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Subcellular Localization of Transmembrane E-cadherin-GFP Fusion Protein and Expression in HeLa Cells

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Abstract - *E-cadherin, transmembrane protein tagged with GFP at its carboxy terminal (pcDNA3.1/CTGFP-TOPO® vector) cloned (TA cloning) in E.coli was expressed in a mammalian cell culture system (HeLa cells). Restriction endonuclease digestion using SmaI and application of colony PCR confirmed the correct orientation of the insert (2650bp) in the vector. The DNA sequencing and nucleotide BLAST analysis was used to investigate the integrity of the CDH1 gene prior to transfection. The vectors were transiently transfected in to 25% and 50% confluence HeLa cell mass aided by lipofectamine2000. Eventually, the fluorescent signals emitted by the successfully transfected cells were visualized using an epifluroscent microscope and confocal microscope. However, the resultant confocal microscopy analysis did not show any significant GFP signals from the transmembrane region of the cell. The GFP-CDH1 transcript expression level was determined by quantitative real time PCR analysis in which the qPCR data showed that GFP-E-cadherin expression did not show much fold difference when the tag location was C- or N-terminal of the protein, implying that the gene expression is independent of the tag position.*

Key Words: GFP-fusion proteins, E-cadherin, Restriction endonuclease digestion, qPCR

1. INTRODUCTION

The Green Fluorescent Protein (GFP) was derived from the jellyfish *Aequorea victoria* (yellow tissue masses comprising about 6000-7000 photogenic cells working with the enzyme aequorin) by Osamu Shimomura in 1972. Aequorin is known to be an enzyme that catalyzes the breakdown of luciferin which is the reaction responsible for the emission of light, bioluminescence [15]. The enzyme aequorin doesn't require a substrate or cofactor [4,9]. The green fluorescence emitted is directly proportional to luminescent energy generated by the induction of the calcium ions (energy is transferred to the protein luciferin which shifts the colour from blue to green) [18]. GFP comprises 238 amino acids in which certain amino acids have special roles, the amino acid serine at position 65, tyrosine at 66 and glycine at position 67 are known to be associated with the functioning of green fluorophore [21]. The significance of GFP as a reporter protein remained imaginative till 1992 but then in next few years' researchers gradually initiated employing GFP to investigate the regulation of gene expression and protein localization [3,5] when Prasher was successful in reporting the cloning of wild type GFP and obtaining the DNA sequence of the same [12]. Eventually, GFP gained popularity through different sectors of science resulting in a huge number of scientists to employ GFP in their research for performing different assays in immunochemistry and genetics [14,20]. GFP is used as fusion protein with many different proteins as they have the advantage of not being degraded by any proteases in the host and thus elevate the chance of stabilization and expression in the host.

E-cadherin, a type-1-transmembrane protein that aids in cell adhesion in association with calcium ions by the formation of adherents in junctions between cells within a tissue. The cell to cell adhesion is related to the coordination of cadherin domains present outside the cell membrane but the intracellular cytoplasmic tail is found to be associated with many adaptors and signalling molecules [7]. Jacob and his colleagues (1983), first described E-cadherin as a glycoprotein present in the cell membrane which is involved in cell compaction [8,11] and the mouse gene was later cloned [13]. All the above mentioned cadherin domains and adaptor proteins in the transmembrane together form the cadherin adhesome. The major components of cadherin superfamily found in most of the organisms are protocadherins, cadherins, desmogleins and desmocollins [1]. The genomic organization of the cadherin family was found to be very diverse, CDH1 gene encodes E-cadherin. Among many cadherins, the E-cadherin and N-cadherin were best characterised. Cadherins were considered as macromolecules that express in the cell surface leading to cell sorting and homophilic interaction specificity in the extracellular region [22]. Further, there are many reports stating the lateral dimerization of Ecadherin and N-cadherin having much similar crystal structures [10,16]. However, one of their domains were purely responsible for the crystal packing but not the others due to the absence of the calcium ions binding site.

Cancer and metastasis are found to inhibit the functioning of E-cadherin so these proteins are found degraded or in inactive stage in cancer cells [2]. The down regulation of E-cadherin eventually lowers the strength of cellular adhesion in a tissue, leading to increased cellular motility which is the reason behind spread of cancer cells from one organ or site to another affecting the tissues of new site. E-cadherin was found to be effective indicative molecule to detect breast cancer by pathologists. In a study related to immunohistochemistry, it was found that E-cadherin expression was remarkably reduced in the case of lobular carcinoma and severe ductal carcinoma [17].

The present study focused on C-terminal GFP tagged GFP-E-cadherin fusion protein localization in the transmembrane of the cell. CDH1 gene (2650bp) was cloned into the TOPO vector (uses topoisomerase that aids in highly efficient cloning) followed by transformation of *E.coli* cells for high copy number resulting in higher number of successful positive transformants screened by colony PCR and analysed by restriction endonuclease digest and sequence analysis. These selected clones with correct insert orientation (clone 7 and 8) were used to transfect HeLa cells and the cellular localization of GFP-E-cadherin fusion protein was studied followed by relative quantification of the GFP-E-cadherin fusion protein in the vector by quantitative real time PCR. Further, the fluorescent signals emitted were observed under confocal microscope.

2. METHODOLOGY

2.1 Amplification of target gene

CDH1 gene (target) was amplified by PCR technique using gene specific primers. The PCR master mix 1 comprised of 10µL (10X) PCR buffer, 8µL (25mM) magnesium chloride, 4µL (10mM) dNTP, 4µL of E-cad forward primer and C-terminal tag E-cad reverse primer, 1µL (1U/µL) Taq polymerase while PCR master mix 2 had an additional 4µL of DMSO.

Note:

E-cad forward primer: 5'-ACCATGGGCCCTTGGAGCCGC-3'

E-cad reverse primer: 5'-CGTCGTCCTCGCCGCCTC-3'

Both master mix 1 and 2 were made up to 92µL with distilled water. 23µL of master mix was added to three eppendorf tubes each to serve as test reactions (Set 1) followed by which the same was repeated with master mix 2 (Set 2). 2µL of E-cad (Gene 3) template DNA was added to tubes 1 and 2 while 2 µL of nuclease free water was added to tubes 3 in both the sets. The reactions were run in the thermocycler with initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 30 secs, 60°C for 30 secs, and 72°C for 1.5 min. Final extension of amplicons were performed at 72°C for 7min. The resultant PCR products were subjected to Gel electrophoresis for confirmation of the valid amplicon using 1% w/v agarose gel and 1X TBE. The gel was stained with SYBR green and run at 100V for 45 min (GeneRulerTM 1kb DNA Ladder was used as a DNA). The gel was visualized under UV light and the gel purified PCR products were quantified using NanoDrop spectrophotometric technique (Thermo Scientific NanoDrop 1000).

2.2 Ligation and Transformation

The GFP Fusion TOPO Cloning strategy was carried out by adding 4µL of fresh PCR product with 1µL of salt solution and 1µL of TOPO vector. The final volume was made to 5µL with addition of water. The mixture was incubated for 20 minutes at 37°C in order to ligate the PCR product in to the TOPO vector (6155bp). The transformation was carried out using One Shot TOP10 cells. 2µL of TOPO cloning reaction mixture was added to a vial containing the chemically competent TOP10 *E.coli* cells and incubated for 30 min in ice followed by another 30 sec incubation at 42°C (heat shock). The vial was immediately transferred to ice and left in shaker for about 1 hour at 37°C. 50µL, 100µL and 150µL of cells from the vial were plated on 3 LB agar plates containing ampicillin respectively. All 3 agar plates were incubated for 24 hours at 37°C.

2.3 Screening for transformants – Colony PCR

From the 3 agar plates, 10 transformant colonies were randomly selected and the insert was screened using T7 Forward and GFP Reverse primer. A fragment of 2650bp was expected to confirm the presence of the recombinant plasmid. PCR reactions were carried in the same way as described in the previous section (with DMSO) with cycling conditions as 94°C for 10 min; 30x (94°C, 30 secs; 60°C, 30 secs; 72°C, 2 min) and a final extension of 72°C for 10 min. Simultaneously, a LB agar plate was divided into 10 parts and labelled from 1-10, the colonies were streaked on corresponding patches on LB plate. The patch plate was incubated for 24 hours at 37°C.

2.3 Investigation on insert orientation

Colony PCR was performed to investigate the insert orientation by using two primer combinations (Gene specific Forward primer + GFP Reverse primer and Gene specific Reverse primer + GFP Reverse primer). Two master mixes were prepared in which the master mix 1 and the master mix 2 contained the primer combination 1 and 2 respectively with the total volume of 200µL for 8 reactions. Small amount of the cell mass from the patches of the patch plate was transferred to the corresponding PCR tubes containing the master mix and were labelled 1-6 for master mix 1 set and 1'-6' for master mix 2 set (one no template control for each set). The samples were amplified during PCR with reaction conditions 94°C for 10 min; 30x (94°C, 30 secs; 60°C, 30 secs; 72°C, 2 min) and a final extension of 72°C for 10 min. Finally, the samples were casted on an 1% agarose gel with 12 well comb fixed at the end of the well plate and a 9 well comb at the middle of the well plate to accommodate all the samples in one gel. The agarose gel electrophoresis was performed at 120V for 40 minutes and the gel was viewed under UV transillumination.

Culturing of plasmids with correct orientation

The two clones (colonies 7 and 8) identified to have the right orientation were inoculated in to 2mL of LB broth and were incubated at 37°C in a shaker for 200rpm for 24 hours.

Inspection of plasmid constructs

For both the culture tubes, plasmid extraction was performed. The cultures were centrifuged at 3000rpm for 2

minutes and then the supernatant containing the culture media was discarded.

The pellet containing the cells with our desired plasmids were resuspended in 250µL of buffer P1 containing RNase followed by addition of same amount of buffer P2 containing SDS (Sodium dodecyl sulphate) and sodium hydroxide (alkaline). The eppendorf tubes were gently mixed by inversion till attaining a clear solution. 350µL of Buffer N3 which acts as a neutralizing buffer was added and centrifuged at 3000 rpm for 10 minutes. The supernatants obtained were transferred into two spin columns (QIAprep) and were centrifuged at 13000 rpm for 1 minute. The flow through at the bottom of the column was discarded from both the columns and 500µL of buffer PB was added and again centrifuged at 13000 rpm for 1 minute followed by immediate removal of flow through and addition of 750µL of buffer PE, centrifugation repeated at same condition. The spin columns were transferred into two fresh 1.6mL eppendorf tubes and 50µL of sterile water was added to the membrane and left undisturbed for 1 minute followed by a final centrifugation at 13000 rpm for 1 minute. The spin column was discarded and the eluted plasmid DNA was stored on ice at -20°C. Nanodrop spectrophotometer analysis was performed to quantify the eluted DNA and simultaneously agarose gel electrophoresis was performed using three different volume of samples (1µL, 2µL and 4µL) for both the tubes and the gel was run at 120V for 60 minutes (1kB ladder was used for reference).

2.4 Restriction enzyme analysis

The restriction enzyme analysis of plasmid DNA extracted from clones 7 and 8 was carried out using 1µL of SmaI restriction enzyme with 10X buffer 4 (CutSmart) for about 5µL of plasmid DNA made up to a final volume of 20µL using sterile water. The digests were analyzed on agarose gel electrophoresis by casting the samples with 4µL of loading dye on 1% agarose gel run at 120V for 40 minutes.

2.5 DNA Sequencing

The clone 7 was taken for sequencing based on its 260/280 ratio (nanodrop spectrophotometer analysis). 7.12µL of plasmid DNA was transferred to two fresh eppendorf tubes labelled as 7001_47 and 7001_48 followed by addition of 1µL of T7 forward primer and GFP reverse primer respectively. Both the tubes were sequenced by Genome Research Facility and the sequences obtained were analyzed by ClustalX (multiple sequence alignment). The sequence results were further subjected to nucleotide blast (Blastn) to compare our sequence encoding E-cadherin with the nucleotide sequence database.

2.6 HeLa cell culture and transient transfection

HeLa cells grown in a T25 cell culture flask was provided. The confluency of the cells was recorded by visualization of the cells under inverted microscope. The culture medium was removed aseptically and the monolayer (adherent) cells washed with 5mL of pre-warmed 1X PBS (Phosphate buffered saline) was followed by addition of 2mL of trypsin and the flask was incubated at 37°C for 5 minutes for the cells to detach. The resultant single cell suspension was confirmed by checking the cells under inverted microscope. The contents were centrifuged at 1200rpm for 3 minutes. The pellet was resuspended in 5mL of fresh culture medium after discarding the supernatant. The cells obtained were counted using a haemocytometer and the percentage cell viability was calculated. 110µL of cell suspension was added to the first row of 24 well plate containing 390µL of culture media to have 50% confluence while in the second row of same plate, 94µL of cells were added to 406µL culture media to have 25% confluence.

Similarly, 110µL of cell suspension was added to the first row of 8 well plate containing 190µL of culture media to have 50% confluence while in the second row of same plate, 94µL of cells were added to 206µL culture media to have 25% confluence. The transfection of the Hela cells was performed by the treatment of cells with two different volumes of Lipofectamine2000 resulting in 2µL and 4µL transfections. 4µL (500ng) of plasmid DNA was added to The 2µL and 4µL of lipofectamine2000 diluted with 2mM Opti-MEM/L.Glutamax were mixed with 50µL of culture media without serum (contains no antibodies or antifungal agents) each resulting in mixture 1 and 2. The mixture 1 was added to the first two wells in the columns 2 and 3 of the 24 well plate and in first two wells of column 2 in 8 well culture plate with 54µL of plasmid DNA preparation in each well. Similarly, the mixture 2 was added to the first two wells in columns 4 and 5 of the 24 well plate and in first two wells of the columns 3 and 4 in 8 well culture plate with 54µL of plasmid DNA preparation in each well. The column 6 in 24 well plate and column 1 in 8 well plate served as no transfection control and the plates were incubated at 37°C for 48 hours in an incubator set up with 5% CO2 atmosphere.

2.7 Determination of mRNA expression levels

The 24 well culture plate was viewed under inverted microscope for selection of 2 wells with highest confluency and the first two wells of column 4 and 5 were taken for RNA extraction. The culture medium was removed and the cells were washed with with 500µL PBS solution twice followed by the addition of 175µL of SV RNA Lysis Buffer to break open the cell membrane. The genomic DNA released was sheared using a 1.5mL syringe by repeated draws through the 25-gauge needle and the whole content of the wells was transferred in to a fresh 1.6mL eppendorf tube. 350µL of SV RNA Dilution Buffer was added and mixed by inversion. The tube was subjected to heat treatment at 70°C for 3 minutes followed by centrifugation at 13000rpm for 10 minutes. The clear lysate was transferred into a new tube and treated with 200µL of 100% ethanol, whole content of the tube was transferred to a spin basket assembly and centrifuged at 13000rpm for a minute. The eluate was discarded and 600µL

of SV RNA wash solution was added and same centrifugation step repeated. The eluate was discarded and 50µL of DNase incubation mixture was added followed by 15minutes incubation at 37°C. Then 200µL of SV DNase Stop Solution was added and centrifuged for 1 minute at 13000rpm followed by the addition of 600µL of SV RNA wash solution, centrifuged at 13000rpm for 1 minute. Supernatant was discarded and the wash step repeated with 250µL of SV RNA wash solution, centrifuged at 13000rpm for 2 minutes. Again, the supernatant was discarded and about 100µL of nuclease free water was added to the membrane, centrifuged at 13000rpm for 1 minute. The eluted RNA was quantified using a nanodrop spectrophotometer. 4.5µL of this RNA was used for cDNA synthesis using 0.5µL of of random hexamers (50µM), 0.5µL of dNTP's (10mM), 0.1µL of DTT and 2µL of reaction buffer (5X). 0.5µL of Superscript II RNase minus Reverse Transcriptase was used to aid in reverse transcription. Finally, Q-PCR of cDNA samples was performed to elucidate on mRNA expression levels using GFP master mix and GAPDH master mix.

2.8 Confocal microscopy and image acquisition

In 8 well culture plate, the culture media was removed from the wells and the transfected wells were washed with 400µL of PBS. The cells of these wells were fixed using 400µL of 100% ethanol. 350µL of Hoescht 33342 stain was added to the first well in column 1(No transfection) and both the wells of column 3 (4µL transfection) and left undisturbed for 5 minutes followed by PBS wash twice. 3µL of mounting medium was added to each well and a rectangular coverslip was placed on it and the slide was visualized under OLYMPUS BX51 Epifluorescent upright microscope and ZEISS LSM 510 META Confocal microscope.

3. RESULTS

3.1 Amplification of the target gene

The amplification of gene encoding E-cadherin was performed by PCR in two sets in which the resultant bands in agarose gel were in the form of thick smears of size 8000bp for samples without DMSO but bands of two samples added with DMSO around 2650bp confirmed the presence of target gene in the sample amplified (figure 1). These bands were sliced and weighed to be 0.207g. The DNA recovered from the gel was purified and quantified having the concentration of 123.4ng/µL.

3.2 Ligation and Transformation

Ligation was successful and the colonies selected transferred to the patch plate did show growth after the incubation period.

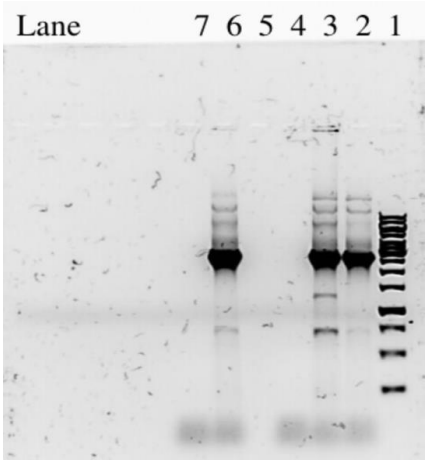

Fig -1: Agarose gel image of PCR amplified products of size ranging from 2000bp to 3000bp corresponding to CDH1 gene (2650bp). GeneRulerTM 1kb DNA Ladder (lane 1)

3.3 Screening for transformants – Colony PCR

Positive transformants were screened by non-directional colony PCR. The gel run did not show any bands under UV transillumination but the DNA ladder was run perfectly which may be due to improper voltage supply and reduced time for agarose gel electrophoresis that the larger gene could not diffuse out through the well (figure 2).

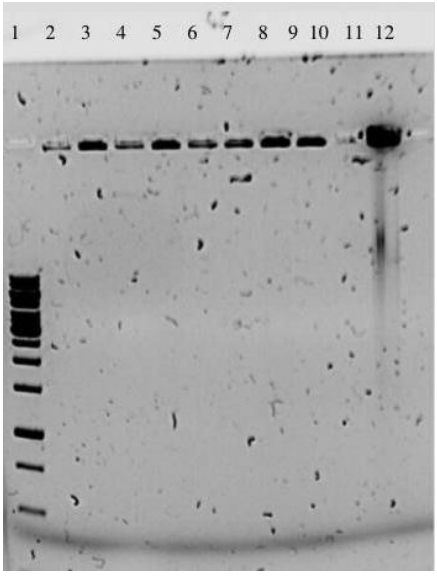

Fig -2: Agarose gel image of non-directional Colony PCR amplified product to screen the colonies with insert. GeneRulerTM 1kb DNA Ladder (lane 1) was used as marker.

3.4 Investigation on insert orientation

The positive clones 6, 7 and 8 were found to have correct orientation in directional colony PCR. No template controls for both sets did not show any bands confirming that there are no contaminants. The GeneRulerTM 1Kb DNA ladder (lane1 and 6') provides the evident that bands obtained for the positive clones corresponds to our target gene (figure 3).

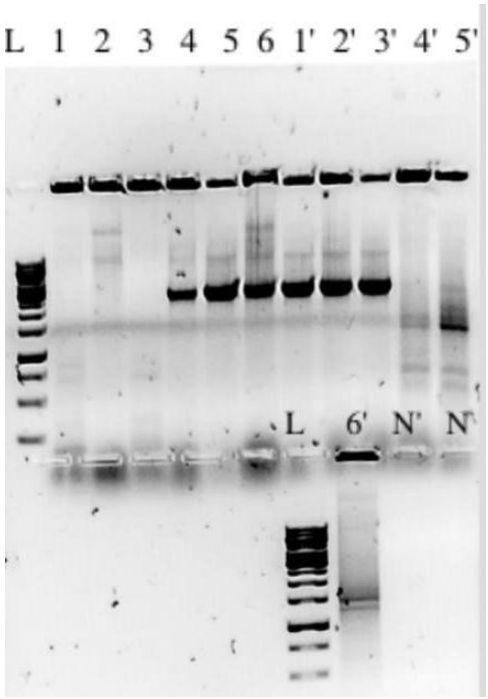

Fig -3: Agarose gel image of directional Colony PCR amplified product to determine orientation of the insert. GeneRulerTM 1kb DNA Ladder (lane L) was used as marker.

3.5 Inspection of plasmid constructs

The DNA was eluted from the selected plasmid constructs and their quantification by nanodrop spectrophotometer resulted in yield of 6309ng for clone 7 and 5409.7 for clone 8 with a total volume of eluted DNA being 45µL and 47µL respectively. In agarose gel electrophoresis, the increase in volume of sample (1µL, 2µL and 4µL) showed increase in number of bands which were seen as thick smears. For clone 7, 1µL of plasmid (lane 1) resulted in a single band of 9100bp while 2µL (lane 2) and 4µL (lane 3) resulted in thick smears of range 8500bp and 8900 respectively. For clone 8, the two volumes 1µL and 2µL (lane 4 and 5) resulted in thin smears of size 9000bp but 4µL (lane 6) resulted in thick smear of size 8000bp.

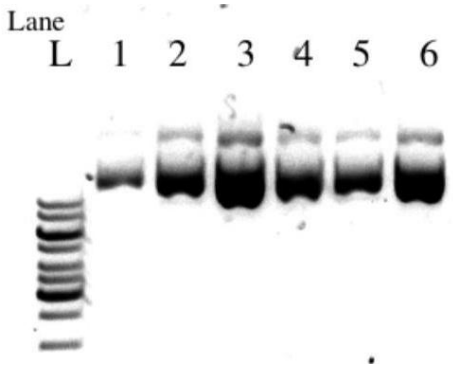

Fig -4: Agarose gel image of plasmid DNA of different volumes extracted from clones 7 and 8. GeneRulerTM 1kb DNA Ladder (lane L) was used as marker.

3.6 Restriction enzyme analysis

The restriction digest resulted in fragments of size 6553bp as bands in the gel for clone 7 and 8 (lanes 1 and 2) which confirmed that the positive clones were in the correct orientation but did not exactly match the expected result (bands at 6553bp and 3251bp) derived for SmaI digest of target gene that excise the plasmid at 2 sites.

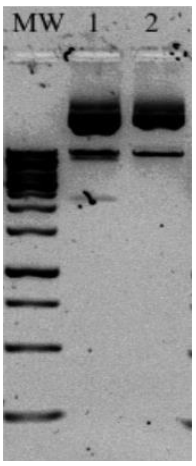

Fig -5: Restriction endonuclease digest analysis of the plasmid with the insert using SmaI (MW lane refers to the molecular weight marker, restriction enzyme digest of clone 7 and 8 in lanes 1 and 2 respectively). GeneRulerTM 1kb DNA Ladder (lane 1) was used as marker.

3.7 DNA Sequencing

The DNA sequence of clone 7 was amplified using T7 forward and GFP reverse primer which matched the consensus sequence of Homo sapiens mRNA for E-cadherin, AB25106.1 having 97% identity (E = 0) which confirmed that the vector contained the target gene (CDH1).

3.8 HeLa cell culture and transient transfection

The cell culture obtained in T25 flask had about 98% confluence and the culture plates prepared meet the expected confluence levels (50% and 25%). The viable cell count was found to be 904000 cells and 12000 non-viable cell count per 5 (large) squares counted resulting in a percentage viability of 98.7%.

3.9 Determination of mRNA expression levels

The 24 well culture plate was used for this study, mRNA extracted was quantified using nanodrop spectrophotometer and was found to have a concentration of 5.7ng/µL for 2µL transfection and 20.4ng/µL for 4µL transfection. The mRNA expression levels were insistent by the incorporation of quantitative real time PCR using cDNA synthesized. The relative quantification of GFP-CDH1 transcripts based on tag position within the plasmid resulted in a fold difference of 1.020 (Table 1).

Table -1: Relative quantification of GFP-CDH1 transcripts based on tag position within the plasmid

Tag Position	Sample	GFP	GAPDH	ΔCt	ΔΔCt (ΔCtCT - ΔCtNT)	Fold Difference 2-ΔΔCt
CT	1	18.73	21.92	-3.19	-0.029	1.020
	2	18.72	21.56	-2.83		
	3	19.04	21.38	-2.34		
NT	1	17.49	20.29	-2.80	0.066	0.955
	2	17.61	20.36	-2.75		
	3	17.82	20.44	-2.61		

3.7 Confocal microscopy and image acquisition

The 8 well culture plate was used for preparing the slide for epifluorescent microscopy. The slide was initially observed in OLYMPUS BX51 Epifluorescent upright microscope in which a huge mass of cells was observed but no fluorescence emitted. When the same slide was observed under ZEISS LSM 510 META Confocal microscope, using different filters again there was no significant fluorescent signals identified for both 50% and 25% confluence regions with 4µL transfection.

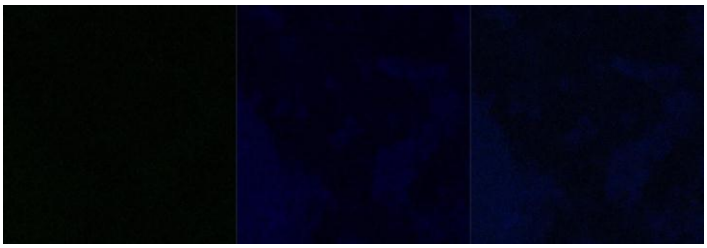

Fig -6: HeLa cells with 50% and 25% confluence after transfection with GFP-CDH1 Gene (E-cadherin) transmembrane signals were absent when visualized under confocal microscope.

4. DISCUSSION

The CDH1 gene (E-cadherin) was successfully cloned into pcDNA3.1/CT-GFP-TOPO vector generated from transformant *E.coli* colonies. The amplification of the gene by PCR showed that specific bands at 2650bp were seen when the gene was amplified in the presence of DMSO which is due to the fact that DMSO prevents the formation of secondary structures. The quantification of the desired DNA band recovered from the gel slice by nanodrop spectrophotometer served as an evidence of good amount of pure DNA sample (260/280 ratio = 1.97) and concentration of the DNA (123.4 ng/µL). The ligation of the vector and transformation was successful even though the colony PCR did not show any bands which may be due to irregular voltage supply and reduced time for the run of the gel as the same sample resulted in characteristic bands while performing colony PCR for confirming the insert orientation. The method of transformation employed was TA cloning method so there is a 50% chance for insert to be in correct orientation or flip and fix in wrong orientation [6]. Therefore, the result obtained was reasonable depicting 3 colonies in correct (clone 6,7 and 8) and the other 3 (clone 1,2 and 3) in wrong orientation. As the analysis of construct proved clone 7 to have higher yield with higher amount of pure DNA, it was selected for use for further analysis of the E-cadherin coding gene. The agarose gel electrophoresis of the eluted DNA proved that increase in volume of sample that is directly proportional to its concentration did increase the band thickness. The bands representing the vector (6155bp) with the insert (2650bp) were close to that expected (8805bp). However, the bands for 1µL and 2µL showed similar bands due to small difference in DNA concentration. The restriction endonuclease digest analysis of the eluted DNA matched the expected results only to a certain limit (Figure 7).

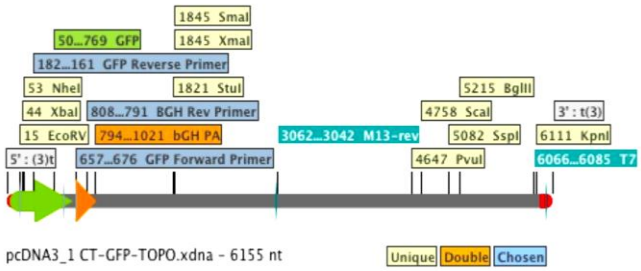

Fig -7: Restriction enzyme map of E-cadherin in expression vector (Designed using Serial cloner 2-6-1)

The bands to be found at 2251bp were missing as the restriction enzyme provided was not sufficient resulting in higher concentration of uncut plasmid seen as a thick smear of size 8804bp [19]. There is also a chance that the 2251bp bands got folded and were seen as a single band of size 6553bp (lane 6). The DNA sequencing process resulted in a sequence of length 1751bp, which was used as the template or query sequence in nucleotide BLAST search that identified the sequence to show 97% similarity with Homo sapiens mRNA for E-cadherin, AB25106.1 with a few nucleotide substitutions. However, these substitutions did not change the amino acids coded by the region and hence the protein encoding gene was taken for expression analysis by transfection in HeLa cells. Two different volumes (2µL or 4µL) of transfection with Lipofectamine2000 corresponding 25% and 50% confluent cells were analyzed. The HeLa cells being transfected with plasmid containing the CDH1 gene tagged with GFP should give fluorescence in the transmembrane region taking up the Hoescht stain. There was no characteristic fluorescence signal emitted by the cells when observed in epifluorescent or confocal microscope which may be due to the fact that the cells did not take up the Hoescht stain. The quantitative real time PCR data demonstrated that GFP-CDH1 transcript expression levels were same irrespective of the tag position with no significant fold difference.

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