

# A Cell-Based Assay to Assess Hemichannel Function

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Activation of connexin hemichannels is involved in the pathophysiology of disorders that include deafness, stroke, and cardiac infarct. This aspect makes hemichannels an attractive therapeutic target. Unfortunately, most available inhibitors are not selective or isoform specific, which hampers their translational application. The absence of a battery of useful inhibitors is due in part to the absence of simple screening assays for the discovery of hemichannel-active drugs. Here, we present an assay that we have recently developed to assess hemichannel function. The assay is based on the expression of functional human connexins in a genetically modified bacterial strain deficient in  $K^+$  uptake. These modified cells do not grow in low- $K^+$  medium, but functional expression of connexin hemichannels allows  $K^+$  uptake and growth. This cell-growth-based assay is simple, robust, and easily scalable to high-throughput multi-well platforms.

## INTRODUCTION

Membrane proteins correspond to ~30 percent of genomes and are frequently expressed at low levels [1]. However, they are the targets of the majority of drugs currently on the market and drug transporting membrane proteins are also important targets to affect pharmacokinetics [2,3]. Ion channels constitute a subgroup of membrane proteins characterized by their conductance, gating, and selectivity. When open, ion channels form a hydrophilic pathway across the membrane through which ions flow at a high rate by electrodiffusion, determining their conductance, whereas the relative permeability of different ions, due to differences in charge and/or size, determines selectivity. Finally, factors such as voltage, ligands, and post-translational modifications produce conformational

changes that open and close the channels (gating). Some ion channels are highly selective (e.g., voltage-gated  $K^+$  channels), while others are not. The latter group includes gap-junction channels (GJCs†) and hemichannels (HCs) formed by connexins.

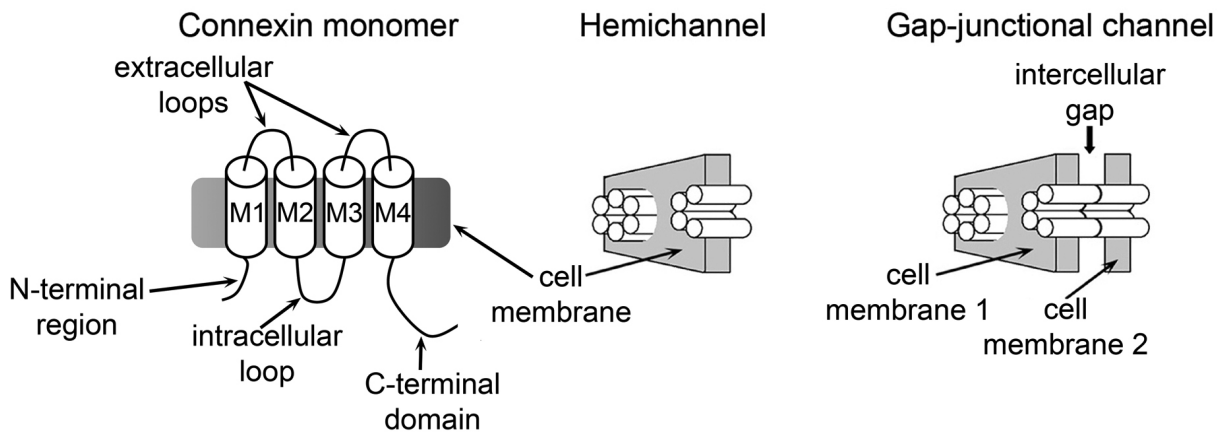
There are 21 human connexin isoforms, with lengths between 226 and 543 amino acids [4,5]. Connexins can oligomerize to form homomeric or heteromeric HCs (Figure 1) of varying permeability properties, regulation, and associations with other proteins [4,6]. Each connexin has four transmembrane helices (M1 to M4), two extracellular loops, and cytoplasmic hydrophilic regions (N- and C-terminal regions and intracellular loop) (Figure 1) [4-6]. Sequence analysis shows that the intracellular regions are poorly conserved, whereas the M1-extracellular loop

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†Abbreviations: Cx26, connexin 26; Cx43, connexin 43; GJC, gap-junction channel; HC, hemichannel; HTS, high-throughput screening; OD600, absorbance measured at 600 nm.

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**Figure 1.** Connexin channels and hemichannels. Schematic representation of a connexin subunit (monomer), a hemichannel (hexamer), and a gap-junctional channel (dodecamer). M1 to M4: transmembrane helices. Each monomer is depicted as a cylinder in the hemichannel and the gap-junctional channel. This figure is reproduced from one originally published in the *J Biol Chem* [30], and is reproduced with permission from the American Society for Biochemistry and Molecular Biology.

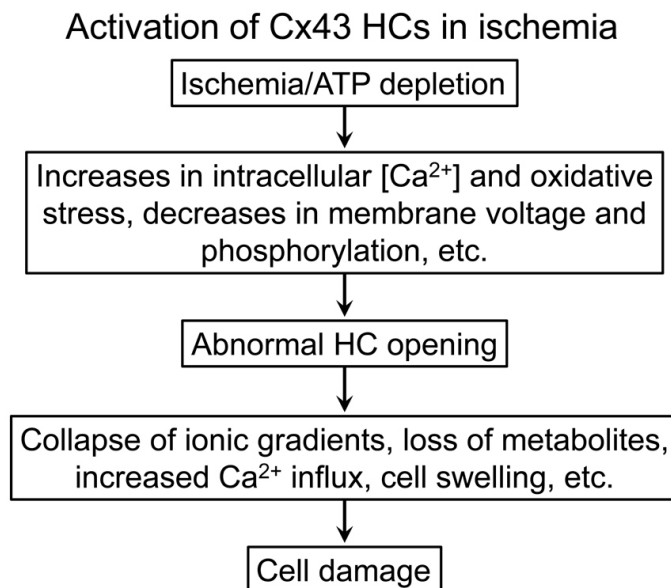
1-M2 sequence is well conserved, especially the M1 sequence [5]. Six connexins subunits assemble as hexamers to form HCs [6], and head-to-head docking of HCs from adjacent cells forms GJCs (Figure 1) [6].

Because of their large pore size, GJCs are permeable to small hydrophilic molecules of up to 400-800 Da (including many second messengers) [6]. Therefore, they not only mediate cell-to-cell electric coupling, but are involved in chemical coupling between neighboring cells. The general properties of GJCs and HCs in terms of pore size and selectivity are quite similar [6,7], with the pore formed by M1 and M2, and the narrowest region of the pore near the extracellular side of the membrane [4,8,9]. Since uncharged hydrophilic molecules can diffuse through GJCs and HCs, they are often referred to as channels, as opposed to ion channels.

## CONNEXINS IN HEALTH AND DISEASE

HCs are mostly closed under normal conditions, but they play an important role in autocrine and paracrine signaling, by mediating the transmembrane fluxes of signaling molecules/metabolites such as ATP,  $\text{NAD}^+$ , glutamate, glutathione,  $\text{PGE}_2$ , and glucose [10]. Cx43, a 382-amino acid connexin, is expressed in parenchymal cells of a variety of organs, such as cardiac muscle, brain and kidney, as well as in capillary endothelial cells [11]. Connexins are abundantly expressed in the excitation-conduction system and in the contractile myocar-

dium, and Cx43 GJCs mediate the cell-to-cell conduction of the electrical impulse generated by the sinoatrial node, essential for the coordinated contraction of the heart [12-16]. Heart disease is the most common cause of death in the U.S., and many of these deaths are caused by cardiac ischemia and arrhythmias. Cx43 GJCs and HCs have important roles in the damage of the heart muscle elicited by ischemia, and the genesis and maintenance of arrhythmias [14,15,17-20]. Cx43 also plays important roles in ventricular arrhythmias, including the most lethal one, ventricular fibrillation [12,14,15,17,19]. Cx43 HCs play a pathophysiological role in ischemic damage of the heart (myocardial infarction), brain (stroke), and kidneys (ischemic renal tubule necrosis) [17,18,21-26]. The activation of Cx43 HCs in cardiomyocytes, astrocytes, and renal proximal tubule cells under conditions that mimic ischemia contributes to the cell damage [17,18,21-26]. Under physiological conditions several factors maintain the HCs mostly closed. These include normal extracellular  $\text{Ca}^{2+}$  in the low millimolar range, the cell-negative membrane voltage, and phosphorylation by PKC [11,27-35]. However, HCs open in ischemia, even in the continuous presence of millimolar extracellular  $[\text{Ca}^{2+}]$  [11,21-26,28,30-33,35]. The mechanism is not completely understood, but seems to involve changes in post-translational modifications (decreased phosphorylation due to ATP depletion and increased phosphatase activity, and/or changes in nitrosylation due to the oxidative stress), although the increase in cytosolic  $[\text{Ca}^{2+}]$  can also play a



**Figure 2.** Activation of Cx43 hemichannels under ischemic conditions. Activation of Cx43 hemichannels (HCs) participates in the damage of cardiomyocytes, glia and renal tubule cells in ischemia. The pathways of activation have not been clearly defined, but there is evidence for all those indicated in the figure.

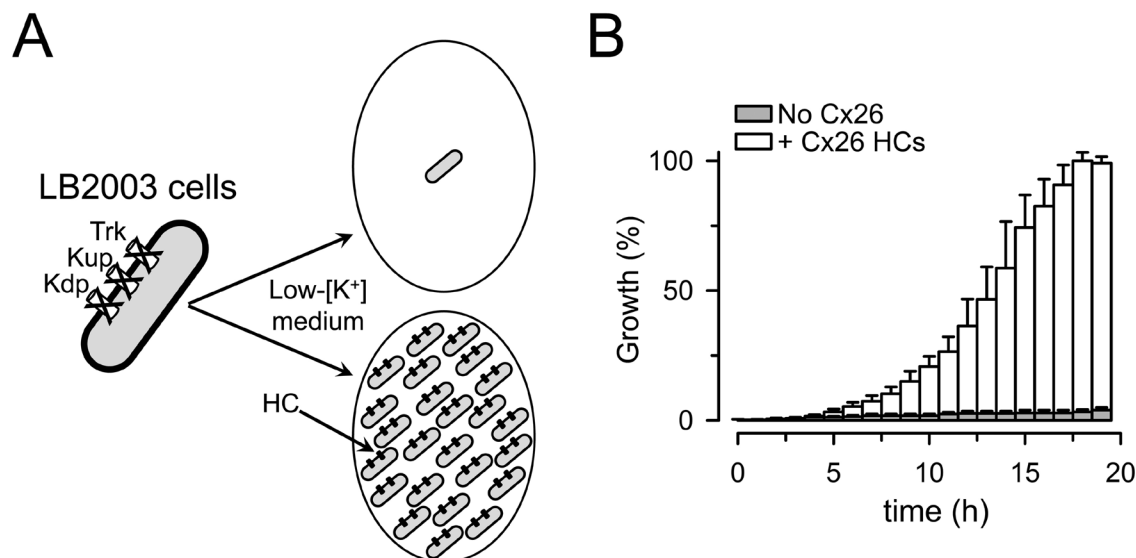
role [11,18,22,24,25,33,35-41]. GJCs link the cytoplasm of adjacent cells, compartments of very similar composition, whereas plasma membrane HCs link the intracellular and extracellular fluids, which have very different compositions. Abnormal HCs opening can lead to losses of metabolites,  $\text{Ca}^{2+}$  influx with alterations of signaling and protease activation, equilibration of ionic gradients, and cell swelling. Therefore, abnormal HC opening can result or contribute to cell damage and/or death in a number of disorders (Figure 2).

Connexin 26 (Cx26) has paramount importance in the inner ear. Hearing loss is very common and can occur at any time from infancy to old age [36,42-47]. Approximately 1/1,000 infants have profound hearing impairment, and ~50 percent of the cases are due to single gene mutations, mostly Cx26 mutations [36,42-46]. In addition, a role of Cx26 in non-genetic deafness is also likely [42,44,46,48,49]. Cx26, the main connexin in the inner ear, is smaller (226 amino acids) than Cx43 (382 amino acids) and their primary sequences display < 30 percent amino-acid identity. Cx26 has a very short C-terminal domain (< 20 residues vs. > 150 residues in Cx43). Based on their lower permeability to fluorescent dyes and metabolites, it seems that GJCs and HCs formed by Cx26 have a smaller apparent pore size than those formed by Cx43 [6].

In the inner ear, the cochlea houses the organ of Corti, a narrow spiral containing the hair cells that transduce sound into electrical impulses. The cochlear gap-junctional communication network is essential for hearing

[45,46,50]. Cx26 mutations may cause deafness by reducing gap-junctional communication with decreased  $\text{K}^+$  recycling into the endolymph [50,51], a mechanism recently questioned [52], or by selectively reducing cell-to-cell permeability to signaling molecules such as inositol trisphosphate (IP3) [53-56]. Deafness due to “leaky” HCs has also been proposed [43,57,58]; in this case, cell damage, with the resulting deafness, would occur as a consequence of uncompensated water and solute fluxes (Figure 2). In addition, it has been shown that some deafness-associated mutants display increased  $\text{Ca}^{2+}$  permeability, and it has been speculated that the increased  $\text{Ca}^{2+}$  influx results in apoptosis and death of hair and supporting cells [59,60]. Although the detailed mechanisms of deafness are not definitively understood, Cx26 HC inhibitors are potential therapeutic leads for deafness mediated by leaky HCs, including HCs that display increased  $\text{Ca}^{2+}$  permeability [43,57-62].

There are many additional disorders where targeting connexin HCs may prove useful. These include HCs in the central nervous system, where under some abnormal conditions activated microglia can release massive amounts of glutamate through connexin HCs, which damages neural cells. This process could play a major role in the neuronal damage of a variety of neurodegenerative diseases, and targeting of HCs has been proposed for therapy [63-65]. Another potential use of HC inhibitors is for the inhibition of neovascularization in the treatment of cancer [65].



**Figure 3.** Growth complementation by expression of Cx26 HCs in LB2003 cells. (a) Schematic representation of the LB2003 cells. (b) Growth complementation by Cx26 shown as percent of the maximal growth in the Cx26-expressing cells. Data are means  $\pm$  SEM. Adapted from J Biomol Screen [79] with permission from SAGE Publications. See [79] for details.

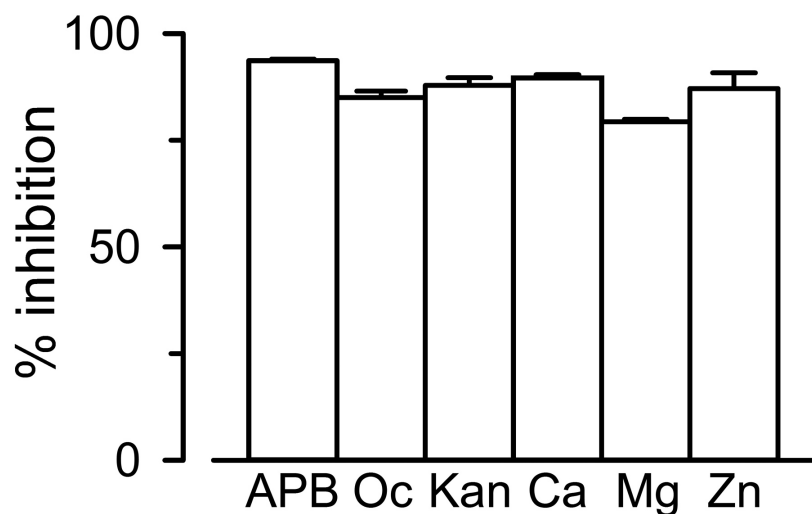
### HIGH-THROUGHPUT ASSAYS TO ASSESS GAP-JUNCTION CHANNEL FUNCTION

Dye transfer experiments have been traditionally used to assess GJC functionality and have been recently exploited for high-throughput screening (HTS) assays aimed at discovering GJC inhibitors. These assays are based on the transport from donor to acceptor cells via GJCs. In one assay, donor cells were loaded with calcein, a fluorescent probe, and its transfer to acceptor cells was imaged using automated fluorescence microscopy [66]. Another assay was based on the permeability of GJCs to  $\text{Ca}^{2+}$  [67]. Donor cells co-expressing Cx43 and  $\alpha 1\text{A}$  adrenergic receptors were paired with acceptor cells co-expressing Cx43 and aequorin, a  $\text{Ca}^{2+}$ -sensitive luminescent protein. Activation of  $\alpha 1\text{A}$  receptors led to  $\text{Ca}^{2+}$  waves that spread to the acceptor cells via GJCs and were detected by the increase in aequorin luminescence. A rather similar principle was used in another assay, where donor cells co-expressed a  $\text{I}^-$  transporter and connexins, the acceptor cells co-expressed connexins, and a yellow fluorescent protein variant whose fluorescence emission is quenched by the anion [68]. In this assay, influx of  $\text{I}^-$  into the donor cell is followed by diffusion through GJCs into the acceptor cell, where it quenches the yellow fluorescent protein emission. In all these assays, changes in the signal that reflect decrease donor/acceptor transport were used to identify new GJC inhibitors [66-68]. These assays have been proven useful, but have limitations such as the relative complexity of sample preparation and data analysis. They are also focused on GJCs and cannot be

used to assess the function of HCs. There are many HC assays based on methodologies such as dye uptake and electrophysiology [6,10], but complexity and cost make their adaptation for HTS of large chemical libraries costly and difficult.

### EXPRESSION OF HUMAN CONNEXIN HEMI-CHANNELS IN BACTERIA

Studies with isolated systems where experimental conditions are controlled are important to understand the bases of normal function and the molecular mechanisms of diseases. Recombinant connexins for research studies are generally expressed in mammalian cells, insect cells or frog oocytes. Until our recent report, the insect cell/baculovirus expression system was the only available system that yielded purified connexins in the amounts necessary for detailed biochemical and biophysical studies [8,31,69-73]. In our recent report, we developed and optimized an *Escherichia coli*-based expression/purification system that yields milligram amounts of functional human Cx26 HCs [74]. Bacteria were transformed with a plasmid containing *E. coli*-optimized DNA for the expression of human Cx26 with a C-terminal poly-His tag preceded by a protease cleavage site (TEV protease) to remove the tag after purification. Cx26 was purified by immobilized metal affinity chromatography based on the affinity of the Cx26 poly-His tag for  $\text{Co}^{2+}$ , followed by size-exclusion chromatography. The highly-purified Cx26 formed very stable HCs in detergent [74]. The hu-



**Figure 4.** Effects of HC inhibitors on Cx26-dependent growth complementation. Reduction in growth complementation by connexin HC inhibitors in LB2003 cells expressing Cx26. Cells were grown without any additions (control, not shown), or in medium supplemented with 50  $\mu\text{M}$  2-aminoethoxydiphenyl borate (APB), 1 mM 1-octanol (Oc), 100  $\mu\text{M}$  kanamycin A, 1 mM  $\text{Ca}^{2+}$  (Ca), 20 mM  $\text{Mg}^{2+}$  (Mg), or 100  $\mu\text{M}$   $\text{Zn}^{2+}$  (Zn). Data are means  $\pm$  SEM. Adapted from J Biomol Screen [79] with permission from SAGE Publications. See [79] for details.

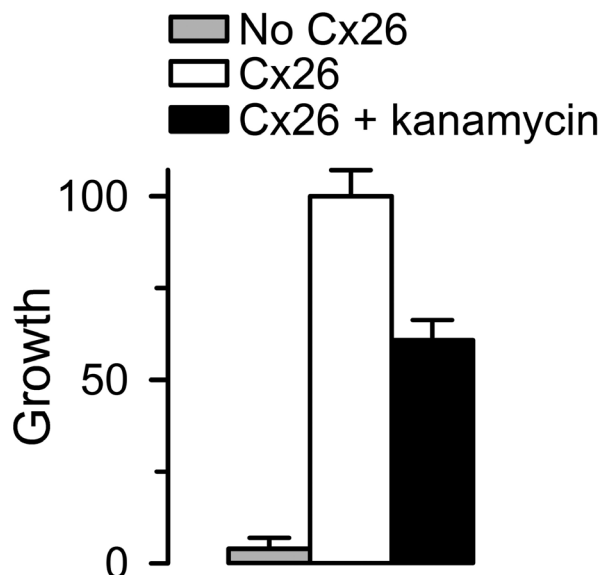
man Cx26 HCs purified from bacteria were structurally and functionally identical to those purified from insect cells and essentially all HCs reconstituted in unilamellar liposomes were functional [72,74]. Although differences between bacterial and mammalian post-translational modifications could be an issue, the problem is minimized because there is no evidence of direct regulation of Cx26 by post-translational modifications and connexins are not glycosylated. In contrast, phosphorylation of the C-terminal domain of Cx43 clearly regulates function [11,31,33,35,73], but it is possible to phosphorylate purified connexins *in vitro* [31,73]. The bacterial expression system has the potential to accelerate the pace of structural and functional connexin research.

## AN ASSAY FOR HEMICHANNEL FUNCTION IN BACTERIA

Independently of the usefulness of the bacterial expression system, our report showed that it is possible to express functional human connexin HCs in *E. coli* [74]. We took advantage of that observation to develop a new cell-based assay to evaluate the function of human HCs expressed in bacteria. We used an *E. coli* strain (LB2003) with deletion of three  $\text{K}^+$  uptake systems (Kdp, Kup and Trk) [75,76]. LB2003 cells do not grow in low- $[\text{K}^+]$  medium, but grow under conditions where  $\text{K}^+$  influx and intracellular  $[\text{K}^+]$  are expected to increase as increasing  $[\text{K}^+]$  in the growth medium or expressing recombinant  $\text{K}^+$ -selective channels [75,77,78].  $\text{K}^+$  is nec-

essary for growth because of its involvement in many important cellular processes such as maintaining turgor pressure, activation of enzymes and intracellular pH regulation. We were able to achieve growth of LB2003 cells in low- $[\text{K}^+]$  medium by expressing human Cx26 (Figure 3), Cx43, or Cx46 [74,79]. On one hand, this phenomenon of growth recovery (growth complementation) was expected because HCs provide a pathway for  $\text{K}^+$  influx, as  $\text{K}^+$  channels do. On the other hand, HCs are “large” and poorly-selective channels that can also have deleterious effects on the cells (e.g., depolarization, alterations in metabolites homeostasis; see Figure 2). Since there is a favorable electrochemical driving force for  $\text{K}^+$  electrodiffusion across the *E. coli* inner membrane, it is expected that the increased  $\text{K}^+$  permeability elicited by HC expression will produce growth complementation by increasing  $\text{K}^+$  influx and steady-state cytosolic  $[\text{K}^+]$ . In fact, our recent data support such a mechanism by showing that intracellular  $[\text{K}^+]$  is increased by  $\sim 30$  mM in LB2003 cells expressing Cx26 HCs that are grown in 4 mM  $[\text{K}^+]$  [79].

Two sets of results support the notion that growth complementation by connexin expression is the result of the presence of functional HCs: 1) Growth complementation was blocked by known HC inhibitors that included divalent cations, 2-aminoethoxydiphenyl borate, octanol, and aminoglycosides (Figure 4) [74,79]. Inhibitors of connexin-formed channels with affinities in the low-nM range are not available, but these compounds at the concentrations employed are known to inhibit HCs [80-83]. Although non-specific, divalent cations are well-known



**Figure 5.** Evaluation of assay performance. Values are presented as means  $\pm$  SD of data from multi-well plates from 276 (No Cx26 and Cx26) and 72 (Cx26 + kanamycin) measurements performed in four independent experiments. Growth was normalized to the mean of Cx26-expressing cells under control conditions. The HC inhibitor kanamycin was used at a concentration of 10  $\mu$ M, which produces ~50 percent inhibition. The calculated  $Z'$  factors for Cx26 vs. No Cx26 and Cx26 + kanamycin A vs. No Cx26 were 0.8 and 0.6, respectively. Adapted from J Biomol Screen [79] with permission from SAGE Publications. See [79] for details.

inhibitors of HCs [84]; 2) The sensitivity of growth complementation by Cx26 mutants to the inhibitory effect of  $Ca^{2+}$  was altered [79]. The Cx26 mutants G45E and D50N form functional HCs with low sensitivity to the inhibitory effect of extracellular  $[Ca^{2+}]$  [59,61,62]. These mutants were able to produce growth complementation, but inhibition by external  $[Ca^{2+}]$  was severely hampered (D50N) or abolished (G45E) in the cells expressing the mutants [79]. Our assay complements more complex methodologies that can provide far more detailed mechanistic information, such as permeability assays and electrophysiological studies [6,10].

The  $Z'$  factor is a commonly used statistical parameter to assess the quality of a HTS assay [85]. It is a dimensionless parameter that takes into account the day-to-day and well-to-well variability of the samples. It is calculated from the sample means ( $\mu$ ) and standard deviations ( $\sigma$ ) as:  $Z' = 1 - (3\sigma_s + 3\sigma_c) / |\mu_s - \mu_c|$ , where the subscripts s and c denote sample and control, respectively. An assay with  $Z' \geq 0.5$  is best, whereas values between  $0 < Z' < 0.5$  point to a marginal assay that requires optimization, and assays with  $Z' < 0$  are not suitable for HTS [85]. With a  $Z'$  of 0.8, our assay has a great potential for HTS (Figure 5) [79].

Because of its simplicity, low-cost, easy scalability, reproducibility, and sensitivity, the assay presented here should be useful for the discovery of new and better HC inhibitors [79]. However, it can miss inhibitors because

of factors such as limited access to the periplasmic space due to the presence of the outer membrane, indirect effects that need interaction of inhibitors with proteins not present in bacteria, and partial HC inhibition that is insufficient to decrease cell  $[K^+]$  to impair cell growth.

The HTS HC function assay presented here has the potential for screening large chemical libraries to discover new, effective, and specific HC inhibitors for research and therapy. To accomplish this aim, scaling up the assay from 96-well to 384- or 768-well plates will be desirable. Since Cx26 and Cx43/Cx46 are among the most dissimilar connexin isoforms [4-6], it seems likely that our assay can be used to assess the function of HCs formed by most or all connexin isoforms.

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