

# A Novel Fluorescence Assay for the Determination of Ligand Binding Constant for the Human 5-HT<sub>3a</sub> Serotonin Receptor

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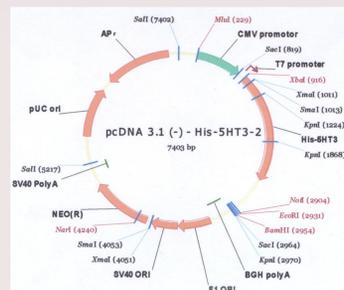


## Abstract

The 5-Hydroxytryptamine type-3 (5-HT<sub>3a</sub>) serotonin receptor is a ligand gated ion channel found in both the central (CNS) and peripheral nervous system (PNS). It is the principle target receptor for the relief of nausea and vomiting caused by chemo- and radio-therapies in cancer patients. This makes the study of both agonist and antagonist ligands important as the knowledge gained can lead to better pharmacological manipulation of this receptor in the creation of antiemetic and other drugs, as well as further developing cost-effective rapid techniques that can be applied to other receptors. The most common ligand binding assays are radio-assays in which a radio labeled ligand is used. This technique is not only prohibitively expensive but requires the use of radioactive agents and creates hazardous waste. Fluorescence microscopy is a powerful tool with a broad scope of applications easily adaptable from single molecule experiments to macroscopic observations. This coupled with the ability to create fluorescently labeled ligands allows for the creation of novel ligand binding assays using fluorescence microscopy. In creating an innovative protocol for the 5-HT<sub>3a</sub> receptor, it is necessary to correlate new data with established assays using traditional ligands, as well as creating optimal conditions to allow for high-throughput methods, and ensuring receptor functionality using a biomimetic environment.

## 5-HT<sub>3a</sub> Expression

The 5-HT<sub>3a</sub> serotonin receptor was expressed in human embryonic kidney cells (HEK-293) using cDNA prepared from plasmid cDNA expressed in E.coli



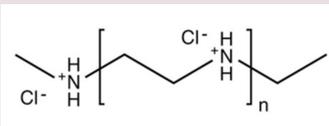
Plasmid complimentary DNA expressed in E.coli for use in transfection

The pcDNA then underwent endotoxin removal and digestion to attain only the cDNA. (Done by Elsa Silva-Lopez)



## 5-HT<sub>3a</sub> Expression

HEK cells are transfected using branched polyethylenimine (PEI) /DNA complexes

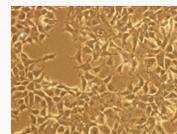


Polyethylenimine

The PEI complexes well with DNA and induces the cell to incorporate the resulting complex through endocytosis.



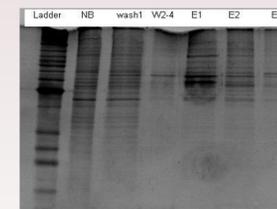
Condensed DNA/PEI polyplex



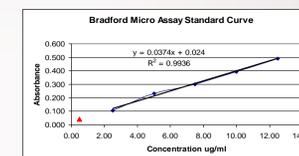
HEK-293 cells adhering to media bottle surface<sup>2</sup>

## Purification/Characterization

After transfection the receptor is purified from the cell membrane and the purity confirmed using SDS-PAGE.



After confirmation that the receptor was successfully expressed, the protein concentration is determined using a Bradford assay.



Total protein calculations allow for precise control of the amount of protein included in later steps.

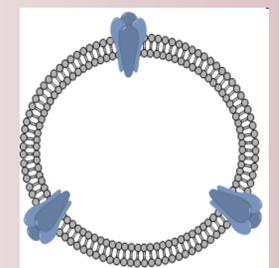
## Proteoliposome Formation

In an effort to approximate legitimate ligand binding constants the receptor is incorporated into a large ~800nm unilamellar vesicle using a detergent.

Uniform vesicle size distribution can be checked using dynamic light scattering before and after protein incorporation



Large Unilamellar Vesicle



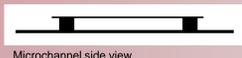
Proteoliposome

## Fluorescence Assay

Rhodamine B was used to create consistent optimized conditions as it is a good analogue to the fluorescent tags used in currently available ligands

The Laser wavelength is set to 532nm at 190μw

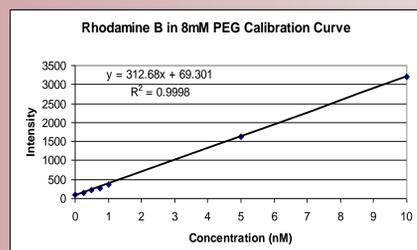
Path length is controlled by creating uniform microchannel slides with a microchannel length of 100microns.



Microchannel side view

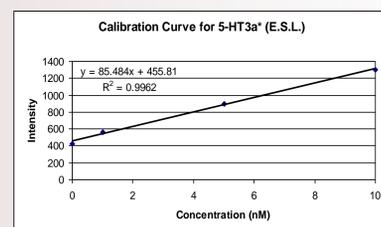
Rhodamine B solutions were prepared in 8mM polyethylene glycol 8,000 (PEG) to prevent binding to the glass surfaces

Using these parameters an initial calibration curve was created



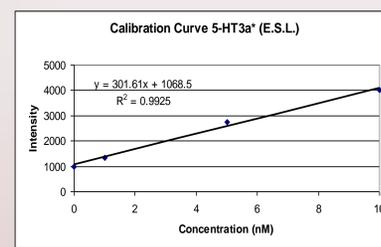
## Fluorescence Assay cont...

Following the initial Rhodamine B curve a new curve was created using a fluorescently tagged 5-HT<sub>3a</sub>\* (ligand also in 8mM PEG) created by Elsa Silva-Lopez



gain set at 28.0

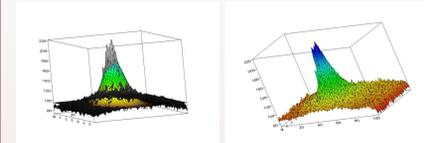
This ligand did not fluoresce as brightly requiring adjustment increasing gain. The following graph shows that intensity is still reliably measured at higher gain



gain set at 70.0

## Results/Discussion

During purification of the receptor an excess amount of buffer was added which diluted the protein to lower than usual levels. However, there was still enough total protein to continue with proteoliposome formation it remains to be seen if this coupled with "long" term storage will result in varied results. Data analysis of intensity is still a point of contention the current calibration curves



Raw data fitted with a 2D-gaussian curve

Raw Data

use averages of maximum intensities. One could also integrate the overall intensity after fitting to an appropriate Gaussian function, which may give higher sensitivity. Initial tests using Rhodamine B showed that the dye had an affinity for the surfaces of the microchannel which at low concentrations (250-1,000pM) interfered with accurate measurement. Using PEG alleviated this problem with the dye, stressing that care must be taken to ensure non-specific binding is minimized.

## Results/Discussion cont...

Preliminary results indicate that fluorescence appears linear within the expected concentration range of analytical importance.

A lot of time and effort is dedicated to creating biomimetic environments that can be used to accurately measure things like ligand binding constants. These range from creating lipid bilayers on optical surfaces to more complex biomimetic layered systems, and in either system the lipid environment can be manipulated depending on the protein's origin. While the current proposed proteoliposome method may give good results, an efficient assay will allow for quick testing of various environments to achieve values closer to in-vivo measurements in pursuit of better biomimetic systems.

## Conclusion

While creating a protocol for protein expression and purification resulting in a functional protein can be very challenging. Once the protocol is well established and tested the same method can be used to reliably continue producing the protein of interest. This leaves more time to focus on measuring biologically relevant qualities and designing new experiments. As protein expression becomes more efficient it is crucial to have a sensitive and adaptable probe in this case the use of fluorescence microscopy.

## References

- 1.
2. <http://www.tgrbio.com/cancer-cell-lines-primary-cell-cultures/cell-models-hek-293-cells.html>

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