

Flux Assays in High Throughput Screening of Ion Channels In Drug Discovery-A Review

Abstract:

Ion channels have been identified as therapeutic targets in various disorders such as cardiovascular disease, neurological disease and cystic fibrosis. Flux assays to detect functional ionic flux through ion channels are becoming increasingly popular as tools for screening compounds. In an optimized flux assay, modulation of ion channel activity may produce readily detectable changes in radiolabeled or non-radiolabeled ionic flux. Technologies based on flux assays are currently available in a fully automated high-throughput format for efficient screening. This application offers sensitive, precise, and reproducible measurements giving accurate drug rank orders matching those of patch clamp data. Conveniently, the flux assay is amenable to adaptation for different ion channels such as potassium, sodium, calcium, and chloride channels by employing suitable tracer ions. The non-radiolabeled rubidium based flux assay coupled with the Ion Channel Reader technology has become very successful in ion channel activity analysis and is emerging as a popular technique in modern drug discovery.

Introduction:

Recent years have seen pharmaceutical companies investing significant portions of research funds towards ion channel drug discovery and development. This investment is not a surprise as ion channel blockbuster drugs generate over 6 billion dollars in sales per annum and 15% of the top selling drugs are targeted for ion channels.^{1, 55, 15} Ion channels are seen as key molecular targets because of their immense physiological significance. Channel dysfunction has been linked to such diseases as cardiac arrhythmias, cystic fibrosis, familial paralyses, myasthenia gravis, epilepsy, ataxia, multiple sclerosis and diabetes.^{39, 18, 19, 46, 48, 50, 56}

Interest in ion channels also extends beyond drug discovery. There is a growing recognition of a connection between the hERG potassium channel and long QT syndrome. Sanguinetti *et al.* (1995) first suggested a link between hERG channel inhibition and drug induced long QT syndrome.³⁹ Support for this link was further strengthened by publications from regulatory bodies worldwide after a number of drugs, including terfenadine and cisapride, were pulled off the market following several incidences of drug-induced fatal ventricular fibrillation.²⁷ Particular drug classes of concern with regards to hERG-safety are antiarrhythmics, antibiotics, antihistamines, antipsychotics and gastric prokinetics.^{9, 10, 11, 31} There is, therefore, pressure on the pharmaceutical companies to invest in drug safety procedures to screen their compound libraries and eliminate potential hERG blockers early in the drug development process.

One approach pharmaceutical companies have developed for ion channel drug discovery and drug safety has been to develop assays for high-throughput screening. Such assays should ideally be functional in nature; however, the development of a high-throughput functional assay for

studying ion channel modulation has been hindered by a lack of reliable detection methods. This issue has been of particular importance with regards to voltage gated ion channels. The biggest hurdle has been to generate sufficient high throughput with acceptable information content and reliability. Traditionally, ion channel screening has relied on methods based upon functional measurements including (1) ion fluxes and corresponding ion concentration changes and (2) membrane potential changes. Non-functional methods, such as radioligand binding, have also been used. Presently, technologies employed for ion channel screening include binding assays, ion-flux assays, fluorometric imaging, and patch clamping. All of these methods are amenable to scale-up, automation and miniaturization, however, the pharmaceutical industry prefers to employ functional assays. This review will assess the status of the flux assays used in HTS of ion channel in drug discovery.

Flux-assays:

Radiotracing is a powerful *in vitro* method, allowing for functional detection of ion channel modulating activities of potential pharmaceuticals and has long been used to monitor ionic flux through ion channels.¹² Radiolabeled flux assays study ion channel activity by chemically initiating the opening of channels in the presence of a fixed amount of a radiolabeled cation, or anion, and a potential ion channel modulator compound.⁴³ After flux activation, cells are separated from their medium and lysed. The radiolabeled ions in both the lysate and supernatant are measured by either electron spin resonance (ESR) or scintigraphy. Inhibition or activation of the channel by the channel modulator compound will affect the concentration of radiolabeled ions, thereby changing the ratio of intracellular radiotracers (lysate) to extracellular radiotracers

(supernatant). These concentration changes are compared to the flux ratios found in the absence of the test compound to indicate the potential of the compound for modulating ion channels.

The activity of most ion channels can be studied by employing the appropriate radiotracers. For example, radiotracers sodium-22 (^{22}Na) and carbon-14-guanidinium (^{14}C -guanidinium) are suitable for monitoring flux through sodium ion channels for pharmacological and toxicological studies.^{1, 12} Potassium channels can be studied with either rubidium-86 (^{86}Rb) or potassium-42 radiotracers while calcium and chloride ion channels can be studied with calcium-45 and chloride-36 or iodine-125.^{4, 13} Radiotracer flux assays are reliable, direct and flexible since they are easily performed with miniaturization and automation. In fact, results from compound potency studies with radiotracer flux assays are highly comparable to results provided by patch clamp.⁴

Radioactive rubidium-86 is often used to study potassium channel activity since K^+ channels are permeable not only to K^+ ions, but also to Rb^+ ions.^{7, 32} Rb^+ is an excellent tracer ion for K^+ channels due to their similarity in size and charge. An added advantage of using Rb^+ as a tracer is the fact that mammalian cells do not contain significant amounts of Rb^+ ions, therefore, providing a very low background noise in the assay. The principal drawback of radiotracers lies in the potential toxicity and health hazard associated with radioactivity. As a result, many HTS labs are reluctant to use the Rb^{86} -based radioactive flux assay format.

The development of a non-radioactive Rb^+ assay has greatly enhanced the ease of using this assay.⁴⁵ The non-radioactive Rb^+ flux assay, also known as a cold rubidium flux assay, has been

well-described in the scientific literature and has been widely applied for the detection of potassium channel activity.^{45, 44, 49} In the cold tracer method, Rb^+ is quantified by atomic absorption spectroscopy (AAS), an accurate and robust technique for elemental analysis. Further examples of channels screened with this method are voltage-gated K^+ channels including inward rectifiers, delayed rectifiers, Ca^{2+} -activated potassium channels and ligand-gated channels.^{45, 44}

Cold rubidium flux is particularly useful for the high throughput screening of hERG blockers. Using an atomic absorption spectrometer (Aurora Biomed, Vancouver), several known hERG channel blockers have been studied and the results compared to those of electrophysiological patch clamp. The results obtained have proven to offer a new and improved approach to ion channel screening as the rank order based on IC_{50} values for the known hERG blockers closely match the order determined with patch-clamp.⁴⁵ The potency estimates obtained with the two technologies is shown graphically in Figure 1. Aurora Biomed's Ion Channel Reader (ICR) couples the cold Rb^+ flux method with flame atomic absorption spectroscopy (FAAS), and is capable of detecting minute concentrations of ions; thereby providing sensitivity levels unequaled by other AAS.

HTS and miniaturization:

The pharmaceutical industry is faced with two central issues: increased throughput of new chemical entities (NCEs) and greater originality in terms of addressable biological targets. The advent of new chemical synthesis procedures and combinatorial chemistry has resulted in a large increase in the availability of compounds for HTS.^{5, 29, 30} Concurrently, genomics has been used to identify previously unknown ion channels, thereby providing additional targets for HTS.

These developments together have dramatically increased the number of targets and the number of chemical compounds. For example, in the early 1990's a typical pharmaceutical company was expected to screen about 75,000 samples through each of its 20 or so drug targets in any given year.^{29, 14} In 2000, the average number of compounds tested per screen for an average pharmaceutical/biotech company was 270,000 and this number is expected to swell to approximately one million compounds per screen per average company in the year 2005.

The pharmaceutical industry is under constant pressure to reduce the cost of drug discovery and development. To provide new drugs, it has relied almost exclusively upon the screening of compound libraries. The HTS systems used to study ion channels are similar to conventional biochemical and cellular *in vitro* assays used with large sample sizes. With respect to ion channel modulators, the development of HTS methods has proven to be difficult and time-consuming, especially for voltage gated ion channels. Presently, the focus in improving HTS methods has shifted to increasing information content in conjunction with time and cost.⁶

Developments in high throughput ion channel research such as novel assay formats, assay miniaturization, and automation have all played key roles in improving the cost effectiveness and speed of drug discovery.^{17, 29} Miniaturization addresses the pharmaceutical industry's three basic needs: lower cost, faster turnaround, and reduced space. The combined cost of chemicals and biological reagents used is approximately 75% of the total cost of the assay. Miniaturization reduces this cost by proportionately reducing the volume, compressing a larger number of samples onto one plate. Sample compression reduces the distance and, hence, the time of reading between the samples.^{12, 29} Miniaturization also leads to a reduction in the amount of facility space

required for screening libraries. Unfortunately, although miniaturization may rapidly reduce the need for workspace, the caveat to miniaturization in HTS is that it may limit the scope of HTS. The reason being that not every HTS assay may be suitable in a miniaturized format due to an inadequate detection signal. This is especially true for cell-based flux assay where signal is dependent on ion concentration and cell numbers.

The advancement of automation is also contributing to HTS and miniaturization by enabling larger number of samples to be processed per day at a lower cost. Automation has evolved from plate readers with stacking devices in the early 1990's to integrated systems and workstation approaches.^{12, 29} This integrated approach is now preferred as it incorporates robot-compatible assays with instruments such as the ICR series. Automation allows continuous 24-hour operation and data tracking, thereby significantly increasing the throughput.

Requirements for an ideal assay:

There are several important aspects and attributes of the assay and associated technology to consider when evaluating a technology for ion channel screening. Jia Xu discusses these aspects in detail in his paper on "Ion-Channel Assay Technologies: quo vadis?"⁵¹ In brief, important features to consider are sensitivity, specificity, throughput, robustness, information content, flexibility, cost and physiological relevance of the technology. Flux assays when evaluated against the different characteristics mentioned above are likely a good choice for many HTS laboratories as they are functional in approach, high throughput in nature, robust, sensitive, specific and cost effective.

Firstly, the results generated by the Rb^+ flux assay are comparable to patch clamp from mammalian cells as well as *Xenopus* oocytes^{28, 35, 42}. Secondly, the flux assay is amenable to automation; giving speed to precision in screening therapeutic agents.⁷ Flux assay technology is also relatively inexpensive for use over time, at less than 10 cents per data point, in comparison with alternative technologies. As an economical screening tool for drug discovery and drug safety analysis, it is unparalleled, and as a result, many pharmaceutical leaders worldwide are currently using flux assay systems.^{38, 44, 70, 72} One outcome of this interest is that flux assays are presently being developed for several ion channel and transporter candidates, using suitable tracers (Table 1).

Comparison with other technologies:

Apart from flux assays, various other techniques are also popular among screening laboratories. A summary comparison of these current and emerging technologies is provided by Gonzales et al. (1999) and Gonzales and Tsien (1997).^{23, 24}

Electrophysiology:

The patch clamping technique is considered the gold standard of ion channel technologies. It is sensitive, reliable, and offers the most accurate and high content data. However, based on many factors like cell type and the type of ion channel under study, the patch clamping is labor-intensive and has a very low throughput. For single dose screening, the throughput of this method is at best in the order of 10-20 compounds per day, depending upon the efficiency and expertise of the researcher.¹² Thus, the patch clamp techniques only allows for slow profiling of primary hits in drug discovery and safety screening.

Ever since the development of whole-cell recording of ion channel activity, a number of automated patch clamp (auto-patch) technologies are being developed to meet HTS requirements.^{26, 12, 33, 34, 41, 51, 53} Planar-array based HT patch clamp provides two orders of magnitude increase in throughput over traditional patch clamp.^{34, 40} In addition to its high throughput, planar-array based patch clamp provides a platform for assessment of state-dependent binding of ion channel modulating compounds. The high throughput of these instruments coupled with the high information content makes them very valuable tools for ion channel drug discovery. However, the complexity and amount of data generated may lead to other issues such as data management, interpretation and documentation. Another new technology that has been developed involves direct coupling of ion channels to semiconductors on silicon chips. Currently, this platform is only restricted to the academics and has not yet attracted the attention of the pharmaceutical industry.⁴¹

Radioligand binding assays:

Radioligand binding assays require previous knowledge of binding sites on the target and the generation of a radiolabeled ligand specific for those binding sites.^{51, 20} The method of detection is based on competition of the test compounds with the radiolabeled ligand for similar binding sites. Displacement of the radiolabeled probes from the target is an indication of the interaction of the test compound with the target. Binding assays have the advantage of being low cost and amenable to high throughput formats, however, the assay measures the binding affinity of the compound, rather than the ion channel modulating ability and therefore gives very low information content.¹² This method is also prone to both false positive and negatives as undetected binding at the same site, or allosterically-linked sites, does not guarantee that the

compound has not bound to other sites on the target and binding itself does not guarantee functional modulation of the ion channel. Furthermore, the scope of use of the binding assay is limited by the availability and affinity of radiolabeled ligands.^{12, 16, 20}

Fluorescence-based assays:

Fluorescence-based assays are widely used for screening various ion channel targets, as they are robust and easy to setup. However, these assays are only indirect methods of measuring membrane potential-dependent or ion concentration-dependent fluorescent signal changes, rather than a direct measure of changes in ionic current. Fluorescent dyes are generally categorized into two groups – membrane potential sensitive dyes and FRET (fluorescence energy transfer – based) dyes.

Membrane potential sensitive dyes can be further divided into two groups – the slow oxonol dyes, such as bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄), and the fast dyes such as the Fluorescent Membrane Potential (FMP) dyes.^{47, 2, 3} Both dyes are lipophilic and distribute themselves across the cell membrane according to changes in cumulative charge within the cell dyes and are detectable on the FLIPR system. The main difference between DiBAC₄ and FMP dyes is the response time. FMP has a response time in tens of seconds, whereas DiBAC₄ generally takes minutes to response to changes in membrane potential.²

FRET dyes couple voltage-sensing oxonol dyes to voltage insensitive donor fluorophores anchored in the cell membrane.^{24, 25} Changes in ionic concentration within the cell membrane affect the mobility of the oxonol molecule, thereby causing changes in fluorescent emission.

FRET-based dyes are fast response dyes as well, giving sub-second temporal resolutions to allow kinetic reading.² A Voltage Ion Probe Reader (VIPR) allows for high throughput fluorescence detection in 96 and 384 well format.^{2, 16}

Ion-specific fluorescent probes, such as calcium indicator dyes, are also available for measuring the ionic concentrations in cells.²⁴ Upon binding with fluorophores, the fluorescent signal of calcium is altered and is detectable with a FLIPR system. Calcium indicator dyes are available in different affinities, membrane permeabilities and excitation and emission spectra. Similarly, fluorescence based assays have been used for screening compounds against CFTR chloride transport.^{22, 36, 47}

Unfortunately, these fluorescence-based assays have several drawbacks.^{2, 44, 47} They are prone to false positives and negatives. Such assays are prone to false positives as they only measure changes of membrane potential. The dyes can, therefore, potentially select for compounds that only change the membrane potential, but not channel activity. Furthermore, autofluorescent compounds, which are not uncommon in compound libraries, also contribute to the high rates of false positives. False negatives often result from the quenching effect of the dyes and high background noise levels of the assays. Fluorescent dye assays often have high background noise giving high noise to signal ratios, thereby masking weaker signals and adding to the number of false negatives detected.

Correlation of flux assays with electrophysiology and *in vivo* data:

Ion Channel technologies are typically compared to patch clamping, the gold standard method of electrophysiology. For flux assays, the IC₅₀ values are similar to those reported with electrophysiology studies conducted on *Xenopus* oocytes.^{35, 42} On the other hand, several drugs tested with the Rb⁺ flux assay generate a 4 to 20 fold potency shift when compared to electrophysiology patch clamp data from mammalian cells.^{44, 7} Both methods however, provide similar rank order for various test compounds, including known potent blockers of hERG (Table 2). This suggests that although the relationship between the two technologies indicates that Rb⁺ flux values for IC₅₀ are typically higher than that of patch clamping, statistically, the flux assay may be acceptable as an alternate and/or complimentary method.

Additional support for this postulation is the high value of the correlation coefficient (0.88) for a linear fit to the data points in this comparison (Table 2). Therefore, even though estimates obtained using the flux assay approach do not give a one-to-one correlation with patch clamp, flux assays can still provide valuable information on compound activity and have provided another platform for screening compounds. The rightward shift in potency given by this assay does not alter the rank order of test compounds between flux assay and patch clamp.^{7, 44}

Suitability or quality of the screening assay determines the confidence in the capability of HTS assay to screen compounds. This can be ascertained by determining screening window coefficient known as Z factor.⁵² An assay with Z value of 0.5 or higher is designated as an excellent assay. The Z-value for the flux assay matches that of an excellent assay.^{7, 44} Moreover, the screening of sample groups of compound libraries from diverse sources with flux assays

suggests that the Rb⁺ flux assay is reliable for positive predictions of hERG channel blocking activity with a low false hit rate. While considering the patch clamp IC₅₀ as the gold standard for studying the biophysical properties of compounds, flux assays can be used as a primary filter to screen a significant number of compounds to reduce the burden on patch clamping.⁷ Moreover, the HTS format of this assay makes it amenable to screen very large chemical libraries.

Limitations of flux assays:

Despite providing an HTS format for drug screening, flux assays have some inherent limitations described by Owen and Silverthorne 2002:³⁸

1. **Weak signals:** Indeed, some ion channels generate very weak signals, below the detectable flow/amount of the desired ion through endogenous channels. However, AAS has very good sensitivity to detect weak signals. Moreover, the heterologous expression provides for a sufficient number of the desired ion channels in the cell membrane to produce a detectable signal.
2. **Transient signals:** Some ion channels like NaV1.5, NaV1.2a, and SCN5A are activated only transiently for less than 10 milliseconds.^{21, 53, 37 43, 12} On the other hand Flux assays, have a slow temporal response, ranging from seconds to minutes, which can also create detection difficulties. Transient signals also pose a detection problem for fluorescence methods as they have a resolution of 1-10 seconds. Fast-inactivating channels can be manipulated to remain open with pore binding compounds, like batrachotoxin, veratridine, aconitine,

and grayanotoxin.⁸ However, the impact of opening channels in this way on the screening of compounds needs to be viewed from a pharmacological perspective.

3. ***Control of membrane potential:*** The suitability of controlling membrane potential is also a matter of concern in all the assays except electrophysiology. Consequently, uncontrolled potential and driving force lead to a non-linear relationship between the signal and channel conductance.⁵⁰
4. ***“State Aware” screening:*** The screening of state-specific binding compounds is a problematic where blockers have a preference for the inactivated state of the channel rather than open or closed states. An example of this detection problem occurs with sodium channel screening.
5. ***Limited time resolution:*** Since ion fluxes are determined from concentration changes, therefore, have limited time resolution. This problem is also shared by membrane potential based assays.^{2, 50}
6. ***Lower throughput:*** flux assays have lower throughput in comparison to fluorescence-based assays. However, flux assays enjoy higher throughput than patch clamping.

Conclusion:

The recent understanding that ion channel modulators offer significant therapeutic solutions to a variety of pathophysiological conditions has led to the development of specific drug and safety screens targeting ion channels. Technologies based on electrophysiology offer superior sensitivity and information content but lack the throughput required by the pharmaceutical industry as throughput is in more demand than high content information. The latter requirement may be met with electrophysiology when the compound advances in the drug discovery pipeline. Radiotracer flux assays undoubtedly suffer from bio-safety aspects, while non-radioactive assays offer the advantages of avoiding any toxicity hazards and the concern for isotope half-life. Moreover, data generated from non-radioactive flux assays identify this method as highly sensitive. Indeed, the Z Factor values correlate with an excellent assay with a large detection window; this allows for a range of drug potencies to be determined. Flux assays are also easy to perform and are amenable to automation. Flux assays stand in deep contrast to fluorescence assays that have been shown to have high background noise, high rates of false positives and negatives, dye half-life concerns, and expensive consumables. Furthermore, most available dyes only measure indirect processes such as membrane potential changes or calcium influx. Among the available technologies, flux assays can be used as primary screens when looking for functional ion channel targets or as secondary screens for drug safety analysis.

Various technologies and assays are evolving to fulfill the needs of high throughput screening of voltage-gated ion channels. This has contributed to the popularity of the flux assay in the biopharmaceutical industry. A close integration of biology, chemistry, and engineering may be

deciding the future of modern drug discovery. Moreover, automated HTS systems will have to compete with systems involving miniaturization, which provide for extremely cost effective and physiologically relevant screening systems. Currently, there is an overwhelming need to discover an HTS technology that is accurate, reliable and cost effective.

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Table 1: Various target classes of ion channels/transporters that may be screened with flux assay using tracer ions.

Target Class	Screening by Flux Assay	Tracer Ion/Ion	Reference
Ion Channel			
Potassium channels			
Voltage gated	+	Rb ⁺	45, 44, 70
Ligand gated	+	Rb ⁺	45
Mechano-sensitive	+	Rb ⁺	
Ca ²⁺ activated	+	Rb ⁺	61*, 71
Sodium channels			
Voltage gated	+	Li ⁺	59, 60
Nicotine Acetylcholine Receptors	+	Li ⁺	60
Chloride channels	+	♦Cl ⁻	59
Calcium channels			
Voltage-gated	+	Zn ²⁺	57, 62
	+	Ba ²⁺	63
G-Protein Coupled Channels			
Ca ²⁺	+	Ba ²⁺	66
K ⁺	+	Rb ⁺	
Transporters			
CFTR	+	I ⁻	4*
	+	♦Cl ⁻	59
GABA Receptors	+	♦Cl ⁻	
Na ⁺ -K ⁺ -ATPase	+	Rb ⁺	58, 59
	+	Li ⁺	
K ⁺ -Cl ⁻ Cotransporter	+	Rb ⁺	65*
	+	♦Cl ⁻	
	+	Rb ⁺	67*, 68*
Na ⁺ -K ⁺ -Cl ⁻ Cotransporter	+	Rb ⁺	64*, 69*
	+	Li ⁺	
	+	Cl ⁻	69*
H ⁺ - Cl ⁻ Cotransporter	+	♦Cl ⁻	
Na ⁺ -Ca ²⁺ Cotransporter	+	Li ⁺ , Ba ²⁺	
Cl ⁻ -HCO ³⁻ Cotransporter	+	♦Cl ⁻	

*Radiotracer used

♦Analysis of free Ag after precipitation of Cl⁻ with fixed concentration of AgNO₃

Table 2: Comparison of patch clamp and ICR based Rb flux assay IC-50 values.

Drug	Electrophysiology IC₅₀ (nM)	ICR IC₅₀ (nM)	Electrophysiology Drug Rank	ICR Drug Rank
Astemizole	2	30	1	1
Cisapride	5	100	2	2
E-4031	10	155	3	3
Terfenadine	79	253	4	4

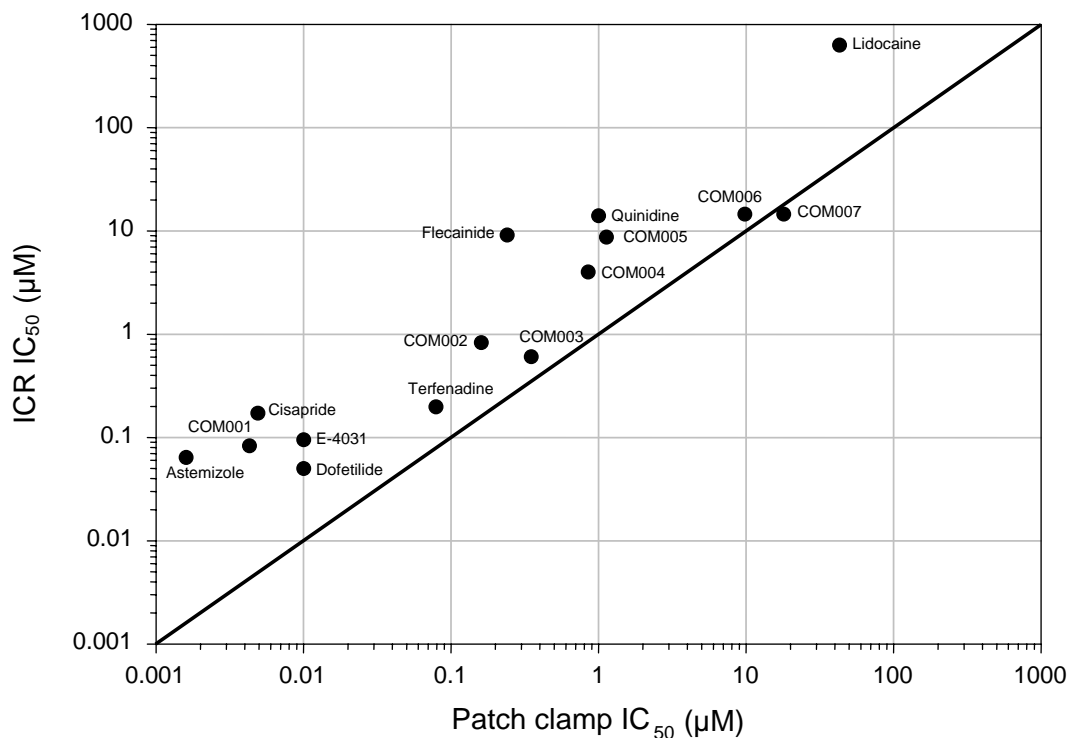


Figure 1– Comparison of potency estimates for hERG block between patch clamp and ICR. Patch clamp potency estimates were obtained using the peak tail current obtained at -50 mV following a 4 second depolarization to $+20$ mV in escalating concentration of drug. The same cell line (HEK-293 expressing cloned hERG ion channels) was used for both patch clamp and the ICR assay. The thick diagonal line represents the line of unity. The correlation coefficient for a linear fit to the data is 0.88 (not shown).