

Development of HTS Cell Based Efflux Assay for CFTR using Ion Channel Reader

Sikander Gill*, Raj Gill, David Wicks, Soo Sen Lee and Dong Liang
Aurora Biomed Inc, 1001 E Pender Street, Vancouver, BC, V6A 1W2, Canada

I. Abstract

Cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP regulated, protein kinase A chloride channel. Mutation of CFTR causes various degrees of progression of Cystic Fibrosis (CF) disease and the most severe form of mutation results in a complete loss of channel function. Any pharmacological intervention that can restore CFTR activity will greatly improve the quality of life of CF patients.

High throughput screening (HTS) of chloride channel remains a challenge for CF drug development. Here we report the development of a specific and highly sensitive HTS assay for CFTR. The protocol has been developed in a cell line endogenously expressing CFTR and the chloride efflux was precipitated with silver nitrate. Activation and inhibition of CFTR with agonist and standard blockers, respectively has been successfully studied using Aurora Biomed's Ion Channel Reader-8000. These results indicate that silver precipitation method is a reliable method for screening CFTR as well as other chloride channels.

II. Introduction

Chloride (Cl⁻) channels play an important role in the regulation of electrical excitability in muscles and neurons, fluid transport in epithelial tissues and pH regulation in intracellular organelles. The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a Cl⁻ channel that becomes activated after phosphorylation by cAMP-dependent protein kinase. Several hundred mutations in this channel have been found to result in cystic fibrosis (CF).

A high throughput screening (HTS) format for screening CFTR as a molecular target for drug discovery is currently not available. The patch clamp technique¹ which represents the gold standard for determining ion channel activity suffers from low throughput. As such the drug discovery industry has turned to using radiotracer flux methods with either ³⁶Cl or ¹²⁵I or using fluorescent methods² to determine ion channel function. However, there are many concerns related to the use of radioactivity in large quantities or fluorescence based indicators.

Aurora Biomed describes an HTS assay that is specific and highly sensitive for screening compounds against CFTR. This method is a cell-based flux assay coupled to silver precipitation and atomic absorption spectroscopy (AAS). The results from these preliminary studies are very promising as compound potencies derived by Aurora Biomed's non-radioactive method are very comparable to those derived from radioactive flux assays.

III. Materials and Methods

A T-84 cell line expressing CFTR was grown in 1:1 DMEM and Ham's F-12 medium, supplemented with 10% FCS at 37°C, in 5% CO₂. Cells were plated at a density of 50,000 cells/well in 96-well microplates and incubated at 37°C, 5% CO₂ until 80-90% confluency was attained. The culture medium of the cells was removed and the monolayer of cells was washed three times with 200 µl of the Wash Buffer to remove traces of chloride (Figure 1). The wash buffer was replaced with 200 µl of Channel Open Buffer containing Forskolin. The channel activation is recommended for 5 minutes.

Compounds were added at 2 µl/well of a 100 X stock solution in 100% DMSO along with 25 µM of the agonist Forskolin. The total volume in each well was 200 µl (198 µl of solution containing Forskolin and 2 µl of the 100 X compound). The 100 X compound solution is diluted in the Channel Activation Buffer to 1x and contains less than 1% DMSO. Then extracellular solution was carefully collected into another multi-well plate. The cells were lysed with 200 µl of 1.5% Triton X-100 to release the intracellular chloride.

To 200 µl of the extracellular and intracellular lysate samples, 30 µl of silver (Ag⁺)-Standard Solution (50 ppm) was added (Figure 2). The silver chloride (AgCl) precipitated in the reaction mixture was allowed to settle for 3-4 hours in darkness. The samples were then analyzed on the ICR with Cl⁻ Analysis Buffer for excess Ag in the supernatant. The Cl⁻ concentration was calculated as follows:

$Cl^- = 2.46 - 0.378Y + (0.778/Y)$, where Y = excess of Ag⁺ concentration in the samples. The % efflux was calculated as % of control.

Figure 1. CFTR assay protocol

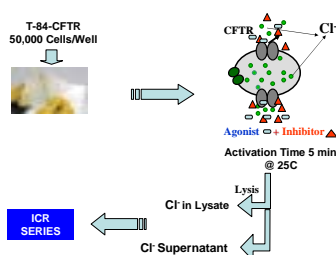


Figure 2: Indirect determination of chloride concentration

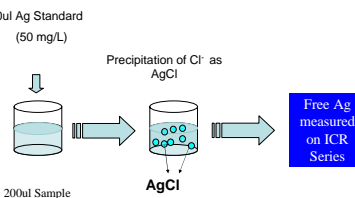


Figure 3. ICR 8000 and ICR 12000



IV. Results

A. Validation of the silver precipitation method for determining chloride concentrations by the ICR

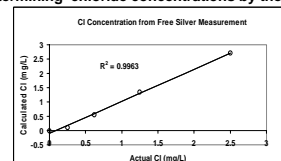


Figure 4. The free silver precipitation measurement demonstrated $r^2=0.996$ against actual concentrations of Cl⁻ indicating that this method can be used for the measurement of Cl⁻ efflux with the ICR series.

B. Optimization of the concentration of agonist Forskolin for channel activation

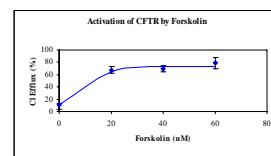


Figure 5. The activity of this channel showed a dose-dependent up-regulation with increasing concentrations of Forskolin. Activated and basal level efflux of about 80% and 20%, respectively was observed. (n=3)

C. Confirming the activation of CFTR by abolishing chloride efflux with standard blockers.

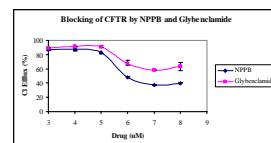


Figure 6. The up-regulated current is abolished by the antagonists 2-(3-phenylpropyl amino)-benzoic acid⁴ (NPPB) and Glybenclamide. These two compounds are known to inhibit chloride efflux⁴⁻⁵. NPPB has been shown to inhibit Cl⁻ up-regulated current by 40% in membrane potential probes⁴.

D. Determining the potency of standard blockers of CFTR.

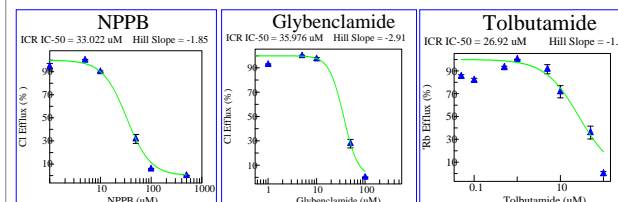


Figure 7. NPPB, Glybenclamide and Tolbutamide showed IC₅₀s of 33.02, 35.97 and 26.92 µM, respectively.

E. Tabulating the IC₅₀ of different blockers from iodide efflux and chloride efflux IC₅₀.

Compound	ICR Flux IC-50 (uM)	¹²⁵ I Flux IC-50 (uM)
NPPB	33.02	400
Glybenclamide	35.98	37
Tolbutamide	26.92	400

V. Conclusion

Both activators and blockers of CFTR chloride channels were easily identified using Aurora Biomed's ICR 8000 and Chloride Flux Assay. Here, the preliminary data on CFTR Chloride Channel Assay has been shown to identify compounds that change channel ion flow of Cl⁻ through CFTR channels. Although more experiments are required, it is evident that Aurora Biomed's ICR technology is adaptable to many ion channel HTS needs.

VI. References

- Gong *et al.* (2002) Measurement of the Permeation and Conduction Properties of the CFTR Chloride Channel using Excised Inside-Out Membrane Patches. The European Working Group on CFTR Expression:14.
- Beq *et al.* (2003) Radiotracer flux method to study CFTR channel activity: Regulation, pharmacology and drug discovery. The European Working Group on CFTR Expression:13.
- Andersson *et al.* (2001) Measurement of chloride efflux from nasal epithelial cells using the fluorescent indicator MQAE. The European Working Group on CFTR Expression:3.
- Kirkup *et al.* (1996) Investigation of the effects of 5-nitro-2-(3-phenyl-propylamino)-benzoic acid (NPPB) on membrane currents in rat portal vein. *Br J Pharmacol.* 117: 175.
- Yamazaki *et al.* (1997). Inhibitory effects of glybenclamide on CFTR swelling, activated and calcium activated chloride channel in mammalian cardiac myocytes. *Circ Res* 81: 101-109.