

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**OPTIMIZATION OF THE RECOMBINANT PD-1 PROTEIN
EXPRESSION IN TRANSIENTLY TRANSFECTED
MAMMALIAN CELL LINES**

VELİCAN ENGİN GAYİR
A THESIS SUBMITTED FOR THE DEGREE OF
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GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

GEÇİCİ OLARAK TRANSFEKTE EDİLMİŞ
MEMELİ HÜCRE HATTINDA PD-1
PROTEİNİ REKOMBİNANT İFADESİ
OPTİMİZASYONU

VELİCAN ENGİN GAYIR
YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

DANIŞMANI
PROF. DR. IŞIL KURNAZ

GEBZE
2022



YÜKSEK LİSANS JÜRİ ONAY FORMU

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SUMMARY

The human PD-1 protein was produced as a project output that can be used for cancer-related analytical purposes. PD-1 is one of the most popular protein that can able to inhibit the activation of T cells thus resulting in the proliferation of cancerous cells. Monoclonal antibody-based drugs which can block the development of cancerous cells by blocking the PD-1 protein, have taken their place on the market. In line with the given information, the PD-1 protein, which was expressed as a project output, was a potential final product that can be used in the bioassay development for monoclonal antibodies and therefore in cancer treatment studies.

The mentioned thesis was based on optimization of expression and analytical potential of human PD-1 protein from the transiently transfected HEK293T (Human Embryonic Kidney) cell line. Cell viability after transfection was demonstrated by cell counter device, protein expression was checked according to GFP luminescence by using fluorescent microscopy, and quantified by using image analysis software. According to yield values, the data was investigated by using “design of experiment” tools to figure out the effects of parameters for higher expression rates. The cells expressed human PD-1 with optimized transfection parameters were used to screen the binding property of blocking antibody, by using a flow cytometry device and an ELISA test kit.

Key Words: Programmed Cell Death-1, Transient Transfection, Recombinant DNA Technology, Mammalian Cell Culture, Blocking Antibodies.

ÖZET

İnsan PD-1 proteini, kanserle ilgili analitik amaçlar için kullanılabilecek bir proje çıktısı ürün olarak üretildi. PD-1 proteini, T hücrelerinin aktivasyonunu inhibe edebilir ve böylece kanserli hücrelerin çoğalmasına yol açabilir. PD-1 proteinini bloke ederek kanserli hücrelerin gelişimini engelleyebilen monoklonal antikor bazlı ilaçlar piyasada yerini almıştır. Verilen bilgiler doğrultusunda proje çıktısı olarak ifade edilen PD-1 proteini, monoklonal antikorların biyo-analitik testlerinde ve dolayısıyla kanser tedavisi çalışmalarında kullanılabilecek potansiyel bir nihai ürün olma potansiyeline sahiptir.

Söz konusu yüksek lisans tezi, geçici olarak transfekte edilmiş HEK293T (İnsan Embriyonik Böbrek) hücre hattında insan PD-1 proteininin ifade değerlerinin optimizasyonunu ve analitik denemelerini kapsamaktadır. Transfeksiyon sonrası hücre canlılığı, hücre sayacı cihazı ile gösterildi, protein ifadeleri floresan mikroskobu kullanılarak GFP ışımaya göre kontrol edildi ve görüntü analiz yazılımı kullanılarak nicelendirildi. Verim değerlerine göre, veriler en iyi parametreleri bulmak için “deney tasarımı” araçları kullanılarak karşılaştırıldı ve gruplandırıldı. Optimize edilmiş transfeksiyon parametreleriyle insan PD-1 proteinini ifade eden hücreler, bir akış sitometri cihazı ve bir ELISA test kiti kullanılarak bloke edici antikorun bağlanma özelliğini taramak için kullanıldı.

Anahtar Kelimeler: Programlanmış Hücre Ölümü-1, Geçici Transfeksiyon, Rekombinant DNA Teknolojisi, Memeli Hücre Kültürü, Bloke Edici Antikorlar

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
CMV	: Cytomegalovirus
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DPBS	: Dulbecco's Phosphate-Buffered Saline
FBS	: Fetale Bovine Serum
FSC-H	: Forward Scatter – Height
FITC	: Fluorescein isothiocyanate
GFP	: Green Fluorescent Protein
HEK 293T	: Human Embryonic Kidney 293 T cell
HRP	: Horseradish Peroxidase
LA	: Luria-Bertani Agar
LB	: Luria-Bertani Broth
mAb	: Monoclonal Antibody
NCBI	: National Center for Biotechnology Information
NK	: Natural Killer
NSCLC	: Non-Small Cell Lung Cancer
ORF	: Open Reading Frame
PBS	: Phosphate-Buffered Saline
PBS-T	: Phosphate Buffered Saline with 0.05 % Tween-20
PCR	: Polymerase Chain Reaction
PEI	: Polyethyleneimine
PD-1	: Programmed Cell Death -1 Protein
PD-L1	: Programmed Cell Death Ligand 1 Protein
PD-L2	: Programmed Cell Death Ligand 2 Protein
rpm	: Revolutions per Minute
SSC-H	: Side Scatter – Height
TMB	: 3,3',5,5'-Tetramethylbenzidine
WB	: Western Blot

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1. INTRODUCTION

1.1. What is PD-1?

As an inflammatory disease, “cancer” is known to trigger the responses by native immunity cells such as macrophages and neutrophils and T and B cells as adaptive immunity. Innate immunity effect “cancer” disease without any specific recognition for immunogenic antigens while adaptive immunity regulates specific response to tumor-associated antigens [Disis, 2010]. The combination of given immune system effects lead to harmonically inhibiting cancer progression. Among the adaptive immune system checkpoints, one of the most popular interaction pathways occurs between the B7 protein family these are transmembrane co-stimulatory ligands which are expressed on antigen-presenting cells, and CD28 protein family co-stimulatory receptors. These checkpoint interactions have several effects on regulating immune cell activation and directing inflammation and autoimmunity [Vesely et al., 2011].

Programmed Cell Death 1 (PD-1) is a member of the CD28 family and is a transmembrane receptor protein expressed by T cells, B cells, Natural Killer cells (NKs), and myeloid cells and has direct effects on the functional situation of immune system cells in abnormal situations such as infectious diseases and cancers. The PD-1 binds to the specific ligands, PD-L1, and PD-L2; the members of the gene family B7. These ligands are usually expressed in the membrane part of several peripheral tissue types such as tumor and hematopoietic cells. The triggered interaction of these proteins directs the immune responses to the way of the inhibition of “T cell receptor-mediated lymphocyte activation” and “cytokine secretion” [Dong et al., 2002]. As such, PD-1 pathway blockade methods, may amplify the T cell activity more specifically to tumor-like tissues and provide an immune control which fastens the destruction of the cancerous cells [Iwai et. al., 2002], [Hirano et. al., 2005].

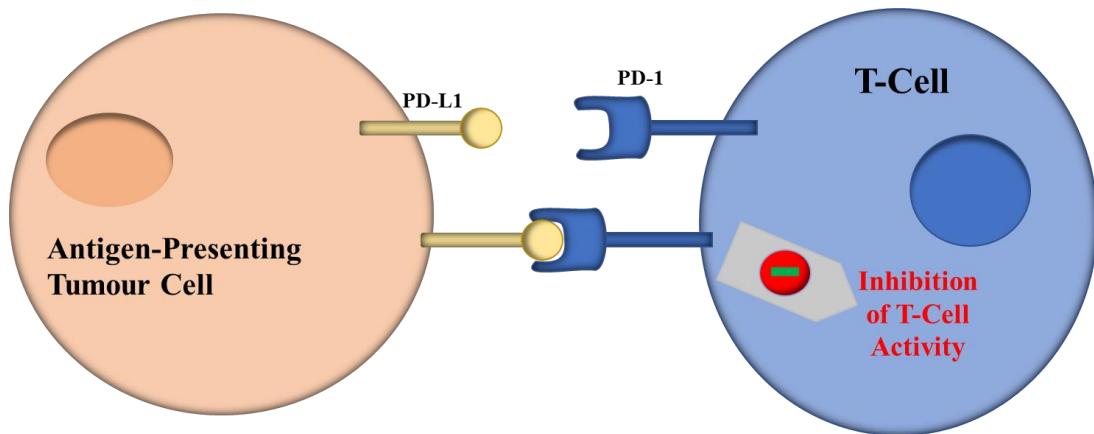


Figure 1.1: Graphical representation for the interaction of PD-1 and PD-L1 and effect to T-cell.

PD-1 protein, otherwise known as programmed cell death protein 1 precursor (*Homo sapiens*; NCBI accession codes: NP_005009.2 for protein sequence, NM_005018.3 for nucleotide sequence) is the focus of this thesis study. Protein contains 288 amino acid residues and is encoded by 2097 base pairs. Topology data covers 4 different regions withstands on protein sequence of PD-1 [Web 1, 2021].

10	20	30	40	50
MQIPQAPWPV	VWAVLQLGWR	PGWFLDSPDR	PWNPPTFSPA	LLVVTEGDNA
60	70	80	90	100
TFTCSFSNTS	ESFVLNWYRM	SPSNQTDKLA	AFPEDRSQPG	QDCFRFVTQL
110	120	130	140	150
PNGRDFHMSV	VRARRNSGT	YLCGAISLAP	KAQIKESLRA	ELRVTERRAE
160	170	180	190	200
VPTAHPSPSP	RPAGQFQTLV	VGTVGGLLGS	LVLVWVLAV	ICSRAARGTI
210	220	230	240	250
GARRTGQPLK	EDPSAVPVFS	VDYGELDFQW	REKTPEPPVP	CVPEQTEYAT
260	270	280		
IVFPSGMGTS	SPARRGSADG	PRSAQPLRPE	DGHCSWPL	

Signal Sequence	Extracellular Domain	Transmembrane	Cytoplasmic Domain
-----------------	----------------------	---------------	--------------------

Figure 1.2: Topology data refers to 4 sections of protein sequence: Green: Signal sequence, Orange: Extracellular Domain, Yellow: Transmembrane section, Blue: Cytoplasmic Domain.

1.2. PD-1 Targeting Therapies

Medical Biotechnology, or “Red Biotechnology”, has partly focused on biomanufacturing of "Blocking antibodies" for PD-1, the main strategy has been to cure inflammatory diseases by the inhibition of immune system checkpoint interactions that occurs between PD-1 and PD-1 ligands [Pardoll, 2012], [Leach et al., 1996]. Since the PD-1 protein expression helps the tissue cells to avoid activated T-cells [Ishida et al., 1992], recently completed as well as ongoing clinical trials target these interactions of these molecules and their ligands and achieve pathway boost with remarkable results in curative clinical responses against cancer [Chinai et al., 2015].

In the global market, immunotherapies that target PD-1 checkpoint protein were estimated to be over USD 19 billion in 2019 and estimations indicate that market volume will be worth over USD 36 billion by 2024 [Web 2, 2020]. Nivolumab (Opdivo), Pembrolizumab (Keytruda), and Cemiplimab (Libtayo) are the first group of immune checkpoint inhibitors specified for Programmed Cell Death-1. These human IgG monoclonal antibodies against PD-1, are currently using for treatments of more than several cancers and tumor-based diseases such as Non-Small Cell Lung Cancer (NSCLC), Melanoma, Brest Cancer, Renal Cancer, Head/Neck Cancer, and squamous cell carcinomas [Vaddepally et al.,2020].

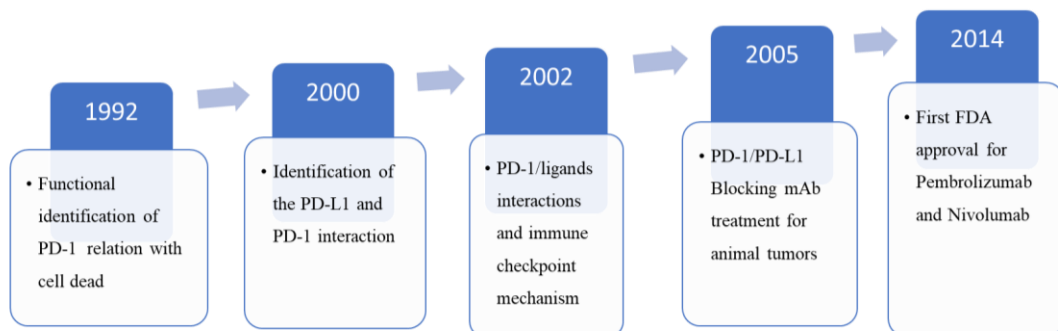


Figure 1.3: Milestones for identifying and exploiting PD-1 in Cancer Treatments.

1.3. Benefits of Recombinant Protein Expression

The increased attention to PD-1 blocking antibodies increased demand for human PD-1 protein as raw material, which is necessary for bioanalytical assay development, thus characterization of antibodies.

Various biotechnology companies produce extracellular domain of PD-1 proteins with affinity tags as recombinant proteins, however, these proteins are not in their natural localization within the membrane, which may interfere with the interaction of anti-PD-1 antibodies with these recombinant PD-1 proteins. This study aims for recombinant production of full-length human PD-1 protein localized on the plasma membrane to devise a cell-based bioassay platform. To that end, a mammalian cell line (HEK293T) is selected for cell factory, the gene of interest is purchased in a mammalian expression plasmid contains CMV promoter, and for transfection of cells, transient transfection method was chosen, as it produces larger quantities of the recombinant PD-1 protein in a relatively short period.

Recombinant protein expression concept is based on the transfection of genetic materials to host organism using vectors such as plasmid DNA as used in this thesis study, and the eukaryotic host cell line chosen in this study can express eukaryotic functional proteins with the appropriate post-translational modifications [Longo et al., 2013]. Transfection systems may be specialized into two groups: stable transfection and transient transfection [Stepanenko and Heng, 2017]. Transient transfection methodology has satisfying advantages in terms of faster production timelines as easier and cheaper pre- and post-bioproduction processes.

Stable transfection, on the other hand, requires the generation of stable cell lines, which in turn requires screening of dozens of transfected cells for integration of the target DNA into the host chromosome, and this process may take several months to identify the best-producing clone(s) [Bandaranayake and Almo 2014]. Furthermore, since the target DNA is integrated into the chromosome, and since this integration is most commonly to only one of the two homologous chromosomes (haploid) and not both (diploid), the amount of protein produces is much less than in transient transfection strategy. Furthermore, the epigenetic state of the integration point also influences the level of protein expression from the target DNA since random integration may be to a “silenced” region of the chromosome. Specific chromosomal

loci can be selected for integration to achieve optimal integration, however, site-specific recombination will make stable cell line generation even longer and more difficult.

On the other hand, since depending on the plasmid copy number a host cell may receive multiple copies of the plasmid, transcription, and translation from all plasmids within the cell will ensure high levels of protein expression. Yet, the downside with the transient transfection method is that the cells lose the vectors during different rounds of cell division since the plasmids will not replicate themselves and be eventually diluted when divided among the progeny. Still, in small-scale protein expression studies as well as for our bioassay platform study, it is a faster and more economical method compared to stable transfection.

Chemical transient transfection reagents are generally based on lipid materials which show cationic properties for efficient DNA clustering and pH buffering potential that can protect DNA/reagent complex from being attacked by nucleases when lysosomal swelling occurs [Boussif et al., 1995]. Polyethyleneimine (PEI), one of the most used transient transfection reagents, has promising features in terms of stabilization periods in different storage parameters and cost-effectiveness of experiments [Ehrhardt et al., 2006]. Moreover, PEI enables higher transfection efficiencies when compared to other commercial transfection reagents for HEK293 cells that we have chosen for recombinant expression of PD-1 protein [Yang et al., 2017].

1.4. Aim of the Thesis

PD-1 is a critical protein for investigating and developing curative proteins towards the therapy of inflammatory diseases such as cancer. Therefore, this thesis aims to express PD-1 protein localized on cells, as rapidly and cost-effectively for establishing a bioassay platform to test various anti-PD-1 antibodies. Despite extensive academic literature on the PD-1 mechanism of action, there is a lack of data towards basic know-how of expression optimization of human PD- 1 protein in transiently transfected cells. The present thesis' aim is the optimization of human PD- 1 protein expression in transiently transfected HEK293T mammalian cell lines and the potential of these transfected cells for use in bioassay development.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Laboratory Materials and Equipment

2.1.1.1. Laboratory Materials

Petri dishes (100 mm) (Lamtek), Tissue culture plates (6-well, 24-well, 60 mm - TRP), tissue culture flasks (T25, T75, T150 - TRP), Falcon tubes (15 ml, 50 ml CAPP), serological pipettes (5 ml, 10 ml, 25 ml - CAPP), Microcentrifuge tubes (1.5 ml, 2ml), Cryotubes (1ml, 2ml), micropipette tips (10 μ L, 100 μ L, 1mL)

2.1.1.2. Laboratory Devices and Equipment

Micropipettes (Eppendorf), -80°C freezer (VWR), CO₂ incubator (Stericycle i160 - Thermo Scientific), Laminar Flow Cabinet – Safe 2020 (Thermo Scientific), Spectrophotometer – NanoDrop (Shimadzu), Spin (Sprout), Vortex (JeioTech), ZOE Fluorescent Cell Imager (Bio-Rad Laboratories), Heat Block (JeioTech), Liquid nitrogen tank (Taylor-Wharton, LS4800), Hemocytometer (Marienfeld Improved with Bright Line), 10X Light microscope (used for Cell counting), Water Bath (JeioTech), -20°C freezer, +4°C freezer.

2.1.2. Bacterial Materials

Escherichia coli DH5 α bacterial strain was selected for cloning and amplifying of the expression plasmid.

Luria-Bertani Agar (Caisson Labs), Luria-Bertani Broth (Caisson Labs), Kanamycin (Biomatik), CaCl₂ (Acros Organics), Incubator Shaker (BTLab Systems), Plasmid isolation kit (DNA-Spin Plasmid Purification Kit, Intron Biotechnology).

2.1.3. Plasmid and Transfection Reagent

Transfection was done with polyethyleneimine (PEI) transfection reagent. Expression plasmid pCMV3-PDCD1-GFPSpark is purchased from Sino Biological Inc. Detailed information related to plasmid is given in Table 2.1, Figure 2.1, and Figure 2.2.

Table 2.1: Information of purchased plasmid.

Catalog Number	HG10377-ACG
Gene	Programmed cell death1(PDCD1)
Source	Human
cDNA Size	867bp
RefSeq ID	NM_005018.2
Plasmid Name	pCMV3-PDCD1-GFPSpark
Vector Size	6848bp
Antibiotic Resistance	Kanamycin

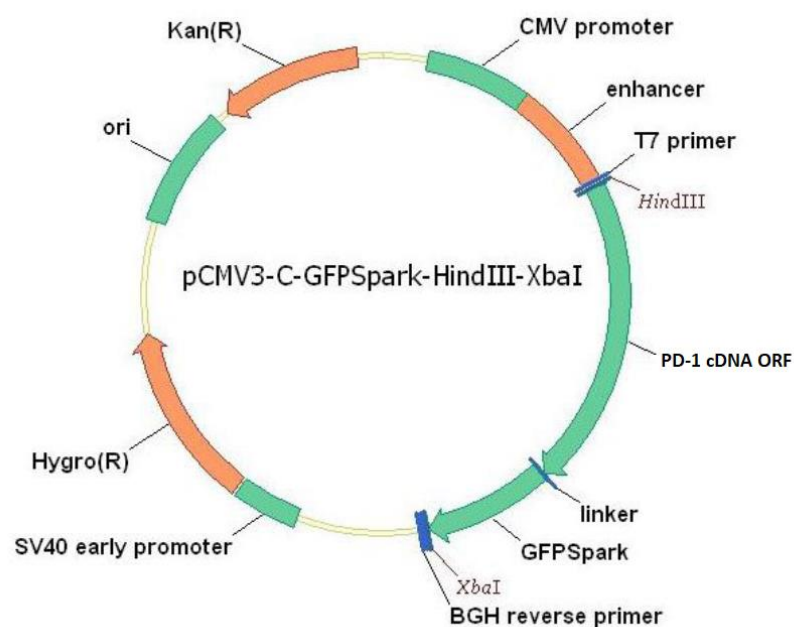


Figure 2.1: Plasmid map.

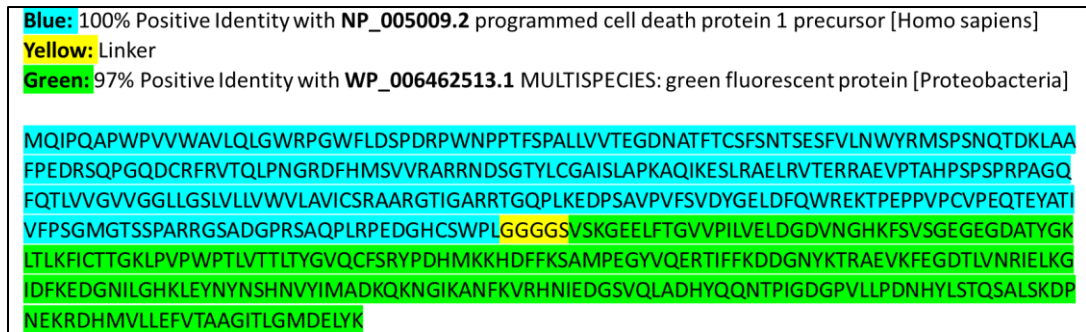


Figure 2.2: Protein FASTA sequence of ORF within the plasmid. BLAST tool was used to investigate protein sequence which was coded by the plasmid (Blue: gene of interest, PD-1; Yellow: linker sequence; Green: GFP protein).

2.1.4. Cell Line and Culture Consumables

HEK293T (Human Embryonic Kidney 293T) cells were stocked in our laboratory. The origin of the cell line is ATCC with ID: CRL-3216 [Web 3, 2021].

Dulbecco's Modified Eagle Medium (DMEM) 1X with L-glutamine and 4,5g/L Glucose, without Pyruvate (Gibco), Dulbecco's Modified Eagle Medium (DMEM) 1X with L-glutamine, pyruvate and 4,5g/L Glucose (Gibco), Fetal Bovine Serum (FBS) (Gibco), Dulbecco's Phosphate Buffered Saline 1X (DPBS) (Gibco), 0.05% Trypsin-EDTA Solution 1X (Gibco), Penicillin + Streptomycin (10000U-ml Penicillin, 10mg/ml Streptomycin)(PAN Biotech), Dimethyl sulfoxide (DMSO) (BIOFROXX).

2.2. Methods

2.2.1. Competent Cell Preparation

E. coli DH5 α colony glycerol stock sample was taken out from -80°C freezer and waited for defrosting at room temperature (25°C). The dissolved cell mixture was put into 10mL of LB Broth medium within a 15mL falcon tube and incubated at 37°C - 180 RPM overnight. The next day, 1mL of growth medium was added to 44mL of LB Broth medium and incubated at 37°C - 200 RPM for 3 hours. Afterward, the inoculum was centrifuged at 25°C - 5500 RPM for 10 minutes. The cell pellet was dissolved with 1mL ice-cold CaCl₂ and completed to 5mL with ice-cold CaCl₂ and incubated in ice for 1 hour. Then, centrifuged at 4°C - 3500 RPM for 5 minutes, the pellet was dissolved with 1mL of supernatant and put into a microcentrifuge tube to store at 4°C freezer. Competent cells were used within 1 to 2 days for transformation.

2.2.2. Plasmid Resuspension

The microcentrifuge tube, which was containing lyophilized plasmid, was centrifuged at 25°C – 5000xg for 5 minutes. 100 μ L of water for injection (WFI) was added and incubated for 10 minutes at room temperature. Then, the tube was mixed with vortex and a quick spin run was applied at 25°C – 5000x g. The plasmid-containing solution was aliquoted to 4 microcentrifuge tubes, divided into 2-2 groups to store them in -20°C and -80°C.

2.2.3. Plasmid Transformation into *E.coli* DH5 α

50 μ L of freshly prepared component cells were mixed with 2 μ L of suspended plasmids in a microcentrifuge tube. For negative control, the same amount of component cells was taken into a microcentrifuge tube. Both tubes were incubated in ice for 15 minutes, incubated at 37°C for 90 seconds, and then incubated in ice again for 2 minutes. 200 μ L of LB Broth medium was added and mixtures were incubated at 37°C – 200RPM for 1 hour in a shaker incubator. After incubation, the transformation

mixture and negative control sample were poured and spread onto LB Agar plate that contained 15µL Kanamycin antibiotic. Plates were incubated at 37°C overnight.

2.2.4. Plasmid Isolation

Given methodology was used for each sample, if more amounts of plasmid are needed, the same procedure can be multiplied for several samples.

A single colony was picked from the transformation LB Agar plate and incubated at 37°C – 200RPM for 16 hours. After incubation, bacteria culture was centrifuged at 25°C – 13000RPM for 30 seconds, and the supernatant was discarded. The bacterial pellet was resuspended with 250µL of RNase added resuspension buffer by vortexing. 250µL of lysis buffer was added, the tube inverted gently 10 times and incubated at room temperature for 3 minutes. Then, 350µL of neutralization buffer was added, the solution was mixed by inverting the tube 10 times and incubated in ice for 5 minutes and the mixture was centrifuged at 4°C – 13000RPM for 10 minutes. The obtained supernatant was transferred to a plasmid filtration column that was placed into a collection tube, assembly was centrifuged at 4°C – 13000RPM for 60 seconds, the filtrate was discarded, the step was repeated with 500µL of washing buffer A, 700µL of washing buffer B, and without any solution, was completed with dried filtration column. The filtration column was incubated with 50µL of water for injection pre-heated to 60°C and plasmid DNA was collected by centrifugation at 25°C – 13000RPM for 60 seconds. The collected solution (50µL) was measured by Spectrophotometer- Nanodrop for plasmid DNA quantities.

2.2.5. Cell Culture Defrosting and Seed Inoculation

HEK293T cell line was removed from the liquid nitrogen tank and placed at 37°C for thawing. Then, defrosted cells were incubated in a T-25 flask with complete DMEM medium (High Glucose medium + %10 FBS and %1 Penicillin Streptomycin) at 37°C - %5 CO₂ for 4 hours. Afterward, the medium was refreshed with a fresh medium. Each time point that the cell confluency was reached 80%, cells were treated with trypsin, collected, and counted with Hemocytometer (Marienfeld Improved) that was cleaned with 70% alcohol before taking into laminar airflow cabinet. 10µL of cell

growth medium was placed onto the hemocytometer glass, covered with a coverslip, and examined by a 10x light microscope. Cells in the 4 groups of 16 squares at the corners are counted, averaged, and multiplied by 10,000 which was resulted in the number of cells in a milliliter of cell growth medium. According to the surface area of cell culture flasks or dishes, the required numbers of cells passaged for proliferation.

2.2.6. Cell Culture Passaging and Proliferation

When T-25 and T-75 flasks were reached to >80% cell confluency, the medium was pulled out, cells were rinsed with 1mL 1X DPBS (3mL for T-75) and the flask was tilted flatwise to wash out all remaining medium from the flask, then DPBS solution was collected out. 500 μ L of Trypsin (1mL for T-75) was added and the flask tilted again, incubated the flask at 37°C for 1 minute. 1mL of complete DMEM medium (5-10mL for T-75) was added to inactivate trypsin. The flask was hit by sides to successful detachment of cells from flask surface. The cell mixture was collected and centrifuged at room temperature – 1500RPM for 5 minutes. The cell pellet was dissolved with a complete DMEM medium; cell counting was applied, required number of cells were taken to be incubated before transfection or for passaging to obtain cell proliferation.

2.2.7. Transient Transfection of Cells

According to the optimized number of cells per surface area of flask/plate (cell confluency >40%), cells were counted and injected into new flask/plate (24-well and 6-well flasks, 60mm plate) and incubated at 37°C – 5% CO₂ for overnight. The next day, the required amount of PEI (1mg/mL stock) was mixed with the serum-free DMEM medium to 25% of the total working volume of the flask or plate. The PEI mixture was incubated at room temperature for 5 minutes. Then, the required amount of plasmid DNA was added to the mixture and vortexed for 10 seconds and incubated at room temperature for 20 minutes. By the time, the growth medium was gently removed from the cell culture flask/plate and complete DMEM medium was added with an amount of 25% of the total working volume. When incubation time ended, the DNA+PEI mixture was dropped over cell culture as snail motif slowly, then incubated

flask/plate at 37°C – 5% CO₂ for 2 hours. After 2 hours of incubation, the cell culture was filled to 100% of total working volume with complete DMEM medium and incubated at 37°C – 5% CO₂ for 48 hours.

2.2.8. Transfection Efficiency Quantification

When 48 hours incubation time ended, protein expression was followed with GFP luminescence by using ZOE Fluorescent Cell Imager (Bio-Rad). Full bright and fluorescence images of the same frame of cells were taken and analyzed with ImageJ software [Schneider et al., 2012]. The idea behind the quantification of expression using images was based on the determination of “area” which resulted as “rates”. Full bright images were used to determine cell cultures' confluency rate within the taken frame, cells were selected with lines, and surface areas were determined by using the “Measure” command. Afterward, threshold limits were set to greenfield images that show GFP luminescence, the surface area of luminescence spots was determined with the "Analyze particles" command. The surface area of the luminescence spot was divided by the surface area of total cells, the result was multiplied by 100 to obtain the percentage result which gave transfection efficiency. At the same time, both images were superimposed for visualization of percentile results in a single image.

2.2.9. Antibody Binding Assay

Flow cytometer and ELISA assays were performed with commercial mAb against PD-1 protein to show PD-1 interaction. Cell lines were prepared in our laboratory and experiments were built and applied with help of Ayça SÖYLEMEZ, Junior Bioassay Specialist within Bioassay Laboratory of Nobel Pharma Biotech.

2.2.9.1. Sample Preparation

Flow cytometry was used to determine binding behaviors of commercial mAb (Merck Sharp & Dohme Corp) with HEK293T cells expressed PD-1 (HEK+) and stock HEK293T cells (HEK-) as the negative control (TT-60 Experiment).

The growth medium was discarded and both cells (HEK+ and HEK-) were harvested by gently scraping them into complete DMEM media. The cell culture mixtures were centrifuged at 400 g for 5 min. The supernatants were discarded, and pellets were resuspended in fresh, room temperature complete DMEM medium to wash off any remaining cell debris and proteins. Another centrifugation was applied at 400 g for 5 minutes at room temperature. The supernatants were discarded, and pellets were resuspended in the appropriate amount of room temperature complete DMEM medium. Cells were counted using a hemocytometer with Luna Cell Counter (Logos Biosystems), cell viability was also obtained using the same equipment. Collected cells were used for mAb binding determination.

2.2.9.2. Flow Cytometer Analysis for mAb Binding

Once counted, the cells were diluted with cold (4°C) complete DMEM medium to a minimum concentration of 1×10^5 cells/ml. 400 µl of the cell suspensions were aliquoted into microcentrifuge tubes. Antibody was added at the rate of vendor-recommended dilution (2000 µg/mL) and mixed well. The mixtures were incubated at 4°C for 1 hour while avoiding direct light. 50µL of samples were injected into a flow cytometer (Novocyte Quanteon Agilent).

2.2.9.3. ELISA Analysis

Once counted, the cells were diluted with a cold (4°C) complete DMEM medium. Cells were (HEK+ and HEK-) diluted to wells as following given table 3.3. The plate was incubated at 37°C for 4 hours in a CO₂ incubator to made cells confluent. TT-2A, TT-2B, and TT-ELISA plates were directly jumped to the below steps. All volume amounts were given for 96-well plate at the below steps, for 24-well plate experiments, all volumes were multiplied with “6”. When confluency was obtained, the growth medium was removed, and cells were washed with 1X DPBS. Then DPBS was removed, 100µL of PBS containing %4 paraformaldehyde was added and incubated for 15 min at room temperature. After incubation, cells were washed with PBS 3 times, and PBS was removed.

200µL of blocking buffer (Thermo Scientific) was added. Cells were incubated for 2 hours at room temperature. Blocking buffer was removed and cells were washed with PBS-T (%0.05) (Phosphate Buffered Saline with 0.05% Tween 20 – Sigma Aldrich) 3 times. 100µL of different serial dilutions of Keytruda prepared incubation buffer (prepared with complete DMEM medium) (varying concentration values were mentioned in results part) were added to each well (for TT-2A and TT-2B plates, mAb concentration was applied at constant value of concentration). Incubation was applied for 1 hour at room temperature at 500rpm shaking. Then cells were washed with PBS-T (%0.05) 3 times. Secondary antibody (HRP conjugated Human Fc (1/60000 dilution) - Sigma Aldrich) was prepared, added as 100µL, and cells were incubated in a dark place for 30min at room temperature at 500rpm shaking. Cells were washed again with PBS-T (%0.05) 3 times. 100mcl of TMB substrate was added and color change was followed. Readings were performed at 655nm, and reaction stop was planned as at OD value of 0.700-0.800 stop the reaction with stop solution. Reading was performed at 450nm.

3. RESULTS

3.1. *E.coli* Transformation and Plasmid Isolation

E. coli DH5 α cells are transformed with pCMV3-PDCD1-GFPSpark plasmids, and Kanamycin antibiotic was used for selection.

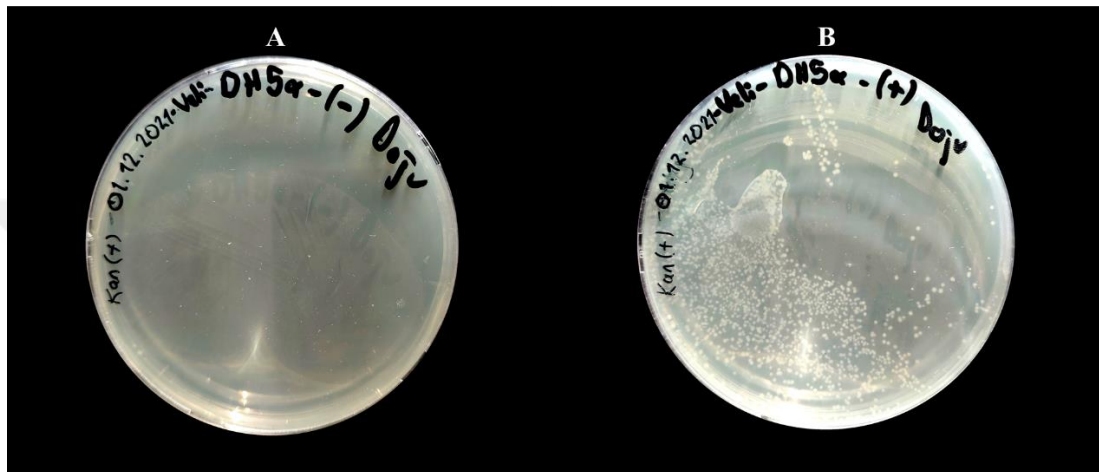


Figure 3.1: *E.coli* DH5 α cells were inoculated on LB Agar plates: A) Negative control plate (Stock cells without plasmid transformation); B) Transformation plate (cells with plasmid transformation).

In Figure 3.1, visible single colonies were observed for transformed cells, despite cells were not observed for the negative control plate. Variable numbers of colonies were inoculated in LB Broth media to grow biomass for plasmid isolation. Plasmid isolation was applied 3 times. The first experiment was set with 3 colonies, second and third experiments were set with 5 colonies to obtain a higher amount of plasmid DNA. Plasmid concentration results were obtained was satisfactory as given in Table 3.1.

Table 3.1: Plasmid isolation nanodrop results. Sample 1: First set of plasmid isolation experiment with three samples; Sample 2: Second set of plasmid isolation experiment with five samples; Sample 3: Last set of plasmid isolation experiment with five samples.

Sample	Plasmid Concentration	Sample	Plasmid Concentration	Sample	Plasmid Concentration
1A	99.2 ng/ μ L	2A	143.0 ng/ μ L	3A	77.0 ng/ μ L
1B	114,4 ng/ μ L	2B	118.7 ng/ μ L	3B	102.6 ng/ μ L
1C	110,5 ng/ μ L	2C	143.8 ng/ μ L	3C	85.3 ng/ μ L
		2D	109.4 ng/ μ L	3D	86.0 ng/ μ L
		2E	100.0 ng/ μ L	3E	78.1 ng/ μ L

3.2. Transient Transfection Optimization - Transfection Efficiencies

HEK293T cells were transiently transfected with pCMV3-PDCD1-GFPSpark purified plasmids and PEI as transfection reagents. 3 repeats of transfection were performed in a 24-well plate, labeled as TT-1, TT-2A, and TT-2B for better explanation within the results section. DNA/PEI ratio, number of cells, and DNA amounts were the parameters changed crossly to screen transfection efficiencies. Incubation was continued at 37°C – 5% CO₂ for 48 hours. Experiment parameters that were set up, as given in Figure 3.2.

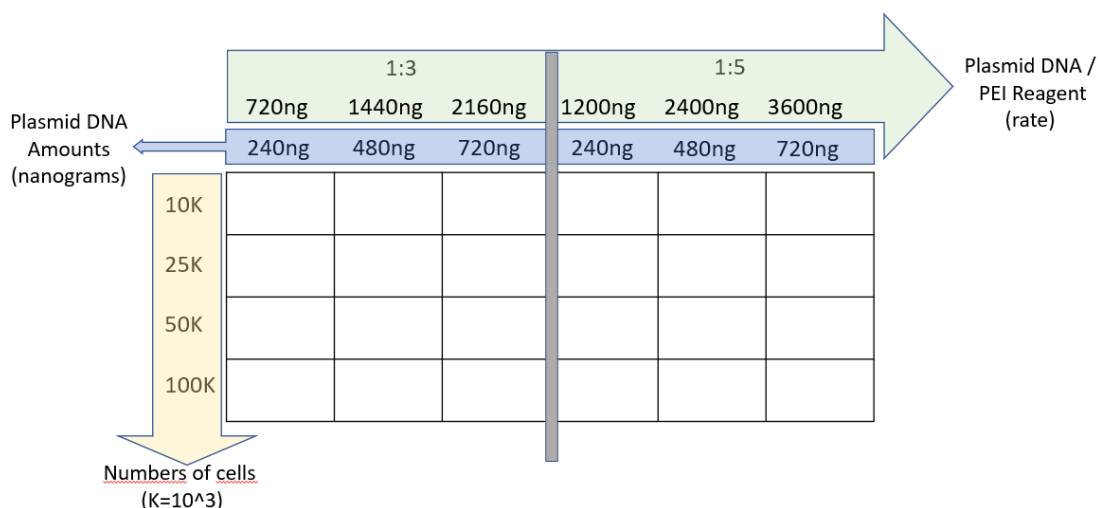


Figure 3.2: Transient transfection experiment parameters set up in a 24-well plate. Green arrow: DNA amount rates per PEI reagent amounts (upper row) and determined PEI reagent amounts (ng per well- lower row); Blue arrow: Plasmid DNA amounts (ng per well); Yellow arrow: Cell numbers (per well).

After 48 hours of incubation, expression was detected with GFP fluorescence responses. Images that had better frames were taken in full bright and ultraviolet format at a scale of 100 μ m with ZOE Fluorescent Cell Imager. Microscope images were used for visualization with respect to the result and ImageJ software was used for area measurements, as described in the methodology. Figure 3.3 was prepared with the purpose to demonstrate the usage of ImageJ software over microscope images of the TT-1 experiment as an example. Superimposition was also demonstrated for visualization of transfection efficiency differences over well plates, therefore superimposed images of all transient transfection experiments (TT-1, TT-2A, and TT-2B) are attached to the Appendices section.

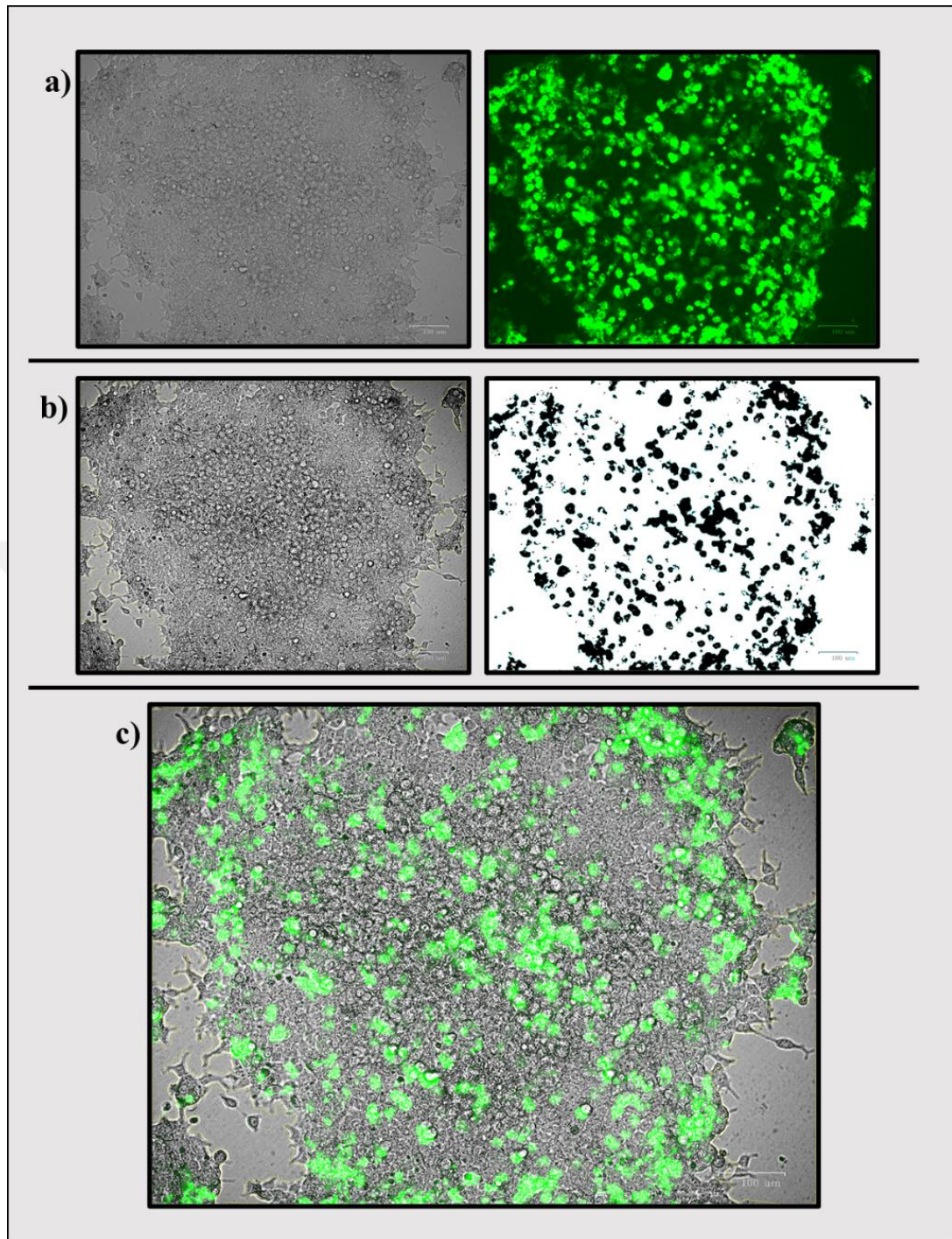


Figure 3.3: TT-1 experiment: Images of the well with highest transfection efficiency percentage (DNA: 720ng, PEI:3600ng, Cell: 100×10^3). a) Default images as full bright (left), ultraviolet (right); b) Images processed with using ImageJ; c) Superimposed images.

3.2.1. TT-1 Experiment

TT-1 was the first experiment set up, which was a non-repetitive experiment. Transfection efficiency results were given in Table 3.2 and GFP fluorescence areas were given in Table 3.3.

Table 3.2: TT-1 experiment: Transfection efficiencies as percentage in each cell of the 24-well plate. Comma signs were used as decimal separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “ $\times 10^3$ ”.

TT-1 Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0%	5,67%	7,33%	8,84%	11,44%	17,08%
Cells: 25K	5,81%	4,51%	15,84%	7,99%	13,57%	7,97%
Cells: 50K	2,06%	4,05%	10,18%	9,60%	3,11%	7,14%
Cells: 100K	3,37%	6,36%	14,93%	3,40%	15,18%	22,75%

The results given in Table 3.2 are showing the variation of transfection efficiencies. 100×10^3 cells /720ng DNA /3600ng PEI has presented the highest transfection efficiency.

Table 3.3: TT-1 experiment: GFP fluorescence areas as μm^2 of each cell on the 24-well plate. Dots were used as thousand separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “ $\times 10^3$ ”.

TT-1 Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0	3.577.562	1.981.628	7.362.763	7.316.311	2.968.448
Cells: 25K	17.466.309	2.599.665	14.982.915	15.424.920	45.173.742	24.965.309
Cells: 50K	13.190.928	25.894.436	51.473.941	56.267.364	18.776.200	31.756.183
Cells: 100K	25.134.683	46.174.174	93.402.171	23.513.113	87.773.699	157.123.711

The results given in Table 3.3 are showing GFP fluorescence areas of transfected cells in each well. The data collection is not accurate enough to comment about DNA and PEI amount parameters over transfection, however, wells with 100×10^3 cells presented dramatically higher areas over remaining wells, especially 100×10^3 cells / 720ng DNA / 3600ng PEI ($157.123.711 \mu\text{m}^2$)

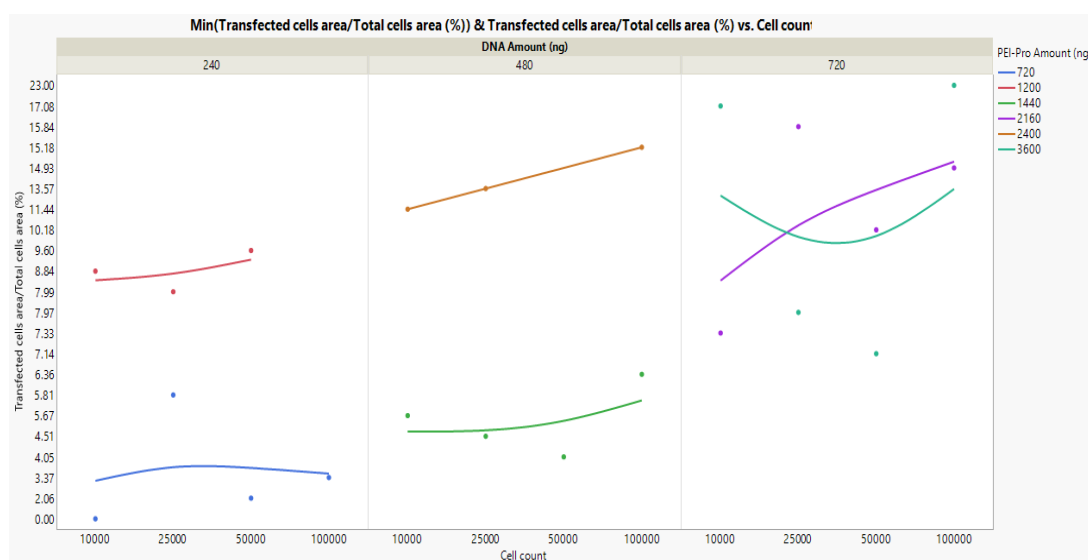


Figure 3.4: TT-1 Experiment: Transfection efficiency percentages against cell count graph. Points were indicated for yield percentages per cell count and colors were indicated for 2 different rates of PEI over DNA (3:1 and 5:1). Colorful lines were indicated for mean values of yields for each cell count and PEI rate.

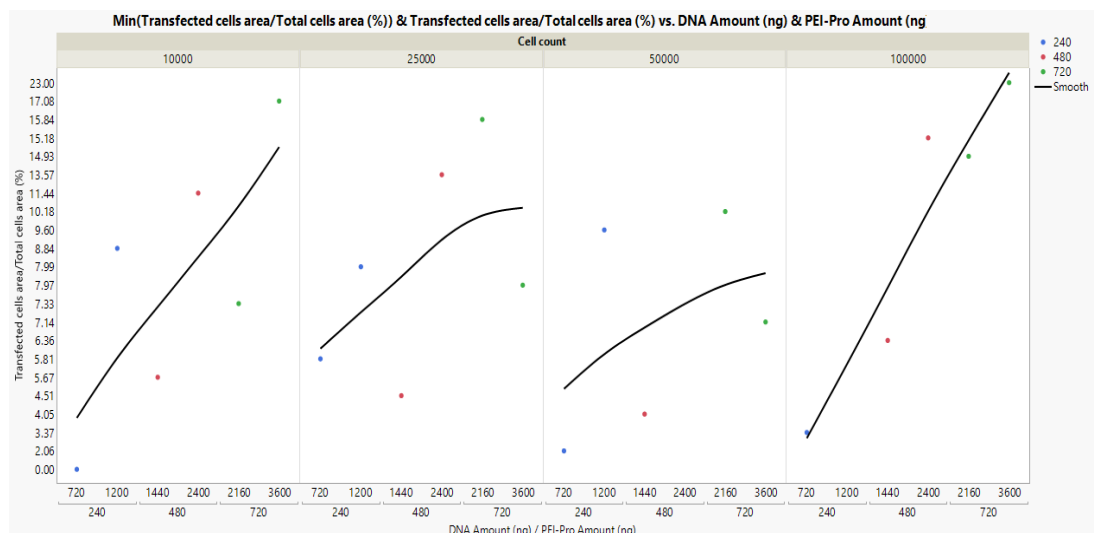


Figure 3.5: TT-1 Experiment: Transfection efficiency graph against the combination of DNA amounts & PEI amounts. The Y-axis was indicated for yield percentages. The X-axis was indicated with DNA amounts & PEI amounts combination to better visualization of DNA amount effect on yield. Colors were added to make the graph easier to be read, which were also indicating DNA amounts.

3.2.2. TT-2A and TT-2B Experiments

TT-2A and TT-2B are two replicate experiments based on identically the same parameters and values with TT-1.

Table 3.4: TT-2A and TT-2B experiments: Transfection efficiencies as percentage in each cell of the 24-well plate. Comma signs were used as decimal separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “x10³”.

TT-2A Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0%	2,39%	3.04%	0%	1.73%	14.73%
Cells: 25K	0%	0.43%	1.02%	0.34%	4.64%	14.77%
Cells: 50K	0.06%	10.54%	17.84%	6.09%	18.21%	18.06%
Cells: 100K	0.50%	1.85%	4.19%	2.01%	8.34%	13.16%

TT-2B Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0%	2.88%	4.26%	1.10%	2.31%	5.58%
Cells: 25K	0%	9.70%	11.37%	0.50%	6.87%	17.10%
Cells: 50K	1.37%	9.14%	14.85%	1.90%	9.61%	16.50%
Cells: 100K	1.58%	7.49%	3.99%	0.28%	2.79%	2.41%

TT-2A – TT-2B Average Efficiency Percentanges	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0,00%	2,64%	3,65%	0,55%	2,02%	10,16%
Cells: 25K	0,00%	5,07%	6,20%	0,42%	5,76%	15,94%
Cells: 50K	0,72%	9,84%	16,35%	4,00%	13,91%	17,28%
Cells: 100K	1,04%	4,67%	4,09%	1,15%	5,57%	7,79%

The results given in Table 3.4 are showing the variation of transfection efficiencies over two replicate experiments applied in 24-well plates (TT-2A and TT-2B). For TT-2A, 50x10³ cells /480ng DNA /2400ng PEI presented highest transfection

efficiency (18.21%), and for TT-2B, 25×10^3 cells /720ng DNA /3600ng PEI presented highest transfection efficiency of plate (17.10%). Average efficiency values were also mentioned within Table 3.4, in which 50×10^3 cells /720ng DNA /3600ng obtained the highest transfection efficiency (17.28%).

Two replicates of the experiment enable the investigation of parameters' effectiveness on transient transfection as visualized in Figures 3.6, 3.7, and 3.8. To obtain valuable results, duplicate results of each well were handled to calculate mean and standard deviation values. Respected results of each parameter were handled, the mean value was calculated and represented as “Blue Bar”. Lines that were drawn onto bars indicated standard deviations. Numerical data of the “mean” has been given as red and “standard deviation” data has been given in brackets as red.

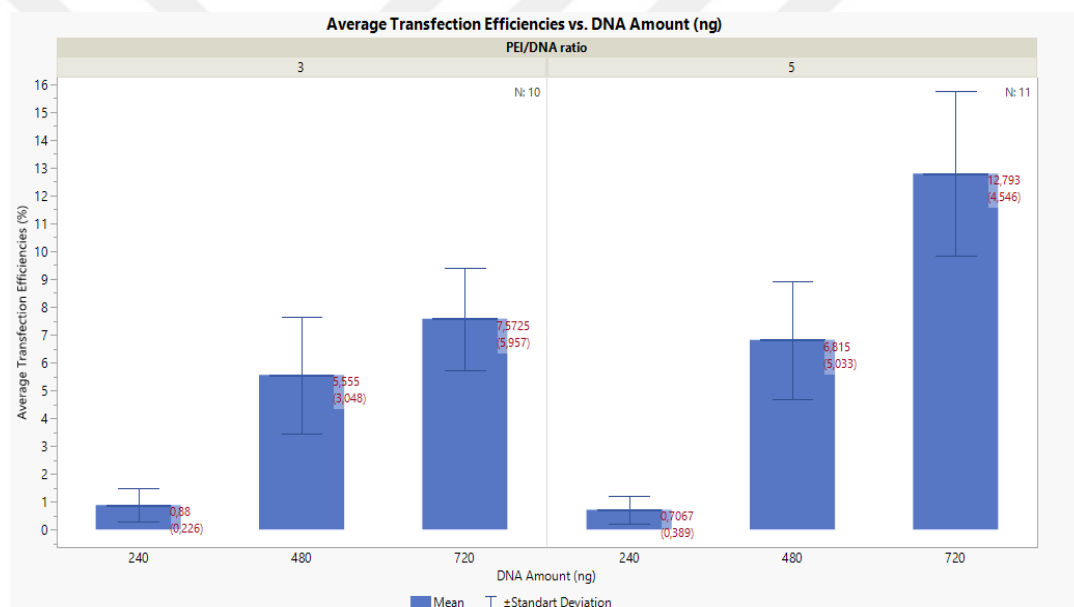


Figure 3.6: TT-2A and TT-2B experiments: Average transfection efficiencies as a percentage against DNA amounts which have been grouped by PEI/DNA ratio. Blue bar: Mean of results respective to the parameter.

Figure 3.6 shows that increment in DNA amount (240ng to 720ng), results in an exact increment in transfection efficiencies. Moreover, an increase of PEI/DNA ratio (3 to 5) affected transfection efficiency as a significant increment for the 720ng DNA amount variable (7.57% to 12.79%).



Figure 3.7: TT-2A and TT-2B experiments: Average transfection efficiencies as a percentage against cell count/well.

Figure 3.7 indicates the effect of cell numbers within each well on transfection efficiency. There is a linear increment in transfection efficiency from 10×10^3 to 50×10^3 numbers of the cell, however, 100×10^3 cells resulted in lower efficiency values.

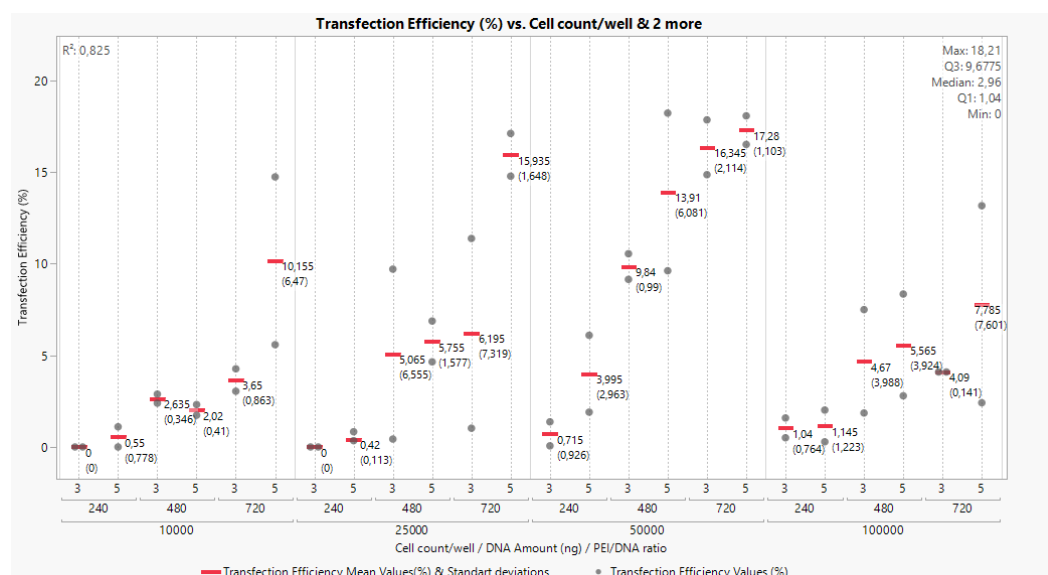


Figure 3.8: TT-2A and TT-2B experiments: Transfection efficiency as a percentage against cell count, DNA amount, and PEI/DNA ratio. Grey dots are representing duplicate transfection efficiency results; Red line represents mean values of replicated results; Numerical values indicate mean values; Brackets indicate standard deviations.

Figure 3.8 was built to represent overall parameters' effects on transfection efficiency. Each gridline was divided to consist of the exact parameters of each well of the plate. Duplicate results were not processed, mean and standard deviation were determined within each duplicated result and presented accordingly to visualize the distribution of results per well better.

Table 3.5: TT-2A and TT-2B experiments: GFP fluorescence areas as μm^2 of each cell on the 24-well plate. Dots were used as thousand separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “ $\times 10^3$ ”.

TT-2A Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0	1.158.295	7.125.166	0	1.257.211	6.650.834
Cells: 25K	0	2.212.138	7.115.549	903.501	3.462.648	50.816.687
Cells: 50K	341.472	67.063.981	127.760.628	44.714.321	133.682.184	127.968.497
Cells: 100K	3.686.263	13.581.809	30.290.630	14.486.187	61.251.091	92.149.356

TT-2B Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	2.176.301	2.176.301	13.252.579	3.255.492	4.394.121	31.775.103
Cells: 25K	0	71.216.996	81.923.402	3.611.967	48.533.159	123.810.078
Cells: 50K	8.130.499	67.103.604	97.462.409	13.923.722	59.327.977	120.520.514
Cells: 100K	11.453.732	54.620.933	26.643.987	2.074.909	20.484.523	16.217.006

The results given in Table 3.5 are showing GFP fluorescence areas of transfected cells in each well for 24-well plates. Data collections are enough to comment about DNA and PEI amount parameters over transfection, which shows an increment of these parameters values have resulted in an increment in GFP fluorescence areas. The wells with 50×10^3 cells presented significantly higher areas over the remaining wells, for the TT-2A plate. 25×10^3 cells row was also satisfied beside 50×10^3 cells for TT-2B plate.

3.2.3. TT-ELISA Experiment

TT- ELISA experiment was set to demonstrate the effects of changing concentrations of mAb, for the development of ELISA assay which can be used for anti-PD-1 antibodies.

Table 3.6: TT- ELISA experiment: Transfection efficiencies as percentage in each cell of the 24-well plate. Comma signs were used as decimal separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “x10³”.

TT-ELISA Experiment	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng
Stock Cells: 100K	0%	0%	0%	0%	0%	0%
GFP(+) PD-1(-) Cells: 100K	14,38%	11,61%	10,63%	13,20%	11,36%	20,05%
GFP(+) PD-1(+) Cells: 100K	16,29%	14,30%	12,59%	12,22%	14,78%	9,39%
GFP(+) PD-1(+) Cells: 100K	16,93%	10,62%	9,95%	11,16%	11,67%	11,45%

Table 3.7: TT-ELISA experiment: GFP fluorescence areas as μm^2 of each cell on the 24-well plate. Dots were used as thousand separators. Data were calculated using ImageJ software. In the “Cells” column “K” stands for “x10³”.

TT-ELISA Experiment	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng	DNA: 720 ng PEI: 3600 ng
Stock Cells: 100K	0	0	0	0	0	0
GFP(+) PD-1(-) Cells: 100K	100.279.270	82.654.849	73.871.708	90.665.900	80.808.232	140.076.918
GFP(+) PD-1(+) Cells: 100K	107.935.441	98.826.261	87.372.388	83.844.334	104.227.909	66.161.639
GFP(+) PD-1(+) Cells: 100K	115.786.962	73.196.786	68.048.494	76.434.221	79.253.105	78.627.701

3.2.4. TT-60 Experiment

Another set of experiment was set to produce PD-1 as much as possible which would be used for antibody binding assay. In addition to the 24-well plate experiment, stock HEK293T cells are proliferated as the negative control. The surface area increases 12 times fold through 24- well plate [Web 4, 2021] to 60mm plate [Web 5, 2021]. The selected parameter set with the highest transfection efficiency was amplified according to the 12-fold increment, to keep cell confluency as same as possible. Incubation conditions were not changed and applied as same into the 60mm plate with 5 mL total medium volume. After 48 hours of incubation, cell growth and expression were checked as image forms of full bright and ultraviolet images.

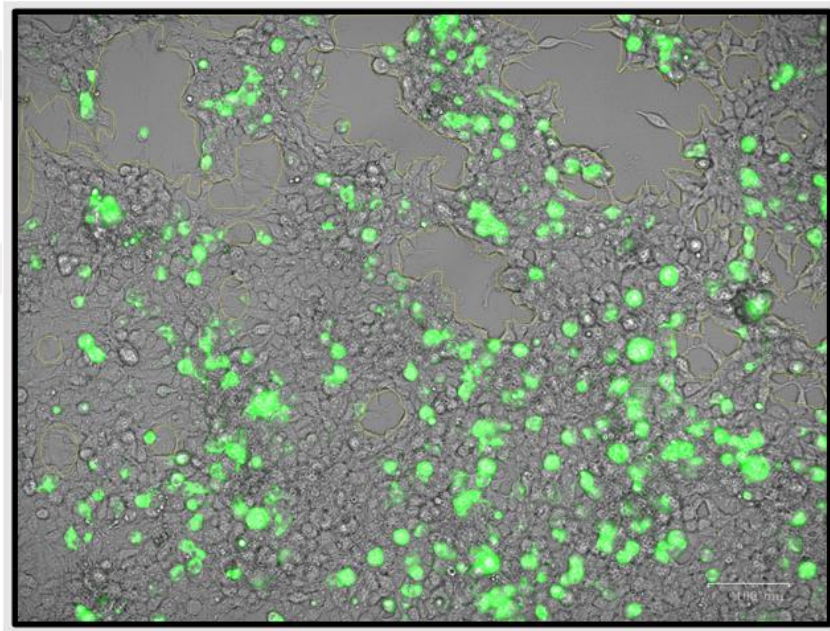


Figure 3.9: Superimposed image of the transfected cells in 60mm plate with selected parameters (DNA: 720ng, PEI:3600ng, Cell: 100×10^3).

ImageJ analysis resulted in transfection efficiency as 20.19% at 83% cell confluency rate and viability was checked with Luna cell counter, which was resulted in 72% cell viability. The negative control sample was had no green luminescence under ultraviolet light, and the data obtained for cell confluency as ≈ 90 -95%, and as 82.1% for cell viability. The biomass was grown, used for further experiments to perform the binding assay.

3.3. Flow Cytometer Applications

The flow cytometer assay was prepared using cells from the TT-60 experiment. HEK- and HEK+ cells were counted and viability was checked. Samples were fed to a flow cytometer. The areas of the events to be selected were observed with the Forward Scatter – Height (FSC-H) versus the Side Scatter – Height (SSC-H) detectors for cell population data. Then, the area was determined with the FITC channel (Absorbance Wavelength: 495nm / Emission Wavelength: 518nm), close to the GFP channel (Absorbance Wavelength: 395nm / Emission Wavelength: 508nm), and the count graph was created against FITC-H channel.

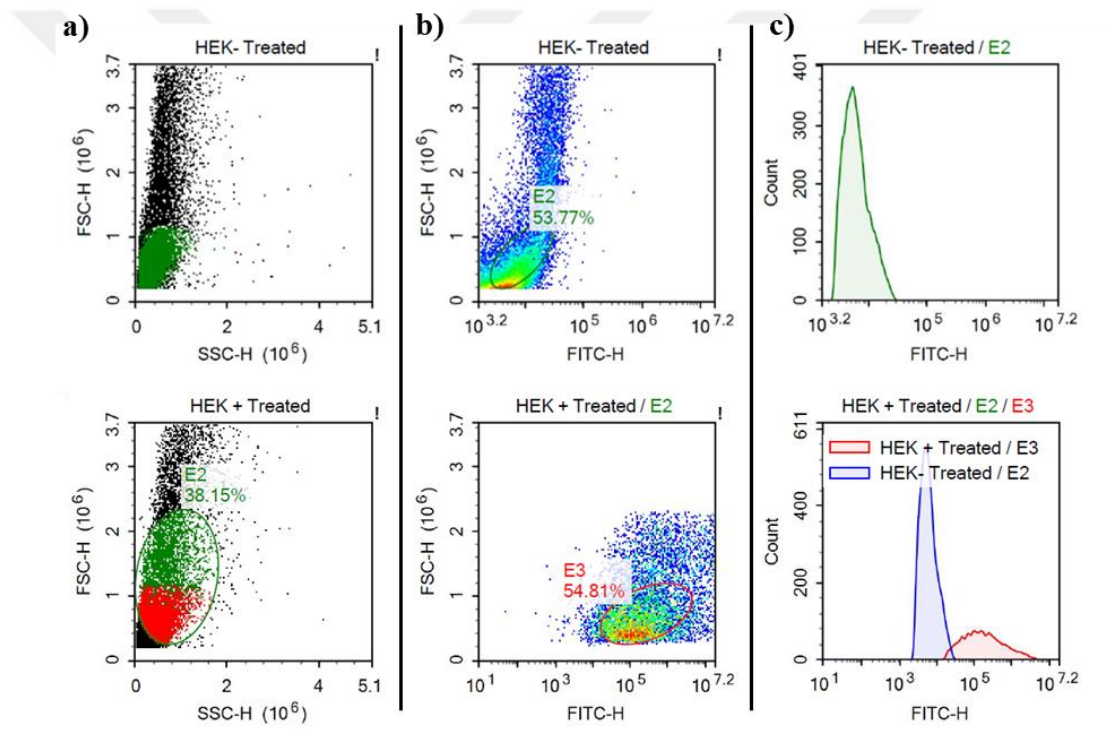


Figure 3.10: Flow cytometer analysis of mAb treated samples (HEK- and HEK+). a) FSC-H versus SSC-H graph was obtained to select optimum cell population, HEK+ samples graph was overlaid on HEK- sample's graph; b) Cell population areas were detected with FITC-H channel; c) Count graphs were represented to show a shifting of area.

3.4. ELISA Assay

ELISA assay was based on the interaction between commercial anti-PD-1 mAb with expressed PD-1 protein from transiently transfected cells. The PD-1 protein was supplied within cells, without any additional purification from the cell factory, since the purpose of the thesis study was the development of a cell-based ELISA test beside optimization of transient transfection of these cells.

The ELISA protocol was started by placing PD-1 expressed cells within plate wells. The commercial mAb was treated as single or varying concentrations with cells. HRP conjugated anti-mAb secondary antibody was used to interact with mAb that bonded to PD-1 expressed cells. The potential complex of PD-1 – mAb – secondary antibody was treated with TMB substrate. Results were planned to be obtained with the mechanism of TMB producing a deep blue color during the enzymatic degradation of hydrogen peroxide by HRP. Finally, the addition of a stop solution was made the assay able to give a clear yellow color that is available to measure absorption at 450nm.

The first set of ELISA assay was prepared using cells from the TT-60 experiment. HEK- and HEK+ cells were prepared for ELISA analysis with parameters given in the table below.

Table 3.8: The mAb concentrations, sample types, and cell counts representation for 96 well plate.

mAb concentrations (ng/mL)	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
	800	800	800	800	800	800	800	800	800	800	800	800
	320	320	320	320	320	320	320	320	320	320	320	320
	128	128	128	128	128	128	128	128	128	128	128	128
	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2	51.2
	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6	25.6
	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2	10.2
	0	0	0	0	0	0	0	0	0	0	0	0
Cell Count	10x10 ³		15x10 ³		20x10 ³		10x10 ³		15x10 ³		20x10 ³	
Sample	HEK+						HEK-					

No significant color change or absorbance values were observed with the given experiment set.

Another set of experiments were built using TT-2A, TT-2B, and TT-ELISA plates to start another ELISA assays. Related plates were built for transient transfection firstly, after 48 hours of incubation, cells are directly fixed with 4% paraformaldehyde into wells.

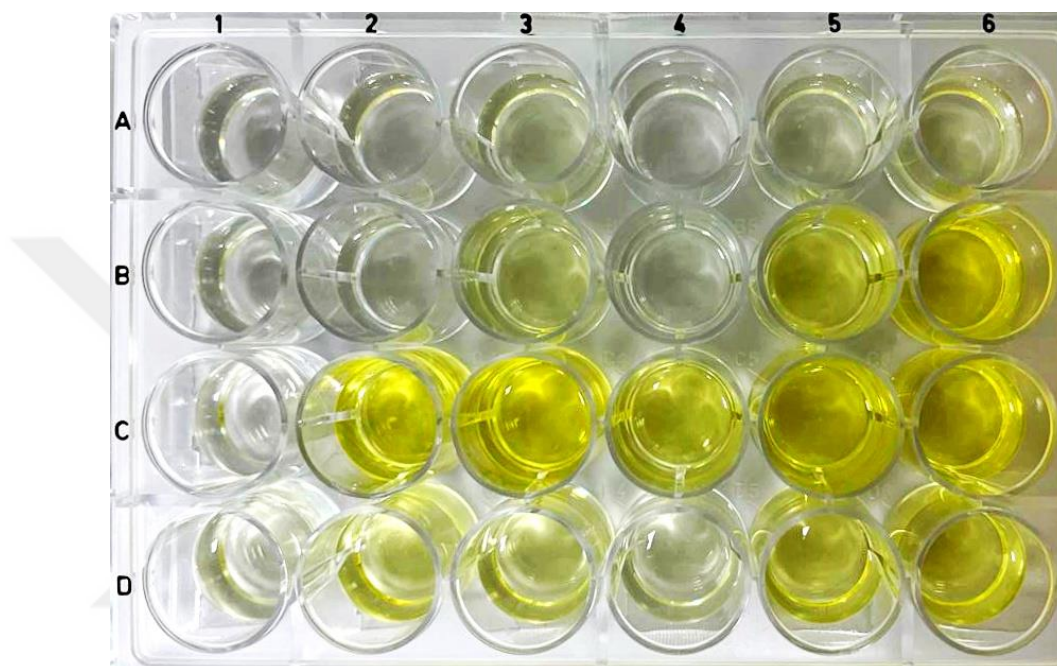


Figure 3.11: TT-2A plate: ELISA assay results as the color change to yellowish.

Table 3.9: TT-2A plate: ELISA assay absorbance results. The mAb concentrations were the same for each well as 20000 ng/mL.

TT-2A Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0.058	0.068	0.118	0.082	0.395	0.708
Cells: 25K	0.071	0.077	0.200	0.103	0.598	0.706
Cells: 50K	0.079	0.592	0.942	0.395	1.007	0.778
Cells: 100K	0.125	0.263	0.213	0.140	0.562	0.846

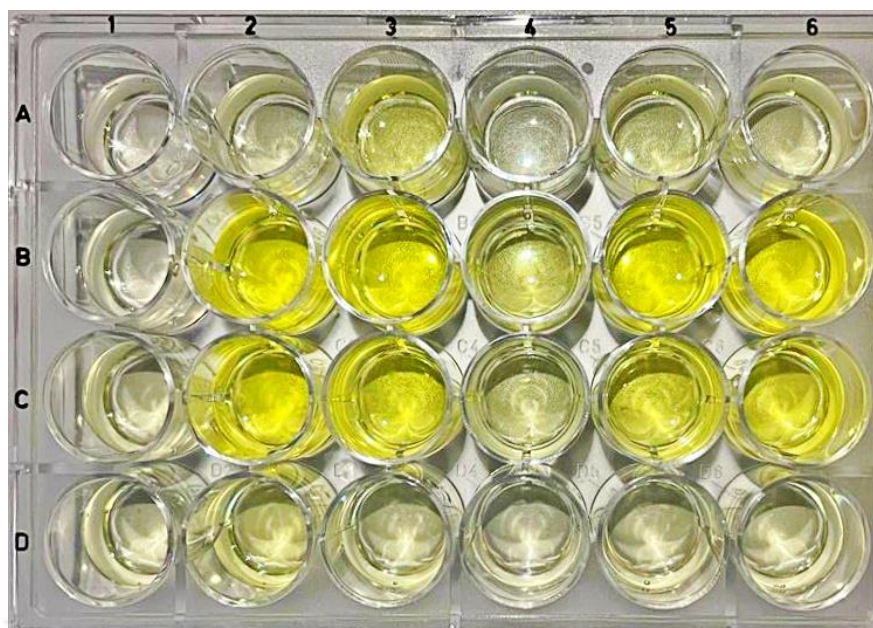


Figure 3.12: TT-2B plate: ELISA assay results as the color change to yellowish.

Table 3.10: TT-2B plate: ELISA assay absorbance results. The mAb concentrations were the same for each well as 20000 ng/mL.

TT-2B Experiment	DNA: 240 ng PEI: 720 ng	DNA: 480 ng PEI: 1440 ng	DNA: 720 ng PEI: 2160 ng	DNA: 240 ng PEI: 1200 ng	DNA: 480 ng PEI: 2400 ng	DNA: 720 ng PEI: 3600 ng
Cells: 10K	0.057	0.120	0.243	0.104	0.437	0.700
Cells: 25K	0.079	0.608	0.658	0.278	0.815	0.799
Cells: 50K	0.134	0.505	0.446	0.185	0.556	0.752
Cells: 100K	0.100	0.203	0.151	0.118	0.358	0.742

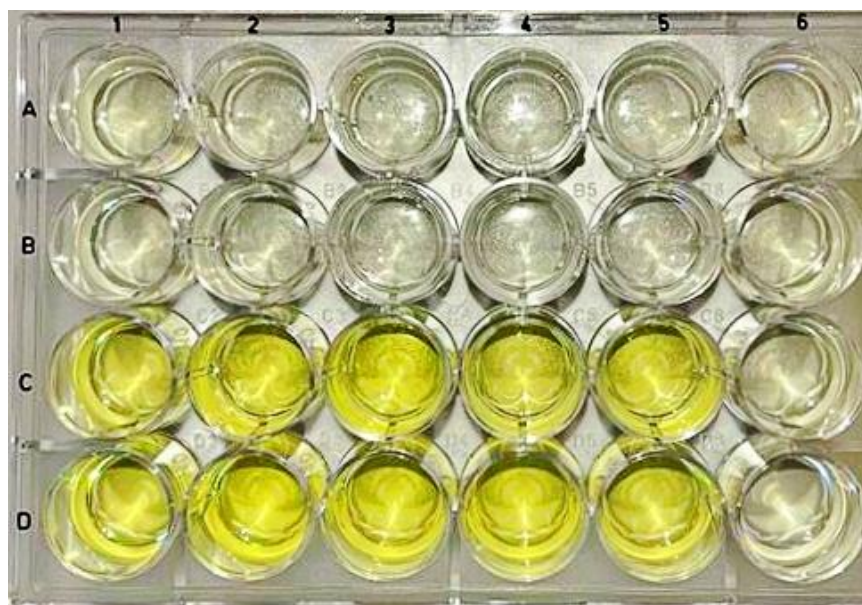


Figure 3.13: TT-ELISA plate: ELISA assay results as the color change to yellowish.

Table 3.11: TT-ELISA plate: ELISA assay absorbance results. The mAb concentrations: 1: 20000 ng/mL; 2: 10000 ng/mL; 3: 5000 ng/mL; 4: 2500 ng/mL; 5: 1250 ng/mL; Blank: No addition.

TT-ELISA ELISA Assay	1	2	3	4	5	Blank
Stock Cells: 100K	0.104	0.077	0.073	0.092	0.059	0.102
GFP(+) PD-1(-) Cells: 100K	0.097	0.094	0.100	0.061	0.062	0.100
GFP(+) PD-1(+) Cells: 100K	0.254	0.292	0.321	0.256	0.244	0.109
GFP(+) PD-1(+) Cells: 100K	0.272	0.357	0.298	0.317	0.255	0.087

TT- ELISA experiment was set up for screening the effects of changing mAb concentrations over replicate wells. The first row contained stock cells without transfection at all, as the negative control. The second row contained transiently transfected cells with GFP plasmid, as negative control but enabled to screen transfection in ultraviolet imaging. The bottom two rows contained transiently transfected cells with GFP+PD-1 plasmid. All transfections were applied with 720ng plasmid DNA and 3600ng of PEI.

4. DISCUSSION and CONCLUSION

In the developing field of biotechnology, studies on cancer are gaining importance day by day. In this direction, bioanalysis methods are of great importance in the development and manufacturing of biotechnological drugs to obtain standardized quality. In this study, the PD-1 protein, with its interaction mechanism that has become a target in many cancer cases, was focused with the aim of developing bioanalytical methods and production by transient transfection. The PD-1-based analytical method kits currently on the market are based on the stable cell line, and the high cost and labor requirement has been the driving force for the development of a different method in this study.

HEK293T cells were transiently transfected with mammalian expression plasmid with human Programmed Cell Death -1 protein as gene of interest fused to Green Fluorescent Protein. Several parameters were applied in variable amounts to screen optimum PD-1 expression through GFP fluorescence. Cell numbers which will be transfected, plasmid DNA amount, and transient transfection reagent (PEI (determined according to the amount of DNA: i.e. DNA: PEI rate was used as 1:3 and 1:5) were used for determining the transfection conditions for optimum efficiency.

The first optimization experiment was set up using a 24-well plate (TT-1). Expression yields were calculated using ImageJ software, results were given in Table 3.2 as yield, Table 3.3 as GFP fluorescence area. The maximum yield was found to be 23.5 % (100×10^3 cells / 720ng DNA / 3600ng PEI). Although the optimization experiment was performed once and therefore statistical analysis could not be performed, it was observed similar results when the TT-60 experiment set within 100×10^3 cells were seeded. Despite, because dramatic the decrease within 25×10^3 and 50×10^3 cells the resulting transfection efficiencies were not consistent. This could be a technical or human error since it was observed that a small number of cells were detached from the well surface during medium addition. Therefore, two replicate experiments (TT-2A and TT-2B) were prepared with identically the same parameters and methods as the TT-1 experiment. Transfection efficiencies were observed consistently, presented as efficiency percentages in Table 3.4 and as GFP fluorescence areas in Table 3.5.

According to consistent results, Figures 3.6, 3.7, and 3.8 were prepared. Figures demonstrated that increment of DNA amount resulted in a slight increase in transfection efficiencies. The ratio of PEI/DNA also showed a similar result, increment of ratio resulted in a minor increase in efficiency percentages. Despite, for cell number parameter, the graph showed that increment of efficiency obtained parallelly to cell number up to 50×10^3 cell number value, but efficiency percentage dropped dramatically for 100×10^3 cell number. There would be a possible reason behind this drop based on completely confluent cells in well. It would affect the transfection of DNA as a heterogeneous distribution, which causes a drop in efficiency percentage because GFP fluorescence areas are divided by higher values of bright field surface areas. The standard deviations were desirable to show reproducibility, but a couple of efficiency percentage deviations distributed into higher values as seen in Figure 3.8, probably reasoned by the human effect to handle experiments, since the methodology consisted of media replacement while preparing cells to transfection.

The TT-60 experiment was analyzed with ImageJ, and analysis showed that cell viability was acceptable and the exogenous expression of the PD-1-GFP fusion protein in these cells did not result in toxicity.

To determine whether a related in vivo expression platform could indeed be used as a bioassay for testing anti-PD-1 antibody binding, we have carried out a series of experiments using TT-60, TT-ELISA, and TT-2A, TT-2B transfection optimization plates.

TT-60 experiment was used for flow cytometer analysis which resulted as given in Figure 3.6. Shifting between areas are significantly visible since this situation was because of the GFP fluorescence of transfected cell, which was not referred to as the binding of the mAb to expressed PD-1 protein. Additionally, an ELISA experiment was prepared and applied from the TT-60 experiment, but color change nor absorbance values were not observed. The sample was prepared for flow cytometer and ELISA from the TT-60 experiment since normally these assays were applied using purified PD-1 protein with higher values of concentration. Possible reason of unsuccessful experiments would be about PD-1 protein amount since only a certain percentage of the cells were expressing the target protein, moreover cells were scraped to be used, that directly reduce the PD-1 protein quantity.

The ELISA experiments repeated with higher amounts of mAb (20000 µg/mL) to the fixed cells of transient transfection plates TT-2A (Figure 3.11, Table 3.7) and TT-2B (Figure 3.12, Table 3.8) to show different rates of transfection effect on mAb binding. Higher rates of mAb, direct fixing of cells into transfection plate resulted in different levels of color change and absorbance at 450nm. These color change and absorbance results showed parallel conclusions as increased efficiency percentages resulted in increment of color change and absorbance. Since anti-PD-1 antibody was used, it could be also commented as transient transfection resulted in PD-1 protein expressed successfully.

Additional ELISA experiment prepared with using TT-ELISA plate, firstly to determine the distribution of mAb concentrations effect on replicates of transfected cells with PD-1 plasmid. Secondly, to demonstrate PD-1 expression by comparison with transfected cells with GFP plasmid. The serial dilutions of mAb with different concentrations did not present a change in absorbance values. Probable reason estimated about PD-1 protein concentration in wells. Normally, these different concentrations of mAb were applied to produce a graph to demonstrate the binding capacity of mAb, therefore higher concentration values of purified PD-1 protein were needed. Since transient transfection applied cells were directly used for this experiment, protein concentration was the bottleneck, therefore each well showed slightly similar absorbance values.

In a conclusion, the study clearly screens the "DNA-PEI-Cell numbers" group of variables' effects on transfection and PD-1 expression. Despite this, analytical methods are supposed to be precisely defined and able to be repeatable with less variation as much as possible. This quality enables screening and investigation of several behaviors of the antibody that wanted to be manufactured without deviation. The obtained data revealed that transient transfection was not a well-defined option to perform bioanalytical tests, because of varying quantities of transfection efficiencies so on protein expression values.

On the other hand, optimized parameters of transient transfection may be used as a quick-response ELISA test for anti-PD-1 antibodies, as developed ELISA plate was successfully showed that PD-1 proteins were expressed and functional.

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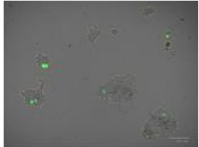
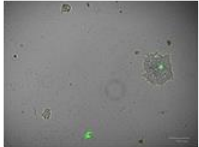
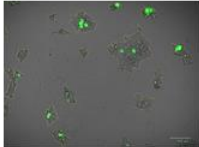
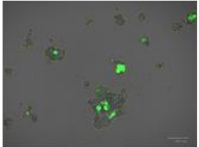
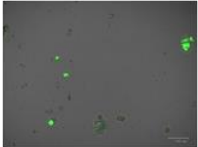
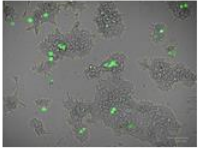
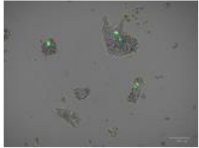
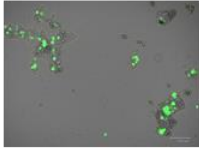
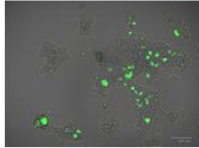
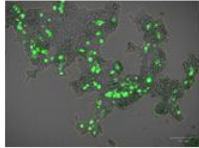
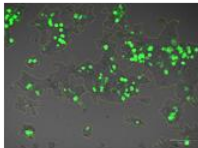
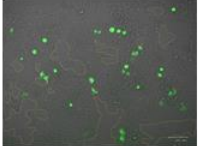
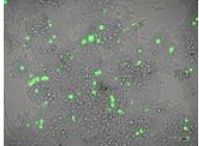
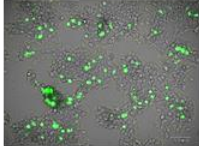
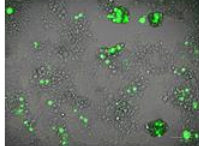
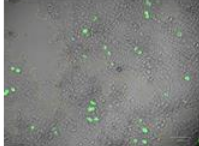
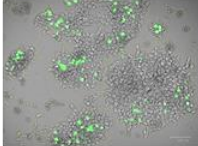
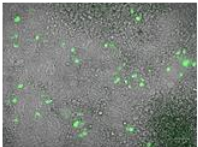
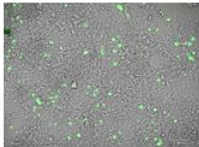
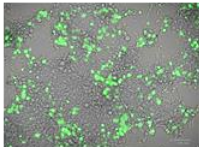
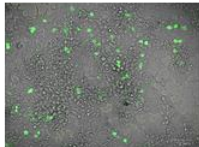
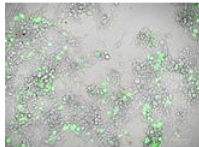
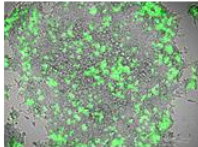
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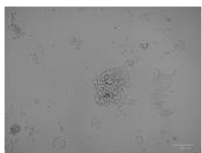
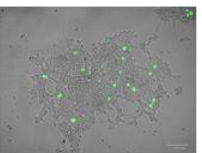
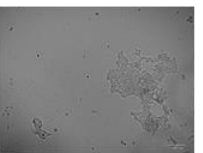
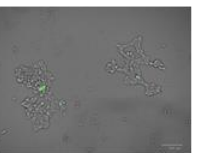
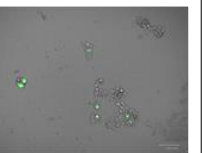
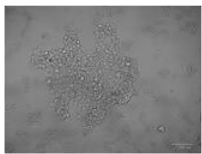
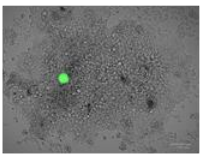
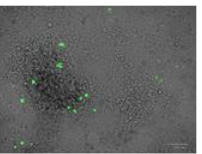
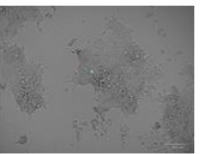
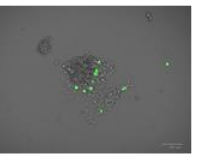
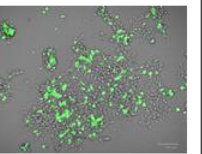
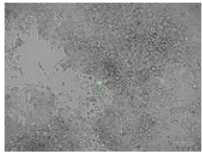
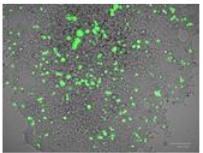
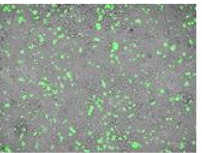
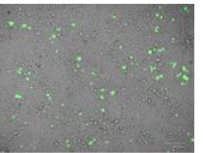
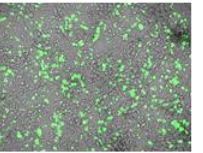
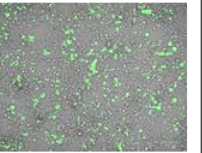
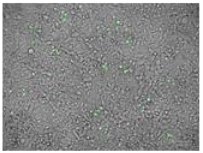
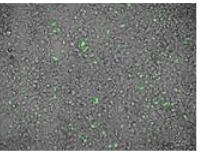
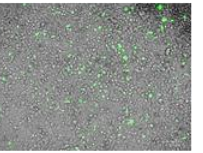
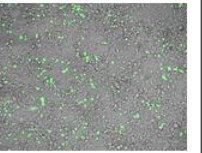


APPENDICES

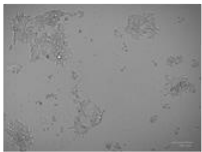
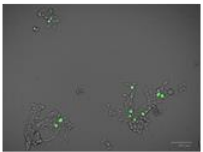
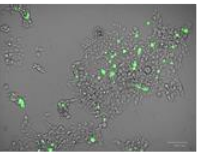
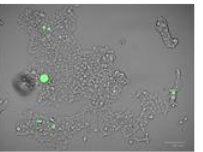
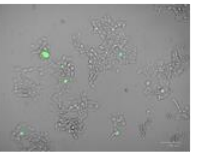
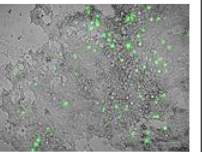
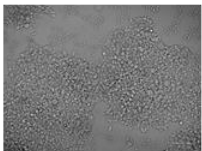
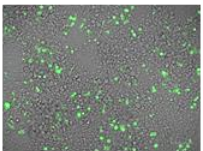
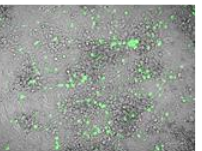
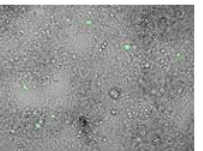
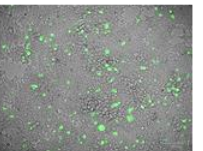
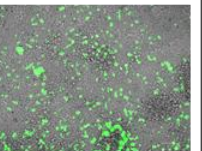
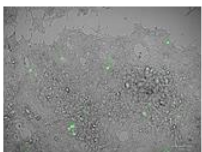
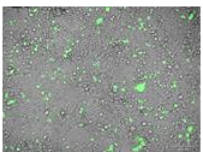
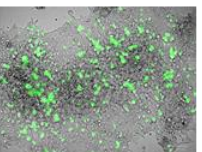
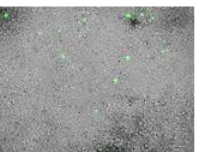
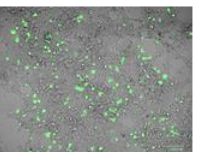
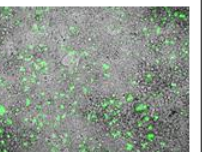
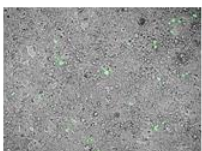
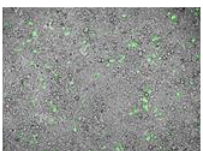
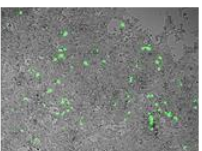
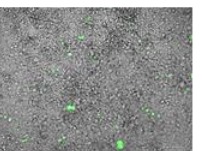
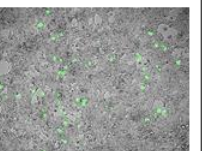
Appendix A: TT-1 superimposed images of expression in 24-well plate.

DNA&PEI Amounts /well Cell Numbers (10³)/well	DNA: 240ng PEI: 720ng	DNA: 480ng PEI: 1440ng	DNA: 720ng PEI: 2160ng	DNA: 240ng PEI: 1200ng	DNA: 480ng PEI: 2400ng	DNA: 720ng PEI: 3600ng
10	Not Available					
25						
50						
100						

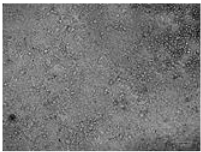
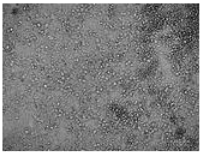
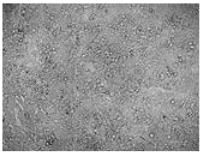
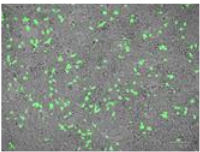
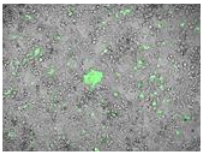
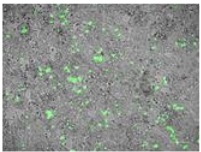
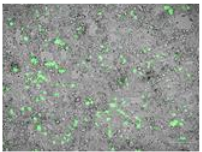
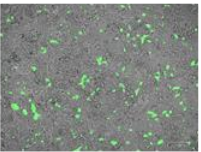
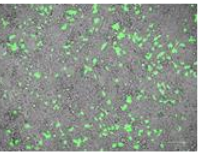
Appendix B: TT-2A superimposed images of expression in 24-well plate.

DNA&PEI Amounts /well Cell Numbers (10³)/well	DNA: 240ng PEI: 720ng	DNA: 480ng PEI: 1440ng	DNA: 720ng PEI: 2160ng	DNA: 240ng PEI: 1200ng	DNA: 480ng PEI: 2400ng	DNA: 720ng PEI: 3600ng
10						
25						
50						
100						

Appendix C: TT-2B superimposed images of expression in 24-well plate.

DNA&PEI Amounts /well Cell Numbers (10³)/well	DNA: 240ng PEI: 720ng	DNA: 480ng PEI: 1440ng	DNA: 720ng PEI: 2160ng	DNA: 240ng PEI: 1200ng	DNA: 480ng PEI: 2400ng	DNA: 720ng PEI: 3600ng
10						
25						
50						
100						

Appendix D: TT-ELISA superimposed images of expression in 24-well plate.

DNA&PEI Amounts /well Cell Numbers 100x(10³) /well	DNA: 720ng PEI: 3600ng					
Stock Cells						
GFP (+) PD-1 (-)						
GFP (+) PD-1 (+)	