

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |           |                                                                                                                                                                                                                                                                         |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>G01N</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | <b>A2</b> | <b>(11) International Publication Number:</b> <b>WO 99/45358</b><br><b>(43) International Publication Date:</b> 10 September 1999 (10.09.99)                                                                                                                            |
| <b>(21) International Application Number:</b> PCT/US99/04549<br><b>(22) International Filing Date:</b> 2 March 1999 (02.03.99)<br><br><b>(30) Priority Data:</b><br>60/076,621                      3 March 1998 (03.03.98)                      US<br><br><b>(71) Applicant (for all designated States except US):</b> ICAGEN, INC.<br>[US/US]; Suite 460, 4222 Emperor Boulevard, Durham, NC<br>27703 (US).<br><br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> SILVIA, Chris [US/US];<br>3105 Broomsedge Way, Durham, NC 27712 (US). YU,<br>Weifeng [CN/US]; 107 Park York Lane, Apex, NC 27502<br>(US).<br><br><b>(74) Agents:</b> PARENT, Annette, S. et al.; Townsend and Townsend<br>and Crew LLP, 8th floor, Two Embarcadero Center, San<br>Francisco, CA 94111-3834 (US). |           | <b>(81) Designated States:</b> AU, CA, US, European patent (AT, BE,<br>CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,<br>NL, PT, SE).<br><br><b>Published</b><br><i>Without international search report and to be republished<br/>upon receipt of that report.</i> |
| <b>(54) Title:</b> IDENTIFICATION AND EXPRESSION OF HUMAN KIR5.1<br><br><b>(57) Abstract</b><br><br>The invention provides isolated nucleic acid and amino acid sequences of hKir5.1, antibodies to hKir5.1, methods of detecting hKir5.1, methods of screening for inward rectifier potassium channel activators and inhibitors using biologically active hKir5.1, and kits for screening for activators and inhibitors of inward rectifier potassium channels comprising hKir5.1.                                                                                                                                                                                                                                                                                                                                 |           |                                                                                                                                                                                                                                                                         |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                          |    |                                              |    |                          |
|----|--------------------------|----|------------------------------------------|----|----------------------------------------------|----|--------------------------|
| AL | Albania                  | ES | Spain                                    | LS | Lesotho                                      | SI | Slovenia                 |
| AM | Armenia                  | FI | Finland                                  | LT | Lithuania                                    | SK | Slovakia                 |
| AT | Austria                  | FR | France                                   | LU | Luxembourg                                   | SN | Senegal                  |
| AU | Australia                | GA | Gabon                                    | LV | Latvia                                       | SZ | Swaziland                |
| AZ | Azerbaijan               | GB | United Kingdom                           | MC | Monaco                                       | TD | Chad                     |
| BA | Bosnia and Herzegovina   | GE | Georgia                                  | MD | Republic of Moldova                          | TG | Togo                     |
| BB | Barbados                 | GH | Ghana                                    | MG | Madagascar                                   | TJ | Tajikistan               |
| BE | Belgium                  | GN | Guinea                                   | MK | The former Yugoslav<br>Republic of Macedonia | TM | Turkmenistan             |
| BF | Burkina Faso             | GR | Greece                                   | ML | Mali                                         | TR | Turkey                   |
| BG | Bulgaria                 | HU | Hungary                                  | MN | Mongolia                                     | TT | Trinidad and Tobago      |
| BJ | Benin                    | IE | Ireland                                  | MR | Mauritania                                   | UA | Ukraine                  |
| BR | Brazil                   | IL | Israel                                   | MW | Malawi                                       | UG | Uganda                   |
| BY | Belarus                  | IS | Iceland                                  | MX | Mexico                                       | US | United States of America |
| CA | Canada                   | IT | Italy                                    | NE | Niger                                        | UZ | Uzbekistan               |
| CF | Central African Republic | JP | Japan                                    | NL | Netherlands                                  | VN | Viet Nam                 |
| CG | Congo                    | KE | Kenya                                    | NO | Norway                                       | YU | Yugoslavia               |
| CH | Switzerland              | KG | Kyrgyzstan                               | NZ | New Zealand                                  | ZW | Zimbabwe                 |
| CI | Côte d'Ivoire            | KP | Democratic People's<br>Republic of Korea | PL | Poland                                       |    |                          |
| CM | Cameroon                 | KR | Republic of Korea                        | PT | Portugal                                     |    |                          |
| CN | China                    | KZ | Kazakstan                                | RO | Romania                                      |    |                          |
| CU | Cuba                     | LC | Saint Lucia                              | RU | Russian Federation                           |    |                          |
| CZ | Czech Republic           | LI | Liechtenstein                            | SD | Sudan                                        |    |                          |
| DE | Germany                  | LK | Sri Lanka                                | SE | Sweden                                       |    |                          |
| DK | Denmark                  | LR | Liberia                                  | SG | Singapore                                    |    |                          |
| EE | Estonia                  |    |                                          |    |                                              |    |                          |

## IDENTIFICATION AND EXPRESSION OF HUMAN KIR5.1

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from USSN 60/076,621, filed March 3, 1998, herein incorporated by reference in its entirety.

### FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

### FIELD OF THE INVENTION

The invention provides isolated nucleic acid and amino acid sequences of hKir5.1, antibodies to hKir5.1, methods of detecting hKir5.1, methods of screening for inward rectifier potassium channel activators and inhibitors using biologically active hKir5.1, and kits for screening for activators and inhibitors of inward rectifier potassium channels comprising hKir5.1.

### BACKGROUND OF THE INVENTION

Potassium channels are involved in a number of physiological processes, including regulation of heartbeat, dilation of arteries, release of insulin, excitability of nerve cells, and regulation of renal electrolyte transport. Potassium channels are thus found in a wide variety of animal cells such as nervous, muscular, glandular, immune, reproductive, and epithelial tissue. These channels allow the flow of potassium in and/or out of the cell under certain conditions. For example, the outward flow of potassium ions upon opening of these channels makes the interior of the cell more negative, counteracting depolarizing voltages applied to the cell. These channels are regulated, e.g., by calcium sensitivity, voltage-gating, second messengers, extracellular ligands, and ATP-sensitivity.

Potassium channels are made by alpha subunits that fall into 8 families, based on predicted structural and functional similarities (Wei *et al.*, *Neuropharmacology* 35(7):805-829 (1997)). Three of these families (Kv, eag-related, and KQT) share a common motif of six transmembrane domains and are primarily gated by voltage. Two other families, CNG and SK/IK, also contain this motif but are gated by cyclic nucleotides and calcium, respectively. The three other families of potassium channel alpha subunits have distinct patterns of transmembrane domains. Slo family potassium channels, or BK channels have seven transmembrane domains (Meera *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94(25):14066-71 (1997)) and are gated by both voltage and calcium or pH (Schreiber *et al.*, *J. Biol. Chem.* 273:3509-16 (1998)). Another family, the inward rectifier potassium channels (Kir), belong to a structural family containing 2 transmembrane domains (*see, e.g.*, Lagrutta *et al.*, *Jpn. Heart. J.* 37:651-660 1996)), and an eighth functionally diverse family (TP, or "two-pore") contains 2 tandem repeats of this inward rectifier motif.

Potassium channels are typically formed by four alpha subunits, and can be homomeric (made of identical alpha subunits) or heteromeric (made of two or more distinct types of alpha subunits). In addition, potassium channels made from Kv, KQT and Slo or BK subunits have often been found to contain additional, structurally distinct auxiliary, or beta, subunits. These subunits do not form potassium channels themselves, but instead they act as auxiliary subunits to modify the functional properties of channels formed by alpha subunits. For example, the Kv beta subunits are cytoplasmic and are known to increase the surface expression of Kv channels and/or modify inactivation kinetics of the channel (Heinemann *et al.*, *J. Physiol.* 493:625-633 (1996); Shi *et al.*, *Neuron* 16(4):843-852 (1996)). In another example, the KQT family beta subunit, minK, primarily changes activation kinetics (Sanguinetti *et al.*, *Nature* 384:80-83 (1996)).

The Kir family of inward rectifier potassium channels includes both heteromeric and homomeric channels that are typically composed of four subunits (*see, e.g.*, Pessia *et al.*, *EMBO J.* 15:2980-2987 (1996)). Inward rectifier channels primarily allow potassium influx, with little potassium outflux. Inward rectifier potassium channels have been found in a wide variety of tissues and cell types. Some subunits of the Kir family, of which the channels are composed, have been cloned, e.g., Kir 1.1, 2.1, 3.1, and 4.1 (*see, e.g.*, Ho *et al.*, *Nature* 362:31-37 (1993); Kubo *et al.*, *Nature* 362:127-133 (1993); Kubo *et al.*,

*Nature* 362:802-806; Morishige *et al.*, *FEBS Lett.* 336:375-380). A rat version of Kir5.1 has been cloned, however, the human Kir5.1 subunit has not been previously identified (*see* Lagrutta *et al.*, *Jpn. Heart. J.* 37: 651-660 (1996)).

## SUMMARY OF THE INVENTION

The present invention thus provides for the first time human Kir5.1, a polypeptide monomer that is a subunit of an inward rectifier potassium channel. Human Kir5.1 has not been previously cloned or identified, and the present invention provides identification and cloning of a nucleic acid encoding Kir5.1.

In one aspect, the present invention provides isolated nucleic acid encoding a polypeptide monomer comprising an alpha subunit of a potassium channel, the polypeptide monomer: (i) forming, with at least one additional Kir alpha subunit, a potassium channel having the characteristic of inward rectification; (ii) having a monomer tail region that has greater than 80% amino acid sequence identity to a human Kir5.1 tail region as measured using a sequence comparison algorithm; and (iii) specifically binding to polyclonal antibodies generated against SEQ ID NO:1.

In one embodiment, the nucleic acid encodes human Kir5.1. In another embodiment, the nucleic acid encodes SEQ ID NO:1. In another embodiment, the nucleic acid has a nucleotide sequence of SEQ ID NO:2.

In one embodiment, the nucleic acid is amplified by primers that selectively hybridize under stringent hybridization conditions to the same sequence as the primers selected from the group consisting of: 5' CCT AAG GGC ACA GCA AAG AAT GAG 3' (SEQ ID NO:3) and 5' GTG TGG CGA AAG TGG TGG TC 3' (SEQ ID NO:4).

In one embodiment, the nucleic acid encodes a polypeptide monomer having a molecular weight of about between 38 kDa to 48 kDa. In another embodiment, the polypeptide monomer comprises an alpha subunit of a homomeric or a heteromeric inward rectifier potassium channel.

In one embodiment, the nucleic acid selectively hybridizes under moderately stringent hybridization conditions to a nucleotide sequence of SEQ ID NO:2

In another aspect, the present invention provides an isolated nucleic acid encoding a polypeptide monomer that specifically hybridizes under highly stringent conditions to SEQ ID NO:2.

In another aspect, the present invention provides an isolated polypeptide monomer comprising an alpha subunit of a potassium channel, the polypeptide monomer: (i) forming, with at least one additional Kir alpha subunit, a potassium channel having the characteristic of inward rectification; (ii) having a monomer tail region that has greater than 80% amino acid sequence identity to amino acids 351-383 of a human Kir5.1 tail region as measured using a sequence comparison algorithm; and (iv) specifically binding to polyclonal antibodies generated against SEQ ID NO:1.

In one embodiment, the polypeptide monomer has an amino acid sequence of human Kir5.1. In another embodiment, the polypeptide monomer has an amino acid sequence of SEQ ID NO:1.

In another aspect, the present invention provides an antibody that selectively binds to the polypeptide monomer described above.

In another aspect, the present invention provides an expression vector comprising the nucleic acid encoding the polypeptide monomer described above.

In another aspect, the present invention provides a host cell transfected with the expression vector described above.

In another aspect, the present invention provides a method for identifying a compound that modulates ion flux through an inward rectifier potassium channel, the method comprising the steps of: (i) contacting the compound with a eukaryotic host cell or cell membrane in which has been expressed a polypeptide monomer comprising an alpha subunit of a potassium channel, the polypeptide monomer: (a) forming, with at least one additional Kir alpha subunit, a potassium channel having the characteristic of inward rectification; (b) having a monomer tail region that has greater than 80% amino acid sequence identity to a human Kir5.1 tail region as measured using a sequence comparison algorithm; and (c) specifically binding to polyclonal antibodies generated against SEQ ID NO:1; and (ii) determining the functional effect of the compound upon the cell or cell membrane expressing the potassium channel.

In one embodiment, the functional effect is determined by measuring changes in current or voltage. In another embodiment, the polypeptide monomer polypeptide is recombinant. In another embodiment, the potassium channel is homomeric or heteromeric.

In another embodiment, the present invention provides a method of detecting the presence of human Kir5.1 in mammalian tissue, the method comprising the steps of: (i) isolating a biological sample; (ii) contacting the biological sample with a Kir5.1-specific reagent that selectively associates with human Kir5.1; and, (iii) detecting the level of Kir5.1-specific reagent that selectively associates with the sample.

In one embodiment, the Kir5.1-specific reagent is selected from the group consisting of: Kir5.1 specific antibodies, Kir5.1 specific oligonucleotide primers, and Kir5.1 nucleic acid probes. In another embodiment, the sample is from a human.

In another aspect, the present invention provides, in a computer system, a method of screening for mutations of human Kir5.1 genes, the method comprising the steps of: (i) entering into the computer system a first nucleic acid sequence encoding an inward rectifier potassium channel protein having a nucleotide sequence of SEQ ID NO:2, and conservatively modified versions thereof; (ii) comparing the first nucleic acid sequence with a second nucleic acid sequence having substantial identity to the first nucleic acid sequence; and (iii) identifying nucleotide differences between the first and second nucleic acid sequences.

In one embodiment, the second nucleic acid sequence is associated with a disease state. In another embodiment, a computer readable substrate comprises the first and the second nucleic acid sequences.

In another aspect, the present invention provides, in a computer system, a method for identifying a three-dimensional structure of Kir5.1 polypeptides, the method comprising the steps of: (i) entering into the computer system an amino acid sequence of at least 10 amino acids of a potassium channel monomer or at least 30 nucleotides of a gene encoding the polypeptide, the polypeptide having an amino acid sequence of SEQ ID NO:1, and conservatively modified versions thereof; and (ii) generating a three-dimensional structure of the polypeptide encoded by the amino acid sequence.

In one embodiment, the amino acid sequence is a primary structure and wherein said generating step includes the steps of: (i) forming a secondary structure from said

primary structure using energy terms determined by the primary structure; and (ii) forming a tertiary structure from said secondary structure using energy terms determined by said secondary structure. In another embodiment, the generating step includes the step of forming a quaternary structure from said tertiary structure using anisotropic terms determined by the tertiary structure. In another embodiment, the methods further comprises the step of identifying regions of the three-dimensional structure of the protein that bind to ligands and using the regions to identify ligands that bind to the protein. In another embodiment, a computer readable substrate comprises the three dimensional structure of the polypeptide monomer.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Functional expression of hKir5.1 and hKir5.1 with hKir4.1.

Cells expressing 1:1 mixtures of hKir5.1 and hKir4.1 (open squares) or cells expressing a tandem dimer of hKir5.1 and hKir4.1 (closed squares) show greater fluorescent dye changes resulting from larger current magnitudes than do cells expressing hKir5.1 alone (open circles).

## DETAILED DESCRIPTION OF THE INVENTION

### I. Introduction

The present invention provides for the first time a nucleic acid encoding hKir5.1, identified and cloned from human tissue. This polypeptide monomer is a member of the "Kir" family of potassium channel monomers. Members of this family are polypeptide monomers that are alpha subunits of inward rectifier potassium channels (K=potassium, ir=inward rectifier). Inward rectifier potassium channels have significant roles in maintaining the resting potential and in controlling excitability of a cell.

The invention also provides methods of screening for modulators, activators, and inhibitors of inward rectifier potassium channels that contain a human Kir5.1 subunit. Such modulators of inward rectifier channel activity are useful for treatment of hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency.

Furthermore, the invention provides assays for hKir5.1 activity where hKir5.1 acts as a direct or indirect reporter molecule. Such uses of hKir5.1 as a reporter molecule in assay and detection systems have broad applications, e.g., hKir5.1 can be used as a reporter molecule to measure changes in potassium concentration, membrane potential, current flow, ion flux, transcription, signal transduction, receptor-ligand interactions, second messenger concentrations, *in vitro*, *in vivo*, and *ex vivo*. In one embodiment, hKir5.1 can be used as an indicator of current flow in a particular direction (inward potassium flow), and in another embodiment, hKir5.1 can be used as an indirect reporter via attachment to a second reporter molecule such as green fluorescent protein.

The invention provides for methods of detecting hKir5.1 nucleic acid and protein expression allows investigation of the channel diversity provided by hKir5.1 and the regulation/modulation of heteromeric channel activity provided by hKir5.1, as well as disease diagnosis for conditions such as hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency

Finally, the invention provides for a method of screening for mutations of hKir5.1 genes or proteins. The invention includes, but is not limited to, methods of screening for mutations in hKir5.1 with the use of a computer. Similarly, the invention provides for methods of identifying the three-dimensional structure of hKir5.1, as well as the resulting computer readable images or data that comprise the three dimensional structure of hKir5.1. Other methods for screening for mutations of hKir5.1 genes or proteins include high density oligonucleotide arrays, PCR, immunoassays and the like.

Functionally, hKir5.1 is an alpha subunit of an inward rectifier potassium channel. Such inward rectifier channels are heteromeric or homomeric and contain typically contain four alpha subunits or monomers each with two transmembrane domains. Inward rectifier potassium channels comprising hKir5.1 can be heteromeric and may contain one or more alpha subunits of Kir5.1 along with one or more other alpha subunits from the Kir family, e.g., Kir4.1. Kir5.1 can also comprise a homomeric inward rectifier channel. The presence of hKir5.1 in an inward rectifier potassium channel modulates the activity of the

heteromeric channel and thus enhances channel diversity. For example, when hKir5.1 associates with hKir4.1, the resulting channel has an increased single channel conductance.

Structurally, the nucleotide sequence of hKir5.1 (SEQ ID NO:2) encodes a polypeptide monomer of approximately 384 amino acids with a predicted molecular weight of approximately 43 kDa (SEQ ID NO:1) and a predicted range of 38-48 kDa. In particular, the amino acid sequence of hKir5.1 consists of a carboxy-terminal "tail" region (approximately amino acids 352 to the C-terminal end of hKir5.1) that has a divergent amino acid sequence as compared to Kir5.1 proteins from other species. For example, the hKir5.1 tail region (amino acids 352-384 of SEQ ID NO:1) shares only about 10% amino acid identity with the tail region of rat Kir5.1.

The present invention also provide polymorphic variants of hKir5.1: variant #1, in which an A residue is substituted for a G residue at amino acid position 214; variant #2, in which a D residue is substituted for an N residue at amino acid position 161; and variant #3, in which an E residue is substituted for a Q residue at amino acid position 180.

Specific regions of the hKir5.1 nucleotide and amino acid sequence may be used to identify polymorphic variants, homologues, and alleles of hKir5.1. This identification can be made *in vitro*, e.g., under stringent hybridization conditions and sequencing, or by using the sequence information in a computer system for comparison with other nucleotide sequences. Typically, identification of polymorphic variants and alleles of hKir5.1 is made by comparing the amino acid sequence of the tail region (approximately amino acids 352-384 of hKir5.1, see SEQ ID NO:1 for example). Amino acid identity of approximately at least 60% or above, preferably 80%, most preferably 90-95% or above in the tail region typically demonstrates that a protein is a polymorphic variant or allele of hKir5.1. Sequence comparison can be performed using any of the sequence comparison algorithms discussed below. Antibodies that bind specifically to the tail region of hKir5.1 can also be used to identify alleles and polymorphic variants.

Polymorphic variants and alleles of hKir5.1 can be confirmed by co-expressing the putative hKir5.1 polypeptide monomer and examining whether the monomer forms a heteromeric inward rectifier potassium channel, when co-expressed with another member of the Kir family such as Kir4.1. This assay is used to demonstrate that a protein having about 80% or greater, preferably 90% or greater amino acid identity to the "tail"

region of hKir5.1 shares the same functional characteristics as hKir5.1 and is therefore a species of hKir5.1. Typically, hKir5.1 having the amino acid sequence of SEQ ID NO:1 is used as a positive control in comparison to the putative hKir5.1 protein to demonstrate the identification of a polymorphic variant or allele of hKir5.1.

5 hKir5.1 nucleotide and amino acid sequence information may also be used to construct models of a heteromeric inward rectifier potassium channels in a computer system. These models are subsequently used to identify compounds that can activate or inhibit heteromeric inward rectifier potassium channels comprising hKir5.1. Such compounds that modulate the activity of channels comprising hKir5.1 can be used to investigate the role of  
10 hKir5.1 in modulation of channel activity and in channel diversity.

The isolation of biologically active hKir5.1 for the first time provides a means for assaying for inhibitors and activators of heteromeric inward rectifier potassium channels that comprise hKir5.1 subunits. Biologically active hKir5.1 is useful for testing modulators, inhibitors, and activators of inward rectifier potassium channels comprising subunits of  
15 hKir5.1 and other Kir members using *in vivo* and *in vitro* expression that measure, e.g., changes in voltage or current. Such activators and inhibitors identified using an inward rectifier potassium channel comprising at least one hKir5.1 subunit can be used to further study inward rectification, channel kinetics and conductance properties of heteromeric channels. Such activators and inhibitors are useful as pharmaceutical agents for treating  
20 disorders such as hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency.. Methods of detecting hKir5.1 and expression of channels comprising hKir5.1 are also useful for diagnostic applications. For example, chromosomal localization  
25 of hKir5.1 can be used to identify diseases caused by and associated with hKir5.1. Methods of detecting hKir5.1 are also useful for examining the role of hKir5.1 in channel diversity and modulation of channel activity.

## II. Definitions

30 As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The phrase “inward rectifier” activity or “inward rectification” refers to a characteristic of a potassium channel composed of individual polypeptide monomers or subunits. Inward rectifier potassium channels primarily allow potassium influx, with little potassium efflux. For example, potassium ions flow inward through such channels when the membrane potential is negative to  $E_K$ , while at more positive membrane potentials, outward currents may be diminished (*see, e.g., Pessia et al., EMBO J. 15:2980-2987 (1996)*).  $E_K$  is the membrane potential for potassium, the combination of the voltage potential and  $[K^+]$  potential at which there is no net flow of potassium ion. This value (-98 mV) is also known as the “reversal potential” or the “Nernst” potential for potassium. Typically, the channel is composed of four subunits and the channel can be heteromeric or homomeric. Inward rectifier potassium channels composed of hKir5.1 can be heteromeric, having at least one hKir5.1 and preferably having two hKir5.1 subunits out of a total of four subunits. The remaining subunits are typically other members of the Kir family such as Kir4.1. The characteristic of inward rectification can be measured by a variety of techniques for measuring changes in current flow and ion flux through a channel, e.g., by changing the  $[K^+]$  of the external solution and measuring the activation potential of the channel current (*see, e.g., U.S. Patent No. 5,670,335*), by measuring current with patch clamp techniques under different conditions, and by measuring ion flux with radiolabeled tracers or voltage-sensitive dyes under different conditions.

A “Kir subunit” refers to an alpha subunit from the Kir family, which forms tetrameric potassium channels, either homo- or heteromeric.

“Homomeric” refers to a potassium channel composed of the same type of alpha subunit, while “heteromeric” refers to a potassium channel composed of two or more different types of alpha subunits, e.g., hKir5.1 and Kir4.1. Both homomeric and heteromeric channels can include auxiliary beta subunits.

A “beta subunit” is a polypeptide monomer that is an auxiliary subunit of a cation channel composed of alpha subunits; however, beta subunits alone cannot form a channel (*see, e.g., U.S. Patent No. 5,776,734*). Beta subunits are known, for example, to increase the number of channels by helping the alpha subunits reach the cell surface, change activation kinetics, and change the sensitivity of natural ligands binding to the channels. Beta

subunits can be outside of the pore region and associated with alpha subunits comprising the pore region. They can also contribute to the external mouth of the pore region.

The phrase "tail region" refers to the region of hKir5.1 that structurally identifies this particular protein (approximately amino acids 352-384 of hKir5.1, see SEQ ID NO:1, e.g., about the last 8-10% of the protein at the C-terminus). This region can be used to identify hKir5.1 polymorphic variants and hKir5.1 alleles of hKir5.1, through amino acid sequence identity comparison using a sequence comparison algorithm such as PILEUP.

"hKir5.1" refers to a polypeptide that is a subunit or monomer of an inward rectifier potassium channel and a member of the Kir family of potassium channel monomers.

When hKir5.1 is part of a potassium channel, preferably a heteromeric potassium channel, the channel has inward rectifier activity. The term hKir5.1 therefore refers to polymorphic variants, alleles, mutants, and closely related interspecies variants that: (1) have a tail region that has greater than about 60% amino acid sequence identity, preferably about 80-90% amino acid sequence identity, to a hKir5.1 tail region; or (2) bind to antibodies raised against an immunogen comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1 and conservatively modified variants thereof; or (3) specifically hybridize under stringent hybridization conditions to a sequence selected from the group consisting of SEQ ID NO:2 and conservatively modified variants thereof; or (4) are amplified by primers that specifically hybridize under stringent hybridization conditions to the same sequence as a primer set consisting of SEQ ID NO:3 and SEQ ID NO:4.

The phrase "functional effects" in the context of assays for testing compounds that modulate Kir channels comprising Kir5.1 includes the determination of any parameter that is indirectly or directly under the influence of the Kir channel comprising Kir5.1. It includes changes in ion flux, membrane potential, current flow, transcription, G-protein binding, phosphorylation or dephosphorylation, signal transduction, receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP<sub>3</sub>, or intracellular Ca<sup>2+</sup>), as measured *in vitro*, *in vivo*, and *ex vivo*, and also includes other physiologic effects such as increases or decreases of neurotransmitter or hormone release.

By "determining the functional effect" is meant assays for a compound that increases or decreases a parameter that is indirectly or directly under the influence of Kir5.1. Such functional effects can be measured by any means known to those skilled in the art, e.g.,

patch clamping, voltage-sensitive dyes, whole cell currents, radioisotope efflux, inducible markers, oocyte or tissue culture cell expression of Kir5.1; transcriptional activation; ligand binding assays; voltage, membrane potential and conductance changes; ion flux assays; changes in intracellular second messengers such as cAMP and inositol triphosphate (IP<sub>3</sub>);  
5 changes in intracellular calcium levels; neurotransmitter release, and the like.

“Inhibitors,” “activators,” and “modulators” of the Kir potassium channels comprising Kir5.1 refer to modulator molecules identified using *in vitro* and *in vivo* assays for channel function. Inhibitors are compounds that decrease, block, prevent, delay activation, inactivate, desensitize, or down regulate the channel. Activators are compounds  
10 that increase, open, activate, facilitate, enhance activation, sensitize or up regulate channel activity. Modulators include naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. Assays for inhibitors and activators include, e.g., co-expressing a Kir5.1 subunit with another Kir alpha subunit in cells or cell membranes, applying putative modulator compounds, and then measuring flux of ions through the Kir  
15 channel and determining the functional effect of the modulator as described above.

Samples or assays comprising a heteromeric inward rectifier potassium channel comprising hKir5.1 that are treated with a potential activator or inhibitor are compared to control samples without the inhibitor, to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative hKir5.1 activity value of  
20 100%. Inhibition of channels comprising hKir5.1 is achieved when the hKir5.1 activity value relative to the control is about 90%, preferably 50%, more preferably 25%. Activation of channels comprising hKir5.1 is achieved when the hKir5.1 activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher.

“Biologically active” hKir5.1 refers to hKir5.1 that comprises a potassium  
25 channel having inward rectifier activity tested as described above. Typically, the potassium channel is heteromeric and contains at least one, preferably two subunits of hKir5.1 and subunits of another Kir family member such as Kir4.1.

The terms “isolated” “purified” or “biologically pure” refer to material that is substantially or essentially free from components which normally accompany it as found in  
30 its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid

chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated hKir5.1 nucleic acid is separated from open reading frames that flank the hKir5.1 gene and encode proteins other than hKir5.1. The term “purified” denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The terms “polypeptide,” “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Polypeptides can be modified, e.g., by the addition of carbohydrate residues to form glycoproteins. The terms “polypeptide,” “peptide” and “protein” include glycoproteins, as well as non-glycoproteins.

The term “amino acid” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid,

i.e., an carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group., e.g., homoserine, norleucine, methionine sulfoxide, and methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid.

- 5 Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical  
10 Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes (A, T, G, C, U, etc.).

“Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino  
15 acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol.*  
20 *Cell. Probes* 8:91-98 (1994)). Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon in an amino acid herein, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic  
25 acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally  
30 identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants and alleles of the invention.

The following groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Serine (S), Threonine (T);
- 3) Aspartic acid (D), Glutamic acid (E);
- 4) Asparagine (N), Glutamine (Q);
- 5) Cysteine (C), Methionine (M);
- 6) Arginine (R), Lysine (K), Histidine (H);
- 7) Isoleucine (I), Leucine (L), Valine (V); and
- 8) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

(see, e.g., Creighton, *Proteins* (1984) for a discussion of amino acid properties).

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, e.g., Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor & Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 50 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of  $\beta$ -sheet and  $\alpha$ -helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include <sup>32</sup>P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the polypeptide of SEQ ID NO:1 can be made detectable, e.g., by incorporating a radiolabel into the peptide, and used to detect antibodies specifically reactive with the peptide).

As used herein a "nucleic acid probe or oligonucleotide" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled as with isotopes, chromophores, lumiphores, chromogens, or indirectly labeled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

A "labeled nucleic acid probe or oligonucleotide" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently, through ionic, van der Waals, electrostatic, or hydrogen bonds to a label such that the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-

recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

A “promoter” is defined as an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription. A “constitutive” promoter is a promoter that is active under most environmental and developmental conditions. An “inducible” promoter is a promoter that is active under environmental or developmental regulation. The term “operably linked” refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The term “heterologous” when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

An “expression vector” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

The terms “identical” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences (i.e., a human Kir5.1 monomer tail region) that are the same or have a specified percentage of amino acid residues or nucleotides (i.e., 80% identity, preferably 85%, 90%, or 95% identity)

that are the same, when compared and aligned for maximum correspondence over a comparison window, as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be “substantially identical.” This definition also refers to the complement of a test sequence.

- 5 Preferably, the percent identity exists over a region of the sequence that is at least about 25 amino acids in length, more preferably over a region that is 50 or 100 amino acids in length.

For sequence comparison, one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if  
10 necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, as described herein, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A “comparison window,” as used herein, includes reference to a segment of  
15 contiguous positions having a length of at least about 25 nucleotides, usually about 50 to about 200 nucleotides, more usually about 100 to about 150 nucleotides in which a sequence may be compared to a reference sequence after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology  
20 algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison,  
25 WI), or by manual alignment and visual inspection.

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show relationship and percent sequence identity. It also plots a tree or dendrogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the  
30 progressive alignment method of Feng & Doolittle, *J. Mol. Evol.* 35:351-360 (1987). The method used is similar to the method described by Higgins & Sharp, *CABIOS* 5:151-153

(1989). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences.

5 Two clusters of sequences are aligned by a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. Using PILEUP, a reference sequence is compared to other test sequences to  
10 determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps. PILEUP can be obtained from the GCG sequence analysis software package, e.g., version 7.0 (Devereaux *et al.*, *Nucleic Acids Res.* 12:387-395 (1984)).

Another example of algorithm that is suitable for determining percent  
15 sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which  
20 either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased.  
25 Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a  
30 word length (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc.*

*Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA* 90:5873-5877 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleotide sequences are substantially identical is that the same primers can be used to amplify both sequences.

The phrase "selectively (or specifically) hybridizes to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent hybridization conditions when that sequence is present in a complex mixture (e.g., total cellular or library DNA or RNA).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acid, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting

point ( $T_m$ ) for the specific sequence at a defined ionic strength pH. The  $T_m$  is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium).

- 5 Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.
- 10 For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with a wash in 0.2x SSC, and 0.1% SDS at 65°C.

- Nucleic acids that do not hybridize to each other under stringent conditions are
- 15 still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1
- 20 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency.

- "Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen.
- 25 The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

- 30 An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair

having one "light" (about 25 kDa) and one "heavy" chain (about 50-70 kDa). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

5                   Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $F(ab)'_2$ , a dimer of Fab which itself is a light chain joined to  $V_H-C_H1$  by a disulfide bond. The  $F(ab)'_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region,  
10                   thereby converting the  $F(ab)'_2$  dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also  
15                   includes antibody fragments either produced by the modification of whole antibodies, or those synthesized de novo using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (*see, e.g., McCafferty et al., Nature* 348:552-554 (1990)).

                  An "anti-hKir5.1" antibody is an antibody or antibody fragment that  
20                   specifically binds a polypeptide encoded by the hKir5.1 gene, cDNA, or a subsequence thereof.

                  A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function  
25                   and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

                  The term "immunoassay" is an assay that uses an antibody to specifically bind  
30                   an antigen. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The phrase “specifically (or selectively) binds” to an antibody or “specifically (or selectively) immunoreactive with,” when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to hKir5.1 with the amino acid sequence of the tail region encoded in SEQ ID NO:1 can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with the tail region of hKir5.1 and not with other proteins, except for polymorphic variants and alleles of hKir5.1. This selection may be achieved by subtracting out antibodies that cross react with molecules such as rat Kir5.1 and other Kir molecules such as Kir4.1. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

The phrase “selectively associates with” refers to the ability of a nucleic acid to “selectively hybridize” with another as defined above, or the ability of an antibody to “selectively (or specifically) bind to a protein, as defined above.

By “host cell” is meant a cell that contains an expression vector and supports the replication or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells such as CHO, HeLa and the like.

“Biological sample” as used herein is a sample of biological tissue or fluid that contains hKir5.1 or nucleic acid encoding hKir5.1 protein. Such samples include, but are not limited to, tissue isolated from humans. Biological samples may also include sections of tissues such as frozen sections taken for histological purposes. A biological sample is

typically obtained from a eukaryotic organism, preferably eukaryotes such as fungi, plants, insects, protozoa, birds, fish, reptiles, and preferably a mammal such as rat, mice, cow, dog, guinea pig, or rabbit, and most preferably a primate such as chimpanzees or humans.

### 5    **III. Isolation of the gene encoding hKir5.1**

#### *A. General Recombinant DNA Methods*

This invention relies on routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene*  
10    *Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons  
15    (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

Oligonucleotides that are not commercially available can be chemically synthesized according to the solid phase phosphoramidite triester method first described by  
20    Beaucage & Caruthers, *Tetrahedron Letts.* 22:1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255:137-149 (1983).

The sequence of the cloned genes and synthetic oligonucleotides can be  
25    verified after cloning using, e.g., the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* 16:21-26 (1981).

#### *B. Cloning methods for the isolation of nucleotide sequences encoding hKir5.1*

In general, the nucleic acid sequences encoding hKir5.1 and related nucleic  
30    acid sequence homologues are cloned from cDNA and genomic DNA libraries or isolated using amplification techniques with oligonucleotide primers. For example, hKir5.1

sequences are typically isolated from human nucleic acid (genomic or cDNA) libraries by hybridizing with a nucleic acid probe, the sequence of which can be derived from SEQ ID NO:2, preferably from the tail region.

Amplification techniques using primers can also be used to amplify and isolate hKir5.1 from DNA or RNA. The following primers can also be used to amplify a sequence of hKir5.1: 5' CCT AAG GGC ACA GCA AAG AAT GAG 3' (SEQ ID NO:3) and 5' GTG TGG CGA AAG TGG TGG TC 3' (SEQ ID NO:4). These primers can be used, e.g., to amplify a probe of several hundred nucleotides, which is then used to screen a human library for full-length hKir5.1. Alternatively, the nucleic acid for hKir5.1 can be directly amplified.

Nucleic acids encoding hKir5.1 can also be isolated from expression libraries using antibodies as probes. Such polyclonal or monoclonal antibodies can be raised using the sequence of SEQ ID NO:1.

hKir5.1 polymorphic variants, alleles, and interspecies homologues that are substantially identical to the tail region of hKir5.1 can be isolated using hKir5.1 nucleic acid probes and oligonucleotides under stringent hybridization conditions, by screening libraries. Alternatively, expression libraries can be used to clone hKir5.1 and hKir5.1 polymorphic variants, alleles, and interspecies homologues, by detecting expressed homologues immunologically with antisera or purified antibodies made against the tail region of hKir5.1, which also recognize and selectively bind to the hKir5.1 homologue.

To make a cDNA library, one should choose a source that is rich in hKir5.1 mRNA, e.g., human kidney, thyroid, or pancreas tissue (*see* Table I). The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.,* Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*).

For a genomic library, the DNA, preferably human, is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton &

Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating hKir5.1 nucleic acid and its homologues combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (see U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid sequences of hKir5.1 directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify hKir5.1 homologues using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers.

Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of hKir5.1 encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

Gene expression of hKir5.1 can also be analyzed by techniques known in the art, e.g., reverse transcription and amplification of mRNA, isolation of total RNA or poly A<sup>+</sup> RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density oligonucleotide arrays, and the like.

Synthetic oligonucleotides can be used to construct recombinant hKir5.1 genes for use as probes or for expression of protein. This method is performed using a series of overlapping oligonucleotides usually 40-120 bp in length, representing both the sense and nonsense strands of the gene. These DNA fragments are then annealed, ligated and cloned. Alternatively, amplification techniques can be used with precise primers to amplify a specific subsequence of the hKir5.1 gene. The specific subsequence is then ligated into an expression vector.

The gene for hKir5.1 is typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors.

*C. Expression in prokaryotes and eukaryotes*

To obtain high level expression of a cloned gene, such as those cDNAs encoding hKir5.1, one typically subclones hKir5.1 into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and if for a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.* and Ausubel *et al.* Bacterial expression systems for expressing the hKir5.1 protein are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of the hKir5.1 encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding hKir5.1 and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding hKir5.1 may typically be linked to a cleavable signal peptide sequence to promote secretion of the encoded protein by the transformed cell. Such signal peptides would include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient

termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein Bar virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A<sup>+</sup>, pMTO10/A<sup>+</sup>, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with a hKir5.1 encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of hKir5.1 protein, which are then purified using standard techniques (*see, e.g., Colley et al., J. Biol. Chem.* 264:17619-17622

(1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983).

5                   Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material into a host cell (*see,*  
10 *e.g.*, Sambrook *et al.*, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing hKir5.1.

                  After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of hKir5.1, which is recovered from the culture  
15 using standard techniques identified below.

#### IV. Purification of hKir5.1

                  Either naturally occurring or recombinant hKir5.1 can be purified for use in functional assays. Naturally occurring hKir5.1 is purified, *e.g.*, from human tissue such as  
20 testes tissue and any other source of a hKir5.1 homologue. Recombinant hKir5.1 is purified from any suitable expression system.

                  hKir5.1 may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook *et al.*,  
25 *supra*).

                  A number of procedures can be employed when recombinant hKir5.1 is being purified. For example, proteins having established molecular adhesion properties can be reversible fused to hKir5.1. With the appropriate ligand, hKir5.1 can be selectively adsorbed  
30 to a purification column and then freed from the column in a relatively pure form. The fused

protein is then removed by enzymatic activity. Finally hKir5.1 could be purified using immunoaffinity columns.

*A. Purification of hKir5.1 from recombinant bacteria*

5                   Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction with IPTG is a one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

10                   Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of hKir5.1 inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM ATP, and 1  
15                   mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (*see, e.g., Sambrook et al., supra; Ausubel et al., supra*).

                    If necessary, the inclusion bodies are solubilized, and the lysed cell suspension  
20                   is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for  
25                   example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of  
30                   immunologically and/or biologically active protein. Other suitable buffers are known to

those skilled in the art. hKir5.1 is separated from other bacterial proteins by standard separation techniques, e.g., with Ni-NTA agarose resin.

Alternatively, it is possible to purify hKir5.1 from bacteria periplasm. After lysis of the bacteria, when hKir5.1 is exported into the periplasm of the bacteria, the

5 periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO<sub>4</sub> and kept in an ice bath for approximately 10 minutes.

10 The cell suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

#### *B. Standard Protein Separation Techniques For Purifying hKir5.1*

##### Solubility Fractionation

15 Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the  
20 amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The  
25 precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to  
30 fractionate complex protein mixtures.

### Size Differential Filtration

The molecular weight of hKir5.1 can be used to isolated it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is  
5 ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

### Column Chromatography

hKir5.1 can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these  
15 methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

## **V. Immunological Detection of hKir5.1**

20 In addition to the detection of hKir5.1 genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect hKir5.1. Immunoassays can be used to qualitatively or quantitatively analyze hKir5.1. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

### *A. Antibodies to hKir5.1*

25 Methods of producing polyclonal and monoclonal antibodies that react specifically with hKir5.1 are known to those of skill in the art (*see, e.g.,* Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of antibodies from libraries of

recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g., Huse et al., Science* 246:1275-1281 (1989); Ward *et al., Nature* 341:544-546 (1989)).

A number of hKir5.1 comprising immunogens may be used to produce  
5 antibodies specifically reactive with hKir5.1. For example, recombinant hKir5.1 or a antigenic fragment thereof such as tail region, is isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived  
10 from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

15 Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (e.g., BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to hKir5.1. When appropriately high  
20 titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (*see Harlow & Lane, supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired  
25 antigen are immortalized, commonly by fusion with a myeloma cell (*see Kohler & Milstein, Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the  
30 monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may

isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.*, *Science* 246:1275-1281 (1989).

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of  $10^4$  or greater are selected and tested for their cross reactivity against non-hKir5.1 proteins or even other related proteins from other organisms (e.g., rat Kir5.1 or other Kir family members such as Kir4.1), using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a  $K_d$  of at least about 0.1 mM, more usually at least about 1  $\mu$ M, preferably at least about 0.1  $\mu$ M or better, and most preferably, 0.01  $\mu$ M or better.

Once hKir5.1 specific antibodies are available, hKir5.1 can be detected by a variety of immunoassay methods. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme Immunoassay* (Maggio, ed., 1980); and Harlow & Lane, *supra*.

#### *B. Immunological binding assays*

hKir5.1 can be detected and/or quantified using any of a number of well recognized immunological binding assays (*see, e.g.*, U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991). Immunological binding assays (or immunoassays) typically use an antibody that specifically binds to a protein or antigen of choice (in this case the hKir5.1 or antigenic subsequence thereof). The antibody (e.g., anti-hKir5.1) may be produced by any of a number of means well known to those of skill in the art and as described above.

Immunoassays also often use a labeling agent to specifically bind to and label the complex formed by the antibody and antigen. The labeling agent may itself be one of the

moieties comprising the antibody/antigen complex. Thus, the labeling agent may be a labeled hKir5.1 polypeptide or a labeled anti-hKir5.1 antibody. Alternatively, the labeling agent may be a third moiety, such a secondary antibody, that specifically binds to the antibody/hKir5.1 complex (a secondary antibody is typically specific to antibodies of the species from which the first antibody is derived). Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species (*see, e.g., Kronval et al., J. Immunol.* 111:1401-1406 (1973); Akerstrom *et al., J. Immunol.* 135:2589-2542 (1985)). The labeling agent can be modified with a detectable moiety, such as biotin, to which another molecule can specifically bind, such as streptavidin. A variety of detectable moieties are well known to those skilled in the art.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, antigen, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

#### Non-Competitive Assay Formats

Immunoassays for detecting hKir5.1 in samples may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of antigen is directly measured. In one preferred "sandwich" assay, for example, the anti-hKir5.1 antibodies can be bound directly to a solid substrate on which they are immobilized. These immobilized antibodies then capture hKir5.1 present in the test sample. hKir5.1 is thus immobilized is then bound by a labeling agent, such as a second hKir5.1 antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second or third antibody is typically modified with a detectable moiety, such as biotin, to which another molecule specifically binds, e.g., streptavidin, to provide a detectable moiety.

### Competitive assay formats

In competitive assays, the amount of hKir5.1 present in the sample is measured indirectly by measuring the amount of a known, added (exogenous) hKir5.1 displaced (competed away) from an anti-hKir5.1 antibody by the unknown hKir5.1 present in a sample. In one competitive assay, a known amount of hKir5.1 is added to a sample and the sample is then contacted with an antibody that specifically binds to hKir5.1. The amount of exogenous hKir5.1 bound to the antibody is inversely proportional to the concentration of hKir5.1 present in the sample. In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of hKir5.1 bound to the antibody may be determined either by measuring the amount of hKir5.1 present in a hKir5.1/antibody complex, or alternatively by measuring the amount of remaining uncomplexed protein. The amount of hKir5.1 may be detected by providing a labeled hKir5.1 molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay the known hKir5.1, is immobilized on a solid substrate. A known amount of anti-hKir5.1 antibody is added to the sample, and the sample is then contacted with the immobilized hKir5.1. The amount of anti-hKir5.1 antibody bound to the known immobilized hKir5.1 is inversely proportional to the amount of hKir5.1 present in the sample. Again, the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

### Cross-reactivity determinations

Immunoassays in the competitive binding format can also be used for crossreactivity determinations. For example, a protein at least partially encoded by SEQ ID NO:1, preferably including the tail region, can be immobilized to a solid support. Proteins (e.g., rat Kir5.1 and other Kir family members such as Kir4.1) are added to the assay that compete for binding of the antisera to the immobilized antigen. The ability of the added proteins to compete for binding of the antisera to the immobilized protein is compared to the ability of hKir5.1 encoded by SEQ ID NO:1 to compete with itself. The percent

crossreactivity for the above proteins is calculated, using standard calculations. Those antisera with less than 10% crossreactivity with each of the added proteins listed above are selected and pooled. The cross-reacting antibodies are optionally removed from the pooled antisera by immunoabsorption with the added considered proteins, e.g., distantly related homologues.

The immunoabsorbed and pooled antisera are then used in a competitive binding immunoassay as described above to compare a second protein, thought to be perhaps an allele or polymorphic variant of hKir5.1, to the immunogen protein (i.e., hKir5.1 of SEQ ID NO:1). In order to make this comparison, the two proteins are each assayed at a wide range of concentrations and the amount of each protein required to inhibit 50% of the binding of the antisera to the immobilized protein is determined. If the amount of the second protein required to inhibit 50% of binding is less than 10 times the amount of the protein encoded by SEQ ID NO:1 that is required to inhibit 50% of binding, then the second protein is said to specifically bind to the polyclonal antibodies generated to a hKir5.1 immunogen.

#### Other assay formats

Western blot (immunoblot) analysis is used to detect and quantify the presence of hKir5.1 in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind hKir5.1. The anti-hKir5.1 antibodies specifically bind to the hKir5.1 on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (e.g., labeled sheep anti-mouse antibodies) that specifically bind to the anti-hKir5.1 antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (e.g., antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)).

### Reduction of non-specific binding

One of skill in the art will appreciate that it is often desirable to minimize non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this technique involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

### Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, as long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS<sup>TM</sup>), fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (e.g., <sup>3</sup>H, <sup>125</sup>I, <sup>35</sup>S, <sup>14</sup>C, or <sup>32</sup>P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (e.g., polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to

another molecules (e.g., streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. The ligands and their targets can be used in any suitable combination with antibodies that recognize hKir5.1, or secondary antibodies that recognize anti-hKir5.1.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems that may be used, see U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

## VI. Assays for modulators of hKir5.1

### A. Assays

hKir5.1 and its alleles and polymorphic variants are subunits of heteromeric inward rectifier potassium channels. The activity of an inward rectifier potassium channel comprising an hKir5.1 subunit or monomer can be assessed using a variety of *in vitro* and *in vivo* assays, e.g., measuring current, measuring membrane potential, measuring ion flux, e.g., potassium or rubidium, measuring potassium concentration, measuring second messengers and transcription levels, and using e.g., voltage-sensitive dyes, radioactive tracers, and patch-clamp electrophysiology. Furthermore, such assays can be used to test for inhibitors and activators of channels comprising hKir5.1. Such modulators of inward rectifier channel activity are useful for treating hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism, hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency.). Such modulators are also useful for investigation of the channel diversity provided by hKir5.1 and the regulation/modulation of heteromeric channel activity provided by hKir5.1.

Modulators of hKir5.1 activity are tested using biologically active hKir5.1, either recombinant or naturally occurring. The protein can be isolated, expressed in a cell, or expressed in a membrane derived from a cell. In such assays, hKir5.1 is typically co-expressed with another member of the Kir family such as Kir4.1. Modulation is tested using one of the *in vitro* or *in vivo* assays described above. Samples or assays that are treated with a potential hKir5.1 inhibitor or activator are compared to control samples without the test compound, to examine the extent of modulation. Control samples (untreated with activators or inhibitors) are assigned a relative hKir5.1 activity value of 100. Inhibition of channels comprising hKir5.1 is achieved when the hKir5.1 activity value relative to the control is about 90%, preferably 50%, more preferably 25%. Activation of channels comprising hKir5.1 is achieved when the hKir5.1 activity value relative to the control is 110%, more preferably 150%, more preferable 200% higher. Compounds that increase the flux of ions will cause a detectable increase in the ion current density by increasing the probability of a channel comprising hKir5.1 being open, by decreasing the probability of it being closed, increasing conductance through the channel, and allowing the passage of ions.

Changes in ion flux may be assessed by determining changes in polarization (i.e., electrical potential) of the cell or membrane expressing the channel comprising hKir5.1.

A preferred means to determine changes in cellular polarization is by measuring changes in current (thereby measuring changes in polarization) with voltage-clamp and patch-clamp

5 techniques, e.g., the "cell-attached" mode, the "inside-out" mode, and the "whole cell" mode (see, e.g., Ackerman *et al.*, *New Engl. J. Med.* 336:1575-1595 (1997)). Whole cell currents are conveniently determined using the standard methodology (see, e.g., Hamil *et al.*,

*Pflugers. Archiv.* 391:85 (1981). Other known assays include: radiolabeled rubidium flux assays and fluorescence assays using voltage-sensitive dyes (see, e.g., Vestergaard-Bogind *et*  
10 *al.*, *J. Membrane Biol.* 88:67-75 (1988); Daniel *et al.*, *J. Pharmacol. Meth.* 25:185-193

(1991); Holevinsky *et al.*, *J. Membrane Biology* 137:59-70 (1994)). Assays for compounds capable of inhibiting or increasing potassium flux through the channel proteins comprising hKir5.1 can be performed by application of the compounds to a bath solution in contact with and comprising cells having an channel of the present invention (see, e.g., Blatz *et al.*, *Nature*  
15 323:718-720 (1986); Park, *J. Physiol.* 481:555-570 (1994)). Generally, the compounds to be tested are present in the range from 1 pM to 100 mM.

The effects of the test compounds upon the function of the channels can be measured by changes in the electrical currents or ionic flux or by the consequences of changes in currents and flux. Changes in electrical current or ionic flux are measured by  
20 either increases or decreases in flux of cations such as potassium or rubidium ions. The cations can be measured in a variety of standard ways. They can be measured directly by concentration changes of the ions or indirectly by membrane potential or by radiolabeling of the ions. Consequences of the test compound on ion flux can be quite varied. Accordingly, any suitable physiological change can be used to assess the influence of a test compound on  
25 the channels of this invention. The effects of a test compound can be measured by a toxin binding assay. When the functional consequences are determined using intact cells or animals, one can also measure a variety of effects such as transmitter release (e.g., dopamine), hormone release (e.g., insulin), transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), cell volume changes (e.g., in red blood cells),  
30 immunoresponses (e.g., T cell activation), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as [Ca<sup>2+</sup>].

Preferably, the hKir5.1 subunit of the assay will be selected from a subunit having a sequence of SEQ ID NO:1 or conservatively modified variant thereof.

Alternatively, the hKir5.1 subunit of the assay will be derived from a eukaryote and include an amino acid subsequence having amino acid sequence identity to the tail region of hKir5.1.

5 Generally, the amino acid sequence identity will be at least 60% or 70%, preferably at least 80%, most preferably at least 90-95%.

The hKir5.1 homologues will generally confer substantially similar inward rectification characteristics on a channel comprising such a subunit, as described above. In a preferred embodiment, the cell placed in contact with a compound that is assayed for  
10 increasing or decreasing ion flux is a eukaryotic cell, e.g., an oocyte of *Xenopus* (e.g., *Xenopus laevis*) or a mammalian cell such as a CHO or HeLa cell.

### *B. Modulators*

The compounds tested as modulators of Kir channels comprising a Kir5.1  
15 subunit can be any small chemical compound, or a biological entity, such as a protein, sugar, nucleic acid or lipid. Alternatively, modulators can be genetically altered versions of a Kir5.1 subunit. Typically, test compounds will be small chemical molecules and peptides. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or  
20 organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-  
25 Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such “combinatorial  
30 chemical libraries” or “ligand libraries” are then screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that

display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see* Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (*see, e.g.*, Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially  
5 available (*see, e.g.*, ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, etc.).

In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing a Kir channel comprising a  
10 Kir5.1 subunit is attached to a solid phase substrate. In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter  
15 plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay several different plates per day; assay screens for up to about 6,000-20,000 different compounds is possible using the integrated systems of the invention. More recently, microfluidic approaches to reagent manipulation have been developed, e.g., by Caliper  
20 Technologies (Palo Alto, CA).

## VII. Computer-assisted Drug Design and Diagnostics

Yet another assay for compounds that modulate hKir5.1 activity involves computer assisted drug design, in which a computer system is used to generate a three-  
25 dimensional structure of hKir5.1 based on the structural information encoded by the amino acid sequence. The input amino acid sequence interacts directly and actively with a preestablished algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then examined to identify regions of the structure that have the ability to bind, e.g., ligands or other potassium  
30 channel subunits. These regions are then used to identify ligands that bind to the protein or region where hKir5.1 interacts with other potassium channel subunits.

The three-dimensional structural model of the protein is generated by entering channel protein amino acid sequences of at least 10 amino acid residues or corresponding nucleic acid sequences encoding a hKir5.1 monomer into the computer system. The amino acid sequence of the monomer is selected from the group consisting of SEQ ID NO:1 and conservatively modified versions thereof. The amino acid sequence represents the primary sequence or subsequence of the protein, which encodes the structural information of the protein. At least 10 residues of the amino acid sequence (or a nucleotide sequence encoding 10 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the channel protein is then generated by the interaction of the amino acid sequence and the computer system, using software known to those of skill in the art. The structural model and the sequences used to generate it can also be stored on a computer readable substrate.

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the monomer and the heteromeric potassium channel protein comprising four monomers. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches

hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the hKir5.1 protein to identify ligands that bind to hKir5.1. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

Computer systems are also used to screen for mutations, polymorphic variants, alleles and interspecies homologues of hKir5.1 genes. Such mutations can be associated with disease states. Once the variants are identified, diagnostic assays can be used to identify patients having such mutated genes associated with disease states. Identification of the mutated hKir5.1 genes involves receiving input of a first nucleic acid or amino acid sequence encoding hKir5.1, selected from the group consisting of SEQ ID NO:1 and conservatively modified versions thereof. The sequence is entered into the computer system as described above. The first nucleic acid or amino acid sequence is then compared to a second nucleic acid or amino acid sequence that has substantial identity to the first sequence. The second sequence is entered into the computer system in the manner described above. Once the first and second sequences are compared, nucleotide or amino acid differences between the sequences are identified. Such sequences can represent allelic differences in hKir5.1 genes, and mutations associated with disease states.

### VIII. Cellular Transfection and Gene Therapy

The present invention provides the nucleic acids of encoding Kir5.1 for the transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well known vectors for the transfection of target cells and organisms as described below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The nucleic acids encoding Kir5.1, under the control of a promoter, then expresses a Kir5.1 subunit of the present invention, thereby mitigating the effects of absent, partial inactivation, or abnormal expression of the Kir5.1 subunit gene.

Such gene therapy procedures have been used to correct acquired and inherited genetic defects, cancer, and viral infection in a number of contexts. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* (Doerfler & Böhm eds., 1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994)).

Delivery of the gene or genetic material into the cell is the first critical step in gene therapy treatment of disease. A large number of delivery methods are well known to those of skill in the art. Preferably, the nucleic acids are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds) (1995); and Yu *et al.*, *Gene Therapy* 1:13-26 (1994).

Methods of non-viral delivery of nucleic acids include lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, and agent-enhanced uptake of DNA. Lipofection is described in, e.g., US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (e.g., Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of

polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al.*, *Cancer Gene Ther.* 2:291-297 (1995); Behr *et al.*, *Bioconjugate Chem.* 5:382-389 (1994); Remy *et al.*, *Bioconjugate Chem.* 5:647-654 (1994); Gao *et al.*, *Gene Therapy* 2:710-722 (1995); Ahmad *et al.*, *Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

The use of RNA or DNA viral based systems for the delivery of nucleic acids take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of nucleic acids could include retroviral, lentivirus, adenoviral, adeno-associated and herpes simplex virus vectors for gene transfer. Viral vectors are currently the most efficient and versatile method of gene transfer in target cells and tissues. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vector that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system would therefore depend on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof (*see, e.g.*, Buchscher *et al.*, *J. Virol.* 66:2731-2739

(1992); Johann *et al.*, *J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al.*, *Virol.* 176:58-59 (1990); Wilson *et al.*, *J. Virol.* 63:2374-2378 (1989); Miller *et al.*, *J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).

In applications where transient expression of the nucleic acid is preferred, adenoviral based systems are typically used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and levels of expression have been obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, e.g., in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (*see, e.g.*, West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors are described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.* 63:03822-3828 (1989).

In particular, at least six viral vector approaches are currently available for gene transfer in clinical trials, with retroviral vectors by far the most frequently used system. All of these viral vectors utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

pLASN and MFG-S are examples are retroviral vectors that have been used in clinical trials (Dunbar *et al.*, *Blood* 85:3048-305 (1995); Kohn *et al.*, *Nat. Med.* 1:1017-102 (1995); Malech *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al.*, *Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al.*, *Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al.*, *Hum. Gene Ther.* 1:111-2 (1997)).

Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient

gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al.*, *Lancet* 351:9117 1702-3 (1998), Kearns *et al.*, *Gene Ther.* 9:748-55 (1996)).

Replication-deficient recombinant adenoviral vectors (Ad) are predominantly used transient expression gene therapy, because they can be produced at high titer and they readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and E3 genes; subsequently the replication defector vector is propagated in human 293 cells that supply deleted gene function in trans.

Ad vectors can transduce multiply types of tissues *in vivo*, including nondividing, differentiated cells such as those found in the liver, kidney and muscle system tissues.

Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Stermann *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include

Rosenecker *et al.*, *Infection* 24:1 5-10 (1996); Stermann *et al.*, *Hum. Gene Ther.* 9:7 1083-1089 (1998); Welsh *et al.*, *Hum. Gene Ther.* 2:205-18 (1995); Alvarez *et al.*, *Hum. Gene Ther.* 5:597-613 (1997); Topf *et al.*, *Gene Ther.* 5:507-513 (1998); Stermann *et al.*, *Hum. Gene Ther.* 7:1083-1089 (1998).

Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, and  $\psi$ 2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host, other viral sequences being replaced by an expression cassette for the protein to be expressed. The missing viral functions are supplied in *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess ITR sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is

not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, e.g., heat treatment to which adenovirus is more sensitive than AAV.

In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. A viral vector is typically modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the viruses outer surface. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other pairs of virus expressing a ligand fusion protein and target cell expressing a receptor. For example, filamentous phage can be engineered to display antibody fragments (e.g., FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences thought to favor uptake by specific target cells.

Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (e.g., intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (e.g., lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

*Ex vivo* cell transfection for diagnostics, research, or for gene therapy (e.g., via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a nucleic acid (gene or cDNA), and re-infused back into the subject organism (e.g., patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (see, e.g., Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique*

(3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

In one embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be  
5 differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such as GM-CSF, IFN- $\gamma$  and TNF- $\alpha$  are known (*see Inaba et al., J. Exp. Med.* 176:1693-1702 (1992)).

Stem cells are isolated for transduction and differentiation using known  
10 methods. For example, stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells) (*see Inaba et al., J. Exp. Med.* 176:1693-1702 (1992)).

Vectors (e.g., retroviruses, adenoviruses, liposomes, etc.) containing  
15 therapeutic nucleic acids can be also administered directly to the organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a  
20 particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells. The nucleic acids are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable  
25 methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

## IX. Pharmaceutical Compositions

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention (*see, e.g.*, Remington's Pharmaceutical Sciences, 17<sup>th</sup> ed., 1989).

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The nucleic acids and proteins of the invention, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the packaged nucleic acid with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the packaged nucleic acid with a base, including, for example, liquid triglycerides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of packaged nucleic acid can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by the packaged nucleic acid as described above in the context of *ex vivo* therapy can also be administered intravenously or parenterally as described above.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient.

In determining the effective amount of the nucleic acid or protein to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of the Kir family potassium channel comprising a Kir5.1 subunit, the physician evaluates circulating plasma levels of the compound, toxicities, progression of the disease, and the production of anti-compound antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1  $\mu\text{g}$  to 100  $\mu\text{g}$  for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

For administration, inhibitors and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

Transduced cells are prepared for reinfusion according to established methods (see Abrahamsen *et al.*, *J. Clin. Apheresis* 6:48--53 (1991); Carter *et al.*, *J. Clin. Arpheresis* 4:113-117 (1998); Aebersold *et al.*, *J. Immunol. Meth.* 112:1-7 (1998); Muul *et al.*, *J. Immunol. Methods* 101:171-181 (1987); and Carter *et al.*, *Transfusion* 27:362-365 (1987)).

After a period of about 2-4 weeks in culture, the cells should number between  $1 \times 10^8$  and  $1 \times 10^{12}$ . In this regard, the growth characteristics of cells vary from patient to patient and from cell type to cell type. About 72 hours prior to reinfusion of the transduced cells, an aliquot is taken for analysis of phenotype, and percentage of cells expressing the therapeutic agent.

## X. Kits

hKir5.1 and its homologues are a useful tool for examining expression of and regulation of heteromeric inward rectifier potassium channels. hKir5.1 specific reagents that specifically hybridize to hKir5.1 nucleic acid, such as hKir5.1 probes and primers, and hKir5.1 specific reagents that specifically bind to the hKir5.1 protein, e.g., hKir5.1 antibodies are used to examine expression and regulation.

Nucleic acid assays for the presence of hKir5.1 DNA and RNA in a sample include numerous techniques are known to those skilled in the art, such as Southern analysis, northern analysis, dot blots, RNase protection, S1 analysis, amplification techniques such as PCR and LCR, and *in situ* hybridization. In *in situ* hybridization, for example, the target nucleic acid is liberated from its cellular surroundings in such as to be available for hybridization within the cell while preserving the cellular morphology for subsequent interpretation and analysis. The following articles provide an overview of the art of *in situ* hybridization: Singer *et al.*, *Biotechniques* 4:230-250 (1986); Haase *et al.*, *Methods in Virology*, vol. VII, pp. 189-226 (1984); and *Nucleic Acid Hybridization: A Practical Approach* (Hames *et al.*, eds. 1987). In addition, hKir5.1 protein can be detected with the various immunoassay techniques described above. The test sample is typically compared to

both a positive control (e.g, a sample expressing recombinant hKir5.1) and a negative control.

Human Kir5.1 monomers and the Kir potassium channels containing these Kir5.1 monomers can be used with high density oligonucleotide array technology (e.g., GeneChip™) to identify homologs and polymorphic variants of hKir5.1 in this invention. In the case where the homologs being identified are linked to a known disease, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); and Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

The present invention also provides for kits for screening for modulators of hKir5.1. Such kits can be prepared from readily available materials and reagents. For example, such kits can comprise any one or more of the following materials: biologically active hKir5.1, reaction tubes, and instructions for testing hKir5.1 activity. Preferably, the kit contains biologically active hKir5.1. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. For example, the kit can be tailored for *in vitro* or *in vivo* assays for measuring the activity of a heteromeric inward rectifier potassium channel comprising an hKir5.1 subunit.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

5

Example I: Cloning and expression of hKir5.1*A. Isolation of nucleic acid encoding hKir5.1 and expression vector constructs*

Using PCR and primers, according to standard conditions, hKir5.1 is amplified from human testes cDNA. The following primers were used for amplification: 5' CCT AAG GGC ACA GCA AAG AAT GAG 3' (SEQ ID NO:3) and  
10 5' GTG TGG CGA AAG TGG TGG TC 3' (SEQ ID NO:4).

The cDNA is prepared from total RNA isolated from human testes according to standard methods. hKir5.1 is amplified with the primers described above using the following conditions: 30 seconds at 96°C, 30 seconds at 55°C, and 3 minutes at 72°C for 40 cycles.

15

The PCR products are subcloned into plasmids and sequenced according to standard techniques. The nucleotide and amino acid sequences of hKir5.1 are provided, respectively, in SEQ ID NO:2 and SEQ ID NO:1.

Example II: Expression and inward rectifier activity of heteromeric channels containing

20

hKir5.1 monomers

hKir5.1 monomer was expressed according to standard methodology, to demonstrate its ability to form heteromeric potassium channels with inward rectifier activity (see Figure 1). Changes in current magnitude were indirectly measured using a reporter voltage-sensitive fluorescent dye (*see, e.g., Etts et al., Chemistry and Physiology of Lipids*,  
25 69:137 (1994)). Figure 1 shows that cells expressing 1:1 mixtures of hKir5.1 and hKir4.1 (open squares) or cells expressing a tandem dimer of hKir5.1 and hKir4.1 (closed squares) show greater fluorescent dye changes resulting from larger current magnitudes than do cells expressing hKir5.1 alone (open circles).

Example III: Expression of Kir5.1 in human tissues

RNA expression of hKir5.1 was examined in human tissues according to standard methodology. Table I indicates expression distribution data:

| 5  | <u>Table I</u> |                        |
|----|----------------|------------------------|
|    | <u>Organ</u>   | <u>Relative amount</u> |
|    | brain          | +/-                    |
|    | stomach        | +/-                    |
|    | pancreas       | ++                     |
| 10 | thyroid gland  | +++                    |
|    | salivary gland | +                      |
|    | kidney         | ++++                   |
|    | trachea        | +/-                    |

15           The distribution of hKir5.1 indicates channels comprising this subunit may play an important role in the function of the kidney, thyroid, pancreas, and salivary gland. Agents which modulate channels comprising hKir5.1 are of value as therapeutic treatments for hypertension, acute renal failure, chronic renal failure, diabetes insipidus, diabetic nephropathy, hypothyroidism, hyperthyroidism, goiter, hypoparathyroidism,

20   hyperparathyroidism, pancreatic insufficiency, diabetes, cystic fibrosis, sialorrhea, and salivary insufficiency.

WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a polypeptide monomer comprising an alpha subunit of a potassium channel, the polypeptide monomer:

5 (i) forming, with at least one additional Kir alpha subunit, a potassium channel having the characteristic of inward rectification;

(ii) having a monomer tail region that has greater than 80% amino acid sequence identity to a human Kir5.1 tail region; and

(iii) specifically binding to polyclonal antibodies generated against SEQ ID NO:1.

10 2. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes human Kir5.1.

3. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes  
15 SEQ ID NO:1.

4. The isolated nucleic acid sequence of claim 1, wherein the nucleic acid has a nucleotide sequence of SEQ ID NO:2.

20 5. The isolated nucleic acid of claim 1, wherein the nucleic acid is amplified by primers that selectively hybridize under stringent hybridization conditions to the same sequence as the primers selected from the group consisting of:

5' CCT AAG GGC ACA GCA AAG AAT GAG 3' (SEQ ID NO:3) and

5' GTG TGG CGA AAG TGG TGG TC 3' (SEQ ID NO:4).

25 6. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a polypeptide monomer having a molecular weight of about between 38 kDa to 48 kDa.

7. The isolated nucleic acid of claim 1, wherein the polypeptide monomer  
30 comprises an alpha subunit of a heteromeric inward rectifier potassium channel.

8. An isolated nucleic acid encoding a polypeptide monomer that specifically hybridizes under stringent conditions to SEQ ID NO:2.

9. The isolated nucleic acid of claim 1, wherein said nucleic acid selectively hybridizes under moderately stringent hybridization conditions to a nucleotide sequence of SEQ ID NO:2.

10. An isolated polypeptide monomer comprising an alpha subunit of a potassium channel, the polypeptide monomer:

(i) forming, with at least one additional Kir alpha subunit, a potassium channel having the characteristic of inward rectification;

(ii) having a monomer tail region that has greater than 80% amino acid sequence identity to amino acids 351-383 of a human Kir5.1 tail region; and

(iv) specifically binding to polyclonal antibodies generated against SEQ ID NO:1.

11. The isolated polypeptide monomer of claim 10, wherein the polypeptide monomer has an amino acid sequence of human Kir5.1.

12. The isolated polypeptide monomer of claim 10, wherein the polypeptide monomer has an amino acid sequence of SEQ ID NO:1.

13. The isolated polypeptide monomer of claim 10, wherein the polypeptide monomer comprises an alpha subunit of a heteromeric potassium channel.

14. An antibody that selectively binds to the polypeptide monomer of claim 10.

15. An antibody of claim 14, wherein the polypeptide monomer has an amino acid sequence of SEQ ID NO:1.

16. An expression vector comprising the nucleic acid of claim 1.

17. A host cell transfected with the vector of claim 16.

18. A method for identifying a compound that modulates ion flux through  
5 an inward rectifier potassium channel, the method comprising the steps of:

(i) contacting the compound with a eukaryotic host cell or cell membrane in which  
has been expressed a polypeptide monomer comprising an alpha subunit of a potassium  
channel, the polypeptide monomer:

(a) forming, with at least one additional Kir alpha subunit, a potassium  
10 channel having the characteristic of inward rectification;

(b) having a monomer tail region that has greater than 80% amino acid  
sequence identity to a human Kir5.1 tail region; and

(c) specifically binding to polyclonal antibodies generated against SEQ ID  
NO:1; and

15 (ii) determining the functional effect of the compound upon the cell or cell membrane  
expressing the potassium channel.

19. The method of claim 18, wherein the functional effect is determined by  
measuring changes in current or voltage.

20. The method of claim 18, wherein the potassium channel monomer  
polypeptide is recombinant.

21. The method of claim 18, wherein the potassium channel is  
25 heteromeric.

22. The method of claim 18, wherein the potassium channel monomer  
polypeptide is human Kir5.1.

23. The method of claim 18, wherein the potassium channel monomer  
30 polypeptide has an amino acid sequence of SEQ ID NO:1.

24. A method of detecting the presence of human Kir5.1 in mammalian tissue, the method comprising the steps of:

(i) isolating a biological sample;

(ii) contacting the biological sample with a Kir5.1-specific reagent that selectively associates with human Kir5.1; and,

(iii) detecting the level of Kir5.1-specific reagent that selectively associates with the sample.

25. The method of claim 24, wherein the Kir5.1-specific reagent is selected from the group consisting of: Kir5.1 specific antibodies, Kir5.1 specific oligonucleotide primers, and Kir5.1 nucleic acid probes.

26. The method of claim 24, wherein the sample is from a human.

27. In a computer system, a method of screening for mutations of human Kir5.1 genes, the method comprising the steps of:

(i) entering into the computer system a first nucleic acid sequence encoding an inward rectifier potassium channel polypeptide monomer having a nucleotide sequence of SEQ ID NO:2, and conservatively modified versions thereof;

(ii) comparing the first nucleic acid sequence with a second nucleic acid sequence having substantial identity to the first nucleic acid sequence; and

(iii) identifying nucleotide differences between the first and second nucleic acid sequences.

28. The method of claim 27, wherein the second nucleic acid sequence is associated with a disease state.

29. A computer readable substrate comprising the first amino acid sequence of claim 27.

30. The computer readable substrate of claim 29, further comprising the second amino acid sequence.

31. In a computer system, a method for identifying a three-dimensional structure of Kir5.1 polypeptides, the method comprising the steps of:

(i) entering into the computer system an amino acid sequence of at least 10 amino acids of a potassium channel monomer or at least 30 nucleotides of a gene encoding the polypeptide, the polypeptide having an amino acid sequence of SEQ ID NO:1, and conservatively modified versions thereof; and

(ii) generating a three-dimensional structure of the polypeptide encoded by the amino acid sequence.

32. The method of claim 31, wherein said amino acid sequence is a primary structure and wherein said generating step includes the steps of:

(i) forming a secondary structure from said primary structure using energy terms determined by the primary structure; and

(ii) forming a tertiary structure from said secondary structure using energy terms determined by said secondary structure.

33. The method of claim 31, wherein said generating step further includes the step of forming a quaternary structure from said tertiary structure using anisotropic terms encoded by the tertiary structure.

34. The method of claim 31, further comprising the step of identifying regions of the three-dimensional structure of the protein that bind to ligands and using the regions to identify ligands that bind to the polypeptide.

35. A computer readable substrate comprising the three dimensional structure of the polypeptide of claim 31.

## SEQUENCE LISTING

hKir5.1 amino acid sequence (SEQ ID NO:1)

5 MSYYGSSYHIINADAKYPGYPPPEHIIAEKRRARRRLLHKDGCNVPYFKHIFGEWGSY  
VVDIFTTLVDTKWRHMFVIFSLSYILSWLIFGSVFWLIAFHGDLNDPDITPCVDNV  
HSFTGAFLFSLETQTTIGYGYRCVTEECVAVLMVILQSILSCIINTFIIGAALAKMATA  
RKRAQTIRFSYFALIGMRDGLCLMWRIGDFRPNHVVEGTVRAQLLRYTEDSEGRM  
TMAFKDLKL VNDQIILVTPVTIVHEIDHESPLYALDRKAVAKDNFEILVTFIYTG DSTG  
10 TSHQSRSSYVPRXILWGHRFNDVLEVKRKYKVNCLQFEGSVEVYAPFCSAKQLDW  
KDQQLHIEKAPPVRESCTSDTKARRRSFSAVAIVSSW

hKir5.1 nucleotide sequence (SEQ ID NO:2)

15 TTACTACTACAAAACCTCACCTGGATCCCTAAGGGCACAGCAAAGAATGAGCTATT  
ACGGCAGCAGCTATCATATTATCAATGCGGACGCAAAATACCCAGGCTACCCGC  
CAGAGCACATTATAGCTGAGAAGAGAAGAGCAAGAAGACGATTACTTCACAAAG  
ATGGCAGCTGTAATGTCTACTTCAAGCACATTTTTGGAGAATGGGGAAGCTATGT  
GGTTGACATCTTCACCACTCTTGTGGACACCAAGTGGCGCCATATGTTTGTGATA  
20 TTTTCTTTATCTTATATTCTCTCGTGGTTGATATTTGGCTCTGTCTTTTGGCTCATA  
GCCTTTCATCATGGCGATCTATTAAATGATCCAGACATCACACCTTGTGTTGACA  
ACGTCCATTCTTTCACAGGGGCCTTTTTGTTCTCCCTAGAGACCCAAACCACCAT  
AGGATATGGTTATCGCTGTGTTACTGAAGAATGTTCTGTGGCCGTGCTCATGGTG  
ATCCTCCAGTCCATCTTAAGTTGCATCATAAATACCTTTATCATTGGAGCTGCCTT  
25 GGCCAAAATGGCAACTGCTCGAAAGAGAGCCCAAACCATTTCGTTTCAGCTACTTT  
GCACTTATAGGTATGAGAGATGGGAAGCTTTGCCTCATGTGGCGCATTGGTGATT  
TTCGGCCAAACCACGTGGTAGAAGGAACAGTTAGAGCCCAACTTCTCCGCTATAC  
AGAAGACAGTGAAGGGAGGATGACGATGGCATTTAAGACCTCAAATTAGTCAA  
CGACCAAATCATCCTGGTCACCCCGGTA ACTATTGTCCATGAAATTGACCATGAG  
30 AGCCCTCTGTATGCCCTTGACCGCAAAGCAGTAGCCAAAGATAACTTTGAGATTT  
TGGTGACATTTATCTATACTGGTGATTCCACTGGAACATCTCACCAATCTAGAAG

CTCCTATGTTCCCCGARAAATTCTCTGGGGCCATAGGTTTAATGATGTCTTGGA  
GTAAAGAGGAAGTATTACAAAGTGAAGTCTTACAGTTTGAAGGAAGTGTGGAA  
GTATATGCCCCCTTTTGCAGTGCCAAGCAATTGGACTGGAAAGACCAGCAGCTCC  
ACATAGAAAAAGCACCACCAGTTCGAGAATCCTGCACGTCGGACACCAAGGCGA  
5 GACGAAGGTCATTTAGTGCAGTTGCCATTGTCAGCAGCTGGTGAAAACCCTGAG  
GAGACCACCACTTTCGCCACACATGAATATAGGGAAACACCTTATCAGAAAGCT  
CTCCCTGACTTTAAACAXGAATCCTCTGTWGAATCCCAAATGTTAGTCCTAAAAT  
TGCAATTATGAGGGCTACCACTGAATCATTTTATCTTTCAGCCAATCAAGTCGTT  
GTAAACGTGGCTTTTTTTGAAAGTGTTATGGCTATGTTTTATGATGATGCTGGGTAA  
10 GTAGAGTAAGTTAAACTTGGTAAAAGATAATCTAAAAATTCCATAGTTCTCAGTT  
ATTAAAATTTTTCTTGTTCCGGAATTC

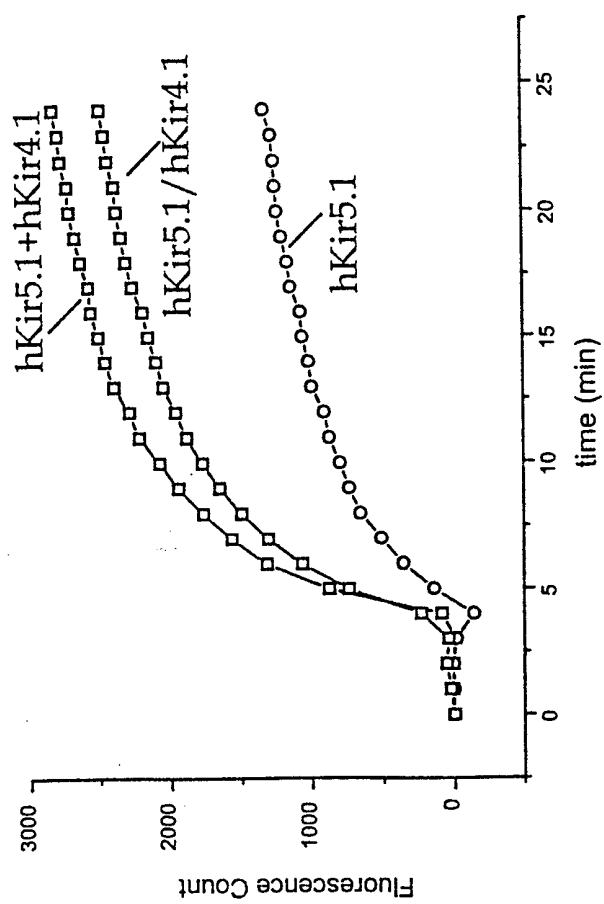


Figure 1