Chapter 5

Optimizing Techniques to Implement Nonsense Suppression in Mammalian Cells*

**This work was done in collaboration with Kristina McCleary.*

5.1 ABSTRACT

Our lab previously used microelectroporation to incorporate unnatural amino acids into ion channels expressed in mammalian cells. While this work represented major progress, this method still faced several limitations. Specifically, microelectroporation is not robust enough for large-scale cellular imaging techniques and requires significant amounts of aminoacylated tRNA. This chapter describes our efforts to further develop transfection techniques for mammalian cells to increase the expression of mutant protein such that functional analysis of several thousand cells simultaneously can be achieved using the FlexStation3. Using HEK293T cells, we have optimized two protocols to efficiently cotransfect DNA and tRNA. These protocols are termed double electroporation using the Neon transfection system and single transfection using *Trans*IT transfection reagent. Both methods successfully suppress an amber stop codon using an *in vivo* aminoacylated tRNA, but fail to achieve suppression using *in vitro* aminoacylated tRNAs. Studies are ongoing to evaluate the cause of the failed suppression experiments using *in vitro* aminoacylated tRNAs. Possible causes include the amino acid falling off the tRNA inside the cell and translational incompetence of the tRNA molecule itself.

5.2 INTRODUCTION

The nonsense suppression methodology, developed by Schultz in 1989,[1] has emerged as a powerful biophysical tool facilitating site-specific incorporation of virtually any unnatural amino acid - as long as it can be synthesized in the chemistry lab - into biologically relevant proteins. This method is particularly attractive because it can conceivably generate an infinite number of proteins with novel chemical properties as well as probe protein structure/function relationships.

Our lab has adapted the nonsense suppression methodology to incorporate unnatural amino acids into proteins expressed in *Xenopus laevis* oocytes.[2, 3] In doing so, we have successfully determined the ligand binding mechanism and channel gating properties of numerous ion channels and neuroreceptors.[4-9] While heterologous expression in *Xenopus* oocytes has proven an optimal model system to efficiently express hundreds of mutant proteins, we are currently interested in expanding this technology to a mammalian expression system. One advantage of implementing a mammalian expression system is that one can study mammalian proteins in a biologically relevant context (*i.e.*, one can study a *human* protein in a *human* cell line). Additionally, using a mammalian system will allow for the study of cell-specific signal transduction pathways, and one can imagine designing experiments using unnatural amino acids (*e.g.*, photocaged amino acids) to exert temporal control of various signaling cascades.[10]

Progress towards Unnatural Amino Acid Mutagenesis in Mammalian Cells

One of the key challenges in mammalian protein expression is determining the appropriate transfection method that will maximize protein expression, limit cell toxicity, and consume the least amount of nucleic acid. Several transfection techniques have been

developed to effectively introduce nucleic acids into cells, such as chemical-based methods (*e.g.*, calcium phosphate, cationic polymers, cationic liposomes, and cationic activated dendrimers), non-chemical methods (*e.g.*, electroporation), particle-based methods (*e.g.*, gene gun), and viral transduction. Each transfection technique is accompanied by inherent advantages and disadvantages depending on the cell line of interest and nucleic acid material being delivered.

In recent years, many researchers have contributed major advances towards the types of transfection techniques amenable to introducing both DNA and tRNA into mammalian cells. For example, RajBhandary and workers successfully suppressed a serine site with tyrosine in chloramphenicol acetyltransferase.[11] This experiment utilized a cationic lipid (Effectene) to co-transfect *in vitro* aminoacylated tRNA with DNA into COS cells.[11] Alternatively, Schultz and workers took a different approach employing directed evolution of suppressor tRNA-aminoacyl synthetase pairs to site-specifically incorporate unnatural amino acids into several types of mammalian cells.[12-14] And in 2003, our lab used microelectroporation to cotransfect mRNA and tRNA that had been aminoacylated *in vitro* with an unnatural amino acid to express mutant ion channels in CHO cells and cultured neurons.[15] Whole-cell patch clamp electrophysiology confirmed the presence of functional mutant protein.[15] While this work represents major progress in this field, these methods do not yield robust amounts of mutant protein and require functional analysis on a single cell scale.

We are interested in further developing techniques to increase the expression of mutant protein in mammalian cells to a level that allows functional analysis of several thousand cells simultaneously. This chapter describes our efforts to determine the

optimal transfection protocol for incorporating unnatural amino acids into ion channels expressed in mammalian cells.

5.3 METHODS

5.3.1 Molecular Biology

Human $5HT_{3A}$ subunit gene in pcDNA3.1(+) vector was purchased from Missouri S & T cDNA Research Center. Mouse α4, α4GFP, and β2 genes were in pciNEO vector, and the mouse β4 gene was in pcDNA3.1(+) vector. All mouse constructs were obtained from the Lester lab. All eGFP constructs used in these studies are from Monahan *et al*.[15]

Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene) to incorporate either an amber stop codon (TAG) or a frameshift stop codon (GTTT) at the site of interest within the subunit DNA. THG73[16] was used as the amber suppressor tRNA. Human serine amber suppressor tRNA (HSAS) was used to incorporate serine opposite an amber stop codon.[17] For suppression of the frameshift codon (GTTT), the yeast phenylalanine frameshift suppressor tRNA (YFaFS) was used.[18]

Nitroveratryloxycarbonyl (NVOC) protected cyanomethyl esters of natural or unnatural amino acids and α-hydroxy acid cyanomethyl esters were prepared as previously reported.[2] Aminoacyl tRNA was confirmed by MALDI-TOF MS on 3-hydroxypicolinic acid (3-HPA) matrix. Prior to transfection, the NVOC protecting group on the aminoacyl tRNA was deprotected by photolysis for 5 minutes.

5.3.2 Mammalian Cell Culture

Human embryonic kidney cells (HEK293T) cells were purchased from American Tissue Culture Collection (ATCC). The HEK293T cell line is optimized for increased

efficiency of transient transfections due to expression of the simian virus 40 (SV40) large tumor antigen in the cell.[19, 20] Therefore, transiently transfected plasmids containing the SV40 origin of replication produce extremely high levels of expression.

All cell culture reagents were obtained from Gibco, except for heat-inactivated fetal bovine serum purchased from Sigma. HEK293T cells were grown on sterile 100 mm culture dishes at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were cultured in DMEM:F12 with Glutamax I_{TM} with 10% fetal bovine serum according to literature.[21]

HEK293T cell seeding was optimized for both 100 and 35 mm dishes. **Tables 5.1** and **5.2** indicate the number of cells to seed to achieve the appropriate confluency for day of transfection. According to protocols, 70%-90% confluency is desired to achieve efficient transfection (highlighted in red). 35 mm dishes were used for all transfections and aliquotted into 3 columns of a 96-well plate, unless otherwise specified.

Table 5.1. Optimization of HEK293T cell seeding for 100 mm dishes.

Dilution	Dilution Shorthand	24 hr	36 hr	48 hr	72 hr
10 million cells/dish	1:1	80%	n/a	n/a	n/a
7.5 million cells/dish	1:1.5	70%	-	100%	n/a
5 million cells/dish	1:2	70%	90%	n/a	n/a
3.3 million cells/dish	1:3	25%-30%	-	60%	80%-90%
2.5 million cells/dish	1:4	20%-30%	-	50%	80%-90%
2 million cells/dish	1:5	30%	-	70%	90%
1 million cells/dish	1:10	15%	-	30%	70%

Table 5.2. Optimization of HEK293T cell seeding for 35 mm dishes.

Dilution	24 hr	48 hr	72 hr
100,000 cells/dish	5%	10%	40%
200,000 cells/dish	10%	20%	50%
300,000 cells/dish	15%	25%	70%
400,000 cells/dish	25%	30%	70%
500,000 cells/dish	35%	50%	100%
600,000 cells/dish	30%	70%	n/a
700,000 cells/dish	30%	80%	n/a
800,000 cells/dish	40%	90%	n/a
900,000 cells/dish	50%	100%	n/a
1,000,000 cells/dish	60%	100%	n/a
1,500,000 cells/dish	70%-100%	n/a	n/a

Cells were transiently transfected using various protocols, such as polyethylenimine (PEI), ExpressFect transfection reagent (Denville Scientific), Neon Transfection System (Life Technologies), *Trans*IT-mRNA transfection kit (Mirus), TransMessenger transfection reagent (Qiagen), and Lipofectamine RNAiMAX transfection reagent (Life Technologies). Following transfection, cells were plated (~3 x 10^4 cells/well) onto black 96-well plates with clear bottoms (BD Falcon), incubated for 1-2 days, and then assayed using the Flexstation 3. Unless otherwise indicated, manufacturers' protocols were followed for each transfection method. For PEI transfections in 35 mm plates, 1.5 μg of DNA was transfected with 0.3 mL of DMEM and 9 μL of PEI (in 3 mL DMEM total). For confocal experiments, ExpressFect transfections were performed in 35 mm plates and 1 μg of DNA was transfected with 4 μL of ExpressFect for 4 hr.

5.3.3 The FlexStation 3

The FlexStation 3 is a 96-well fluorescent plate reader used to analyze the functional properties of neurotransmitter-gated ion channels (*e.g.*, human $5HT_{3A}$ and human α4β4 receptors). This device is comprised of two modules - a fluidics module and a detection module (**Figure 5.1**). The fluidics module contains an 8-channel pipette that delivers solutions of neurotransmitter from the source plate to the 96-well sample plate containing HEK293T cells expressing ion channels. The detection module contains an excitation source and a PMT detector that detects the change in fluorescence in response to neurotransmitter activation.

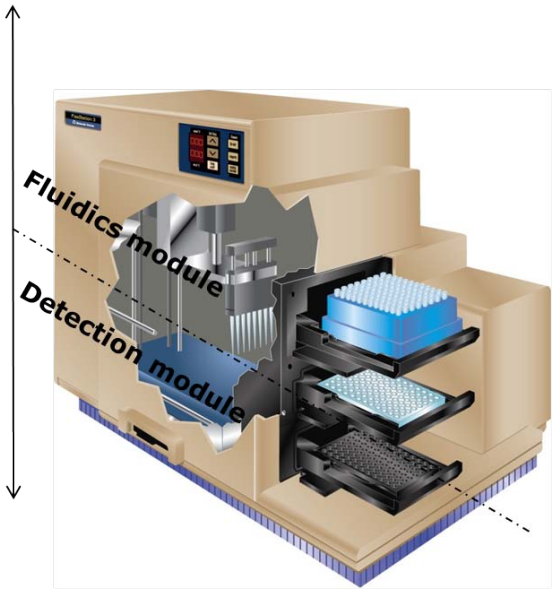


Figure 5.1. Schematic of the FlexStation 3 microplate reader. © Molecular Devices.

Following a two day incubation period, transfected cells in a 96-well plate were washed twice with flex buffer (115 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.9 mM glucose, 10 mM HEPES, pH 7.4) at room temperature.[21] FLIPR membrane potential assay dye (blue kit, Molecular Devices) was diluted 1:10 (unless otherwise specified) in flex buffer and loaded onto washed cells (100 μL/well). Cells were incubated at 37 °C for 45-60 min, and then assayed using the FlexStation. Fluorescence was measured every 1.5 sec for 180 sec, and at 20 sec, 100 μL of neurotransmitter (*e.g.*, 5-HT or ACh) was added to each well. 5-HT and ACh were purchased from Sigma, and neurotransmitter solutions were prepared in flex buffer.

In response to neurotransmitter application, ion channels expressed on the cell membrane bind to the neurotransmitter, undergo a conformational change allowing ions to enter the cell, and cause a change in membrane potential. This change in membrane potential is proportional to the relative fluorescence measurements detected by the FlexStation. Prior to neurotransmitter application, the cell membrane is polarized and the

quenched fluorescent dye is located outside of the cell membrane (**Figure 5.2**). In response to neurotransmitter stimulation, ion channels expressed on the cell membrane open, thereby depolarizing the membrane. The dye enters the cell and fluoresces.

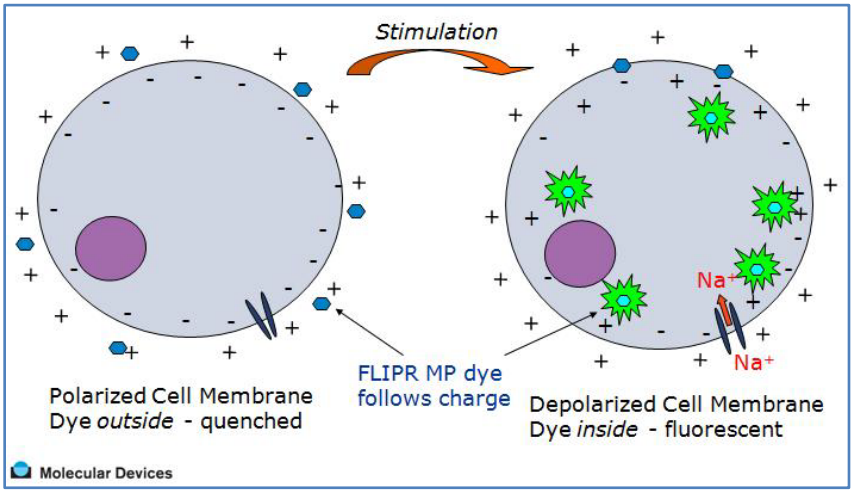


Figure 5.2. Membrane potential assay. Schematic depicting the change in fluorescence in response to cell membrane depolarization. © Molecular Devices.

SOFTmax Pro (Molecular Devices) was used for data analysis (**Figure 5.3A**). Dose-response data were obtained for at least 3 columns of cells (unless otherwise specified). For each column, reduction analysis was performed and the baseline fluorescence at 20 sec was subtracted from the peak fluorescence for each concentration of neurotransmitter (**Figure 5.3B**). These values were normalized to the maximum fluorescence (fluorescence at maximal dose of neurotransmitter). EC_{50} and Hill coefficient (n_H) were calculated by fitting the averaged, normalized dose-response relation to the Hill equation.

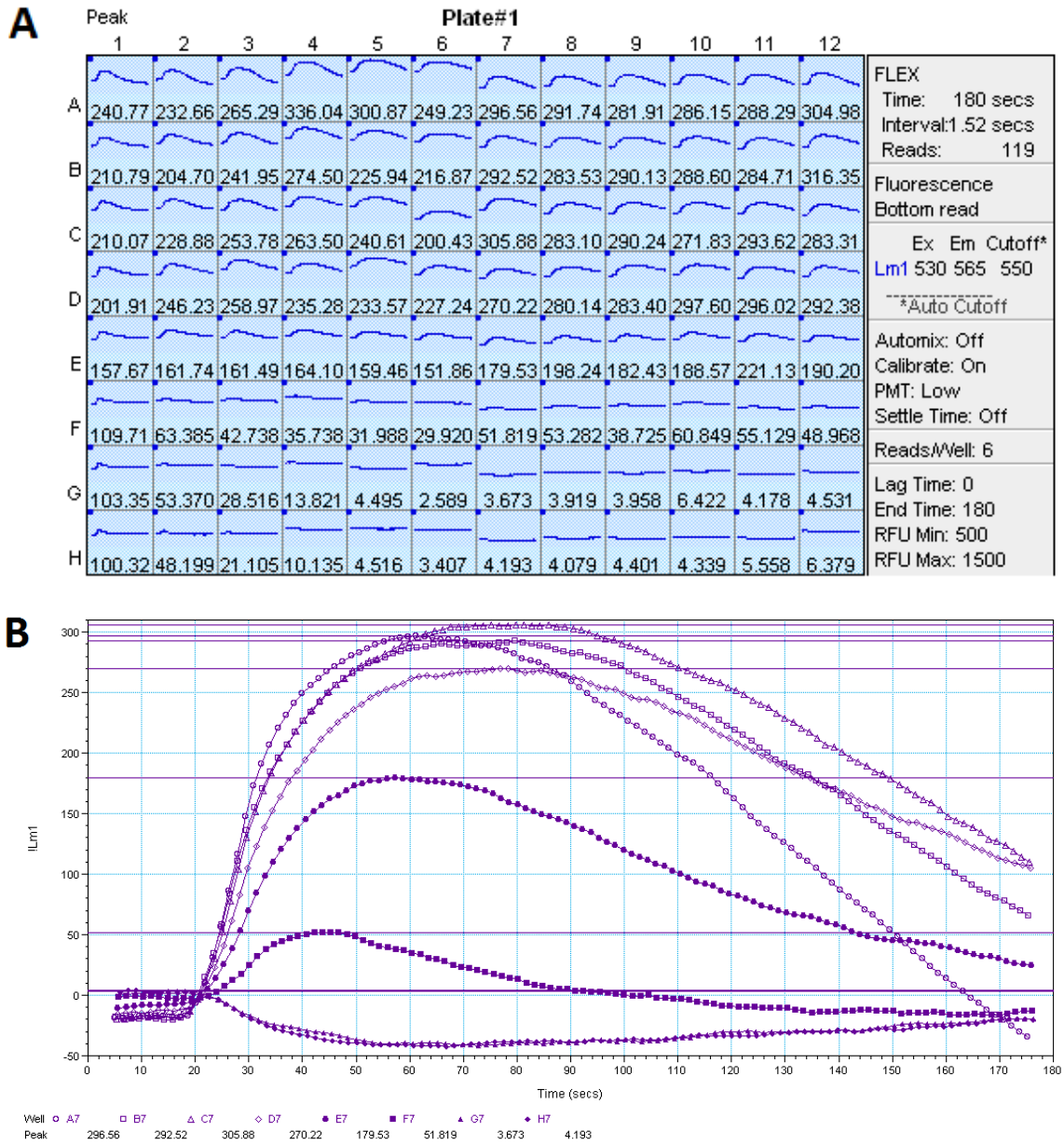


Figure 5.3. SOFTmax PRO data analysis of a 96-well plate. A. Example plate containing HEK293T cells expressing human $5HT_{3A}$ receptors is shown (data collected in house). The columns are labeled 1-12 and the rows are labeled A-H. The drug plate is prepared by serial dilution with row A containing the highest drug concentration, and row H containing the lowest. Each row receives a specific drug concentration. The peak relative fluorescence is reported for each well of the plate. B. Reduction analysis of column 7 (from the plate in A), indicating the peak relative fluorescence per drug concentration.

5.3.4 Fluorescent Dye Experiments

Several concentrations of rhodamine B (Sigma) were prepared in a drug plate by 1:1 serial dilutions of rhodamine B:water starting with a 50 mM stock of rhodamine B. 100 μL of dye was added to 100 μL of water in the assay plate for each concentration and the area under the curve of the emission spectrum was measured. This was repeated for each PMT setting (low, medium, high).

Note that eosin Y and fluorescein (purchased from City Chemical Company and Sigma, respectively) were also tested with the low PMT setting. Interestingly, both dyes displayed a maximal RFU of 50,000 indicating that the FlexStation is capable of detecting very high fluorescence signals.

5.4 RESULTS AND DISCUSSION

5.4.1 Determining the Optimal PMT Setting to Detect Small Fluorescence Signals

It is possible that even under optimal transfection conditions, the fluorescence signal of mutant ion channels containing unnatural amino acids will be significantly lower than for wild type ion channels. Thus, we needed to determine the appropriate FlexStation PMT setting to maximize the relative fluorescence unit (RFU) signal detected. The RFU dependence on PMT setting was measured for a serial dilution of rhodamine B at each of the PMT settings (low, medium, high).

Using the low PMT setting, the FlexStation was unable to detect fluorescence for high rhodamine B concentrations (50 – 0.39 mM), which is likely due to self-quenching of the dye. A maximal signal of 10,000 RFU was detected for 0.19 mM rhodamine B and each subsequent serial dilution produced a fluorescence signal decreased by approximately half (**Table 5.3**). A similar trend was observed for the medium PMT

setting. Here, the maximal signal is 6500 RFU detected for 0.049 mM rhodamine B, followed by a decrease in RFU proportional to dye concentration. Under the high PMT setting, 1.52 μM rhodamine B was the highest concentration that produced a fluorescence signal (300 RFU). Similarly, as the dye concentration decreased, so did the RFU.

Table 5.3. Comparison of RFU signal detected by the FlexStation 3 for each PMT settings for a serial dilution of rhodamine B.

Rhodamine B Concentration (μM)	Low PMT Setting	Medium PMT Setting	High PMT Setting
0.763	191.47	144.91	124.55
0.381	77.67	65.71	47.34
0.191	44.83	27.18	22.21
0.095	20.88	16.13	12.56
0.048	17.04	8.56	6.44
0.024	10.07	5.52	4.34
0.012	7.88	4.64	2.89
0.006	5.87	3.42	2.25

For any given rhodamine B concentration, the low PMT setting produced the greatest RFU signal. As shown in **Table 5.3**, we compared RFU values detected at various PMT settings for very low concentrations of dye. We anticipate that small RFU signals (produced by very low dye concentrations) are likely to mimic those of a mutant ion channel expressing an unnatural amino acid. We, therefore, chose to use the low PMT setting for FlexStation assays of transfected cells.

5.4.2 Transfection of Mammalian Cells

ExpressFect and PEI Transfections of Neuronal Nicotinic Acetylcholine Receptors

To establish a positive control, functional ion channels were expressed in HEK293T cells and detected using the FlexStation. Neuronal α4β2 and α4β4 receptors were tested as ion channel targets for FlexStation analysis. Using ExpressFect

transfection reagent, HEK293T cells were transfected with α4 DNA tagged with green fluorescent protein (α4-GFP) and either β2 or β4 wild type DNA. Confocal microscopy revealed robust membrane expression of α4GFPβ4 receptors, but not α4GFPβ2 receptors (**Figure 5.4**). The α4GFPβ4 image clearly shows membrane expression by the rough edges and the bright dots as ER exit sites, both of which are absent in the α4GFPβ2 image. This observation was not surprising since literature supports that α4β2 receptors become trapped in the endoplasmic reticulum (ER) due to the presence of an ER retention motif and lack of an ER export motif.[22]

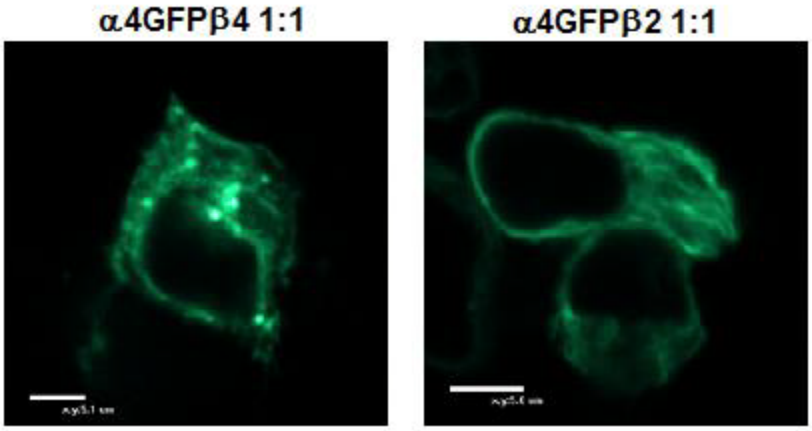


Figure 5.4. Confocal images of HEK293T cells expressing mouse α4β4 or α4β2 receptors containing GFP in the α4 subunit.

We previously reported that biasing α4:β4 subunit mRNA in *Xenopus* oocytes can control α4β4 receptor stoichiometry shown by electrophysiology (Chapter 4).[7] We therefore tested the ability of the FlexStation to detect changes in receptor stoichiometry using a similar experiment. HEK293T cells were transfected with several ratios of α4:β4 subunit DNA and functionally analyzed using the FlexStation (**Table 5.4**). Transfections were performed using both PEI and ExpressFect protocols. Consistent results were obtained using both protocols.

Table 5.4. Human α4β4 receptors expressed in HEK293T cells for given ratios of α4:β4 DNA. EC_{50} measurements and RFU values were collected using the FlexStation.

α4:β4 Ratio	EC_{50} (μM)	Hill	N	Average Max RFU
2000:1	1.79 ± 0.23	1.16 ± 0.14	6	40.4
1000:1	1.46 ± 0.23	1.14 ± 0.17	5	52.9
500:1	0.75 ± 0.04	1.67 ± 0.13	17	104.4
200:1	0.70 ± 0.02	1.49 ± 0.05	17	106.2
100:1	0.72 ± 0.07	1.13 ± 0.10	20	144.6
50:1	0.70 ± 0.05	1.24 ± 0.09	20	155.4
30:1	0.65 ± 0.04	1.20 ± 0.07	20	163.8
10:1	0.66 ± 0.04	1.23 ± 0.07	21	125.0
3:1	0.75 ± 0.07	1.14 ± 0.11	19	80.1
1:1	3.14 ± 0.24	0.98 ± 0.06	23	67.0
1:3	5.51 ± 0.62	2.55 ± 0.48	2	47.3
1:10	7.45 ± 1.60	3.83 ± 3.23	2	16.1

Multiple stoichiometries of the α4β4 receptor were observed. The most abundant α4β4 population was observed for transfection of α4:β4 DNA ratios ranging from 500:1 to 3:1, which likely represents $(\alpha4)_3(\beta4)_2$. These receptors displayed a characteristic EC_{50} of approximately 0.7 μM ACh and an average maximal RFU of 100-150. Alternatively, $(\alpha4)_2(\beta4)_3$ receptors were observed for α4:β4 DNA ratios biasing the β4 subunit, such as 1:1 to 1:10. This second receptor population was accompanied by a right-shifted EC_{50} and decreased average maximal RFU compared to $(\alpha4)_3(\beta4)_2$. It appears that agonist sensitivity for these two α4β4 stoichiometries differs depending on whether the receptors are expressed in HEK293T cells or *Xenopus* oocytes. In HEK293T cells, $(\alpha4)_3(\beta4)_2$ receptors cells display a lower, more sensitive EC_{50} than $(\alpha4)_2(\beta4)_3$ receptors, while the reverse is observed for oocyte expression.[7] We also note the presence of a third α4β4 population for DNA ratios extremely biasing α4 (*e.g.* 2000:1 and 1000:1), which is probably a physiological irrelevant stoichiometry (*e.g.*, $(\alpha4)_4(\beta4)_1$) resulting from excessive subunit bias.

ExpressFect and PEI Transfections of the 5-HT$_{3A}$ Receptor

While the FlexStation can discriminate between receptors that experience variable stoichiometries, we anticipated that attempting nonsense suppression in such a receptor was unnecessarily complicated. We moved forward with the homopentameric serotonin-gated ion channel, the 5HT$_{3A}$ receptor. HEK293T cells were transfected with human 5HT$_{3A}$ DNA using either ExpressFect or PEI transfection protocols. Both transfection methods produced similar results. EC$_{50}$ (0.3 μM 5-HT) and average maximal RFU were consistent with each other and literature values (**Figure 5.5**).[23]

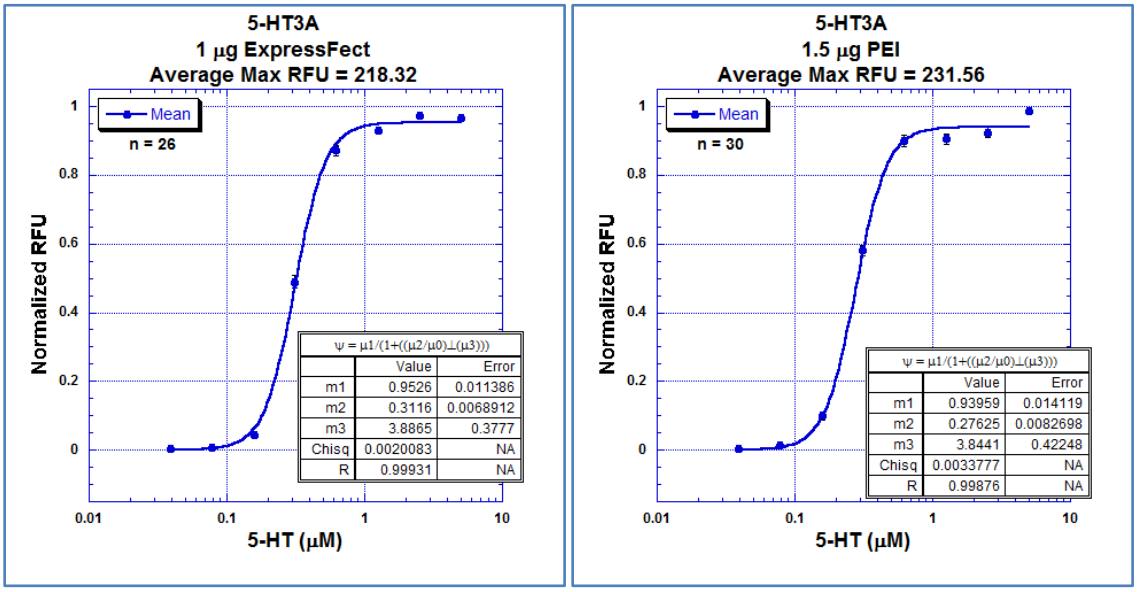


Figure 5.5. Dose-reponse curves of human 5HT$_{3A}$ receptors expressed in HEK293T cells. Cells were transfected using either ExpressFect transfection reagent (left) or PEI (right).

Expression of conventional mutant 5HT$_{3A}$ receptors in HEK293T cells was also examined. Mutation of W183 in the 5HT$_{3A}$ receptor was a logical starting point since our lab previously reported that this residue participates in a cation-π interaction with serotonin and mutation of this residue results in large shifts in EC$_{50}$.[4, 23] Using either ExpressFect or PEI, 5HT$_{3A}$ W183A and W183Y mutant receptors were expressed in cells

and assayed using the FlexStation. Results were consistent with literature values (**Figure 5.6**). The slight differences in absolute EC_{50} value are likely due to species variation; we used human $5HT_{3A}$ constructs while the literature values are based on mouse $5HT_{3A}$ constructs (**Table 5.5**).

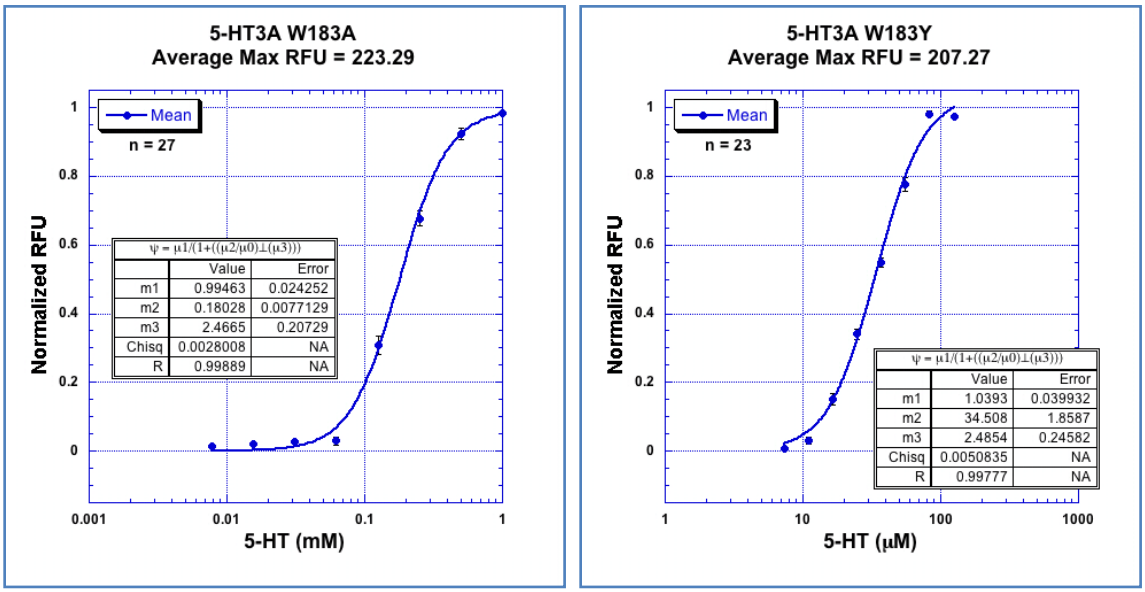


Figure 5.6. Dose-response curves of $5HT_{3A}$ W183A (left) or W183Y (right) mutant receptors expressed in HEK293T cells.

Table 5.5. Conventional $5HT_{3A}$ mutant receptors expressed in HEK293T cells. *Mouse $5HT_{3A}$ data is shown for comparison and referenced from Thompson 2008.[23]

	Human $5HT_{3A}$			**Mouse $5HT_{3A}$***	
	EC_{50} (μM)	Fold Shift	Hill	EC_{50} (μM)	Fold Shift
Wild Type	0.28 ± 0.01	-	3.84 ± 0.42	0.24	-
W183A	180 ± 7	652	2.47 ± 0.21	39.8	166
W183Y	34.5 ± 1.9	125	2.49 ± 0.25	5.25	22

Since mRNA injection is routinely used in oocyte experiments and mRNA is one step further in protein translation compared to DNA, we explored the possibility that transfection of mRNA might yield increased receptor expression in HEK293T cells. *In vitro* transcription $5HT_{3A}$ mRNA from the pcDNA3.1(+) construct was unsuccessful. So the $5HT_{3A}$ gene was subcloned from pcDNA3.1(+) into the pGEMhe vector, which

facilitates *in vitro* mRNA transcription. Oocytes injected with $5HT_{3A}$ mRNA prepared from the pGEMhe vector expressed functional protein. HEK293T cells were transfected with the same $5HT_{3A}$ mRNA using TransMessenger, a kit developed for efficient mRNA transfection. However, no protein expression was detected. This is likely to reflect an mRNA polyadenylation issue rendering the mRNA less stable in a mammalian cell system. We concluded that transfection of mRNA was inferior to DNA transfection, especially considering that the HEK293T cell line was chosen based on its ability to replicate plasmid DNA and yield increased expression.

ExpressFect and PEI Transfections: Attempted Suppression in the $5HT_{3A}$ Receptor

Given that transfection of wild type $5HT_{3A}$ DNA in HEK293T cells proved successful, several attempts were made to incorporate tryptophan at position W183 in the $5HT_{3A}$ receptor. Since W183 makes a cation-π interaction to serotonin, lack of W incorporation or incorporation of any other endogenous amino acid (*e.g.*, readthrough) should be easily detected by the FlexStation. $5HT_{3A}$ W183TAG DNA and THG73 tRNA *in vitro* aminoacylated with W (THG73-W) were cotransfected into cells using either ExpressFect or PEI. Despite transfecting various amounts of $5HT_{3A}$ W183TAG DNA and/or THG73-W, no functional receptor expression was detected for either transfection protocol (**Tables 5.6** and **5.7**). Robust expression was detected from wild type $5HT_{3A}$ DNA transfected into the same batch of HEK293T cells establishing cell viability. Additional trials using new reagents and different batches of nucleic acid materials still resulted in no receptor expression. As such, ExpressFect and PEI transfection methods are not suitable for nonsense suppression in mammalian cells.

Table 5.6. Using ExpressFect to perform wild type recovery of tryptophan at position W183 in the $5HT_{3A}$ receptor. *DNA amount is per 3 columns of a 96-well plate.

W183TAG DNA (μg)*	THG73-W (μg)	ExpressFect, Avg Max RFU
0.5	1.25	No Expression
1	1.25	No Expression
2	1.25	No Expression
5	1.25	No Expression

Table 5.7. Using PEI to perform wild type recovery of tryptophan at position W183 in the $5HT_{3A}$ receptor. *DNA amount is per 3 columns of a 96-well plate.

W183TAG DNA (μg)*	THG73-W (μg)	PEI, Avg Max RFU
Vary DNA, wide range of tRNA		
0.5	0.5	No Expression
1	0.5	No Expression
1	2	No Expression
1	3	No Expression
tRNA constant (1μg)		
0.25	1	No Expression
0.5	1	No Expression
1	1	No Expression
2	1	No Expression
Vary DNA and tRNA, but keep total nucleic acid constant (1μg)		
0.25	0.75	No Expression
0.75	0.25	No Expression
0.1	0.9	No Expression
0.9	0.1	No Expression

Neon Transfections of the 5-HT_{3A} Receptor

Electroporation was explored as an alternative transfection technique amenable to nonsense suppression in mammalian cells. The Neon Transfection System was used to electroporate various amounts of $5HT_{3A}$ DNA. The average maximal RFU was monitored for one- and two-day incubation of transfected cells (**Table 5.8**). Regardless of incubation time, 2 μg of wild type $5HT_{3A}$ DNA per 3 columns of a 96-well plate yielded the highest expression. Cells incubated for two days gave higher expression than for one-day incubations. Several electroporation conditions (*e.g.*, voltage, pulse width, and number of pulses) were tested to determine the most effective transfection protocol

(**Table 5.9**). Conditions 6, 7, and 8 produced the highest average maximal RFU. Condition 7 (highlighted in red) was selected as the optimal condition for further electroporation transfections.

Table 5.8. Optimizating wild type $5HT_{3A}$ DNA transfection using the Neon Transfection System. *DNA amount is per 3 columns of a 96-well plate.

DNA (μg)*	1 Day Incubation, Avg Max RFU	2 Day Incubation, Avg Max RFU
1	145.0	232.0
2	300.1	359.7
4	137.4	309.3
8	185.1	304.5

Table 5.9. Optimization of Neon electroporation protocol. Maximum RFU signals are reported for each of the 24 preprogrammed Neon transfection conditions. 2 μg of DNA was transfected per 3 columns of a 96-well plate.

Condition	Voltage	Width	# Pulses	Max RFU	Condition	Voltage	Width	# Pulses	Max RFU
1	0	20	1	n/a	**13**	1100	20	2	242
2	1400	20	1	294	**14**	1200	20	2	311
3	1500	20	1	299	**15**	1300	20	2	280
4	1600	20	1	174	**16**	1400	20	2	115
5	1700	20	1	130	**17**	850	30	2	265
6	1100	30	1	342	**18**	950	30	2	254
7	1200	30	1	341	**19**	1050	30	2	301
8	1300	30	1	322	**20**	1150	30	2	238
9	1400	30	1	216	**21**	1300	10	3	265
10	1000	40	1	321	**22**	1400	10	3	273
11	1100	40	1	298	**23**	1500	10	3	270
12	1200	40	1	256	**24**	1600	10	3	87

Neon Transfections: Attempted Suppression in the $5HT_{3A}$ Receptor

Using the Neon Transfection System, cells were challenged to incorporate tryptophan at position W183 in the $5HT_{3A}$ receptor. Master mixes containing various amounts of $5HT_{3A}$ W183TAG DNA and THG73-W were prepared, one of which contained THG73-76mer to test for readthrough. HEK293T cells were electroporated with each master mix and the average maximal RFU was monitored (**Table 5.10**). No

functional receptor expression was detected for any of the DNA/tRNA master mixes tested.

Table 5.10. Using Neon electroporation for wild type recovery incorporating tryptophan at position W183 in the $5HT_{3A}$ receptor. *DNA and tRNA amounts are per 3 columns of a 96-well plate.

W183TAG DNA (μg)*	THG73-W tRNA (μg)*	Avg Max RFU
Constant DNA		
2	0	No Expression
2	2 (THG73-76mer)	No Expression
2	0.1	No Expression
2	0.5	No Expression
2	1	No Expression
2	2	No Expression
2	4	No Expression
Constant tRNA		
1	2	No Expression
2	2	No Expression
4	2	No Expression
8	2	No Expression
16	2	No Expression
32	2	No Expression

Here, we show that transfection of DNA containing an amber stop codon and *in vitro* aminoacylated THG73 tRNA does not produce receptor expression for any transfection method employed. These findings differ from previous work by Monahan *et al*. demonstrating that THG73 does in fact incorporate unnatural amino acids into ion channels expressed in mammalian cells.[15] Monahan's studies used whole-cell patch clamp electrophysiology to functionally analyze a single cell,[15] but the FlexStation detects fluorescence signals from thousands of cells in a single well of a 96-well plate. As such, the signal obtained using the FlexStation can be easily diluted by non-expressing cells under conditions of low expression. So it follows that if THG73 is poorly recognized by the mammalian translational machinery resulting in limited protein

expression, this signal might be detected using single-cell detection techniques, but not by the FlexStation.

Given the negative results with THG73, we explored using an alternative suppressor tRNA, the yeast phenylalanine frameshift suppressor tRNA (YFaFS). Cells were electroporated with 2 μg of $5HT_{3A}$ W183GGGT DNA and varying amounts of YFaFS-W tRNA or YFaFS-76mer (**Table 5.11**). Cells appeared healthy by visual inspection, but no expression was detected. Control RFU levels were observed from electroporation of wild type $5HT_{3A}$ DNA. Several trials produced similar results indicating that YFaFS tRNA provides no additional benefit over THG73 tRNA.

Table 5.11. Using Neon electroporation for wild type recovery of a frameshift codon at position W183 in the $5HT_{3A}$ receptor. *DNA and tRNA amounts are per 3 columns of a 96-well plate.

Trial Date	W183GGGT DNA (μg)*	YFaFS-W tRNA (μg)*	Avg Max RFU
Trial 1	2 (WT)	0	572.6
	2	8 (YFaFS-76mer)	No Expression
	2	8	No Expression
Trial 2	2 (WT)	0	301.2
	2	8 (YFaFS-76mer)	No Expression
	2	4	No Expression
	2	8	No Expression
Trial 3	2 (WT)	0	443.7
	2	8 (YFaFS-76mer)	No Expression
	2	4	No Expression
	2	8	No Expression

Using Human Serine Amber Suppressor tRNA to Suppress an Amber Codon in eGFP

The endogenous aminoacyl tRNA synthetase in the cells charges the human serine amber suppressor tRNA (HSAS) with serine, which can then be incorporated opposite an amber stop codon.[17] This human-derived tRNA is recognized by the HEK293T cells, and so we explored using HSAS to optimize co-transfection of DNA and tRNA materials.

We targeted residue A37 in the enhanced green fluorescent protein (eGFP) since this site is located well before the chromophore. If suppression does not occur, then even expression of a truncated protein will not give a fluorescent signal. Both electroporation and ExpressFect were used to transfect cells with eGFP A37TAG DNA and HSAS, and suppression efficiency was monitored by confocal microscopy. Wild type recovery of eGFP A37TAG using THG73-Ala was attempted, but results were indistinguishable from 76mer control experiments. Cells transfected with eGPF A37TAG and HSAS revealed improved eGFP expression relative to wild type recovery, however approximate expression was <1% of wild type expression (**Figure 5.7**). Electroporation gave slightly lower expression than ExpressFect. These experiments provided the initial confirmation that successful transfection of tRNA had occurred, but that protein expression was significantly hindered when using a stoichiometric tRNA (*e.g.*, THG73 tRNA).

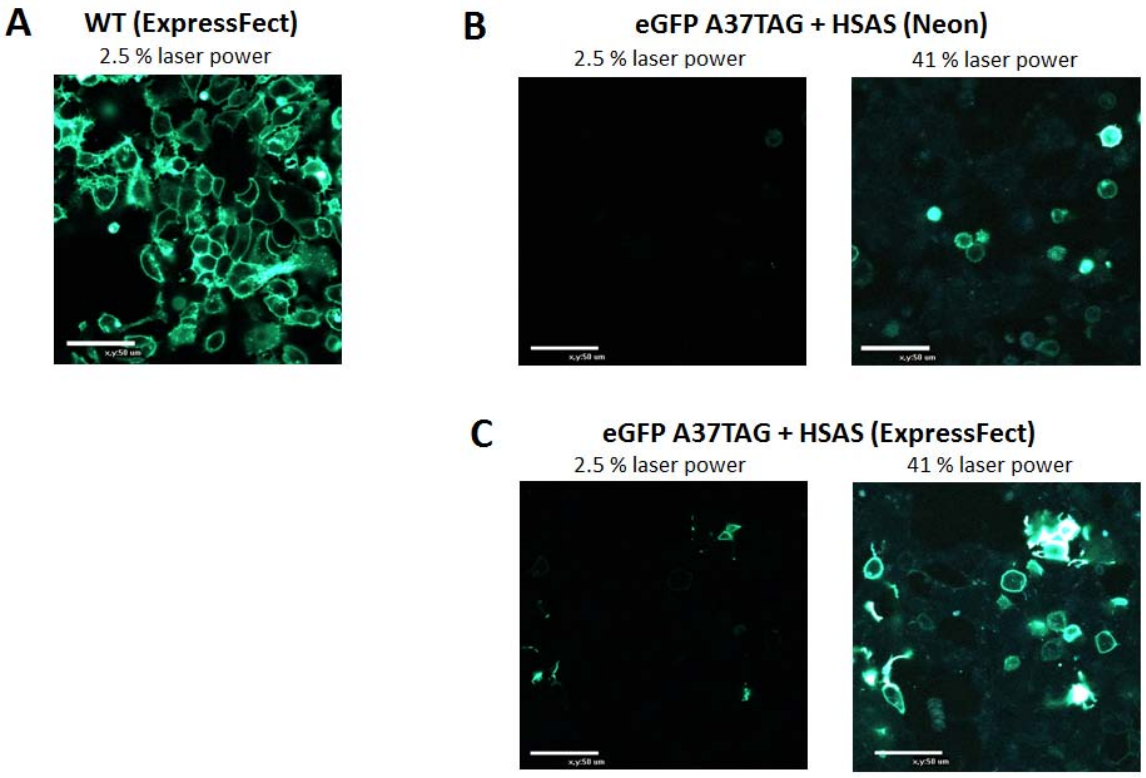


Figure 5.7. Confocal images of HEK293T cells expressing eGFP. A. Wild type eGFP expression (ExpressFect). B. Neon electroporation; suppression of eGFP A37TAG DNA by HSAS. C. ExpressFect; suppression of eGFP A37TAG DNA by HSAS. Images obtained using 2.5% laser power contain 90% saturation. Images obtained using 41% laser power contain 96% saturation.

Using HSAS to Suppress an Amber Codon in the $5HT_{3A}$ Receptor

Returning to the $5HT_{3A}$ receptor, we attempted to perturb receptor function by using HSAS to incorporate serine at position W183, the critical cation-π interaction residue. Both electroporation and PEI were used to transfect a constant amount of $5HT_{3A}$ W183TAG DNA with varying amounts of HSAS (**Table 5.12**). Electroporation of 2 and 4 μg of HSAS produced functional receptors with an EC_{50} of 370 μM 5-HT (1200-fold shifted from wild type). It is likely that challenging the translational machinery to suppress a functionally important site with a non-native amino acid in all five subunits of the receptor can account for the observed low expression. However, this provided

additional evidence that tRNA was successfully delivered to the cells and protein translation occurred. No expression was detected from PEI transfection of HSAS.

Table 5.12. Comparison of Neon electroporation and PEI transfection using HSAS to incorporate serine at position W183 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	HSAS (μg)*	Neon, Avg Max RFU	PEI, Avg Max RFU
2 (WT)	0	180.6	73.9
2 (W183TAG)	0.5	No Expression	No Expression
2 (W183TAG)	2	14.9	No Expression
2 (W183TAG)	4	33.5	No Expression

Suppression with HSAS was further optimized by attempting wild type recovery of residue S61 in the $5HT_{3A}$ receptor. Electroporation, PEI, and ExpressFect were used to transfect a constant amount of $5HT_{3A}$ S61TAG DNA and varying amounts of HSAS (**Table 5.13**). Transfection efficiency was compared by monitoring the average maximal RFU. HSAS suppression was most efficient with electroporation, since this method required the least amount of HSAS. In fact, protein expression decreased with increasing amounts of HSAS. Variable expression was detected from ExpressFect and required more HSAS than electroporation. Cells appeared viable after PEI transfection, but no expression was detected.

Table 5.13. Comparison of Neon electroporation, PEI, and ExpressFect: Wild type recovery using HSAS to incorporate serine at position S61 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	HSAS (μg)*	Neon, Avg Max RFU	PEI, Avg Max RFU	ExpressFect, Avg Max RFU	ExpressFect, Avg Max RFU
		11/15/10	**11/15/10**	**11/15/10**	**12/1/10**
2 (WT)	0	297.1	195.7	320.5	309.5
2 (S61TAG)	2	93.8	No Expression	No Expression	No Expression
2 (S61TAG)	4	37.7	No Expression	80.9	No Expression
2 (S61TAG)	8	No Expression	No Expression	78.3	No Expression

Given that suppression yielded much lower expression compared to wild type, preparation of the membrane potential dye was optimized to a 1:2 dilution in flex buffer to increase signal detection. The average maximal RFU was measured from cells electroporated with 2 μg $5HT_{3A}$ S61TAG DNA and varying amounts of HSAS and incubated with the newly prepared dye (**Table 5.14**). For each HSAS concentration transfected, increased signal was detected using the 1:2 dye dilution compared to the 1:10 dilution (**Table 5.13**). The 1:2 dye dilution was used in all further FlexStation assays.

Table 5.14. Monitoring RFU using the 1:2 dye dilution: Wild type recovery using HSAS to incorporate serine at site S61 in the $5HT_{3A}$ receptor.
*Note that μg of DNA and tRNA is per 3 columns of a 96-well plate.

DNA (μg)	HSAS (μg)	Neon, Avg Max RFU
2 (WT)	0	371.5
2 (S61TAG)	2	112.4
2 (S61TAG)	4	121.4
2 (S61TAG)	8	91.9

Neon Electroporation and ExpressFect: Double Transfections

We next explored the possibility that transfecting a second dose of DNA and/or tRNA to the cells might enhance protein expression. Cells were electroporated with 2 μg of $5HT_{3A}$ S61TAG DNA and 4 μg HSAS using condition 7 (1200 V, 30 ms, 1 pulse). Following 24 hr incubation, 24 preprogrammed electroporation conditions were sampled to electroporate the cells with a second dose of 4 μg HSAS (**Table 5.15**). Condition 22 (1400 V, 10 ms, 3 pulses) resulted in the greatest maximal RFU (390). ExpressFect was also used to doubly transfect cells with DNA and/or HSAS, but these experiments gave significantly lower expression in comparison to electroporation.

Table 5.15. Optimization of Neon electroporation for double transfection of HSAS to incorporate serine at position S61 in the $5HT_{3A}$ receptor. 2 μg of DNA and 4 μg of HSAS was transfected per 3 columns of a 96-well plate.

Condition	Max RFU	Condition	Max RFU
1 (single)	17.4	**13**	No Expression
2	161.1	**14**	118.8
3	158.1	**15**	209.8
4	170.5	**16**	278.4
5	193.4	**17**	54.0
6	172.6	**18**	135.9
7	219.2	**19**	223.5
8	264.5	**20**	279.7
9	315.7	**21**	260.1
10	216.8	**22**	390.0
11	253.7	**23**	273.1
12	277.1	**24**	183.1

Using the electroporation protocol for double transfection, cells were challenged to incorporate tryptophan at position W183 using *in vitro* aminoacylated THG73-W. Cells were electroporated first with 2 μg of $5HT_{3A}$ W183TAG DNA and 8 μg of THG73-W, followed by a second electroporation of the same concentration of DNA and/or tRNA 24 hr later (**Table 5.16**). No readthrough was detected and despite the promising results with HSAS, no expression was observed for electroporation of THG73-W.

Table 5.16. Double Neon Transfection: Wild type recovery incorporating tryptophan at position W183 in the $5HT_{3A}$ receptor. *DNA and tRNA amounts are per 3 columns of a 96-well plate.

DNA (μg)*	tRNA (μg)*	Nucleic Acid Double Transfected	Avg Max RFU (12/21/10)
2 (WT)	0	DNA only	Instrument disrupted, data lost
2 (W183TAG)	8 (THG73-76mer)	tRNA only	No Expression
2 (W183TAG)	8 (THG73-W)	tRNA only	No Expression
2 (W183TAG)	8 (THG73-W)	tRNA + DNA	No Expression

RNA Tranfections of the $5HT_{3A}$ Receptor

Considering that efficient delivery of aminoacylated tRNA into the cells is half the battle, we tested several reagents designed for efficient transfection of RNA material.

*Trans*IT-mRNA, TransMessenger, and Lipofectamine RNAiMAX transfection reagents were used in these studies and are intended for transfection of mRNA or siRNA. Here we describe our efforts to adapt these protocols to cotransfect DNA and tRNA material into HEK293T cells.

According to the *Trans*IT-mRNA transfection protocol, transfection of wild type $5HT_{3A}$ DNA was performed using various ratios of Boost:*Trans*IT reagent (**Table 5.17**). Significant receptor expression, as measured by average maximal RFU, was observed for 2 μg of DNA. Receptor expression was further increased under conditions using a minimum of 7.5 μL of *Trans*IT and a 1:2 ratio of Boost:*Trans*IT.

Table 5.17. Optimization of *Trans*IT Transfection. *DNA amount is per 3 columns of a 96-well plate.

WT DNA (μg)*	Boost Reagent (μL)	*Trans*IT (μL)	Avg Max RFU
0.02	5	7.5	No Expression
0.1	5	7.5	107.0
0.5	5	7.5	308.9
2	5	7.5	467.6
2	1.25	2.5	408.1
2	3.75	7.5	497.8
2	7.5	15	541.8
2	15	30	563.5

After determining the optimal Boost:*Trans*IT ratio, the *Trans*IT protocol was used to suppress an amber stop codon using HSAS. Cells were transfected with various amounts of $5HT_{3A}$ S61TAG DNA and HSAS (**Table 5.18**). Cells experienced decreased viability and reduced protein expression when transfected using large amounts of *Trans*IT transfection reagent (*i.e.*, 15 and 30 μL *Trans*IT). Lowering the amount of *Trans*IT to 7.5 μL improved expression, while 2.5 μL of *Trans*IT gave very low expression. We concluded that 2 μg of S61TAG DNA, a minimum of 4 μg of HSAS, and 7.5 μL of *Trans*IT were necessary to achieve efficient expression.

Table 5.18. Optimization of *Trans*IT Transfection: Wild type recovery using HSAS to incorporate serine at position S61 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

$5HT_{3A}$ DNA (μg)*	**HSAS (μg)**	**Boost Reagent (μL)**	***Trans*IT (μL)**	**Avg Max RFU**
DNA constant, vary HSAS and Boost:TransIT				
2 (WT)	0	7.5	15	471.1
2 (S61TAG)	4	7.5	15	113.1
2 (S61TAG)	8	7.5	15	94.6
2 (S61TAG)	8	15	30	Cells Died
Vary DNA, constant HSAS, vary Boost:TransIT				
0.5 (S61TAG)	4	3.75	7.5	95.2
1 (S61TAG)	4	3.75	7.5	91.1
0.5 (S61TAG)	4	7.5	15	44.8
1 (S61TAG)	4	7.5	15	73.2
Constant DNA, vary HSAS, using 2.5 μL TransIT				
2 (WT)	0	1.25	2.5	167.7
2 (S61TAG)	2	1.25	2.5	No Expression
2 (S61TAG)	4	1.25	2.5	37.4
2 (S61TAG)	8	1.25	2.5	33.5
Constant DNA, vary HSAS, using 7.5 μL TransIT				
2 (WT)	0	3.75	7.5	489.0
2 (S61TAG)	2	3.75	7.5	162.9
2 (S61TAG)	4	3.75	7.5	353.5
2 (S61TAG)	8	3.75	7.5	340.0

Using the optimized *Trans*IT condition, cells were subjected to double transfections, in which the second transfection contained either DNA and HSAS or only HSAS (**Table 5.19**). Double transfection of both DNA and HSAS produced significantly more cell death and lower expression than observed for single transfection. However, when only HSAS was delivered during the second transfection, results were similar to those observed for single transfection, but did not provide any additional benefit over the single transfection. These same conditions were used to doubly transfect cells with $5HT_{3A}$ W183TAG DNA and THG73-W tRNA. Unfortunately, no expression was detected for double transfection of either DNA and tRNA or tRNA only (**Table 5.20**), an observation common to all experiments challenging the cells to express protein using this *in vitro* aminoacylated and stoichiometric tRNA.

Table 5.19. Double *Trans*IT Transfection: Wild type recovery using HSAS to incorporate serine at position S61 in the $5HT_{3A}$ receptor. The first transfection contains both DNA and HSAS, but the second transfection contains *either* DNA and HSAS *or* HSAS only. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	HSAS (μg)*	Boost Reagent (μL)	*Trans*IT (μL)	2X (DNA+HSAS) Avg Max RFU	2X (HSAS only) Avg Max RFU
2 (WT)	0	3.75	7.5	621.6	626.0
2 (S61TAG)	2	3.75	7.5	46.2	177.7
2 (S61TAG)	4	3.75	7.5	51.9	321.4
2 (S61TAG)	8	3.75	7.5	85.0	390.1

Table 5.20. Double *Trans*IT Transfection: Wild type recovery of tryptophan at position W183 in the $5HT_{3A}$ receptor. The first transfection contains both DNA and tRNA, but the second transfection contains *either* DNA and tRNA *or* tRNA only. *DNA and tRNA amounts are per 3 columns of a 96-well plate.

W183TAG DNA (μg)*	tRNA (μg)*	Boost Reagent (μL)	*Trans*IT (μL)	2X (DNA+tRNA) Avg Max RFU	2X (tRNA only) Avg Max RFU
2	8 (THG73-76mer)	3.75	7.5	No Expression	No Expression
2	2 (THG73-W)	3.75	7.5	No Expression	No Expression
2	4 (THG73-W)	3.75	7.5	No Expression	No Expression
2	8 (THG73-W)	3.75	7.5	No Expression	No Expression

Additional RNA transfection methods were tested, but proved inferior to the *Trans*IT method. Suppression experiments using TransMessenger consumed more transfection reagent and produced significantly decreased RFU signals compared to *Trans*IT (*i.e.*, 70 *vs.* 350 RFU, respectively). Alternatively, the Lipofectamine RNAiMAX transfection protocol yielded mediocre expression for transfection of wild type DNA and no expression for suppression experiments. Experimental details for TransMessenger and RNAiMAX transfections are located in **Tables 5.21-5.24**.

Table 5.21. Optimization of TransMessenger transfection conditions. *DNA amount is per 3 columns of a 96-well plate.

WT DNA (μg)*	TransMessenger (μL)	Avg Max RFU
0.1	8	207.6
0.5	8	366.0
2	8	346.8
2	4	No Expression
2	8	376.0
2	16	287.5
4	8	310.0
4	16	361.7

Table 5.22. Optimization of TransMessenger transfection conditions: Wild type recovery incorporating serine at position S61 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	HSAS (μg)*	TransMessenger (μL)	Avg Max RFU
Constant DNA, vary HSAS, constant TransMessenger			
2 (WT)	0	8	274.7
2 (S61TAG)	2	8	No Expression
2 (S61TAG)	4	8	No Expression
2 (S61TAG)	8	8	No Expression
Vary DNA, constant HSAS, vary TransMessenger			
0.5 (S61TAG)	4	8	42.5
1 (S61TAG)	4	8	47.2
0.5 (S61TAG)	4	16	74.1
1 (S61TAG)	4	16	120.4
Constant DNA, vary HSAS, using 16 μL TransMessenger			
2 (WT)	0	16	208.4
2 (S61TAG)	2	16	63.8
2 (S61TAG)	4	16	No Expression
2 (S61TAG)	8	16	No Expression
Constant DNA, vary HSAS, using 32 μL TransMessenger			
2 (WT)	0	32	181.8
2 (S61TAG)	2	32	69.9
2 (S61TAG)	4	32	52.9
2 (S61TAG)	8	32	No Expression

Table 5.23. Optimization of Lipofectamine RNAiMAX transfection conditions. *DNA amount is per 3 columns of a 96-well plate.

WT DNA (μg)*	RNAiMAX (μL)	Avg Max RFU
Vary DNA, constant RNAiMAX		
0.02	7.5	82.3
0.1	7.5	141.5
0.5	7.5	209.1
2	7.5	270.6
Constant DNA, vary RNAiMAX		
2	2.5	162.0
2	7.5	146.1
2	15	163.3
2	30	42.9

Table 5.24. Optimization of Lipofectamine RNAiMAX transfection conditions: Wild type recovery incorporating serine at position S61 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	HSAS (μg)*	RNAiMAX (μL)	Avg Max RFU
2 (WT)	0	15	113.8
2 (S61TAG)	0	15	No Expression
2 (S61TAG)	4	15	No Expression
2 (S61TAG)	8	15	No Expression

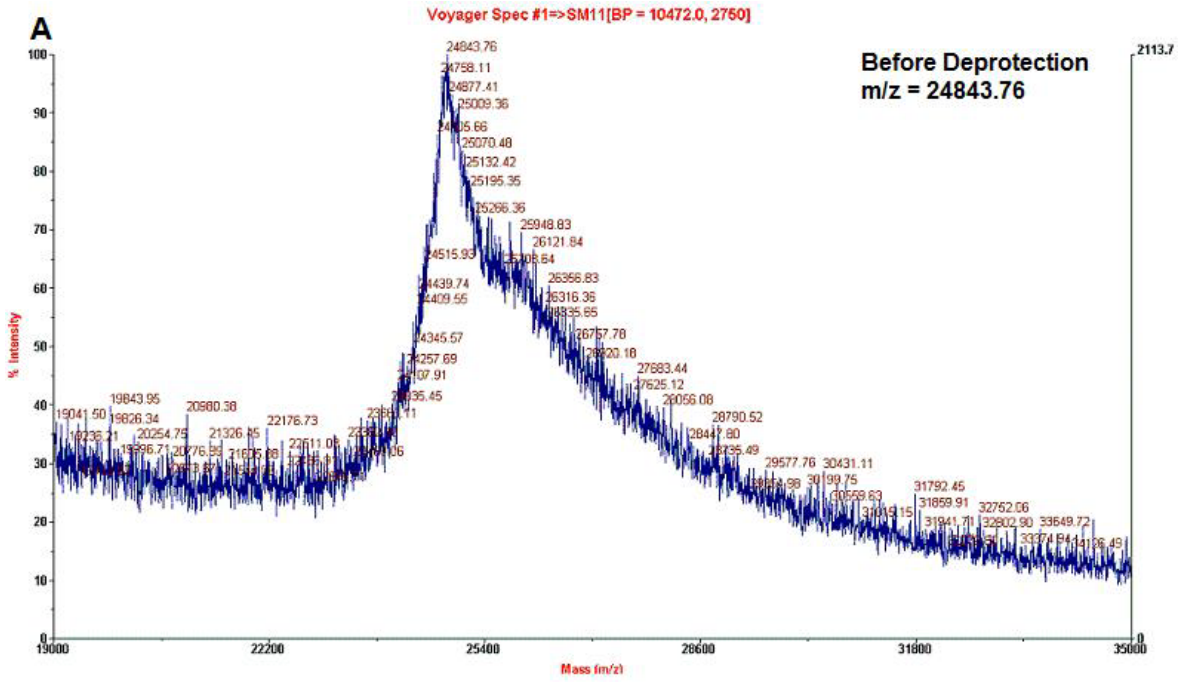
5.4.3 Possible Challenges for Nonsense Suppression in Mammalian Cells

A puzzling result that recurred throughout these studies is that HSAS tRNA easily suppressed the amber stop codon in $5HT_{3A}$ receptors expressed in HEK293T cells, but *in vitro* aminoacylated tRNAs (*e.g.*, THG73 and YFaFS) did not. We then questioned the integrity of the tRNA in response to transfection conditions. Specifically, does the amino acid fall off of the tRNA before entering the cell ***or*** does aminoacylated tRNA enter the cell, but is translationally incompetent? We addressed the first question in two ways.

Analyzing the Effect of Transfection Conditions on tRNA

The integrity of *in vitro* aminoacylated tRNA was analyzed in response to the two most efficient transfection protocols previously identified: double Neon electroporation and single *Trans*IT transfection. To test Neon electroporation, THG73-W was

deprotected for 10 min, electroporated using either condition 7 or 22, and analyzed using MALDI-TOF MS (**Figure 5.8**). To examine the effect of the *Trans*IT protocol, THG73-W was deprotected for 10 min, incubated in the *Trans*IT reagents for 24 hr, and analyzed using MALDI-TOF MS (**Figure 5.9**). As a control, MALDI-TOF MS analysis of THG73-W confirmed the removal of NVOC by deprotection through a mass change of 237.48 (NVOC=239.04) (**Figure 5.10**). Comparison of before and after deprotection for each transfection method did not differ significantly from the control suggesting that neither method should remove the amino acid from the tRNA.



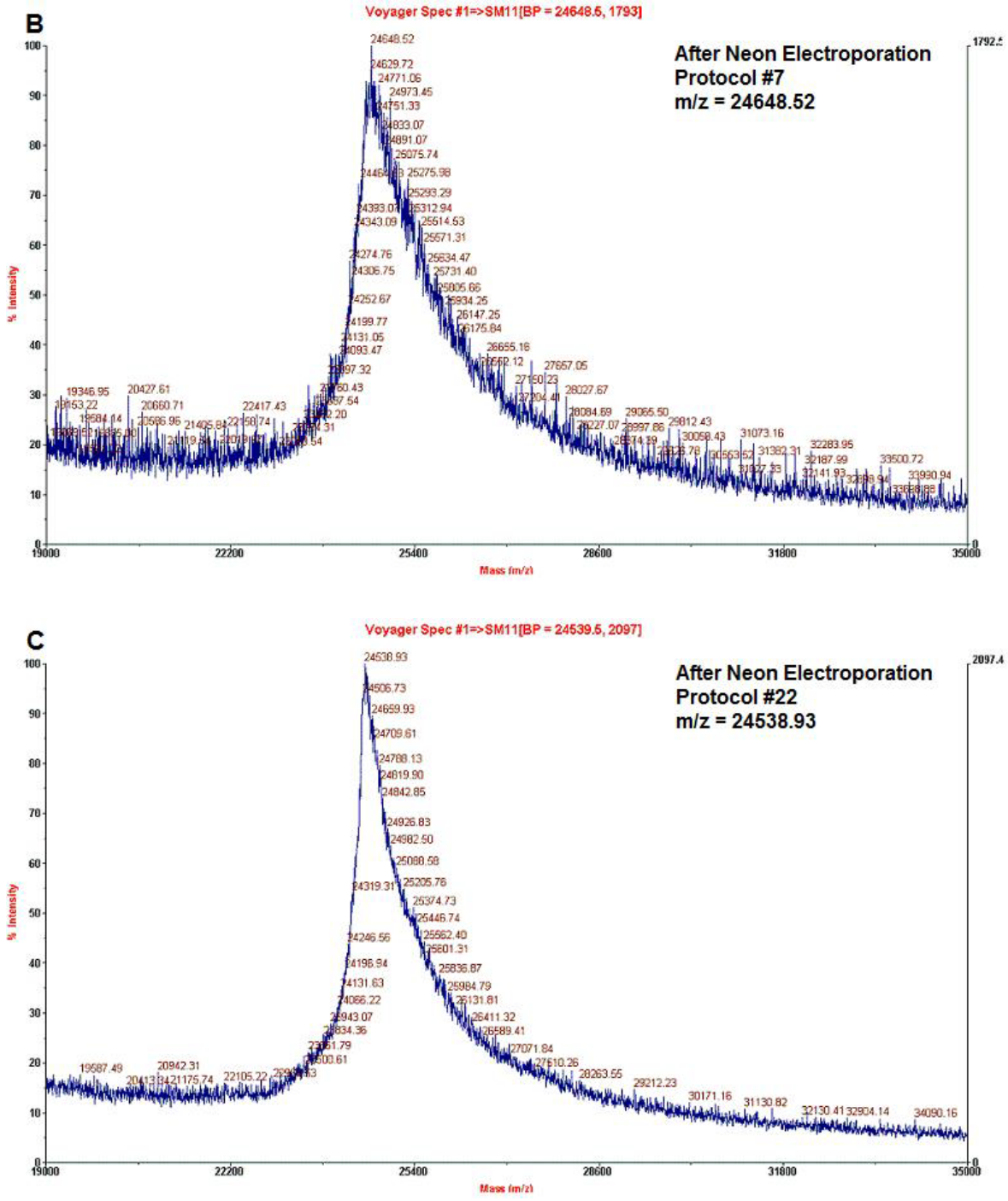


Figure 5.8. THG73-W in response to Neon transfection. A. MALDI of THG73-W-NVOC before deprotection by photolysis. B. MALDI of THG73-W after photolysis and Neon transfection using condition 7. C. MALDI of THG73-W after photolysis and Neon transfection using condition 22.

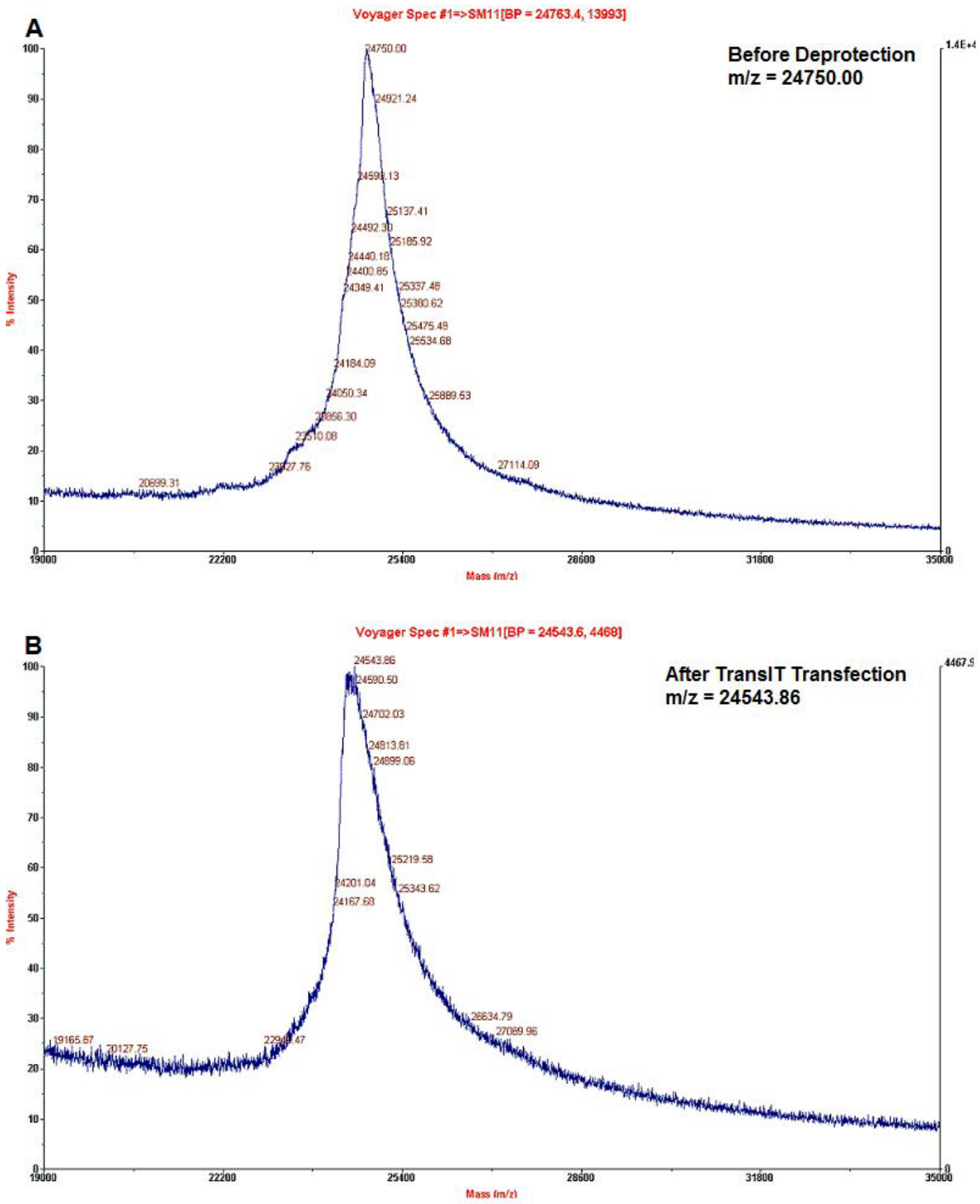


Figure 5.9. THG73-W after *Trans*IT transfection. A. MALDI of THG73-W-NVOC before deprotection by photolysis. B. MALDI of THG73-W after photolysis and *Trans*IT transfection.

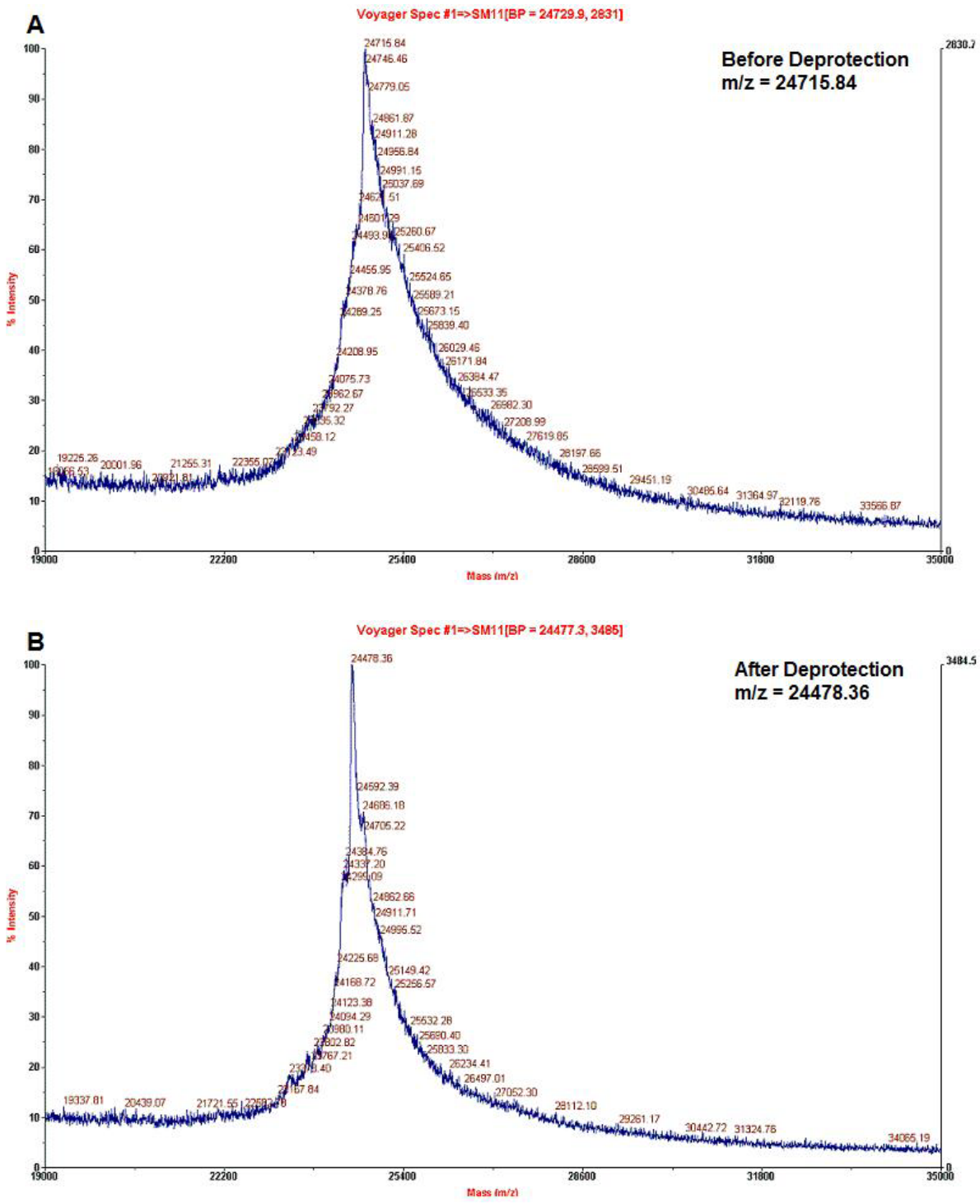


Figure 5.10. Deprotection of THG73-W-NVOC. A. MALDI of THG73-W-NVOC before photolysis. B. MALDI of THG73-W tRNA after photolysis for 5 min.

Transfection of an α-hydroxy Acid

An alternative approach to monitor the integrity of the tRNA is to transfect an α-hydroxy acid, as these are relatively more stable than amino acids and therefore not protected. Cells were challenged to incorporate tryptophan-α-hydroxy (Wah) at position W183 in the $5HT_{3A}$ receptor. Neon electroporation (condition 7) and *Trans*IT transfection protocols were used to transfect 2 μg of $5HT_{3A}$ W183TAG DNA and various amounts of THG73-Wah tRNA (**Table 5.25**). No expression was observed for either transfection protocol.

Table 5.25. Comparison of Neon electroporation and *Trans*IT transfection to incorporate tryptophan α-hydroxy at position W183 in the $5HT_{3A}$ receptor. *DNA and HSAS amounts are per 3 columns of a 96-well plate.

DNA (μg)*	tRNA (μg)*	Neon, Avg Max RFU	*Trans*IT, Avg Max RFU
2 (WT)	0	645.7	407.5
2 (W183TAG)	4	No Expression	No Expression
2 (W183TAG)	8	No Expression	No Expression
2 (W183TAG)	12	No Expression	No Expression

5.5 CONCLUSIONS AND FUTURE DIRECTIONS

This chapter describes the development of methods to enhance nonsense suppression in mammalian cells with the ultimate goal of achieving robust protein expression via transfection of DNA and *in vitro* aminoacylated tRNA. We optimized two protocols to effectively deliver DNA and tRNA to HEK293T cells, such as double electroporation using the Neon transfection system and single transfection using *Trans*IT transfection reagent. Both of these methods successfully suppressed a serine site in the $5HT_{3A}$ receptor using HSAS tRNA, which is aminoacylated with serine *in vivo*. However, both transfection methods failed to produce functional protein when applied to

suppression of an amber stop codon utilizing *in vitro* aminoacylated tRNAs, such as THG73 and YFaFS.

Successful suppression experiments with HSAS tRNA indicated that tRNA is in fact delivered to the cells. Possible issues limiting the functionality of *in vitro* aminoacylated tRNAs could be their stability during the transfection processes and subsequent recognition by the mammalian translational machinery. MALDI-TOF MS analysis of deprotected THG73-W exposed to either transfection method suggested that only the NVOC protecting group was removed and the aminoacyl tRNA remained intact during transfection. These data support the idea that tRNA is delivered to the cell, but that either the amino acid falls off the tRNA inside the cell or the tRNA is translationally incompetent.

Translational incompetence may be attributed to the tRNA identity problem. This concept refers to the specific interactions between the tRNA molecule and its synthetase required for proper activity.[24] The success of nonsense suppression experiments rely on the principle that the mammalian synthetases will not recognize and aminoacylate the tRNA with an endogenous amino acid. One can imagine future experiments designed to bypass the tRNA identity problem entirely. This could be achieved by engineering a novel synthetase that retains tRNA recognition elements specific to the *in vitro* aminoacylated tRNA of interest, but does not recharge this tRNA with any endogenous amino acid. Essentially, this would create a “dummy” synthetase capable of binding the tRNA and “handing it off” to the ribosome. Complementary studies might include engineering the tRNA component to bind the synthetase. Mutagenesis would target the

identity element regions of tRNA, such as the distal ends of the tRNA and the anticodon loop.

Additionally, the possibility that the tRNA is not recognized by essential associated proteins (*e.g.*, EF-Tu) could contribute to hindered translational activity. As such, it may be useful to overexpress the mammalian elongation factor, EF-1α, which is responsible for delivering aminoacyl tRNA to the ribosome. However, we note that several reports have confirmed the high conservation and abundant expression of EF-1α in mammaliam cells.[25]

5.6 ACKNOWLEDGMENTS

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