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K_v1.5 Potassium Channel Assay Using the IonWorks HT System

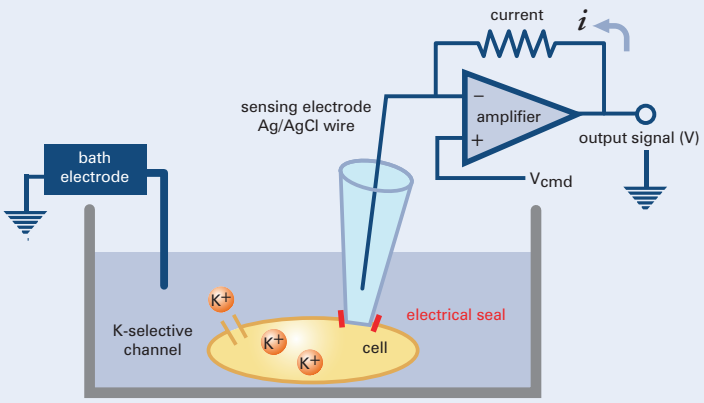
IONWORKS HT APPLICATION NOTE #1

By James Costantin, Ph.D., Andrew Wittel and Wilhelm Lachnit, Ph.D., Molecular Devices, 1311 Orleans Dr., Sunnyvale, CA 94089.

INTRODUCTION

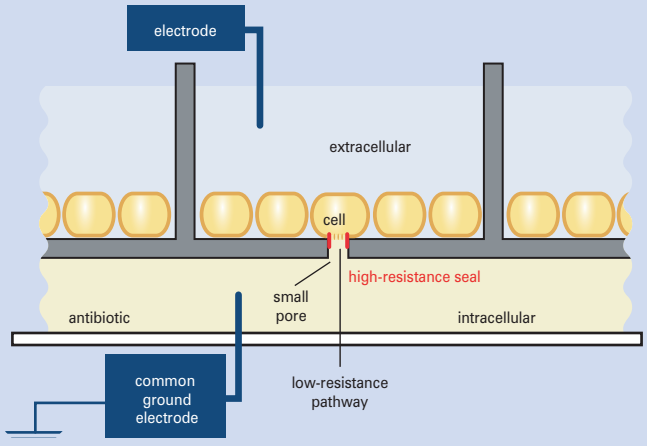
Ionic currents that pass through ion channels are responsible for maintaining and modifying the membrane potential within animal cells from prokaryotes to complex eukaryotes. Voltage-dependent ion channels are especially important in higher animals in the control of cardiac and neuronal activity. Voltage clamp techniques, developed over 40 years ago, allow direct control of the membrane potential inside very large (molluscan) nerve cells and a direct measurement of ionic currents through ion channels. The patch clamp technique (Figure 1a), introduced in 1981, is a refinement of voltage clamp techniques and allows the experimenter to measure ionic currents in much smaller cells, including mammalian cells.[1] Conventional methods of this technique require equipment from multiple vendors and a high degree of technical skill to perform, and are time and labor intensive. Furthermore, this technique does not have the capacity to be significantly automated and scaled-up. The planar patch clamp technique (Figure 1b), using a flat substrate with a hole, reduces some of the technical difficulties. Specifically, the construction of patch pipettes, the use of a microscope and micromanipulators to position the pipettes to the cell, and the need for a vibration isolation table are all eliminated. The planar patch technique also provides the opportunity to scale up the experimental throughput by performing multiple recordings in parallel. The IonWorks® HT System greatly improves throughput by using the PatchPlate™, a 384-well substrate. Parallel recordings are performed by the use of the 48-channel electronic headstage.

Conventional Patch Clamp Technique (Figure 1a)



The conventional technique requires the positioning of a patch clamp pipette onto the cell and an electrical seal formed with the cell membrane. After seal formation, electrical access is obtained using suction or a membrane-perforating agent. The membrane voltages are clamped and the resulting ionic currents are measured with the pipette referenced to the grounded bath solution.

Planar Patch Technique (Figure 1b)



Eliminating the need for patch pipettes and positioning, the cell is drawn to the hole by vacuum. An electrical seal is formed between the cell membrane and the substrate; electrical access is obtained using a membrane-perforating agent. Polarity of the electrodes is opposite compared to the conventional patch clamp technique.

This application note describes the use of the IonWorks HT Platform with a voltage-dependent potassium channel K_v1.5.[2] Voltage-dependent potassium (K_v) channels control the repolarization of action potential waveforms and the patterns of neuronal firing by opening and closing in response to the dynamic voltages present across the cell membrane. K_v1.5 channels are of the delayed rectifier type, meaning that they have slower activation kinetics and very little inactivation when compared to other K_v channels. They are a widely studied channel and a candidate for drug discovery as a target of anti-arrhythmic drugs.

K^+ channels can be divided into two main families based on the structure of their individual pore-forming subunits. K_v channels are a subset of the family with six transmembrane spanning domains (Figure 2); inward rectifiers are the other family with only two transmembrane domains. K_v1.1 to K_v1.9 are a subset of K_v channels with the most homology to the Shaker potassium channel. A functional K_v channel requires four individual pore-forming subunits (Figure 2) while a functional inward rectifier requires a varied stoichiometry of heterologous subunits. The pore-forming or α subunit of K_v channels includes an intrinsic voltage sensor, a K^+ selectivity filter, as well as activation and inactivation gates.

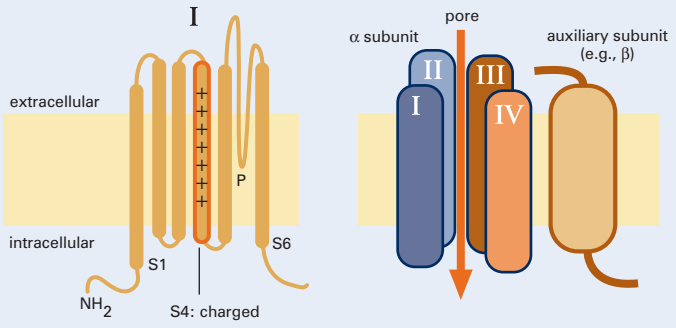
BACKGROUND

K_v1.5 channels were expressed in Chinese hamster ovary (CHO) cells. Ionic currents were recorded on the IonWorks HT Instrument and compared with currents recorded using conventional patch clamp techniques. A comparison of the inhibition of K_v1.5 channel currents by 4-Aminopyridine (4-AP) was also performed using the IonWorks HT Instrument and conventional patch clamp techniques.

MATERIALS

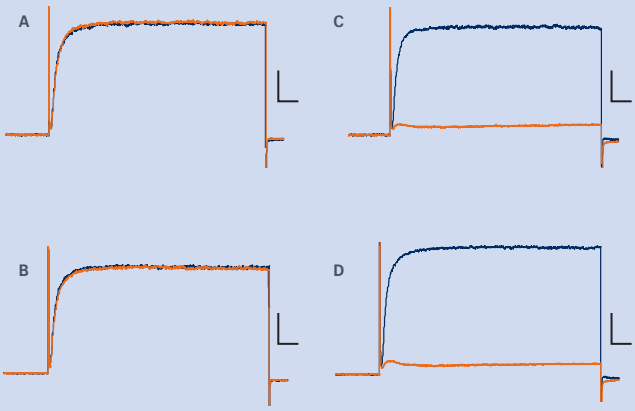
- Cells: Chinese hamster ovary (CHO) cells expressing the K_v1.5 potassium channel
- Reagents and buffers: 4-Aminopyridine (4-AP) (Sigma Cat. # A0152), Amphotericin (Sigma Cat. # A-4888), DMSO (Sigma Cat. # D-2650) were used. Internal Buffer: high K^+, low Cl^- internal buffer containing (in mM): 100 K-Gluconate (Sigma Cat. # G-4500, 40 KCl (Sigma Cat. # P-9333), 3.2 $MgCl_2$ (Sigma Cat. # M-2670), 5.0 EGTA (Sigma Cat. # E-0396), 5.0 Hepes (Sigma Cat. # H-7523) pH 7.25 with KOH. External Buffer: Phosphate Buffered Saline (PBS, Gibco Cat. # 14040)
- Tissue culture flasks: Cells were grown in T-75 flasks (Corning Cat. # 430641)
- Cell culture media: Ham's F-12 (Irvine Cat. # 9058), fetal bovine serum (FBS, Irvine Cat. # 3000), Glutamine/Penicillin/Streptomycin

Schematic Showing the Protein Structure of a Typical Voltage-Dependent (K_v) Channel (Figure 2)



Pore-forming α subunits consist of six membrane-spanning segments (S1–S6). S4 is thought to contain charges contributing to the voltage sensor while the pore region is thought to be located between S5 and S6. A current-passing channel is formed by four α subunits and accessory subunits (e.g. β, etc.).

K_v1.5 Current Tracings Collected on the IonWorks HT Instrument (Figure 3)



A & B: K_v1.5 current stability, assessed by measuring control (pre-compound) currents (blue) and post-compound currents (orange) in presence of "mock compound." C & D: Control currents (blue) and currents in the presence of 3.9 mM 4-AP (orange). Vertical scale bars are 500 pA, horizontal scale bars are 10 ms.

(G/P/S) (Irvine Cat. # 9316), Geneticin (G-418, Gibco Cat. # 10131) to grow the cells; Versene™ (Gibco Cat. # 15050) was used to remove the cells from the flasks

→ PatchPlates: Molecular Devices Cat. # 9000-0688

→ Compound plates: Costar® 96-well plate Cat. # 3355

METHODS

Cell culture

CHO cells were grown in F-12 media supplemented with 10% fetal calf serum, G/P/S (1%) and G-418 (1%) as a selection antibiotic. Cells were plated at a density of ~1-3 x 10^6 cells/ml and passaged at regular intervals when the flasks reached 80-100% confluence. To prepare the cells for experimental runs, they were passaged at 1:4 to 1:10 (flasks) 3-5 days prior to their use on the instrument.

Preparation of antibiotic solution

Aliquots of amphotericin (5 mg) were pre-weighed and stored at 4°C. Prior to cell preparation, 180 µL of DMSO was added to an aliquot of amphotericin. Amphotericin/DMSO solution was vortexed until clear (~2 minutes) and then added to 50 ml of Internal Buffer and vortexed for ~1 minute. Solution was placed in the dark until ready to use.

Cell preparation procedure

Note: This step should be performed after the preparation of all other reagents.

Step 1: Cells (80-95% confluence) were removed from the incubator 3-5 days after being plated.

Step 2: Growth media was removed from the culture flasks and cells were gently rinsed with 4 ml Versene solution for less than 10 seconds, then the solution was removed.

Step 3: 4 ml of Versene solution was added and cells incubated for 6-9 minutes (at 37°C) until cells rounded up and could be easily dislodged from the bottom of the flask with a few brief raps on a solid surface. 20 ml of PBS was added to the flask and the cell suspension was added to two 15 ml tubes (divided equally).

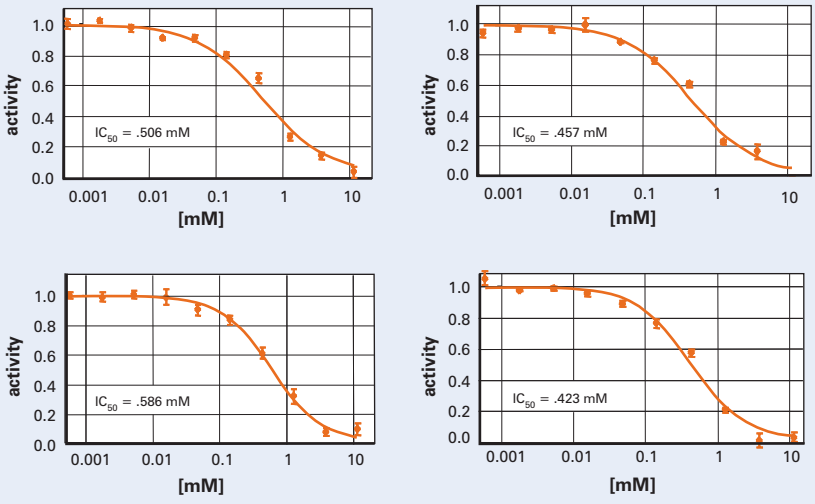
Step 4: The 15 ml tubes were spun down at 800 rpm in a table-top centrifuge. Supernatant was removed and 2.5-3 ml PBS was added per tube and vigorously triturated.

Step 5: A single tube (2.5-3.0 ml) of cell suspension was added to a cell boat on the IonWorks HT Instrument just prior to beginning the experimental run.

Preparation of compound plate

A 105 mM stock solution of 4-AP was prepared in 1% DMSO. This was diluted 1:3 in PBS and 390 µL of the resulting 35 mM solution

IC_{50} Inter-Plate Comparisons (Figure 4)



IC_{50} curves generated from four runs on the IonWorks HT Instrument. Each curve consists of over 250 data points with individual data points obtained from separate cells (< 45 minutes/run, mean ± SEM).

Compound Plate Preparation (Figure 5)

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
B	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
C	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
D	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
E	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
F	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
G	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0
H	3.5E-2	1.2E-2	3.9E-3	1.3E-3	4.3E-4	1.4E-4	4.8E-5	1.6E-5	5.3E-6	1.8E-6	3.5E-2	0

Values in table are [4-AP] in M.

was added to each of the wells in column 1 and column 11 of the compound plate. 260 µL of PBS was then added to columns 2-10 and 12. 130 µL of solution was then simultaneously aspirated out of each of the wells in column 1, dispensed into column 2, and subsequently mixed ten times using the pipettor. This procedure was repeated, transferring 130 µL of solution from column 2 to 3, 3 to 4, etc. through column 10. The pipette tips were replaced after each mixing step. The resulting layout of the compound plate is shown in Figures 5 and 8. The concentrations will be diluted 3-fold on the instrument by the addition of 3.5 µL of compound stock to 7 µL of the buffer/cell solutions in the PatchPlate.

Electrophysiology

K_v1.5 currents were elicited by a voltage step from the holding potential of -70 mV to +40 mV for 100 ms. A modified P/N subtraction[3] was used whereby a linear leak current is estimated by applying a voltage step from -70 to -80 mV. The values from this measurement are then used to extrapolate a leak I/V curve over the entire voltage range. Leak current values are then estimated from this I/V curve and subtracted digitally from all current recordings. There is no subtraction of the capacitive transients as in traditional P/N subtraction. 4-AP was incubated for 180 to 229 seconds. Similar protocols were used for the conventional patch clamp recordings with leak subtraction disabled. Pipettes were pulled from TW-150 glass (WPI) with tip sizes ranging between 3-5 MΩ. Bath perfusion of compound (180-240 seconds) was used on the conventional patch clamp setup. A PC-505 amplifier (Warner) was used in all conventional experiments.

IC_{50} curve generation

Concentration-response curves for 4-AP were fitted to a four-parameter logistic equation:

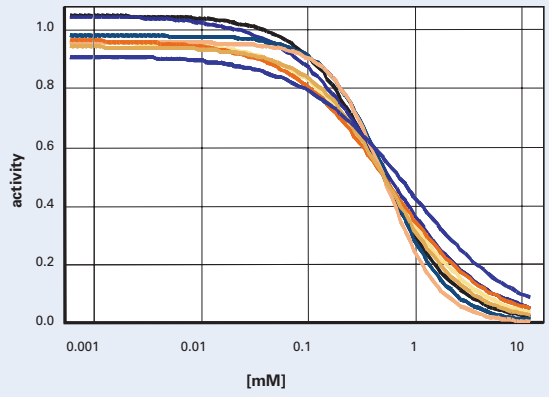
$$\% \text{ of control} = 100\ (1 + ([4\text{-AP}]/IC_{50})^{p})^{-1}$$

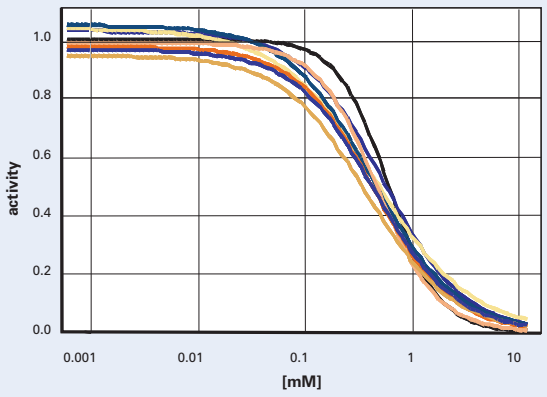
where IC_{50} is the concentration of 4-AP required to inhibit current by 50% and p is the Hill slope.

RESULTS

IC_{50} curves for 4-AP were obtained on the IonWorks HT Instrument to compare reproducibility across different experimental runs and within experimental runs. These data were also compared to that obtained using conventional patch clamp techniques. Curves were generated using cells that received solution from all of the wells of the compound plate

IC_{50} Intra-Plate Comparisons (Figure 6)





row	A	B	C	D	E	F	G	H
IC_{50}	0.461	0.518	0.556	0.547	0.529	0.547	0.559	0.865
mean	0.573			S.D.	0.122			

row	A	B	C	D	E	F	G	H
IC_{50}	0.597	0.538	0.449	0.424	0.473	0.438	0.377	0.446
mean	0.468			S.D.	0.069			

Sixteen IC_{50} curves obtained from two runs. Eight individual 10-point curves/run, from eight compound plate rows. All concentrations for each curve are from a maximum of four replicates or eight replicates for highest concentration (< 45 minutes/run).

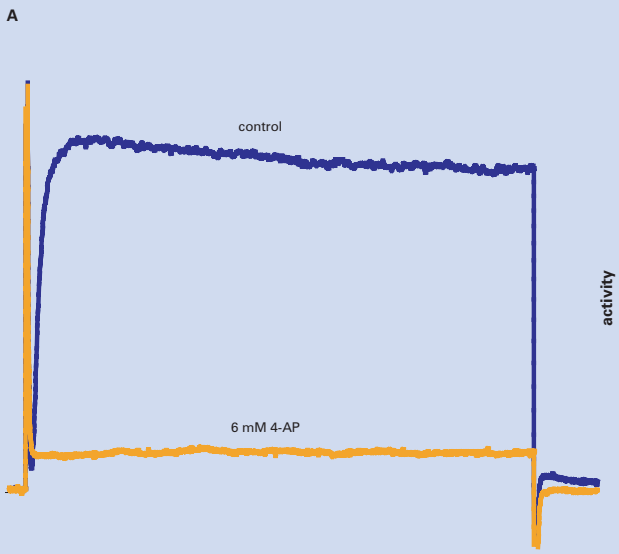
(n = max. of 32 cells/concentration) or from a subset of a single row of the compound plate (n = max. of 4 cells/concentration).

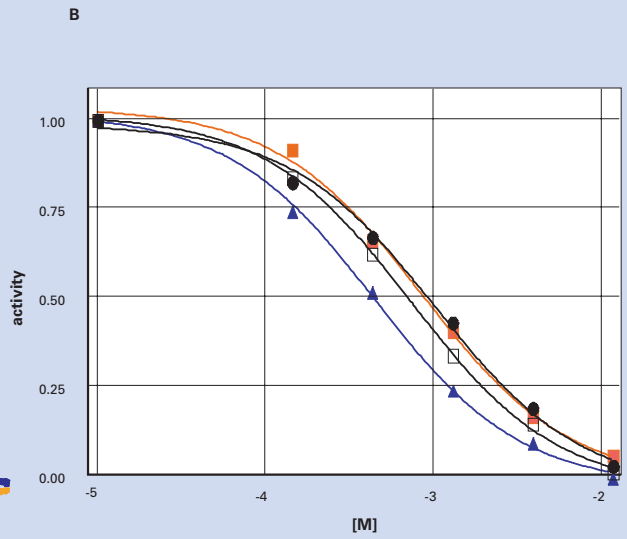
Raw current tracings are shown in Figure 3 before and after the addition of "mock" compound (negative control) and before and after the addition of 3.9 mM 4-AP. The negative controls are located on the PatchPlate where the fluidics head dispenses solution from column 12 of the compound plate. IC_{50} curves generated from individual runs on the IonWorks HT Instrument are shown in Figure 4. Each graph is collected from a single 45-minute run and is constructed from recordings of over 250 cells in each run. The number of possible cells used for each data point is 32, except for the highest concentration of compound where the number is 64. Figure 6 shows two sets of eight IC_{50} curves, each generated from an individual run. Each of the eight IC_{50} curves shown in the two plots is constructed from wells on the PatchPlate that received compound from an individual row of the compound plate. The number of possible cells used for each data point is four, except for the highest concentration of compound where the number is eight. The value for the IC_{50} curves obtained in each row are shown in Figure 6, the mean and standard deviation is also shown for all eight curves from a single 45-minute run.

Conventional patch clamp recordings were performed to compare the results obtained using the IonWorks HT Instrument. Raw data traces are shown in Figure 7 (Panel A) prior to the addition of 4-AP and also in the presence of 6 mM 4-AP. IC_{50} curves were generated using the conventional patch clamp technique (Figure 7, Panel B). The mean value obtained for the IC_{50} for 4-AP using the conventional technique was .773 mM (± .244).

Figure 8 shows the data display from the IonWorks HT software at the end of a run. Panel A shows the PatchPlate view, and Panel B shows the Compound Plate view. The number shown in each compound plate well (B) is the "valid-cell count," i.e., the number of successful recordings obtained (out of a maximum of four replicates). The PatchPlate display uses red squares without an "X" to indicate wells that lost the seal during the experimental run. A red square with an "X" indicates that the well was filtered with a user-defined metric (in this case, due to low expression; ≤ 300 pA). Panel C is the statistical display, which tabulates the number of "no seals" and the number of cells that were filtered out with a user-defined metric. Panel D shows the "configure hits display." A comparison can be made between the user-defined metrics of two scans and differences above (or below) a certain level can be defined as a "hit."

$K_v1.5$ IC_{50} Curves Collected Using the Conventional Patch Clamp Configuration (Figure 7)





$K_v1.5$ 4-AP IC_{50}

cell #	IC_{50} (mM)
1	0.863
2	0.453
3	1.030
4	0.744
mean	0.773
SD	0.244

A: Current tracings of $K_v1.5$ channels collected using conventional patch clamp, voltage steps –70 to +40 mV. Control currents and currents in presence of 6 mM 4-AP.
B: IC_{50} curves from four cells using conventional patch clamp. Fitted curves and IC_{50} values for individual cells also shown. Mean and SD of IC_{50} values also shown.

CONCLUSIONS

The IonWorks HT Instrument delivers reliable and reproducible results in the measurement of IC_{50} curves for 4-AP blockade of K_v1.5 channels. The results are also in agreement with the results obtained using the conventional patch clamp method. The ease in obtaining the data and the throughput of the IonWorks HT Instrument when compared to the conventional technique make it a very appealing instrument for automated high throughput electrophysiology.[4,5]

REFERENCES

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4. Kiss, L., Bennett, P. B., *et al.* (2003). High throughput ion-channel pharmacology: planar-array-based voltage clamp. *Assay and Drug Development Technologies* 1(1-2): 127-135.
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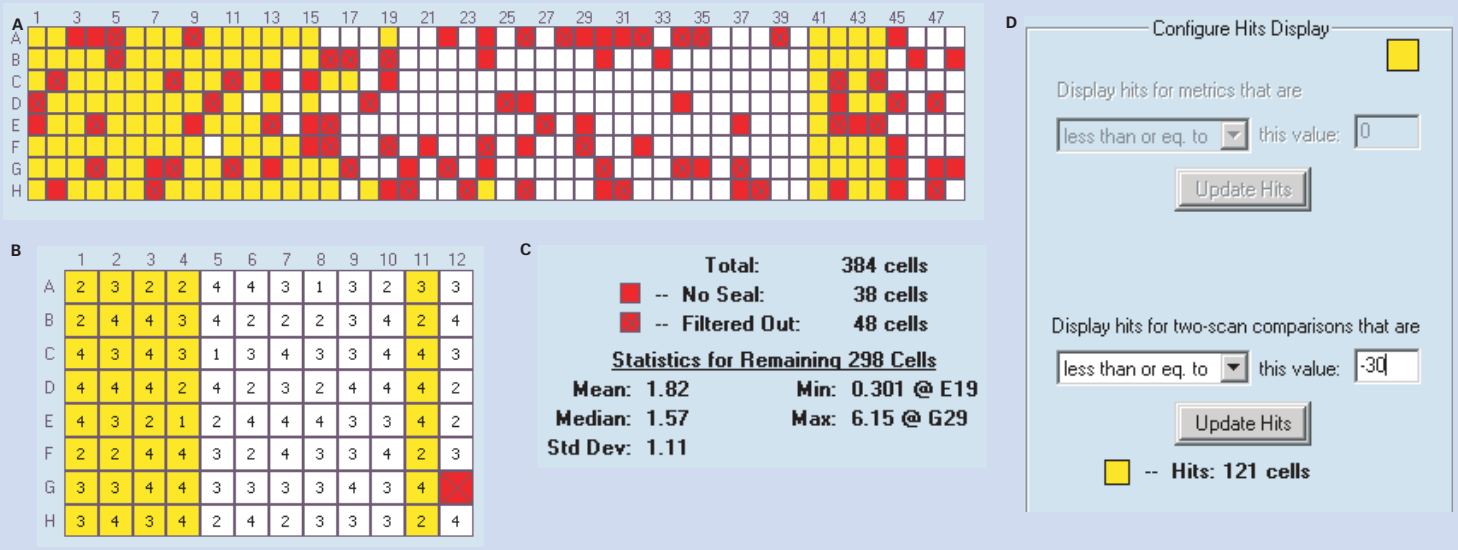
Screen Displays From IonWorks HT Software (Figure 8)



Display of the PatchPlate (A) and compound plate (B) after a run. The key to the color-coded graphic display is described in Panels C and D. Statistics display (C) of the experiment and "configure hits display" (D) showing user-defined hits. See text for a detailed description.

Molecular Devices

SALES OFFICES

United States & Canada
Molecular Devices
Tel. +1-800-635-5577
Fax +1-408-747-3601

Brazil
Molecular Devices Brazil
Tel. +55-11-3616-6607
Fax +55-11-3616-6607

China
Molecular Devices Beijing
Tel. +86-10-6410-8669
Fax +86-10-6410-8601

Molecular Devices Shanghai
Tel. +86-21-6887-8820
Fax +86-21-6887-8890

Germany
Molecular Devices GmbH
Tel. +49-89/96-05-88-0
Fax +49-89/9-62-02-34-5

Japan
Molecular Devices Japan, Osaka
Tel. +81-6-6399-8211
Fax +81-6-6399-8212

Molecular Devices Japan, Tokyo
Tel. +81-3-5282-5261
Fax +81-3-5282-5262

South Korea
Molecular Devices Korea, LLC
Tel. +82-2-3471-9531
Fax +82-2-3471-9532

United Kingdom
Molecular Devices Ltd.
Tel. +44-118-944-8000
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