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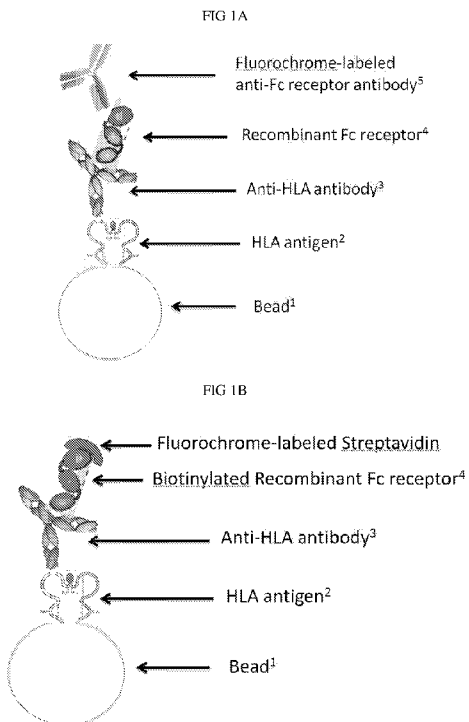
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[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS FOR THE DETECTION OF FC RECEPTOR BINDING ACTIVITY OF ANTIBODIES



(57) Abstract: Disclosed are kits and methods for determining the presence or absence of an antibody of interest in a biological sample of a subject. In particular, the methods may detect either pathological or beneficial antibodies. The method may include the step of contacting a biological sample from a subject with a substrate conjugated to an antigen and an Fc receptor operatively linked to a detectable label. Detection of the label may indicate the presence or absence of an antibody of interest.

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**METHODS AND COMPOSITIONS FOR THE  
DETECTION OF FC RECEPTOR BINDING ACTIVITY  
OF ANTIBODIES**

**Cross Reference to Related Applications**

**[0001]** This application claims priority to and the benefit of US Provisional Applications 62/208,938, filed August 24, 2015 and entitled “Methods and Compositions for the Detection of FC Receptor Binding Activity of Antibodies” and 62/263,862, filed December 7, 2015 entitled “Fc Receptor Binding Assay for Functional Assessment of HLA Antibodies: Initial Clinical Validation,” the contents of which are incorporated herein in their entirety by reference.

**Background of the Invention**

**[0002]** Antibodies develop when the immune system encounters molecular entities that are “non-self”. Antibodies have diverse functions in normal immunologic responses and in disease states that include immunomodulatory effects (both stimulatory and inhibitory) and mediation of immune injury. Antibodies may be protective, such as those that result from vaccines or infection may protect the host from subsequent pathogen exposure. Antibodies may also be pathogenic, as in autoimmune diseases where autoantibodies such as anti-platelet antibodies (in idiopathic thrombocytopenic purpura) or anti-acetyl choline receptor antibodies (as in myasthenia gravis) mediate disease. As explained below, antibodies against allogeneic HLA antigens can be pathogenic in transplant recipients during antibody mediated rejection (AMR). Interactions of antibodies with Fc receptors mediates a substantial proportion of these widely varying functions. Facilitation of the ability to assess antibody-Fc receptor interactions with in vitro assays may provide a useful means for determining clinical relevance of an antibody population.

**[0003]** Rejection remains a major cause of organ transplant failure. In humans, the molecular targets for rejection are a group of polymorphic cell surface proteins

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termed HLA antigens. Developments in the ability to detect antibodies against HLA antigens over the past several years have greatly enhanced the ability to detect and diagnose AMR. Clinical application of these technologies has resulted in widespread recognition that antibodies against donor HLA antigens (termed donor-specific antibodies (DSA)) are likely the major reason for failure of kidney transplants, and also a major cause of failure of heart, lung, and transplants (Loupy et al, N Engl J Med 2013; 369:1215-1226). Current therapies for antibody-mediated rejection (AMR) although often effective, remain suboptimal, particularly in late AMR or in chronic AMR (Sadaka et al., Expert Opin Investig Drugs. 2011; 11:1535-42).

**[0004]** HLA antibodies develop when humans are exposed to tissues or organs from a genetically dissimilar person via blood transfusion, organ transplantation, or pregnancy. HLA antibody prevalence in the general population and in the transplant candidate population are high: over 20% of healthy individuals (higher percentages occur in women because of pregnancy) and over 60% of kidney transplant candidates have HLA-antibodies. In addition, 8-25% of kidney transplant recipients will develop *de novo* DSAs following transplantation. When present at very high levels prior to transplantation, DSA can cause hyperacute rejection with almost immediate loss of the kidney allograft. However, hyperacute rejection is rarely observed because HLA antibodies are efficiently detected by the single HLA antigen bead assay (SAB assay) and these transplants can be avoided. Importantly, kidney (and other organ) transplants are performed regularly in the presence of lower DSA levels, and AMR risk correlates to some degree with DSA levels. Although modest advances have been made in developing therapies for preventing and treating DSA responses, acute and chronic AMR continue to occur with significant frequency in transplant recipients, thereby presenting a major barrier that limits long-term success rates.

**[0005]** HLA single antigen bead (SAB) assays have been developed as a means for detecting anti-HLA antibodies. HLA single antigen beads are constructed by

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attaching recombinant-derived HLA antigens to microbead particles. Although HLA SABs provide a means to detect anti-HLA antibodies, they do not provide a functional assessment, or rather the pathogenic or immune-protective capacity of an antibody. One approach for assessing the pathogenic potential of HLA antibodies has been the C1q assay. However, the C1q assay remains to be proven as a clinically useful tool, and is presently not broadly adopted in the transplant community. The C1q assay has also been noted to have important limitations, including its inability to detect HLA antibody function when HLA antibodies are at moderate or low concentration. Further, it is not uncommon for AMR to occur in the absence of detectable complement activation (assessed by C4d staining of renal allograft biopsies), wherein C1q assays are often negative. In this setting, AMR diagnosis requires a renal allograft biopsy with demonstration of microvascular inflammation (peritubular capillaritis (PTCitis or glomerular capillaritis) Therefore, the avoidance of invasive procedures is a need in the art.

**[0006]** Estimation of the functional and pathogenic potential of HLA antibodies remains an important challenge. Sensitive and early detection of pathogenic antibodies during, before, or after transplant remains a critical need in the art. Lacking in the art is a suitable assay capable of determining the pathogenic capability of HLA antibodies and/or nonpathogenic antibodies, particularly when HLA antibodies are present at low or moderate levels in the serum. The instant invention addresses one or more of the aforementioned problems in the art.

### **Brief Summary of the Invention**

**[0007]** Disclosed are methods for determining the presence or absence of an antibody of interest in a biological sample of a subject. In particular, the methods may detect either pathological or beneficial antibodies. The method may include the step of contacting a biological sample from a subject with a substrate conjugated to an antigen and an Fc receptor operatively linked to a detectable

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label. Detection of the label may indicate the presence or absence of an antibody of interest.

### **Brief Description of the Drawings**

[0008] FIG 1A depicts a diagram of the Fc receptor assay. The single HLA antigen bead (SAB) is shown expressing a recombinant derived HLA antigen. Currently available SABs from commercial vendors each express a single HLA antigen, as an example with some test reagents consisting of 98 Class I and 96 Class II unique SABs, which differ based on the HLA antigen that is expressed. Currently available SAB preparations express HLA antigens from the most frequent HLA gene loci including HLA A, HLA B, HLA C, HLA DR $\beta$ 1, DR $\beta$ 3/4/5, HLA DQ $\alpha$ , HLA DQ $\beta$ , DP $\beta$ , and DP $\alpha$  loci. Multiple unique HLA alleles may exist at each genetic locus. The anti-HLA antibody depicted in FIG 1 derives from the serum of a patient. This in turn, binds the soluble Fc receptor (which can be the Fc $\gamma$ R1 (CD64) molecule, or the Fc $\gamma$ RIIa molecule (CD32A), or the Fc $\gamma$ RIIb molecule (CD32B), or the Fc $\gamma$ RIIc molecule (CD32c), or the Fc $\gamma$ RIIIa molecule (CD16A), or the Fc $\gamma$ RIIIb molecule (CD16B), or the Fc $\mu$ R molecule, or the DC-SIGN molecule (CD209), and which can be recombinant or patient-derived). The final step in the assay involves binding of the Fc receptor molecule by a fluorochrome-labeled anti-Fc receptor secondary antibody. The amount of bound Fc receptor may then be assayed by measuring fluorescence (most commonly on the Luminex platform (Luminex corporation, Austin, Texas)). FIG 1B depicts a schematic of the general approach underlying Example 2 using a biotinylated recombinant Fc receptor.

[0009] FIG 2 depicts the correlation between the CD64 Fc receptor assay and the SAB assay (left panel) and the correlation between the C1q assay and the SAB assay (right panel). Stronger correlation with the SAB assay is observed for the CD64 Fc receptor assay than that observed for the C1q assay.

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- [0010] FIG 3 depicts the abilities of the CD64 Fc receptor assay and the C1q assay to discriminate between strong (i.e., high level) DSA and weak (i.e., low level) DSA. Statistically significant differences are observed with the CD64 Fc receptor assay between strong and weak DSA but not with the C1q assay.
- [0011] FIG 4 depicts the abilities of the CD64 Fc receptor assay and the C1q assay to discriminate between moderate (i.e., moderate level) DSA and weak (i.e., low level) DSA. Statistically significant differences are observed with the CD64 Fc receptor assay between moderate and weak DSA but not with the C1q assay. FIG 4, lower panel shows that moderate strength DSA are associated with marked reduction in allograft survival.
- [0012] FIG 5 depicts testing of sera from 14 patients known to have low or moderate levels (i.e., weak) of HLA antibody as determined by the SAB assay. When tested by the C1q and CD64 Fc receptor assays, C1q binding is almost universally undetectable, whereas significant CD64 Fc receptor binding is reliably detected, demonstrating the superior sensitivity of assaying for Fc receptor binding rather than C1q binding.
- [0013] FIG 6 depicts results of a single patient's sera tested by the SAB assay (top), the C1q assay (middle), and the CD64 Fc receptor assay (bottom). By noting the mean fluorescence intensity (MFI) on the y scale, one can see that the C1q assay does not detect binding by the DR53 antibody, which clearly binds CD64 Fc receptor.
- [0014] FIG 7 depicts results of a single patient's sera tested by the SAB assay (top), the C1q assay (middle), and the CD64 Fc receptor assay (bottom). By noting the mean fluorescence intensity (MFI) on the y scale, one can see that the C1q assay does not detect binding by the DQ2 antibody, which clearly binds the CD64 receptor.
- [0015] FIG 8 depicts assay results showing that HLA antibodies bind to very small areas on the surface of a protein, which are called epitopes. Such epitopes can be expressed by several unique HLA antigens. In this figure, the patient's sera

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binds to an epitope on the HLA B 27 molecule, which is found in the 7 CREG group and the 5 CREG group (CREG = cross reactive groups). As one can observe in the top panel which represents the SAB assay, if the HLA antigens that are circled in blue all express a common epitope and are clustered together, therefore an epitope binding pattern is noted to have been detected. Results for the SAB assay (top), C1q assay (middle), and CD64 Fc receptor assay (bottom) demonstrate that the CD64 Fc receptor assay, but not the C1q assay, retain the epitope binding patterns detected in the SAB assay.

- [0016] FIG 9 depicts the relationship between patients with a Banff g score component of 0 (indicating an absence of glomerulitis on biopsy) and those with a Banff g score component of 3 (indicating severe glomerulitis on biopsy) and mean MFI values by the CD64 Fc receptor assay and by the C1q assay. These studies demonstrate that the CD64 Fc receptor assay results are more reflective of the degree of glomerulitis on kidney allograft biopsy than are C1q assay results.
- [0017] FIG 10 shows that FcR assay may correlate better than does the C1q assay with C4d staining in biopsy-proven AMR.
- [0018] FIG 11 shows that that renal allograft survival after early AMR correlates with the FcR Assay and not the C1q Assay.
- [0019] FIG 12 depicts Banff component scores (g (glomerulitis), ptc (peritubular capillaritis), and g + ptc (composite microcirculatory inflammation) which are indicative of AMR, and how they correlate with DSA levels (weak, moderate, and strong) in the CD64 Fc receptor assay.
- [0020] FIG 13 depicts poorer allograft survival that is seen observed with higher MFI values in the CD64 Fc receptor assay.

### **Detailed Description of the Invention**

- [0021] **Definitions/Acronyms**



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- [0022] ABMR/AMR - antibody-mediated rejection
- [0023] B27 DSA – donor specific antibody that binds to the HLA B27 antigen.
- [0024] Banff component scoring- Banff criteria consist of several components that are evaluated on the biopsy including g (glomerulitis), t (tubulitis), i (interstitial inflammation), v (endotheliitis), ptc (peritubular capillaritis), amongst others. Banff components are then used to make the diagnosis of acute cellular rejection or acute AMR, and also the grade the severity of the rejection (Haas et al, Am J Transplant 2014; 14: 272-283).
- [0025] Bw4 - a public epitope expressed by multiple HLA antigens from the HLA B locus. Bw4 epitope can be demonstrated on SAB and CD64 SAB assays, but not by the C1q assay.
- [0026] CD16 – CD16 is a 50-80 kD protein that is low affinity Fc receptor found on the surface of natural killer cells, neutrophil polymorphonuclear leukocytes, monocytes and macrophages. It can be used to isolate populations of these cells by antibodies directed towards CD16, using fluorescent-activated cell sorting or magnetic-activated cell sorting. CD16 has been identified as Fc receptors Fc $\gamma$ RIIIa (CD16a) and Fc $\gamma$ RIIIb (CD16b). These receptors bind to the Fc portion of IgG antibodies.
- [0027] CD64 - CD64 is a 72 kD single chain type I glycoprotein also known as Fc $\gamma$ RI that possesses high affinity for IgG1 and IgG3 human antibodies. CD64 is a member of the immunoglobulin superfamily and is expressed on monocytes/macrophages, dendritic cells, and neutrophils. The expression can be upregulated by IFN- $\gamma$  stimulation. CD64 also binds IgG immune complexes. CD64 plays a role in antigen capture, phagocytosis of IgG/antigen complexes, cell activation, and antibody dependent cellular cytotoxicity (ADCC).
- [0028] CD32 – CD32 is a surface receptor protein that binds IgG with low affinity expressed by macrophages, neutrophils, eosinophils, and platelets, CD32 may consist of the Fc $\gamma$ RIIA (CD32A which mediates activation effects) or the

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Fc $\gamma$ RIIB molecule (CD32B, which mediates immune suppressive effects) or the Fc $\gamma$ RIIc molecule (CD32c).

- [0029] C1q - The first subcomponent of the C1 complex of the classical pathway of complement activation.
- [0030] CREG – Cross Reactive Groups
- [0031] DSA – Donor-specific antibody
- [0032] DR53 – An HLA antigen encoded by the DR $\beta$ 3 gene on chromosome 6.
- [0033] The term “reference value” as used herein means a value which can be used for comparison with a biomarker under investigation. In one case, a reference value may be the level of a biomarker under investigation from one or more individuals without any known disease. In another case, a reference value may be the level of the biomarker in an individual's sample collected at a different time.
- [0034] “Sample” or “patient sample” or “biological sample” as used herein includes biological samples such as cells, tissues, bodily fluids, and stool. “Bodily fluids” may include, but are not limited to, blood, serum, plasma, saliva, cerebral spinal fluid, pleural fluid, tears, lactal duct fluid, lymph, sputum, urine, amniotic fluid, and semen. A sample may include a bodily fluid that is “acellular”. An “acellular bodily fluid” includes less than about 1% (w/w) whole cellular material. Plasma or serum are examples of acellular bodily fluids. A sample may include a specimen of natural or synthetic origin.
- [0035] The term “body fluid” or “bodily fluid” as used herein refers to any fluid from the body of an animal. Examples of body fluids include, but are not limited to, plasma, serum, blood, lymphatic fluid, cerebrospinal fluid, synovial fluid, urine, saliva, mucous, phlegm and sputum. A body fluid sample may be collected by any suitable method. The body fluid sample may be used immediately or may be stored for later use. Any suitable storage method known in the art may be used to store the body fluid sample; for example, the

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sample may be frozen at about 20° C. to about -70° C. Suitable body fluids are acellular fluids. "Acellular" fluids include body fluid samples in which cells are absent or are present in such low amounts that the peptidase activity level determined reflects its level in the liquid portion of the sample, rather than in the cellular portion. Typically, an acellular body fluid contains no intact cells. Examples of acellular fluids include plasma or serum, or body fluids from which cells have been removed.

**[0036]** The term "label" as used herein, refers to any physical molecule directly or indirectly associated with a specific binding agent or antigen which provides a means for detection for that antibody or antigen. A "detectable label" as used herein refers any moiety used to achieve signal to measure the amount of complex formation between a target and a binding agent. These labels are detectable by spectroscopic, photochemical, biochemical, immunochemical, electromagnetic, radiochemical, or chemical means, such as fluorescence, chemifluorescence, or chemiluminescence, electro-chemiluminescence or any other appropriate means. Suitable detectable labels include fluorescent dye molecules or fluorophores. In some aspects, the detectable label may be an enzyme, a fluorescent molecule, a particle label, an electron-dense reagent, a radiolabel, a microbubble, biotin, digoxigenin, or a hapten or a protein that has been made detectable. In other aspects, the label may be an enzyme that can metabolize a substrate that results in a metabolic product that can be measured (e.g. optical density). As an example, the enzyme alkaline phosphatase can metabolize nitroblue tetrazolium (NBT) to form NBT-diformazan.

**[0037]** "Detecting" as used herein in context of detecting a signal from a detectable label to indicate the presence of a nucleic acid of interest in the sample (or the presence or absence of a protein of interest in the sample) does not require the method to provide 100% sensitivity and/or 100% specificity. As is well known, "sensitivity" is the probability that a test is positive, given that the person has a genomic nucleic acid sequence, while "specificity" is the probability that a test is negative, given that the person does not have the

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genomic nucleic acid sequence. A sensitivity of at least 50% is preferred, although sensitivities of at least 60%, at least 70%, at least 80%, at least 90% and at least 99% are clearly more preferred. A specificity of at least 50% is preferred, although specificity of at least 60%, at least 70%, at least 80%, at least 90% and at least 99% are clearly more preferred. Detecting also encompasses assays with false positives and false negatives. False negative rates may be 1%, 5%, 10%, 15%, 20% or even higher. False positive rates may be 1%, 5%, 10%, 15%, 20% or even higher.

**[0038]** FcR – “Fc Receptor” - Fc receptors (abbreviated FcR) are receptors that bind antibodies, classified based on the type of antibody that they recognize. For example, Fc-gamma receptors (Fc $\gamma$ R) bind IgG, Fc-alpha receptors (Fc $\alpha$ R) bind IgA, and Fc-epsilon receptors (Fc $\epsilon$ R) bind IgE, Fc-mu receptors (Fc $\mu$ R) or DC-SIGN (CD209).

**[0039]** HLA antigens- The human leukocyte antigens (HLA) are encoded at several gene loci on the short arm of human chromosome 6. These genes include two classes (class I and class II) that encode for proteins on the surface of cells that are responsible for regulation of the immune system in humans. Published sequences are available at <https://www.ebi.ac.uk/ipd/imgt/hla/>; <http://hla.alleles.org> and <http://www.anthonynolan.org>

**[0040]** The term “operatively linked” means the linkage between two components in a way that allows a function. For example, in one aspect, a detectable label may be operatively linked to a component desired to be detected, wherein the linkage allows for detection of a detectable label bound, reversibly or otherwise, to the component of interest.

**[0041]** MFI – Mean Fluorescent Intensity

**[0042]** SAB – “Single antigen bead”

**[0043]** SAFR – “Single antigen Fc Receptor”

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- [0044] The term "subject" as used herein, refers to an animal, preferably a mammal, most preferably a human. The term subject may be interchangeably used with the term patient in the context of the present invention.
- [0045] Antibodies mediate cellular and tissue injury by one or more of three mechanisms: 1) complement activation, 2) binding to Fc receptor (FcR)-bearing cells, and 3) by direct binding to cellular membrane proteins (such as HLA class I or class II molecules). For many years, the predominant mechanism for antibody effector function has been thought to be complement activation and, traditionally in transplantation, assessment of donor-specific antibody (DSA) function has been focused solely on complement activation properties. Since the discovery in 1969 that anti-HLA antibodies are lymphocytotoxic, activation of the complement cascade has been considered to be the predominant mechanism by which DSA mediate AMR. C4d, a complement split product, can be detected histologically in AMR, and C4d deposition in peritubular (and/or glomerular) capillaries is now one of the three major diagnostic criteria for AMR. A second major diagnostic criterion has been the demonstration of DSA using the SAB assay (where recombinant derived HLA antigens are bound to polystyrene beads).
- [0046] Complement activation may occur via three pathways: 1) classical pathway, 2) alternative pathway, and 3) lectin-dependent pathway. Of these three pathways, the classical pathway may predominate in AMR. The classical pathway of complement activation is initiated when DSA (that are bound to HLA molecules on the cell surface) bind the complement factor C1q. Historically (and also presently) the predominant mechanism by which DSA mediate allograft injury is thought to be via complement activation. Functional assessment of DSAs to date has therefore focused exclusively on complement activation. For over four decades, anti-HLA antibody function was assessed by complement-dependent cytotoxicity assays. Over the past decade with the advent of SAB assay development, the C1q assay (available via One Lambda/ThermoFisher) was developed as a complementary assay to the SAB

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assay, thereby providing a mechanism for assessing complement binding capabilities of DSA.

**[0047]** The C1q assay is performed using patient sera and allowing these sera to react with SABs, where if present, anti-HLA antibodies will bind to the SABs. Once bound to a SAB, the ability of an anti-HLA antibody to activate complement is assayed by measuring binding of C1q. The C1q assay, however, has several known limitations including: 1) the requirement for substantial biophysical constraints to C1q binding by antibodies (the antibodies must be at high concentrations, and must be bound to target antigens and arranged in a precise hexagonal fashion), and 2) the C1q assay does not replicate *in vivo* complement activation, because HLA antigen expression by SABs does not accurately replicate the antigen density and biophysical properties of HLA antigens *in vivo* (particularly true for SABs expressing HLA DQ molecules, where the HLA DQ antigen density is much greater than *in vivo*). C1q assays are insensitive to HLA antibodies in low to moderate strength range, are oversensitive to DQ antibodies, often does not retain epitope patterns, and a substantial portion of rejections are complement-independent. It is known that approximately half of kidney allograft biopsies in patients with AMR do not exhibit evidence of C4d staining. C4d negative AMR is now a recognized entity within the Banff criteria for AMR diagnosis, where microvascular inflammation (peritubular capillaritis and glomerular capillaritis) can substitute for C4d staining as an AMR diagnostic criterion. Finally, recent studies have shown that relatively small changes in DSA concentration will render the C1q assay from positive to negative. In summary, the C1q assay is not completely validated as a clinically useful tool, and is presently not broadly adopted by the transplant community.

**[0048]** As noted above, when C4d staining is not detectable, AMR diagnosis requires demonstration of microvascular inflammation, (Haas M et al, Am J Transplant 2014; 14L272-283), which unfortunately requires an invasive biopsy procedure. Therefore, the avoidance of invasive procedures is a need in the art.

- [0049] Functional assessment of HLA antibodies has historically been limited to complement-based assays. Solid phase assays assessing the classical pathway of complement activation, however, are limited in that they do not accurately reproduce biophysical constraints of C1q binding by cell surface bound antibody (Ab) (Science 2014; 343(6176):1260). Moreover, HLA Abs are capable of inducing injury by complement-independent mechanisms, including direct signaling via class I and class II cell surface antigens, and via Fc receptor engagement. Applicant has hypothesized that comprehensive assessment of the pathogenic potential of HLA antibodies requires assessment of FcR binding capacity.
- [0050] Multiple end-organ diseases benefit today from transplantation of vascular allografts: kidney, pancreas, islet, heart, lung, intestine, liver, limbs, facial grafts etc. Furthermore, non-vascular tissue grafts, such as hematopoietic and other stem cell transplantation are under substantial expansion. After transplantation, antibody-mediated rejection (ABMR) represents a major risk factor for allograft dysfunction and/or graft loss. Detection of circulating anti-HLA antibody in solid-organ transplantation has continuously improved over the past decade. Both cellular and solid-phase assays are in use for detection of anti-HLA antibodies. Multiplex and flow-based techniques are reported as most sensitive for antibody detection, followed by ELISA and CDC methods. The results of solid-phase methods seem to be less influenced by IgM, auto- and non-HLA antibodies, as well as by cytolytic protocols.
- [0051] Considering the advantages and limitations of each assay, a combination, rather than a single method, may provide the best approach to determine the level of sensitization and the specificity of anti-HLA antibody in transplant recipients.
- [0052] Fc receptor bearing cells have been implicated in AMR of kidney allografts, and by detecting NK cell transcripts in the allograft (as shown by Halloran (Hidalgo et al, Am J Transplant 2009;11: 2532-2541)). The observation that approximately half of AMR episodes in kidney transplants do not appear to

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involve complement, suggest that the other primary mechanisms by which anti-HLA antibodies damage the allograft, viz., via Fc receptor interaction, may be of substantial importance. Until Applicant's invention, there has been no assay available to measure the Fc receptor binding capacity of HLA antibodies, nor was measurement of Fc receptor binding implicated in detection of HLA antibodies.

- [0053]** Disclosed herein are methods and assays for evaluating the Fc receptor binding capacity of an antibody or an antibody preparation, which, in certain aspects, may be derived either artificially (e.g., via recombinant DNA technology or hybridoma technology) or naturally (e.g., human serum or tissues). The present disclosure relates to an assay that can evaluate the pathogenic capabilities of antibodies, and/or, in other aspects, the presence of beneficial antibodies.
- [0054]** Pathogenic antibodies can mediate injury by one of three mechanisms: 1) direct effect on the target cell (by initiating target cell signaling via the target antigen, 2) binding Fc receptors on Fc receptor bearing cells such as macrophage or monocytes or mast cells, and 3) by activating complement. In one aspect, the instant disclosure relates to assays for evaluating the ability of a defined antibody population in human serum (e.g., antibodies that bind to beads expressing HLA A2 antigen) to bind to a defined Fc receptor or group of Fc receptors.
- [0055]** Fc receptors may also mediate immune protection, or downregulation of immune responses, with the classic example being the Fc $\gamma$ RIIb receptor. The Fc $\gamma$ RIIb receptor is known to suppress B cell function (White AL et al, Curr Top Microbiol Immunol. 2014;382:355-72), raising the possibility that Fc receptor assays for Fc $\gamma$ RIIb receptor binding may provide additional insights into the immunomodulatory effects of antibodies of interest.
- [0056]** Determining the Fc receptor binding capabilities of anti-HLA antibodies provides a mechanism for determining the potential pathogenicity or benefit of



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the antibodies in question, as Applicant has observed that Fc receptor bearing cells may provide an important means for mediating the effects of anti-HLA antibodies, in particular, the pathogenic effects.

**[0057]** When antibodies are detected, assessment of their Fc receptor binding and complement activation properties may yield important diagnostic and prognostic information. For many years, the predominant mechanism for antibody effector function has been thought to be complement activation. Traditionally, assessment of donor-specific antibody (DSA) function has been focused solely on complement activation properties. (C1q assay or Complement-dependent lymphocytotoxic assay.) Certain assays may be used to assess the ability of HLA alloantibodies to bind specific complement components (C1q as one example). However, complement binding is inherently more restrictive than Fc receptor binding in terms of the conditions required, including antigen density, antibody density, antibody concentration, antibody three dimensional orientation, and antibody affinity, as compared to Fc receptor binding.

**[0058]** Applicant has found that the FDA approved solid phase single antigen bead-based approaches using recombinant Fc receptor molecules have surprising advantages over traditionally used assays. Applicant has found that C1q and CD64 Fc receptor assays correlate with SAB assay, but CD64 Fc receptor assay has superior sensitivity for weak-moderate Ab strength, and that the CD64 Fc receptor assay described herein provides information unique from that of the C1q assay that is currently in use. Further, Applicant has found that the CD64 Fc receptor assay disclosed herein, correlates with histological changes in the allograft (that are used to diagnose AMR) and allograft survival. These histological changes are termed Banff component scoring. The Banff components comprise the Banff diagnostic system which is the universally accepted criteria for pathologic diagnosis of AMR, and also for acute cellular rejection. The traditionally used C1q assay does not demonstrate correlation with Banff component scoring (Haas, et al *ibid*). Thus, the instant

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disclosure offers novel and improved methods by which detection of pathogenic antibodies involved in AMR may be detected.

**[0059]** In one aspect, a method for determining the presence or absence of antibodies from a biological sample of a subject is disclosed. The method may comprise, for example, the step of contacting a biological sample from a subject with a substrate conjugated to an antigen with an Fc receptor operatively linked to a detectable label. Alternatively, in other aspects, the Fc receptor may be directly conjugated to a fluorochrome or alternatively to biotin, so that an avidin-based conjugate may be used for detection. See FIG 1B.

**[0060]** In certain aspects, when an antibody of interest is present, a complex comprising the substrate conjugated to the antigen and the Fc receptor operatively linked to said detectable label may be formed. Detection of a detectable label on the complex may then be used to indicate that the biological sample may contain an antibody of interest, whether the antibody be pathogenic or beneficial in nature. In one aspect, the absence of detection of a label indicates the absence of an antibody of interest. The antibody of interest may be selected from pathogenic anti-HLA antibodies, non-HLA pathogenic antibodies, or a combination thereof. In one aspect, the biological sample is one in which it is suspected that the subject from which the sample is obtained is suspected of having an antibody of interest, for example, an anti-HLA antibody.

**[0061]** In one aspect, disclosed is an assay for assessing a level of anti-HLA antibodies in a subject. In one aspect, the assay may include a substrate, for example a substrate such as a bead with HLA antigens immobilized on the substrate that serve as an antigen source. The substrate may take a variety of forms, for example, a solid substrate. The solid substrate may be selected from the non-limiting list of beads, fibers, filters, beads, filters, fibers, screens, mesh, tubes, hollow fibers, fluidic channels, microfluidic channels, a plastic substrate, an ELISA plate, or the like.

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**[0062]** The substrate may then be incubated with a biological sample such as test sera from a patient wherein the patient is believed or known to have anti-HLA antibodies. In one aspect, the biological sample may be obtained from a subject selected from a transplant recipient, a transplant candidate, a subject who has had a blood transfusion, a subject who is or has previously been pregnant, and a subject who has previously had an organ, tissue, or cellular transplant. The biological sample may be processed according to what is known in the art, for example, the sample may be immediately analyzed, or analyzed after storage at 4 degrees centigrade, or after storage of about -80 degrees centigrade, or after storage in liquid nitrogen. The sample may be stored for a range of time periods prior to application of the instant methods.

**[0063]** The substrate may then be washed, and incubated with a known recombinant derived Fc receptor (such as, for example, CD16A or CD16B, CD32A, CD32B, CD32C, or CD64) and washed a second time. The substrate may then be incubated with a secondary antibody (either a monoclonal or polyclonal antibody that is specific for the Fc receptor of interest and is labeled with a fluorochrome (or other identifiable marker)) and washed. Incubation and wash times will be readily determined by one of ordinary skill in the art, as will the various markers for labeling the antibody and also for wash solutions (most commonly phosphate buffered saline "PBS").

**[0064]** The substrate may then be analyzed for the presence of the detectable marker, which may take a variety of forms. For example, in one aspect, the substrate may be a bead that is analyzed in a flow cytometer (or on a Luminex platform (Luminex Corporation, Austin, Texas) for fluorescence intensity at an appropriate wavelength for a label that contains a fluorochrome such that the fluorochrome of interest is detected. In this aspect, the intensity of the strength of the signal reflects the strength of the binding of the anti-HLA antibodies and also the strength of binding of the recombinant Fc receptor molecules to the anti-HLA antibodies.

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**[0065]** In one aspect, the recombinant Fc receptor may be an Fc receptor expressed by an immune cell. In one aspect, the Fc receptor may be one or more recombinant Fc receptor selected from CD64, CD16A, CD16B, CD32A, CD32B, CD32C, Fc $\mu$ R, Fc $\epsilon$ R1, and CD23 (Fc $\epsilon$ R1I). In one aspect, the FcR may comprise CD64 (Cluster of Differentiation 64). CD64 (also known as “Fc-gamma receptor 1 (Fc $\gamma$ RI)”) is a type of integral membrane glycoprotein that binds monomeric IgG-type antibodies with high affinity. After binding IgG, CD64 interacts with an accessory chain known as the common  $\gamma$  chain ( $\gamma$  chain), which possesses an ITAM motif that is necessary for triggering cellular activation. Structurally, CD64 is composed of a signal peptide that allows its transport to the surface of a cell, three extracellular immunoglobulin domains of the C2-type that it uses to bind antibody, a hydrophobic transmembrane domain, and a short cytoplasmic tail. CD64 is constitutively found on only macrophages and monocytes, but treatment of polymorphonuclear leukocytes with cytokines like IFN $\gamma$  and G-CSF can induce CD64 expression on these cells. Epitope clustering, interestingly, can be detected by the CD64 assay, but not the C1q assay. HLA antibodies bind to distinctly small areas of HLA molecules that are termed epitopes. Moreover, individually unique epitopes may be expressed by multiple unique HLA molecules (that are encoded by unique HLA alleles at differing loci). Epitopes expressed on more than one HLA antigen are termed “public” epitopes, whereas epitopes expressed on only a single unique HLA antigen are termed “private” epitopes. In SAB assays, HLA antigens expressing a public epitope will tend to cluster together, a phenomenon that is replicated by the CD64 assay, but not the C1q assay.

**[0066]** In one aspect, the biological sample may be contacted to a substrate conjugated to an antigen prior to contact with said Fc receptor operatively linked to a detectable label. The antigen conjugated to the substrate may be selected from an HLA antigen, an antigen that is not an HLA antigen that evokes an antibody response against a transplanted organ or tissue, an antigen that evokes an allergic response, or a combination thereof. The substrate may be washed prior to contact with the Fc receptor operatively linked to a

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detectable label. The biological sample and the substrate conjugated to an antigen may then be incubated for a period of time sufficient to result in a first unit comprising said substrate operatively linked to said anti-HLA antibody. The substrate-anti-HLA complex may then be incubated with a Fc receptor operatively linked to a detectable label for a period of time sufficient to form a second unit comprising a substrate-anti-HLA complex and a Fc receptor operatively linked to said detectable label, wherein the second unit may be detectable. In this aspect, the subject may be positive for anti-HLA antibodies.

**[0067]** In one aspect, when an antibody of interest is detected in a donor subject, a treatment may be administered to a recipient subject who will or has received an organ, cells, or tissue from said donor subject having a antibody. The treatment may be, for example, selected from intravenous immune globulin preparations, plasmapheresis, protein A adsorption columns (or analogous columns), rituximab, obinutuzumab (or other B cell depleting agents), bortezomib, carfilzomib (or other proteasome inhibitors), anti-IL6 antibody (e.g., tocilizumab), anti-BAFF antibody (e.g., belimumab), and combinations thereof.

**[0068]** The disclosed methods may be used for a variety of purposes, for example, to determine the presence of HLA antibodies, to select a donor for transplantation, to identify a suitable organ recipient, to stratify damage potential of an organ recipient to a donor organ, to determine long term prognosis in a donor recipient, or to determine the effectiveness of immunosuppressive agents, wherein said immunosuppressive agent prevents development of DSA with significant damage potential. In one aspect, the assay may be used post-transplantation to determine damage potential due to antibodies, wherein, if a high damage potential is determined, the method may comprise the step of administering to a donor recipient a rejection treatment, wherein the rejection treatment may be selected from one or more of the following treatments: intravenous immune globulin preparations, plasmapheresis, protein A adsorption columns (or analogous columns),

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rituximab, obinutuzumab (or other B cell depleting agents), bortezomib, carfilzomib (or other proteasome inhibitors), anti-IL6 antibody (e.g., tocilizumab), anti-BAFF antibody (e.g., belimumab), and combinations thereof.

**[0069]** In one aspect, the assay may be used to provide additional information beyond the results of the pre-transplant single antigen bead (SAB) assay, such that individual donor specific antibodies could be assessed for their potential to mediate injury to the allograft in either acute, subacute or chronic fashion. For example, if a patient had several potential living donors against which the recipient DSA, a potential for damaging the transplanted organ would exist. The Fc receptor assay may be used to assess such risk. Similarly, the assay results may be used to make decisions on deceased donor kidneys when selecting recipients from the candidate list. In this aspect, for optimal results, one may avoid recipients with DSA determined by the assay to have substantial damage potential (against the donor organ) following transplantation.

**[0070]** In one aspect, the assay results could be used after transplantation to detect DSA that have damage potential. In this aspect, the assay results could aid significantly in the decision whether to treat an individual DSA when detected. Further, the assay results could be used to assess the effects of rejection treatment and thereby aid in the decision as to whether additional treatment is warranted (e.g., with plasmapheresis, IVIG, rituximab, or bortezomib).

**[0071]** In one aspect, the Fc receptor assay may provide prognostic information as to whether DSA that are detected in patients with good function of their transplant, who may be at increased risk for long term, chronic injury. In this aspect, the assay results could help in decision making regarding therapy.

**[0072]** In one aspect, the assay may be a useful biomarker for clinical trial, in that when new immune suppressive agents are developed, an agent that prevents

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development of DSA with significant damage potential would indicate a preferred therapeutic.

**[0073]** Uses for the disclosed FcR assay include, but are not limited to estimation of risk imposed by a population of anti-HLA Abs in a patient who is awaiting transplantation (aid in decision regarding desensitization, aid in decision regarding induction immunosuppression, aid in decision regarding maintenance immunosuppression); Estimation of risk imposed by DSA(s) after transplantation (with AMR, or in the absence of AMR (but is at risk for chronic allograft injury); to guide therapeutic decisions (AMR, desensitization) and in populations (kidney transplant, heart transplant, lung transplant, pancreas transplant, intestinal transplantation, liver transplantation, islet transplantation).

**[0074]** **Examples**

**[0075]** In developing the CD64 FcR assay, samples were run in triplicates and analyzed on both Luminex 200 and 500 platforms. Luminex SA (Single Antigen) beads were tested (from both vendors- One Lambda/ThermoFisher and Immucor) (12 sera for validation, class 1 and II). The intra- and inter-run variability was found to be less than 10%. 60 patient sera from transplant rejection cases were tested in parallel with IgG, IgG1, IgG2, IgG3, IgG4 and C1q assays (720 tests by Luminex SA) along with 10 proficiency test samples and the 12 sera for validation. Patient data from IRB approved UC Transplant Master database was used, which included Banff histologic component scoring, Demographic data, and Renal allograft function and allograft survival data. The data showed that g (Glomerulitis) Banff Component Score in AMR correlated with FcR assay but not the C1q assay. FIG 9.

**[0076]** Example 1.

**[0077]** METHODS: After initial internal validation on PT samples, 60 patients (patients) with antibody mediated rejection (AMR) diagnosed by renal allograft biopsy constituted the study population. Pathologic data included

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Banff component scoring and C4d staining. Serum samples obtained prior to transplantation, at the time of AMR diagnosis and following AMR treatment were analyzed by Luminex based HLA single antigen bead (SAB) microarrays, C1q assay, IgG isotype-specific SAB assays (IgG1, IgG2, IgG3 and IgG4), and Fc Receptor (FcR) binding assay. FcR assays were performed according to standard SOPs for the laboratory-developed test.

**[0078]** RESULTS: FcR assay inter-and intra-run CVs were <20%. Correlation between SAB assay Ab strength and FcR assay was high ( $r=0.70$ ,  $p = 0.0075$ ). In contrast, correlation between IgG assay and C1q assay was lower (0.57), which was primarily due to negative C1q assay results when HLA Ab strength was moderate or low, whereas assays were routinely positive. When anti-HLA-DQ specific HLA Abs were excluded, FcR assay and SAB assay correlations remained high (0.703), whereas C1q assay correlation with SAB assay declined ( $r=0.42$ ). In 14 patients with AMR and low strength donor specific antibodies (DSA) (<2000MFI by SAB assay) C1q assays were routinely negative (14/14), whereas 10/14 (70%) patients were positive by assay ( $p=0.0004$ ). 10 patients with AMR and moderate strength DSA (4000-8000 MFI), 1 of 10 (10%) had positive C1q assay, whereas 8/10 (80%) patients had a positive FcR assay ( $p=0.007$ ). FcR assay results also positively correlated with IgG1 and IgG3 isotype-specific SAB assay strength.

**[0079]** Banff component acute glomerulitis (g) scoring correlated with ( $p = 0.01$ ), but not C1q ( $p=0.92$ ) assays, with similar results for chronic glomerulitis (cg) scoring ( $p=0.033$ ) C1q ( $p =0.49$ ).

**[0080]** Analysis of death censored graft survival following AMR revealed that patients with DSA possessing weak FcR binding activity had substantially extended graft survival as compared to patients with moderate FcR binding DSA or with strong FcR binding DSA.

**[0081]** Conclusions: binding capacity of HLA antibodies provides information unique to that derived from SAB and C1q assays, and correlates with 1) SAB testing,



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2) IgG1 and IgG3 isotype specific SAB testing, 3) g and cg Banff component scoring, and 4) death censored renal allograft survival following AMR. This initial validation analysis indicates that assessment of FcR binding capacity of HLA antibodies provides useful clinical information.

- [0082]** Testing Protocol: The present example is a qualitative microbead multiplex immunoassay for the in vitro diagnostic detection of Fc Receptor binding to HLA-specific antibody in serum. Measurement for the presence of FcR binding antibody is performed as an aid in transplantation patients and/or candidates with humoral allo-response. Positivity is an indicator of allo-antibody binding to FcR.
- [0083]** The assay is optimized for single-HLA antigen bead array. Soluble Fc receptor is commercially available and is derived from recombinant DNA technology. Fluorochrome-labeled anti-Fc receptor antibodies are commercially available either as monoclonal or polyclonal preparations.
- [0084]** The assay is a multiplex bead array assay based on indirect detection of protein binding. Serum is incubated with HLA-coated, internally dye-labeled microbeads. If present, circulating HLA-specific antibody will bind to corresponding epitopes of HLA antigen-coated beads. After washing, soluble recombinant Fc-gamma Receptor is then incubated with the beads. After a second wash, potentially FcR binding to the HLA-specific antibody is further tested by the anti-Fc gamma Receptor -labeled antibody, PE-fluorescence being detected by multiplex (Luminex) platform. Identification of -I binding is obtained by the analysis of fluorescence signal.
- [0085]** FcR binding capacity of HLA antibodies provides information unique to that derived from SAB and C1q assays. Furthermore, analysis of death censored graft survival following ABMR revealed that patients with DSA possessing weak binding activity had substantially extended graft survival as compared to patients with moderate binding DSA or with strong binding DSA.

**[0086]** The main conclusions of the validation studies were: a) Antibody patterns (antibody strength, specificity, class, subclass, C1q binding, Fc Receptor I binding) in early AMR are different from late AMR; b) therapeutic response is influenced by the antibody pattern.

**[0087]** Immediate clinical applications of antibody binding assays will be represented on the one hand by the increase in transplantability of candidates with high levels of antibodies and, on the other hand, by strategies to control the pathology mediated by antibody response against allografts.

**[0088]** Procedure

**[0089]** Method: Microarray

**[0090]** Instrument: LABScan 200 or LABScan 500

**[0091]** ANALYTICAL PRINCIPLE

**[0092]** The method is a multiplex bead array assay based on indirect detection of protein binding. Serum is incubated with HLA-coated, internally dye-labeled microbeads. If present, circulating HLA-specific antibody will bind to corresponding epitopes of HLA antigen-coated beads. After washing, soluble recombinant Fc-gamma Receptor is then incubated with the beads. After a second wash, potentially FcR binding to the HLA-specific antibody is further tested by the anti-Fc gamma Receptor-labeled antibody, PE-fluorescence being detected by multiplex (Luminex) platform. Identification of -I binding is obtained by the analysis of fluorescence signal.

**[0093]** SPECIMEN REQUIRED

**[0094]** Serum (preferred) or Plasma (EDTA or ACD)

**[0095]** Specimen Type & Handling

Criteria	
<b>Type</b>	Serum
<b>-Preferred</b>	Plasma
<b>-Other</b>	
<b>Acceptable</b>	

Criteria	
<b>Collection Container</b>	Red top with no additive(s)
<b>Volume - Optimum - Minimum</b>	8.5 mL draw 150µL of serum
<b>Transport Container &amp; Temperature</b>	Red top vacutainer tube Room temperature or refrigerated
<b>Stability &amp; Storage Requirements</b>	Room 4 days Temperature:
	Refrigerated: 7 days
	Frozen: Indefinitely
<b>Timing Considerations</b>	Samples over 7 days old upon receipt may be unacceptable.
<b>Unacceptable Specimens &amp; Actions to Take</b>	Fibrin may be removed from sample. Lipemic samples may be spun down and fat pulled off.
<b>Compromising Physical Characteristics</b>	Grossly hemolyzed samples are unacceptable.
<b>Other Considerations</b>	Samples should not be heat inactivated, because this might cause a high background in the test.

[0096] REAGENTS AND SUPPLIES

[0097] LABScreen® Beads from One Lambda, Inc. Products are stored at -40°C. In use are thawed and stored at 2-8°C. LS1A04 - LABScreen® Single Antigen HLA Class I – Combi Detection of Class I antibodies and their specificities. LABScreen® Class I Single Antigen Beads (Cat# LSP1AB04) -125 µl per vial LS2A01 - LABScreen® Single Antigen HLA Class II Antibody Detection Test – Group 1; Detection of Class II antibodies and their specificities. LABScreen® Class II Single Antigen Beads –Group 1 (Cat# LSP2AB01) -125 µl per vial; LSNC - LABScreen® Negative Serum Controls LABScreen® Negative -250 µl per vial; LABScreen Wash Buffer 10X (One Lambda Inc. Cat# LSPWABSUF); Dilute to 1X Wash Buffer = 12ml buffer + 108 ml of DI water; Store at 2-8°C, diluted buffer expires in 3 months. DPBS – Dulbecco’s Phosphate Buffered Saline w/o Calcium or Magnesium (Lonza Cat# 17-512F) Store at 2-8°C; Anti-Human Fc gamma Receptor PE, eBioscience; Human FcR Protein (His Tag), Sino Biological Inc.; Sheath fluid Lx100 (Luminex Cat# 40-50000) Store at 15-24°C; Tray seals (One Lambda Inc. Cat#

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SSPSEA300); Whatman Uniplate, 96 wells, 250 µl microplate (Whatman Cat# 7701-3250); Deionized water

**[0098]** INSTRUMENTATION

**[0099]** LABScan 100/200™ or LABScan 3D Flow Analyzer; One Lambda Fusion Software; Fisher Scientific Marathon 16KM Centrifuge; Micro-centrifuge; Vortex mixer with adjustable speed

**[00100]** Equipment Calibration Data

**[00101]** Special instrument Requirements: Luminex Platforms (200 and 500 tested) with a reporter laser wavelength=532nm and a classification laser wavelength=635 nm.

**[00102]** Device Description: The in vitro diagnostic reagent kit contains sufficient reagents for 96 samples. The reagents consist of the following: One vial lyophilized Anti-Human Fc gamma Receptor Phycoerythrin (PE), one vial Human FCGR1A Protein (His Tag) and one vial each of positive and negative controls.

**[00103]** PROCEDURE

**[00104]** 1. Thaw patient serum/plasma and centrifuge 3000rpm for 10 minutes.

**[00105]** 2. Vortex LABScreen® Beads and quick spin to remove from lid top.

**[00106]** 3. Pipette 2.5 µl of appropriate LABScreen® Beads into each well.

**[00107]** 4. Pipette 20 µl of serum into the specified well of the 96-well plate.

**[00108]** 5. Pipette 20 µl of LSNC for each bead type. (This is used when importing into Fusion)

**[00109]** 6. Seal the wells with a tray seal. NOTE: Use a fresh seal for each step that requires application of a tray seal.

**[00110]** 7. Incubate for 30 minutes at room temperature 20-25µC.

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- [00111] 8. After incubation, add 150  $\mu$ l of 1X wash buffer to each well.
- [00112] 9. Cover with tray seal and gently vortex. Centrifuge at 2800 rpms in Thermo IEC Centra-8R or CL40. Centrifuge for 4 minutes.
- [00113] 10. Remove seal from plate and flick out wash solution over trash. After flicking and while tray is still inverted, strike inverted tray 3-6 times onto a paper towel to blot.
- [00114] 11. Add 200  $\mu$ l of 1X wash buffer to each well and repeat steps 9-10.
- [00115] 12. Dilute Fc gamma Receptor Protein with DPBS 1:10.
- [00116] 13. Add 20  $\mu$ l of diluted Fc gamma Receptor Protein into each appropriate well. Gently mix each well with pipette tips and cover.
- [00117] 14. Incubate for 30 minutes at room temperature 20-25°C.
- [00118] 15. Centrifuge at 2800 rpms in IEC Centra-8R Centrifuge for 4 minutes.
- [00119] 16. Remove seal from plate and flick out wash solution over trash. After flicking and while tray is still inverted, strike inverted tray 3-6 times onto a paper towel to blot.
- [00120] 17. Add 200  $\mu$ l of 1X wash buffer to each well and repeat steps 15-16 for a total of three (3) washes.
- [00121] 18. Dilute Anti-Human FcR PE with DPBS 1:10.
- [00122] 19. Add 50  $\mu$ l of diluted Anti- Fc gamma Receptor PE into each appropriate well. Gently mix each well with pipette tips and cover.
- [00123] 20. Incubate for 30 minutes at room temperature 20-25°C.
- [00124] 21. After incubation, add 150  $\mu$ l of 1X wash buffer to each well.
- [00125] 22. Cover with tray seal and gently vortex. Centrifuge at 2800 rpms in Thermo IEC Centra-8R or CL40. Centrifuge for 4 minutes.

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- [00126] 23. Remove seal from plate and flick out wash solution over trash. After flicking and while tray is still inverted, strike inverted tray 3-6 times onto a paper towel to blot.
- [00127] 24. Add 200 l of 1X wash buffer to each well and repeat steps 22-23.
- [00128] 25. Add 80-100 l 1X DPBS to each well and mix with pipette tips in each well.
- [00129] 26. The sample is ready for data acquisition and analysis.
- [00130] Interpretation
- [00131] Internal PC shall be at least 6x the internal NC.
- [00132] Results are established by comparing the FcR results to the standard PE. Review for epitopes and patterns.
- [00133] Use a ratio to the positive control bead:
- [00134] Bead – NC bead
- [00135] PC bead –NC bead
- [00136] ANALYTICAL PERFORMANCE
- [00137] Precision/Reproducibility: Studies were performed using 24 characterized samples and 8 samples from CAP proficiency test samples. Both vendors for Luminex SA beads were tested, for both anti-HLA class I, and anti-HLA class II reactivity.
- [00138] Intra-assay variability was <20%; FcR binding assay was run in triplicates.
- [00139] Inter-assay variability was <20%. Assay was performed on both on Luminex 200 and 500 platforms.
- [00140] Linearity/assay reportable range: Range of results is from 0 to 10000 MFI. Internal PC must be at least 6x the internal NC. Results are established by comparing the FcR results to the standard PE, based on epitope pattern, NBG

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ratio (at least three times higher than NC or self the highest self-antigen bead, whatever is highest) and MFI (at least 10% of PC or the highest reactive bead in the panel, whatever is highest).

[00141] Table. Comparison Studies

Sample #	Antibody Range	FcR	C1q
1	Strong	A2,68,69; B57,58	A2,68,69
2	Strong	Bw6; A33,34,66	A2,68,69, Bw6
3	Strong	B7,81,60,48,27,13,61	B7,81
4	Strong	A23, 24, 25, 32; Bw4	A23,24 Bw4
5	Moderate	B7,81,60,48,41,61,42,47,13,27	NEG
6	Moderate	B57,58,8	B57 B58
7	Moderate	A29, B8,18,35,51,53	NEG
8	Moderate	12 CREG	B41, 44, 45, 46, 49, 50, 60, 61 (12CREG)
9	Weak	A2, B57,58	NEG
10	Weak	Bw4	NEG
11	Weak	A24, A1	NEG
12	Weak	B44,45,76, 82; A2	NEG
13	Strong	DR51,53; DQ2,4,7,8,9	DR53 DQ2,4,7,8,9
14	Strong	DR1,103,15,16, DR51	NEG
15	Strong	DR1,7,9,10,14; DR53; DQ4,5,6,8,9	DR7,10; DR53; DQ4,5,6,8,9
16	Strong	DQ2, DQA1*04,05	DQ2, DQA1*0501
17	Moderate	DR8,11,12,13,14,17,18	NON-SPECIFIC
18	Moderate	DR4	NEG
19	Moderate	DR53	NEG
20	Moderate	DQ4,8,9	NON-SPECIFIC
21	Weak	DQ5, DP1	NEG
22	Weak	DQ3	NEG
23	Weak	DR17,18 DR53	NEG
24	Weak	DR12	NEG
25	Unknown	B:48 60 7 81	B:48 60 7
26	Unknown	A2 23 24 80 B76	A:23 24 80 B:76
27	Unknown	A1 B13 41 44 45 47 49 50 60 61 76 82	B:13 41 44 45 49 50 60 61 76 82

28	Unknown	A:2 24 68 69 B57 58	A:2 24 68 69; B:54 57 58
29	Unknown	DQ:2 4 5 6 7 DQA:04 05 06	DQ:4 5 6; DQA:04 05 06
30	Unknown	DR7	NEG
31	Unknown	DR:1 9 10 103 DRw:51	DR:1 9 10 103 DR51
32	NEG	NEG	NEG

[00142] Table. Matrix Comparison

Epitope concordance in C1q versus FcR		
Antibody Range	FcR	C1q
Strong	8	7
Moderate	8	2
Weak	8	0
PT (strong)	8	7

[00143] QC procedures

[00144] Comparison between SAB and using well defined positive and negative sera from CAP PT. Good correlations, both for positive and negative sera. Comparison between C1q and I-binding assay using well defined positive sera from renal transplant patients with ABMR. Applicant identified 35 patients with antibody mediated rejection (ABMR: 11 early - EAMR + 24 late - LAMR). DSA levels were determined at Day 0 and Day 50 after renal transplantation. All patients had at least 9 months of follow-up after ABMR therapy. The IgG1-4-specific antibody was obtained from SouthernBiotech. All cases had DNA typing for HLA-A, B, C, DRB1,3,4,5, DQA1, DQB1, DPB1 loci and negative flow T- and B-cell crossmatches. All samples were tested for the DSA detection (single-antigen beads Luminex) and post-therapeutic dynamics by IgG1-4 subtype and the disclosed methods. For comparison, all these 70 samples were tested with the commercially available C1q binding assay. Applicant compared results in 33 donor-specific antibody DSAs for early ABMR, and 52 for late ABMR and compared 21 preformed DSAs and 64 de novo DSA. The assay results of the instant disclosure were



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correlated with results from the C1q binding assay, IgG subtype binding assay, public epitope clustering and histo-pathological rejection scores. Correlation between C1q and MFI values ( $R=0.5$ ,  $p<0.05$ ). It can be noticed that FcR MFI  $<2500$  are negative by C1q, which demonstrates higher sensitivity for Applicant's assay. C1q and assays correlate with SAB assay, but FcR has superior sensitivity for weak-moderate Ab strength.

[00145] Example 2. Biotin-conjugated protein (CD64) Assay (See Fig 1B)

[00146] Reagents

[00147] Anti-Human CD64 (Fc gamma Receptor 1) PE, eBioscience # 12-0649 (Concentration: 5 $\mu$ L (0.25  $\mu$ g)/test Clone 10.1 mouse IGG1 0; Human CD64/FCGR1A Protein (His Tag), Sino Biological Inc. #10256-H08H (recommended 380  $\mu$ l of sterile water added to vial); Human CD64.FCGR1 Protein His & AVI Tag, Biotinylated; Sino Biological Inc. #10256-H27H-B; Anti-Human PE Streptavidin; eBioscience #12-4317-87.

[00148] 2-Step Biotin

[00149] 1. Add serum 25  $\mu$ L and beads 1.8  $\mu$ L.

[00150] 2. Incubate for 30 minutes, room temp on shaker

[00151] 3. Using LabScreen Wash Buffer, perform 3 washes (4 min @ 2800 rpm)

[00152] 4. Add 20  $\mu$ L of CD64- biotin conjugated protein @ 1:10

[00153] 5. Add 20  $\mu$ L of Streptavidin-PE @ 1:10

[00154] 6. Incubate for 30 minutes, rm temp

[00155] 7. Using LabScreen Wash Buffer, perform 2 washes

[00156] 8. Add 80  $\mu$ L DPBS and run on Luminex

[00157] 3-Step-Classic CD64 Protein

[00158] 1. Add serum and beads.

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- [00159] 2. Incubate for 30 minutes, room temp in dark on rocker (3-4 min @ 2800 rpm)
- [00160] 3. Using LabScreen Wash Buffer, perform 2 washes
- [00161] 4. Add 20uL of CD64 at dilution of 1:10 using DBPS
- [00162] 5. Incubate for 30 minutes, room temp
- [00163] 6. Using LabScreen Wash Buffer, perform 3 washes (3-4 min @ 2800 rpm)
- [00164] 7. Add 50uL of CD64-PE at 1:10 using wash buffer
- [00165] 8. Incubate for 30 minutes, rm temp
- [00166] 9. Perform 2 washes (3-4 min @ 2800 rpm)
- [00167] 10. Add 80  $\mu$ L DPBS and run on Luminex
- [00168] Example 3. Serum is collected from a transplant recipient in a tube where blood is allowed to form thrombus. Serum is separated from the clot by centrifugation. 50 $\mu$ L of serum is combined with 5 $\mu$ L single HLA antigen beads (from either Immucor or One Lambda/Thermo Fisher. The serum and beads are incubated for 30 minutes at room temperature. Two washes are performed with a wash buffer (as provided by the manufacturer). 50  $\mu$ L of recombinant CD64, diluted 1:50 in wash buffer (usually phosphate buffered saline) is added, and incubation performed for 30 minutes at room temperature. The beads are then washed three times using the wash buffer. 50  $\mu$ L of PE conjugated murine anti-human CD64 antibody (BioLegend) at a dilution of 1:50 is added, followed by a 30-minute room temperature incubation. The beads are washed one time with wash buffer. 80  $\mu$ L of wash buffer is added to the beads and beads are then analyzed on the Luminex platform.
- [00169] In other aspects, other Fc receptor molecules may be used for analysis including: 1) Fc $\gamma$ RIIIa (CD16A), 2) Fc $\gamma$ RIIIb (CD16B), 2) Fc $\gamma$ RIIa (CD32A), 3) Fc $\gamma$ RIIb (CD32B), 4) Fc $\gamma$ RIIc (CD32C), 5) Fc $\mu$ R, 6) Fc $\epsilon$ RI, 6) Fc $\epsilon$ RII, 7)

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Fc $\alpha$ R, or 7) DC-SIGN. Alternatively, an FcR preparation may be derived from a patient (via a process such as immunoprecipitation) and the individual patients FcRs may be used in the assay rather than the recombinant FcR. In one aspect, rather than use a secondary antibody to the recombinant Fc receptor, one may directly conjugate the fluorescent marker (or a molecule such as biotin, where an avidin-linked fluorochrome (or other marker) may be added) directly to the recombinant Fc receptor.

**[00170]** Example 4. Serum is collected from a transplant recipient in a tube where blood is allowed to form thrombus. Serum is separated from the clot by centrifugation. 50 $\mu$ L of serum is combined with 5 $\mu$ L single HLA antigen beads (from either Immucor or One Lambda/Thermo Fisher. The serum and beads are incubated for 30 minutes at room temperature. Two washes are performed with a wash buffer (as provided by the manufacturer). 50  $\mu$ L of recombinant CD16, appropriately diluted in wash buffer (usually phosphate buffered saline) is added, and incubation performed for 30 minutes at room temperature. The beads are then washed three times using the wash buffer. 50  $\mu$ L of PE conjugated murine anti-human CD16 antibody is added at appropriate dilution, followed by a 30-minute room temperature incubation. The beads are washed one time with wash buffer. 80  $\mu$ L of wash buffer is added to the beads and beads are then analyzed on the Luminex platform.

**[00171]** Example 5. Serum is collected from a transplant recipient in a tube where blood is allowed to form thrombus. Serum is separated from the clot by centrifugation. 50 $\mu$ L of serum is combined with 5 $\mu$ L single HLA antigen beads (from either Immucor or One Lambda/Thermo Fisher. The serum and beads are incubated for 30 minutes at room temperature. Two washes are performed with a wash buffer (as provided by the manufacturer). 50  $\mu$ L of recombinant Fc $\gamma$ RIIb, diluted appropriately in wash buffer (usually phosphate buffered saline) is added, and incubation performed for 30 minutes at room temperature. The beads are then washed three times using the wash buffer. 50  $\mu$ L of PE conjugated murine anti-human Fc $\gamma$ RIIb antibody is added at

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appropriate dilution, followed by a 30-minute room temperature incubation. The beads are washed one time with wash buffer. 80  $\mu$ L of wash buffer is added to the beads and beads are then analyzed on the Luminex platform.

**[00172]** Example 6. Exemplary protocol for a CD64 assay using a patient's own Fc Receptors in lieu of a recombinant Fc receptor. As an alternative to using a recombinant Fc receptor (such as CD64), the assay may be personalized by using an individual patients own Fc receptors as follows: 1. Patient's blood may be drawn and a Ficoll separation performed to isolate mononuclear cells. 2. A lineage specific cell isolation (for example, NK, monocytes-macrophages, lymphocytes, etc.) may be performed. 3. Mononuclear cells may be lysed using a non-denaturing lysis buffer. 4. Immunoprecipitation may be performed by incubating overnight at 4°C an anti-Fc receptor antibody (polyclonal or monoclonal, that targets the Fc receptor of interest) with the cell lysate. 5. Incubate with sepharose beads coupled to protein A or protein G (based on the Ig subtype of the anti-Fc receptor antibody). 6. Elute the captured Fc receptor for use in an Fc receptor assay.

**[00173]** All percentages and ratios are calculated by weight unless otherwise indicated.

**[00174]** All percentages and ratios are calculated based on the total composition unless otherwise indicated.

**[00175]** It should be understood that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

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**[00176]** The dimensions and values disclosed herein are not to be understood as being strictly limited to the exact numerical values recited. Instead, unless otherwise specified, each such dimension is intended to mean both the recited value and a functionally equivalent range surrounding that value. For example, a dimension disclosed as “20 mm” is intended to mean “about 20 mm.”

**[00177]** Every document cited herein, including any cross-referenced or related patent or application, is hereby incorporated herein by reference in its entirety unless expressly excluded or otherwise limited. The citation of any document is not an admission that it is prior art with respect to any invention disclosed or claimed herein or that it alone, or in any combination with any other reference or references, teaches, suggests or discloses any such invention. Further, to the extent that any meaning or definition of a term in this document conflicts with any meaning or definition of the same term in a document incorporated by reference, the meaning or definition assigned to that term in this document shall govern.

**[00178]** While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

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### Claims

What is claimed is:

1. A method for determining the presence or absence of an antibody of interest in a biological sample of a subject, comprising the step of contacting said biological sample from said subject with
  - a. a substrate conjugated to an antigen and;
  - b. an Fc receptor operatively linked to a detectable label;wherein, when said antibody of interest is present, a complex comprising said substrate conjugated to said antigen and said Fc receptor operatively linked to said detectable label is formed;  
wherein detection of said detectable label on said complex indicates that said biological sample contains pathogenic or protective antibodies.
2. The method of claim 1, wherein the absence of detection of said label indicates the absence of said antibody of interest.
3. The method of either of claim 1 or 2, wherein said antibody of interest is selected from pathogenic anti-HLA antibodies, non-HLA pathogenic or protective antibodies, or a combination thereof.
4. The method of any preceding claim, wherein said biological sample is suspected of having anti-HLA antibodies.
5. The method of any preceding claim, wherein said substrate is a solid substrate.
6. The method of any preceding claim, wherein said recombinant Fc receptor is an Fc receptor expressed by an immune cell.
7. The method of any preceding claim, wherein said Fc receptor is one or more recombinant Fc receptor selected from CD64, CD16A, CD16B, CD32A, CD32B, CD32C, Fc $\alpha$ R, Fc $\mu$ R, and DC-SIGN, or an Fc receptor derived from a cell or a biological fluid of said subject.

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8. The method of any preceding claim, wherein said biological sample is contacted to said substrate conjugated to an antigen prior to contact with said Fc receptor operatively linked to a detectable label.
9. The method of any preceding claim, wherein said substrate is washed prior to contact with said Fc receptor operatively linked to a detectable label.
10. The method of any preceding claim, wherein said complex is incubated with said Fc receptor operatively linked to a detectable label for a period of time sufficient to form a second unit comprising said complex and said Fc receptor operatively linked to said detectable label, wherein said second unit is detectable where said subject is positive for said antibody of interest.
11. The method of any preceding claim, wherein the Fc is directly conjugated to a biotin or a fluorochrome or other marker/label.
12. The method of any preceding claim, wherein when an antibody of interest is detected in a donor subject, a treatment is administered to a recipient subject who will or has received an organ, cells, or tissue from said donor subject having an antibody, wherein said treatment is selected from intravenous immune globulin preparations, plasmapheresis, protein A adsorption columns, a B cell depleting agent, a proteasome inhibitor, an anti-IL6 antibody, an anti-BAFF antibody, and combinations thereof, and wherein said antibody of interest is a pathogenic antibody.
13. The method of any preceding claim, wherein said antibody of interest is an HLA antibody.
14. The method of any preceding claim, wherein said assay is used to select a donor for transplantation.
15. The method of any preceding claim, wherein said assay is used to identify a suitable organ recipient.
16. The method of any preceding claim, wherein said assay is used to stratify damage potential of an organ recipient to a donor organ.

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17. The method of any preceding claim, wherein said assay is used post-transplantation to determine damage potential due to an antibody of interest, wherein said antibody of interest is a pathogenic antibody, wherein, if a high damage potential is determined, the method further comprises the step of administering to a donor recipient a rejection treatment, wherein said rejection treatment is selected from intravenous immune globulin preparations, plasmapheresis, protein A adsorption columns, a B cell depleting agents, a proteasome inhibitor, an anti-IL6 antibody, an anti-BAFF, and combinations thereof.
  18. The method of any preceding claim, wherein said assay is used to determine long term prognosis in a donor recipient.
  19. The method of any preceding claim, wherein said assay is used to determine the effectiveness of immunosuppressive agents, wherein said immunosuppressive agent prevents development of a donor specific antibody with significant damage potential.
  20. A kit for determining the presence or absence of an antibody of interest in a biological sample of a subject, comprising
    - a. a substrate conjugated to an antigen and;
    - b. an Fc receptor operatively linked to a detectable label;wherein, when said antibody of interest capable of forming a complex comprising said substrate conjugated to said antigen and said Fc receptor operatively linked to said detectable label;
- wherein detection of said detectable label on said complex can be used to determine the presence of pathogenic antibodies in said sample.



FIG 1A

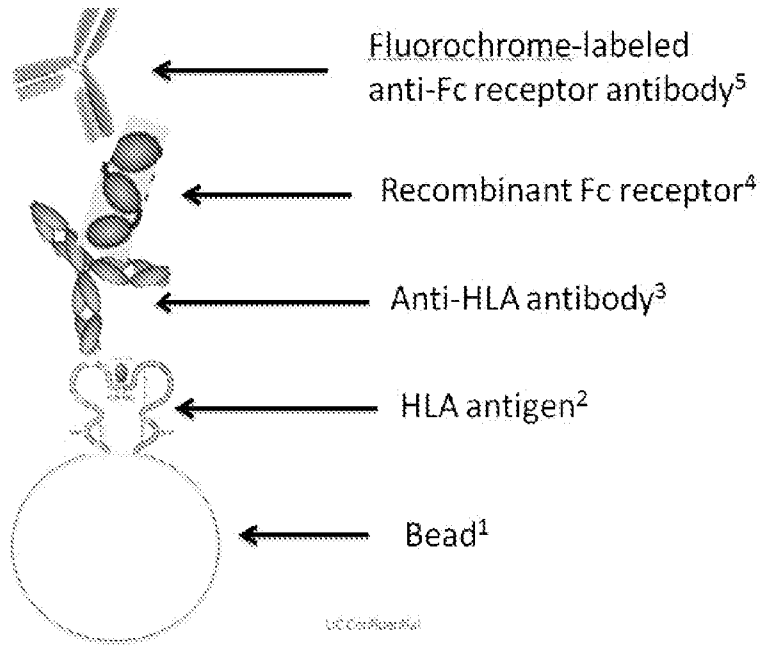


FIG 1B

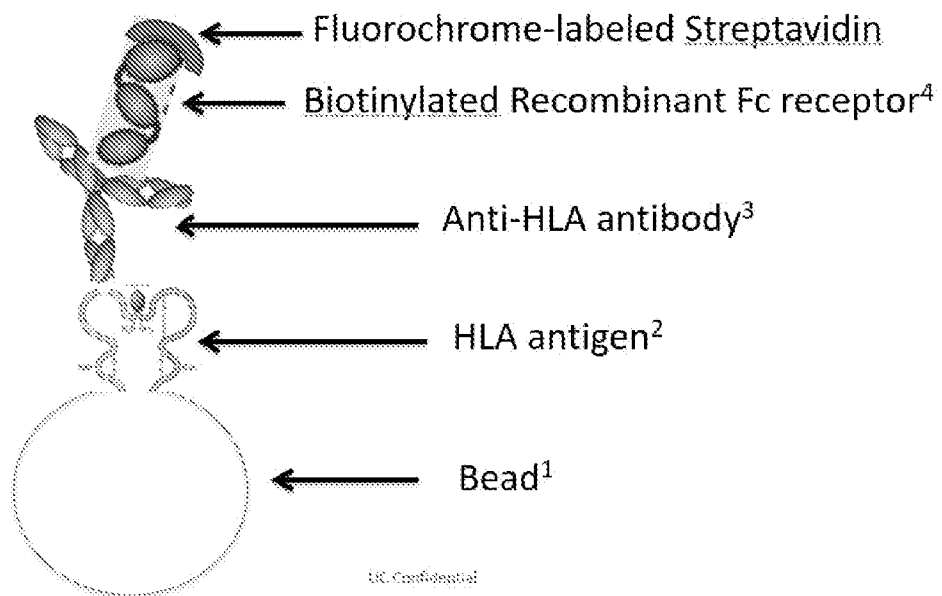
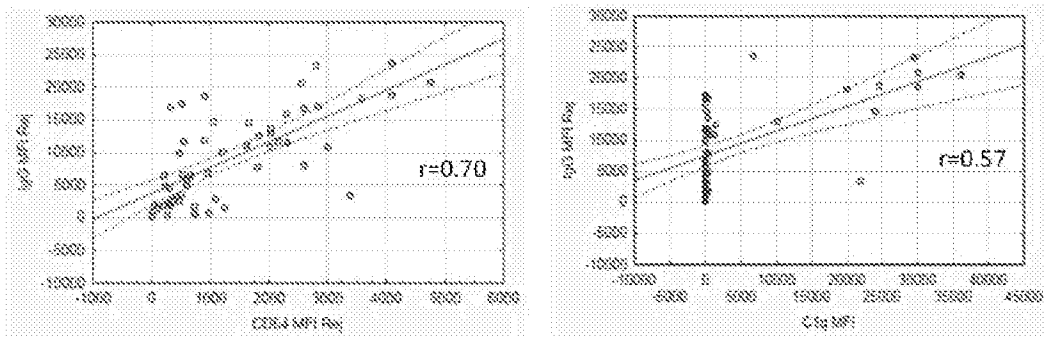


FIG 2

### CD64 FcR Assay Demonstrates Stronger Correlation With SAB Results Than C1q Assay



All cases, n=56

FIG 3

### CD64 FcR Assay But Not C1q Discriminates Between Strong and Weak DSA

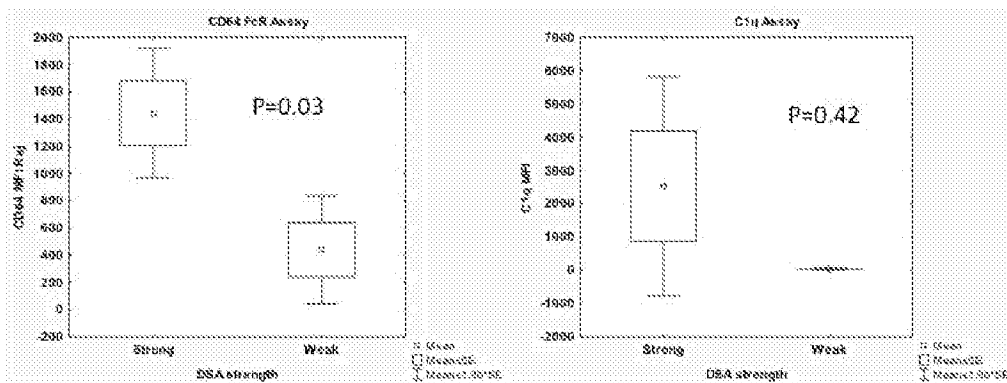
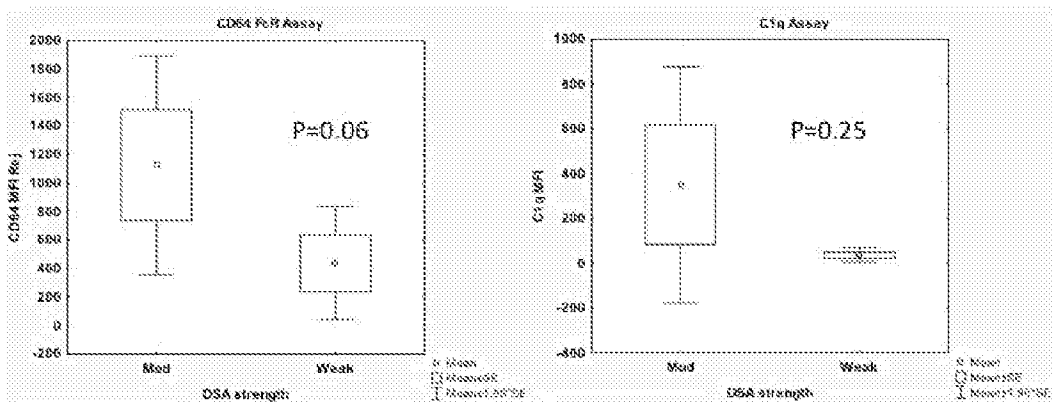


FIG 4

### CD64 FcR Assay But Not C1q Discriminates Between Moderate and Weak DSA



### Moderate Strength DSA Are Associated with Marked Reduction in Allograft Survival

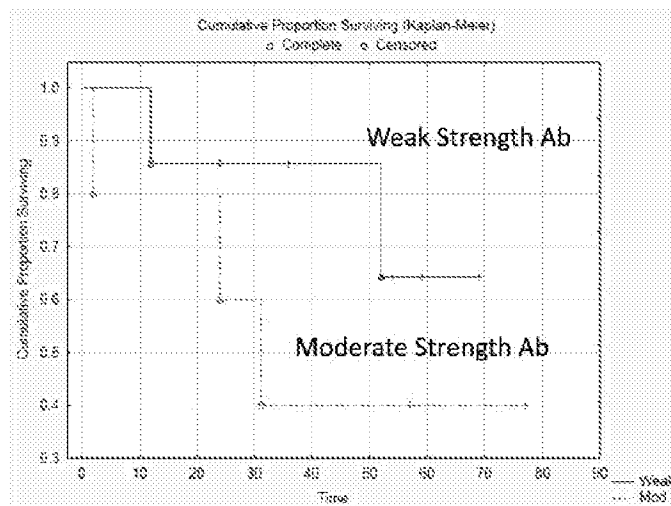


FIG 5

### CD64 FcR and C1q Assay Comparisons: Weak and Moderate Strength Anti-HLA Antibodies

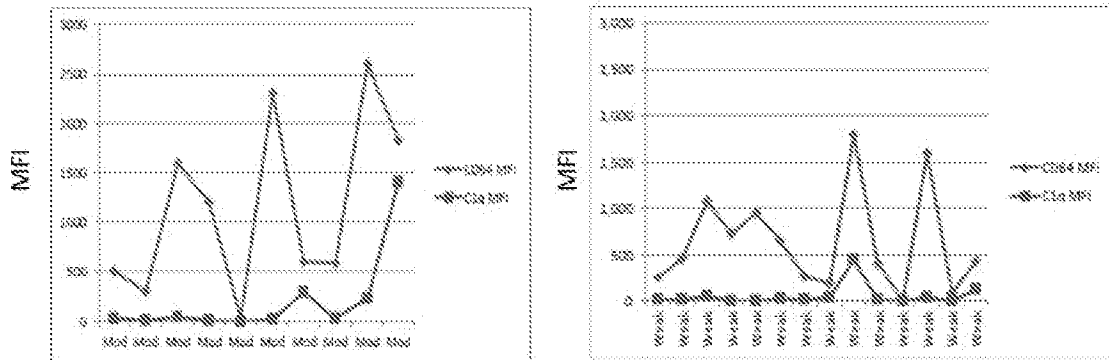


FIG 6

### Public Antigen Epitope Shift Pattern Retained By CD64 FcR Assay

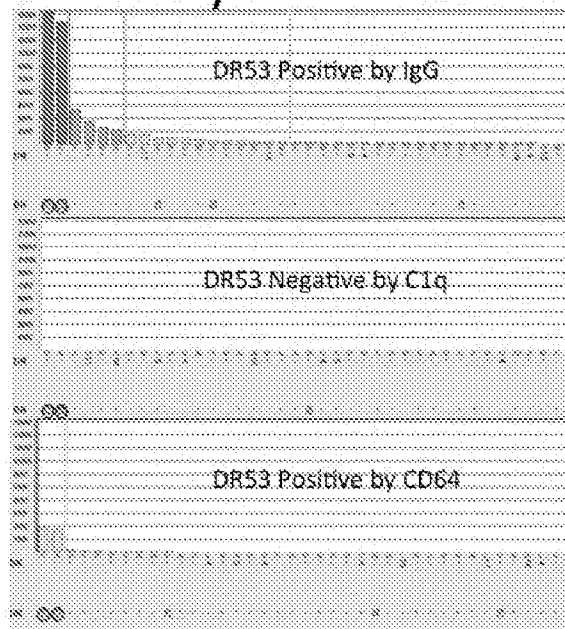
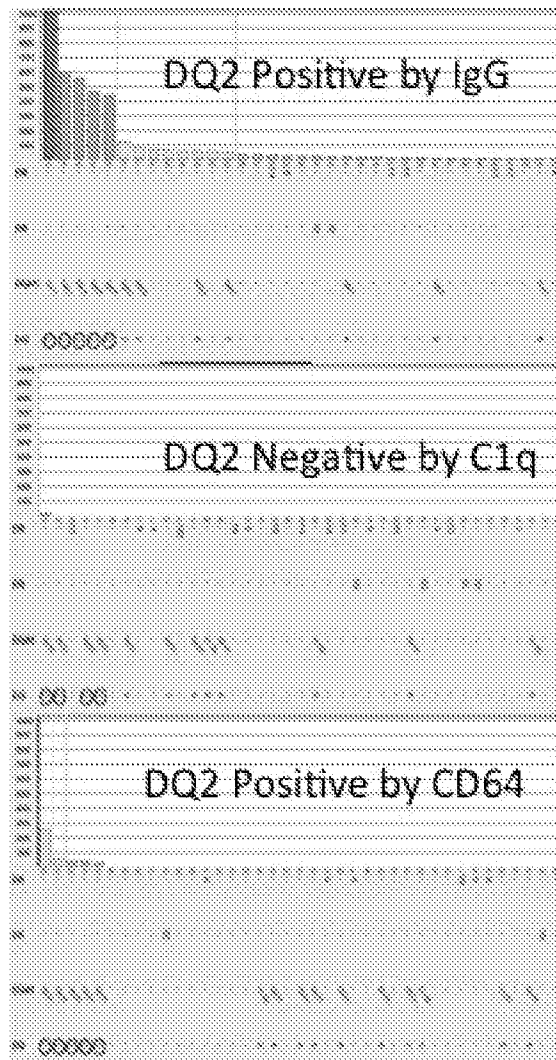


FIG 7

# Private Epitope Shift Pattern Retained by CD64 FcR Assay



HLA B27 DSA With 7 and 5 CREG Epitopes in AMR:

CD64 FcR assay provides results optimal to SAB and C1q Assay

Epitope-based clustering optimally represented by CD64 FcR assay as compared to SAB assay

C1q assay did not provide evidence of epitope clustering

FIG 8

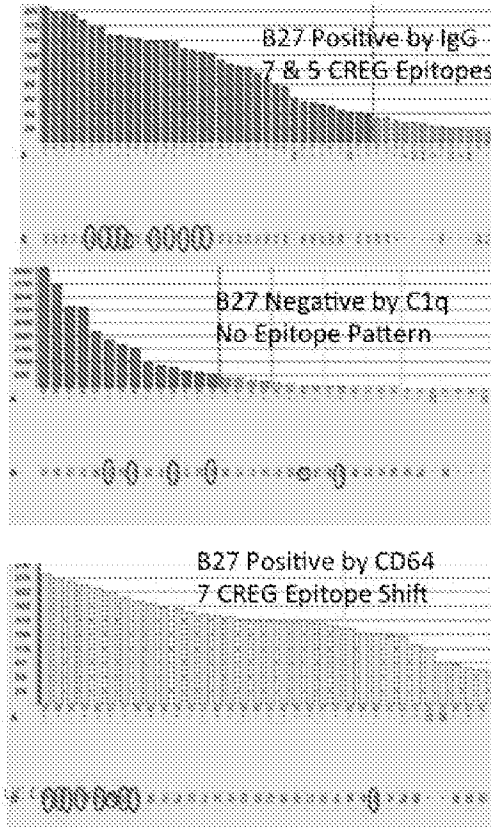


FIG 9

g (Glomerulitis) Banff Component Score in AMR  
Correlation With CD64 FcR Assay but not C1q Assay

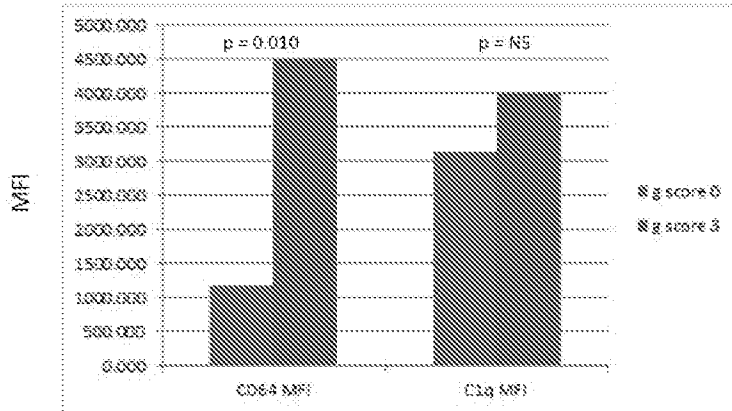
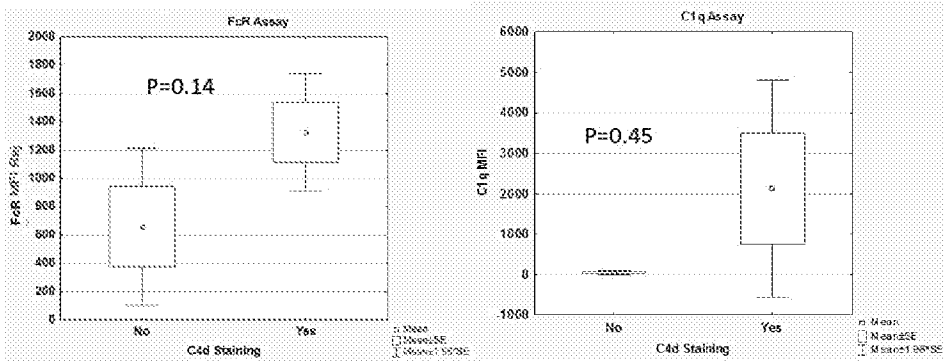


FIG 10

### FcR Assay May Correlate Better with C4d Staining in Biopsy-Proven AMR

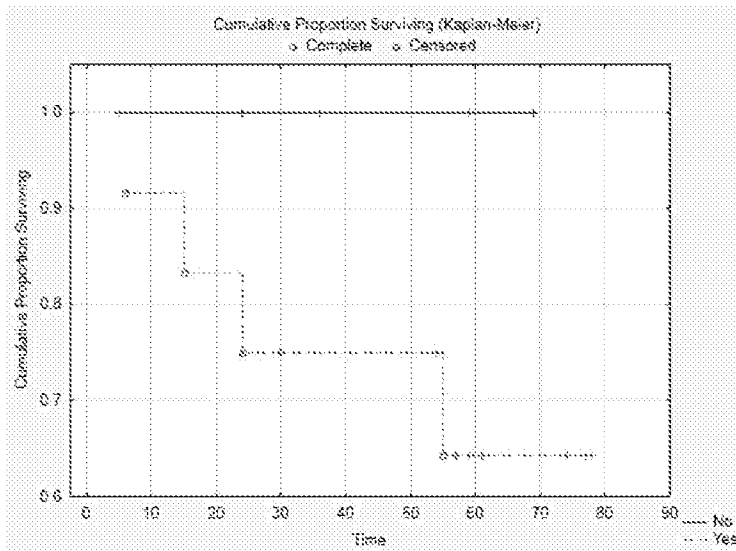


Anti-HLA A, B, DR DSAs



FIG 11

### Renal Allograft Survival after Early AMR: Correlation with FcR Assay



### C1q Assay Does Not Correlate with Allograft Survival

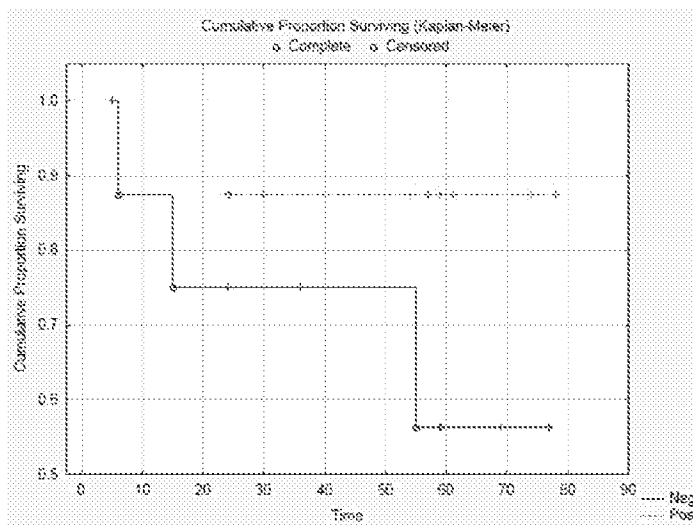
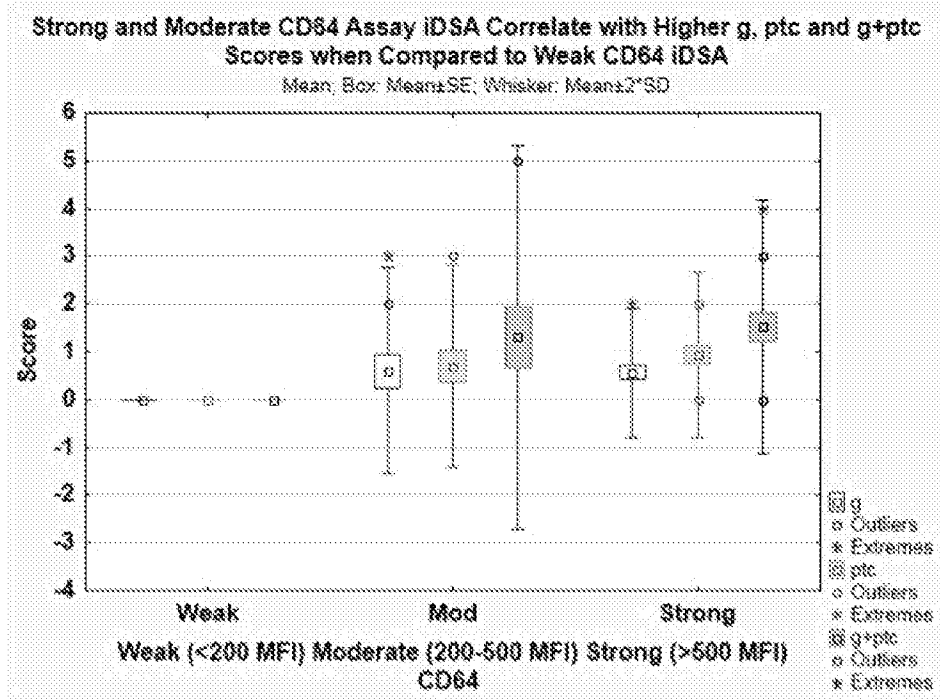


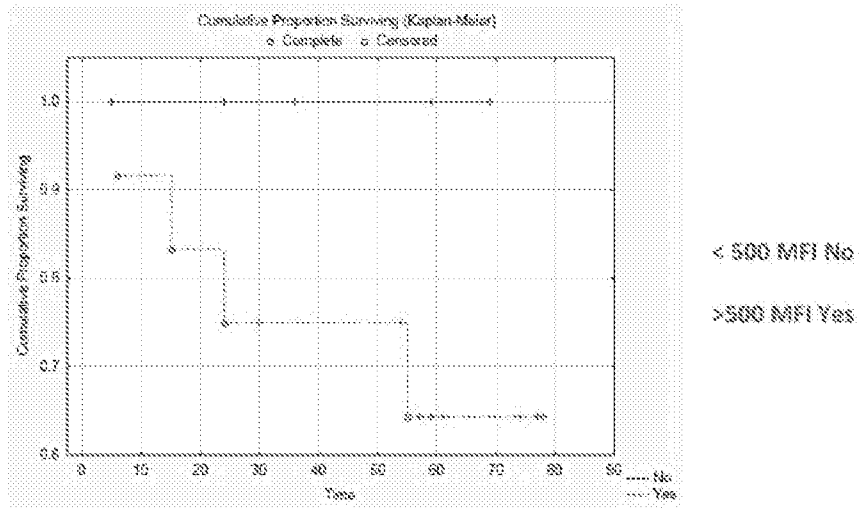
FIG 12



C1q assay shows no correlations

FIG 13

### Cumulative Renal Allograft Survival after Early ABMR Episode in Patients with CD64 < 500 MFI versus >500 MFI



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/48307

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(8) - G01N 33/68 (2016.01) CPC - G01N 33/6857, G01N 33/6854 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC(8) - G01N 33/68 (2016.01) CPC - G01N 33/6857, G01N 33/6854 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC - 436/501; 422/69 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) pubWEST; PatBase; Google Scholar search terms - Antiod\$, detec\$, presenc\$, assay, assay\$, antigen, immobil\$, conjugat\$, surfac\$, support, substrate, Fc, receptor, label, label*, pathogen, pathogenic		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2013/0130404 A1 (MEHRA et al.) 23 May 2013 (23.05.2013) para [0006]; [0022]; [0035]; [0036]; [0046]; [0075]; [0108]-[0113].	1-3, 20
A	US 2009/0124511 A1 (ARCHER et al.) 14 May 2009 (14.05.2009) abstract; para [0012]-[0015].	1
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/>		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 07 October 2016		Date of mailing of the international search report 28 OCT 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/48307

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.: 4-19  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.