

Testing the Effects of Transfection on Mammalian Cytokine RNA Expression

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Abstract—Abstract outline

This experiment aims to study the effect of genetic expression on polyplex treated cells against β -actin and $\text{INF}\alpha$.

I. INTRODUCTION

Gene therapy is an experimental method for treating disease by introducing a healthy copy of a defective gene into the patient's cells to alter the patients genetic material. The alternative is a non viral gene therapy method which can often have simple and large scale production with low host immunogenicity. However, non viral gene therapy can often yield low transfection efficiencies. New advances in technologies have improved transfection efficiency by modifying cytokines. Cytokines are small proteins that are involved in various types of cell signaling. Classifications of these signalings include interferons, chemokines and interleukins. One major factor that often inhibits the usage of non viral gene therapy is the innate immune response. The innate immune response to foreign DNA (often by viruses or bacteria) through cytokines as a trigger that rejects the foreign DNA through one of three possibilities. These possibilities include: inducing cell death, halting the production of protein or recruiting immune cells to initiate an adaptive immune response (e.g. antibody protection). This experiment aims to see if the innate immune response can be inhibited or manipulated to improve gene therapy treatments.

In this experiment, there are two devices used. The first device, the Qubit, is an electronic device that quantifies a substance through fluorescence. The second device is qPCR. Reverse Transcriptase (RT)qPCR is a technique used to describe the level of genetic expression is occurring in vitro by measuring the amount of mRNA within a sample. In this technique, mRNA is reverse transcribed in to complementary DNA by an enzyme labeled reverse transcriptase. For the Real Time PCR experiment, since it is impossible to eliminate all genomic DNA from the cDNA synthesis, two solutions will be prepared. The first solution will be a +RT which will include reverse transcriptase and thus, will have cDNA. The second solution will be a -RT solution which will lack reverse transcriptase. When qPCR is performed on the -RT solution, genomic DNA will be amplified and will be seen in the resulting data.

In this experiment, β -actin and RPL13A will be used for direct controls against the immunological transcripts. $\text{INF}\beta$ will not be used as it will only appear after cycle 40 in the experiment producing undesirable results. The No Template Control will consist of DEPC water. The immunological

transcripts that will be used in this experiment are Interleukin-6 (IL6) and Interferon- α ($\text{INF}\alpha$) Beta actin is a type of actin isoform which is highly involved in cell motility, structure and integrity. RPL13A is a gene that codes for the 60S ribosomal L13a protein. Interleukin-6 (IL-6) is a multifunctional cytokine that defends the host in response to immune and hematopoietic activities. Interferon- α is a part of a large subgroup of interferon proteins that help regulate the activity of the immune system.

II. METHODS

A. RNA Purification

The cells in trizol were thawed before phase separation. During phase separation, the poly treated cells in trizol were incubated at 42° after which an 0.2mL of chloroform was added per 1mL of Trizol. The homogenized sample was incubated at 42° for 2-3 minutes following a vigorous shake for 15 seconds. The sample was then centrifuged 12,000 g for 15 minutes at 4°C before transferring the aqueous phase to a second tube. (100)Again, the sample was centrifuged 12,000 g for 15 minutes at 4°C . The pellet was not washed in fear of losing RNA. The solution then, underwent a series of centrifuges in between the removal of ethanol to result in an RNA pellet. The pellet was then air dried to remove remaining ethanol. The pellet was incubated at 42° following a resuspension in $20\mu\text{L}$ of RNase free water.

B. cDNA Synthesis

For the first step of cDNA Synthesis, two solutions (+RT/-RT) were formed from the following compounds: 10x dsDNase Buffer, dsDNase, Template RNA, polyplex 24hour RNA and nuclease free water. Both solutions were incubated at 42° after being centrifuged. The solutions were then chilled on ice, centrifuged and placed back on ice. For both solutions, 5X Reaction mix and nuclease free water was added. In the +RT solution, Maxima Enzyme mix (reverse transcriptase) was added to the mixture. The -RT solution used DEPC H2O instead of Maxima Enzyme mix so that the -RT solution can simulate the +RT solution without synthesizing RNA into cDNA. When the -RT solution undergoes qPCR, any contaminating genomic DNA will be amplified. The two solutions will be then mixed gently and centrifuged. An incubation period will take place at 25°C for 10 min followed by a second incubation session at 50°C for 15 min. The reaction will be terminated by heating the solutions at 85°C for 5 min. This process was conducted using Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, Cat: K1671)

C. Q_t PCR

Each well will contain 10 μ L of 2X iTaq universal SYBR Green Supermix, 5 μ L of a primer (IL6, INF α , β -actin or RPL13A), and 5 μ L of the respective diluted cDNA. The cDNA needs to be diluted to 4ng/ μ L from their respective concentrations before it is added to the wells. The concentrations of cDNA for both sets of Poly24 and no washed cells were all around 100ng/ μ L. Therefore only one set of calculations were needed for determining the proper dilution of cDNA into water for either INF α and IL6 or β -actin and RPL13A and the respective -RT wells. For just cells, a different set of calculations were needed since the original cDNA concentrations were around 11 ng/ μ L. All wells contained the Green Supermix. The columns were sorted in the following manner: 1-3 contained IL6, 4-6 contained INF α , 7-9 contained β -actin and 10-12 contained RPL13A. Refer to Table II for a compressed map of the PCR wells. The Q_tPCR was ran for 40 cycles.

D. Gel Electrophoresis

In order to further understand the results gained from the Q_tPCR, an 1.2and gel electrophoresis was ran on the PCR product.

E. Analysis

The results from the Q_tPCR produced Ct values from each well. A Ct value is a numeric inverse correlation to the quantity of nucleic acid detected by the apparatus. Comparisons are made between Polyplex treated cells for 24 hours version [AV], Polyplex treated cells for 24 hours version [Ts], Polyplex treated cells for 6 hours with iCRT, Cells that were not washed after 6 hours, cells that were not washed and just cells. Each comparison was conducted by taking the difference between the Ct values of the two primers (immunological transcript minus control) on two different treatments. A second difference was taken between these two treatments (i.e. Poly24-NoWash) and then applied to the $\Delta\Delta$ Ct method to determine the fold induction. This was repeated three times, and thus an average was determined.

III. RESULTS

A. Qubit

Following the cDNA synthesis, the concentrations were measured by the Qubit. The concentrations are as follows: SD1 (standard 1): 54.27ng/ μ L, SD2 (standard 2): 1057.52ng/ μ L, Tk: 8.75ng/ μ L, DA: 68.0ng/ μ L, AV: 487.0ng/ μ L, Ts: 28.0ng/ μ L.

B. Q_tPCR

C. Gel Electrophoresis

The selections of PCR products was based off of melt curves that were inconsistent with surrounding wells of similar product (i.e. β -actin for just cells). Lanes 2-4 (Wells: E1,E2,E3,G1) consists of -RT solutions. These lanes are empty displaying genomic DNA that is almost undetectable. Lane 8 (Well

TABLE I
WELLS FOR IMMUNOLOGICAL RESPONSES

	IL6	INF α	β -actin	RPL13A
Poly24[AV]-NoWash	3.85E-05	7.58E-03	5.09E-05	1.05E-02
Poly24[AV]-Cells	3.10E-02	3.54E-02	3.94E-02	4.27E-02
NoWash-Cells	8.01E+02	4.65E+00	7.01E+02	4.08E+00
Poly24[Ts]-NoWash	8.20E-05	4.64E-03	3.77E-05	6.04E-03
Poly24[Ts]-Cells	6.54E-02	5.90E-02	2.57E-02	2.55E-02
Poly6+iCRT-NoWash6	5.00E+2	4.21E+2	2.58E+1	2.23E+1

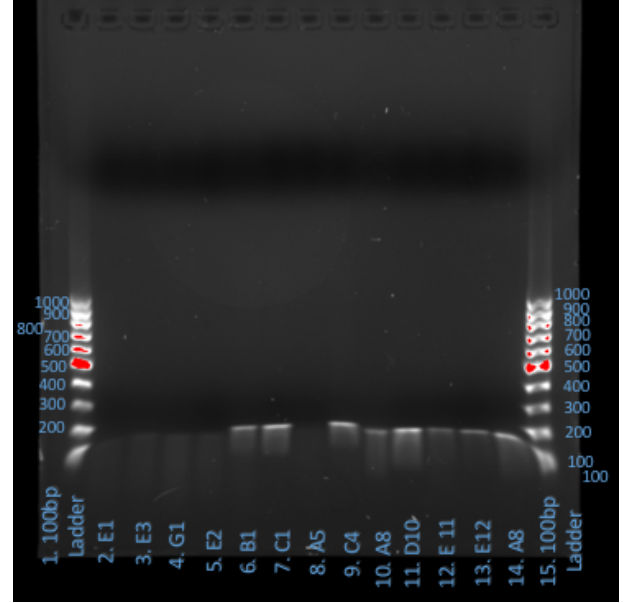


Fig. 1. A 1.2 agarose gel

A5) displays a lack of cDNA for Interferon- α where there should exist amplified cDNA. Lanes 7,9,11 and 14 (Wells: C1,C4,D10,A8) are displaying bands at a greater illumination compared to other lanes. Lanes 7 and 9 consist of Just Cells in Interleukin-6 and Interferon- α respectively. Lanes 11 and 14 are polyplex cells treated for a 24 hour duration in both the [AV] and [Ts] variants for RPL13A and β -actin respectively. Lanes 6,12, and 13 contain smaller amounts of cDNA compared to Lanes 7,9,11 and 14 as they do not contain as great illumination. Lane 6 (Well B1) is cells that were not washed for Interleukin 6. Lanes 12 and 13 (Wells: E11,E12) consist of -RT solutions for RPL13A displaying amplified genomic DNA.

IV. DISCUSSION

The mRNA was purified and converted to cDNA. The resulting concentration was relatively high compared to peers. This eludes that the treatment for these cells of polycationic DNA for 24 hours could result in higher transcription rates.

V. CONCLUSION

To further understand the nature polyplex treated cells in response to a specific duration, it would be suggested that the polyplex cells are treated in 3 hour increments from 3 hours to 24 hours.

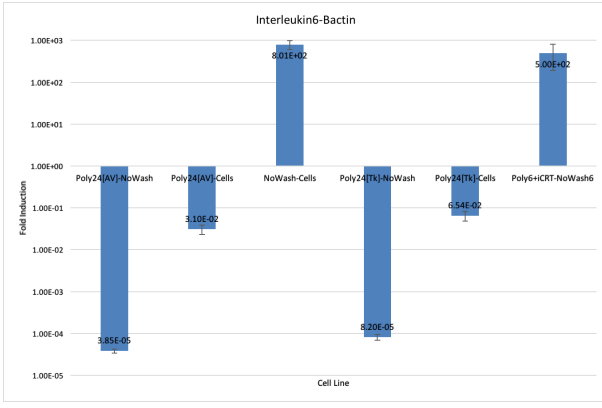


Fig. 2. Logarithmic fold Inductions for Interleukin-6 vs β -actin. Refer to the analysis subsection of the methods section for insight on how this graph was constructed.

VI. FIGURES

TABLE II
PCR WELLS FOR CONTROLS

	Primer	1/4/7/10	2/5/8/11	3/6/9/12
A	Poly24[Tk]	4ng/ μ L	4ng/ μ L	4ng/ μ L
B	Just Cells	4ng/ μ L	4ng/ μ L	4ng/ μ L
C	No Wash	4ng/ μ L	4ng/ μ L	4ng/ μ L
D	Poly24[AV]	4ng/ μ L	4ng/ μ L	4ng/ μ L
E	-RT	Poly24[Tk]	Cells	Poly24[AV]
E	NTC	Poly24[Tk]	Cells	Poly24[AV]
G		NoWash(-RT)	NoWash(NTC)	
H				

TABLE III
LIST OF PRIMERS

Primer Name	Tm	Sequence
RTPCR-IL6-FOR	60	CCTTCCAAAGATGGCTGAAA
RTPCR-IL6-REV	60	CACAGCTCTGGCTGTTCCT
RTPCR-IFNAall-FOR	61	GCACCGAAGCTTACCAGCAG
RTPCR-IFNAall-REV	60	ACAACCTCCAGGCACAA
RTPCR-B-Actin-FOR		TTGCCGACAGGATGCAGAA
RTPCR-B-Actin-REV		GCCGATCCACACGGAGTACTT
RTPCR RPL13A FWD		CCTGGAGGAGAAGAGGAAAGAGA
RTPCR RPL13A REV		TTGAGGACCTCTGTGTATTGTCAA

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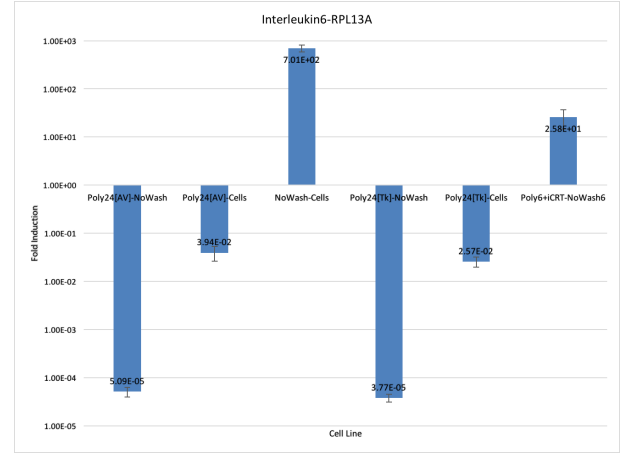


Fig. 3. Logarithmic fold Inductions for Interleukin-6 vs RPL13A. Refer to the analysis subsection of the methods section for insight on how this graph was constructed.

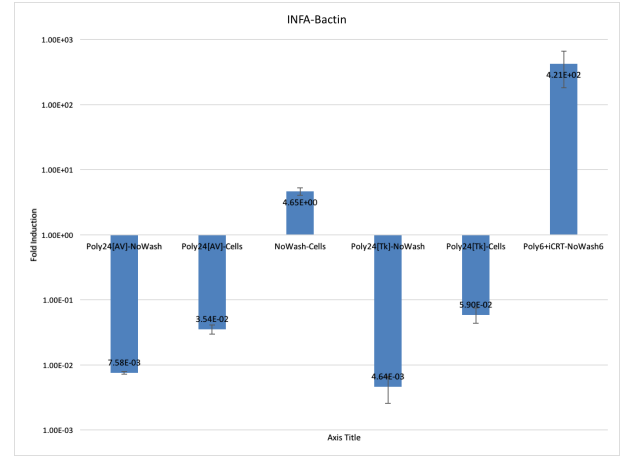


Fig. 4. Logarithmic fold Inductions for Interferon- α vs β -actin. Refer to the analysis subsection of the methods section for insight on how this graph was constructed.

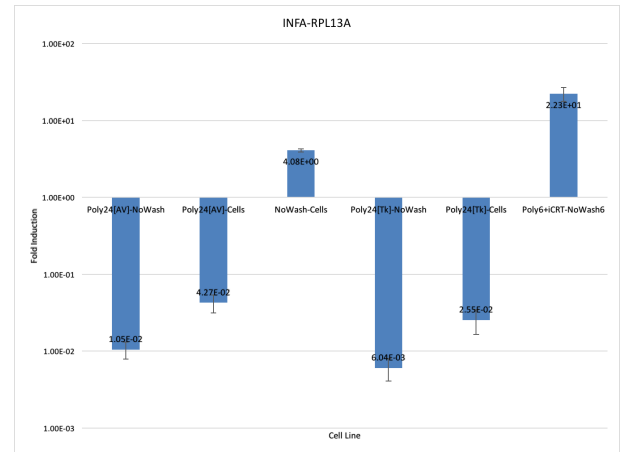


Fig. 5. Logarithmic fold Inductions for Interferon- α an immune cytokine vs RPL13A a standard protein. Refer to the analysis subsection of the methods section for insight on how this graph was constructed.