5-DEST™ vector through the entry clone (pENTR3C™).

3.3 DNA transfection using Lipofectamine™ reagent (Invitrogen)

With the completion of the cloning of the p53-DsRed Monomer N1 into the pAd/CMV/V5-DEST™ vector through pENTR3C™, transfection of Vero cells by Ad-p53-DsRed Monomer N1 was performed. Inverted fluorescence microscope (Nikon T*i* Eclipse) was used to detect DsRed Monomer N1 in Vero cells successfully transfected with Ad5-p53-DsRed Monomer N1 (Figure 14). The transfected cells presented diffuse red fluorescence from the DsRed Monomer N1 that appeared to be present throughout the cytoplasm and nucleus. The presence of DsRed Monomer N1 in cells was a strong indicator of transfection of the recombinant construct and thus possible expression of p53.



A B

Figure 14. (A) Non-transfected and (B) transfected Vero cell with the Ad5-p53-DsRed Monomer N1 plasmid was observed after 24 hours using inverted fluorescent microscope (Nikon T*i* Eclipse) (magnification 40X)

4. Conclusion

A novel recombinant virus (Ad5-p53-DsRed Monomer N1) was successfully constructed. It can be used as an anti-cancer agent in treatment of cancer patients.

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Corresponding Author:
Dr. Salehhuddin Hamdan
Department of Biological Sciences
Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia
81310 Skudai, Johor, Malaysia
E-mail: saleh65@utm.my

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