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(54) Title: DIAGNOSTIC ASSAY FOR TRYPANOSOMA CRUZI INFECTION

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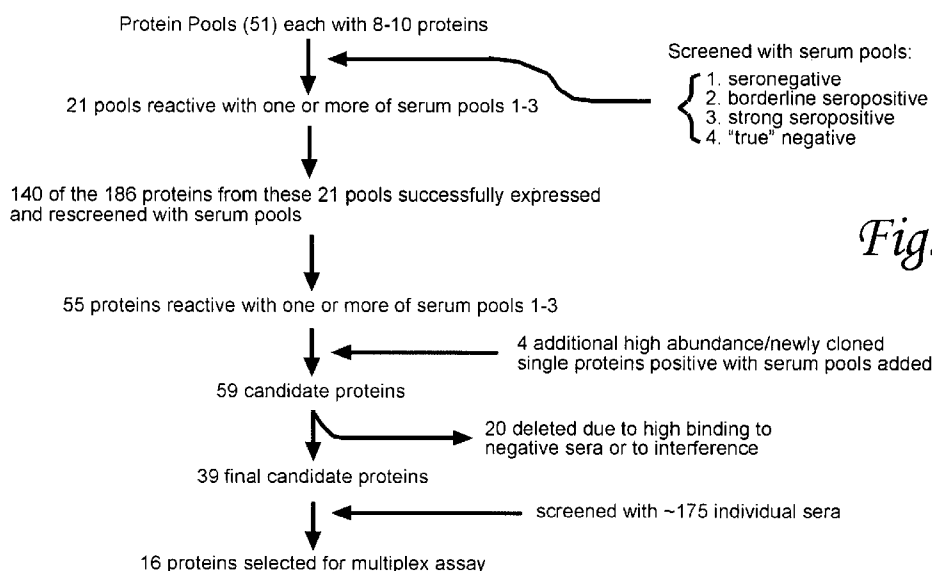


Fig. 7

(57) Abstract: A sensitive, multicomponent diagnostic test for infection with *T. cruzi*, the causative agent of Chagas disease, including methods of making and methods of use. Also provided is a method for screening *T. cruzi* polypeptides to identify antigenic polypeptides for inclusion as components of the diagnostic test, as well as compositions containing antigenic *T. cruzi* polypeptides.

DIAGNOSTIC ASSAY FOR *TRYPANOSOMA CRUZI* INFECTION

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 60/962,498, filed July 30, 2007, and is a continuation-in-part application of U.S. Serial No. 11/587,283, filed October 23, 2006, which is a
10 U.S. National Stage Application of international application PCT/US2005/013777, filed April 22, 2005, which claims the benefit of U.S. Provisional Application Serial No. 60/564,804, filed 23 April 2004, and U.S. Provisional Application Serial No. 60/623,299, filed 29 October 2004, each of which is incorporated herein by reference in its entirety.

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STATEMENT OF GOVERNMENT RIGHTS

This invention was made with government support under Program Project P01 AI0449790 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

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BACKGROUND OF THE INVENTION

Trypanosoma cruzi is an obligate intracellular protozoan parasite. In mammalian hosts *T. cruzi* cycles between a trypomastigote stage which circulates in the blood and the amastigote stage which replicates in the
25 cytoplasm of infected host cells (primarily muscle).

T. cruzi is the etiological agent of Chagas disease and is ranked as the most serious parasitic disease in the Americas, with an economic impact far outranking the combined effects of other parasitic diseases such as malaria, schistosomiasis, and leishmania (Dias et al., Mem. Inst. Oswaldo Cruz, 1999,
30 94:Suppl.1:103). Chagas Disease affects up to 20 million individuals primarily in the Americas where the insect vectors are present and where zoonotic transmission cycles guarantee a steady source of parasites. *T. cruzi* infection has its greatest human impact in areas of Latin America where housing conditions bring people, infected animals, and vector insects into close

proximity. More than 90 million are at risk of infection in endemic areas, and roughly 50,000 children and adults die of chronic Chagas disease every year due to lack of effective treatments. Additionally, 2-5% of fetus carried by infected mothers in endemic areas are either aborted or born with congenital Chagas disease. Loss of revenue in terms of productivity lost due to sickness and medical costs have an overwhelming effect on economic growth of these countries.

Recently, increasing travel and immigration have brought *T. cruzi* infection into the spotlight globally, even in areas where transmission has previously been absent or very low. *T. cruzi* has spread beyond the borders of Latin America and has been detected in Europe, Asia, and the United States (Ferreira et al., J. Clin. Micro., 2001, 39:4390). In the U.S., 50-100 thousand serologically positive persons progressing to the chronic phase of Chagas disease are present, and the number of infected immigrants in developed countries is increasing. It is expected that, due to the exponential increase in emigration from Latin America, Chagas disease may become a serious health issue in North America and Europe in the next decade.

Congenital and transfusion/transplantation-related transmissions are thus becoming increasingly recognized as significant threats. As the number of infected individuals grows, transmission of *T. cruzi* to non-infected individuals through blood transfusion and organ transplants from the infected immigrant donors is emerging as a route for *T. cruzi* transmission in more developed nations (Umezawa et. al. J. Clin. Micro., 1999, 37:1554; Silveira et. al. Trends Parasitol., 2001, 17; Chagas disease after organ transplantation--United States, 2001; MMWR Morb Mortal Wkly Rep. 2002 Mar 15;51(10):210-2). Each year, 15 million units of blood are transfused and approximately 23,000 organ transplants are performed in the United States alone, and presently almost none of the blood supply is tested for *T. cruzi*. A few cases of infection by *T. cruzi* through organ donation have already been reported to United States Centers for Disease Control since 2001. It has therefore become apparent that the screening of blood and organ donors is necessary not only in Latin America but also in developed countries that receive immigrants from endemic areas.

Diagnosis of *T. cruzi* infection is challenging for a number of reasons. The initial infection is seldom detected except in cases where infective doses are high and acute symptoms very severe, as in localized outbreaks resulting from oral transmissions. Classical signs of inflammation at proposed sites of parasite entry (e.g. "Romahia's sign") or clinical symptoms other than fever, are infrequently reported. As a result, diagnosis is very rarely sought early in the infection, when direct detection of parasites may be possible. In the vast majority of human cases, *T. cruzi* infection evolves undiagnosed into a well-controlled chronic infection wherein circulating parasites or their products are difficult to detect even with the use of amplification techniques. A "conclusive" diagnosis of *T. cruzi* infection is often reached only after multiple serological tests and in combination with epidemiological data and (occasionally) clinical symptoms. Further complicating matters, some researchers have reported positive PCR and clinical disease in patients with negative serology. Salomone et al. Emerg Infect Dis. 2003 Dec;9(12):1558-62.

Unfortunately, multiple studies from geographically distinct areas and utilizing a wide range of tests and test formats have shown current diagnostics to be far from dependable (Pirard et al., 2005, Transfusion 45: 554-561; Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Avila et al., 1993, J Clin Microbiol 31: 2421-2426; Castro et al., 2002, Parasitol Res 88: 894-900; Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; Silveira-Lacerda et al., 2004, Vox Sang 87: 204-207; Wincker et al., 1994, Am J Trop Med Hyg 51: 771-777; Gutierrez et al., 2004, Parasitology 129: 439-444; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka et al., 2007, Braz J Infect Dis 11: 226-233; Zarate-Blades et al., 2007, Diagn Microbiol Infect Dis 57: 229-232). Many of the most widely employed serological tests, including one recently licensed by the United States Food and Drug Administration for use as a blood screening test in the U.S. (Tobler et al., 2007, Transfusion 47: 90-96), use crude or semi-purified parasite preparations, often derived from parasite stages present in insects but not in infected humans. The most widely accepted serological tests for *T. cruzi* infection utilize antigens from either whole to semi-purified parasite lysates from epimastigotes that react with anti-*T. cruzi* IgG antibodies. These tests show a degree of variability due to a lack of

standardization of procedures and reagents between laboratories, and a number of inconclusive and false positive results occur due to cross-reactivity with antibodies developed against other parasites (Nakazawa et. al. Clin. Diag. Lab. Immunol., 2001, 8:1024).

5 Other tests have incorporated more defined parasite components, including multiple fusion proteins containing epitopes from various parasite proteins, which, individually have shown some promise as diagnostics (Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; da Silveira JF et al., 2001, Trends Parasitol 17: 286-291; Chang et al., 2006, Transfusion 46: 10 1737-1744). Unfortunately, in the absence of a true gold standard, the sensitivity of new tests is generally determined using sera that have been shown to be unequivocally positive on multiple other serologic tests, but rarely with sera that are borderline or equivocal on one or more tests, an approach that assures only that the test being evaluated is no worse, but not necessarily any 15 more sensitive, than the existing tests.

SUMMARY OF THE INVENTION

The present invention provides new tools for diagnosing and treating *T. cruzi* infections in people and animals. In one aspect, the invention provides a 20 method of screening for antigenic *T. cruzi* polypeptides. First and second substrates are provided that each include a plurality of individually addressable candidate antigens derived from *T. cruzi*. The antigens present on the first and second substrate are substantially the same in order to facilitate comparison. The candidate antigens of the first substrate are contacted with a body fluid of a 25 first mammal known to be positive for *T. cruzi* infection. The candidate antigens of the second substrate are contacted with a body fluid from a second mammal known or reasonably believed to be unexposed to *T. cruzi* infection. At least one antigenic *T. cruzi* polypeptide is then identified using a process in which the antigenic *T. cruzi* polypeptide binds to an antibody present in the 30 body fluid of the first mammal but exhibits little or no binding to an antibody present in the body fluid of the second mammal. Optionally, the first and second mammals may be humans.

Positive evidence of *T. cruzi* infection in the first mammal may, for example, be based on a detection method such as a T cell assay, polymerase chain reaction (PCR), hemoculture or a xenodiagnostic technique. Evidence of negative serology in the second mammal is preferably shown by a negative
5 result when the mammal is tested for *T. cruzi* infection utilizing a conventional serodiagnostic test that relies on antigens from whole or semi-purified parasite lysates from *T. cruzi*, such as, for example, from a *T. cruzi* epimastigote lysate.

More than two substrates that include a plurality of individually addressable candidate antigens may be used. Each substrate is contacted with
10 the body fluid from a mammal which exhibits a different level of serological reaction to *T. cruzi* using a conventional serodiagnostic test that relies on antigens from whole or semi-purified parasite lysates from *T. cruzi*. The method optionally further includes the step of preparing the polypeptide antigens from an expression vector including a nucleotide sequence from *T.*
15 *cruzi*.

Optionally, the screening method may further include a preliminary screening step. The preliminary screening step includes providing a first and a second substrate comprising a plurality of individually addressable antigen pools derived from *T. cruzi* in which the antigen pools present on the first and
20 second substrate are substantially the same. The first substrate is contacted with a body fluid of a first mammal known to be positive for *T. cruzi* infection and the second substrate is contacted with a body fluid from a second mammal known or reasonably believed to be unexposed to *T. cruzi* infection. An antigen pool is then identified that binds to an antibody present in the body fluid of the
25 first mammal but exhibits little or no binding to an antibody present in the body fluid of the second mammal.

In another aspect, the present invention provides an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides. The antigenic polypeptides can be selected from the polypeptides
30 identified in Table 1, 2 and/or 4, and include antigenic analogs or subunits thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a

polypeptide that is not listed in Table 1. The polypeptides are immobilized onto a surface of the substrate. Optionally, the article may include at least one antigenic *T. cruzi* polypeptide identified according to the screening method described above, or antigenic analogs or subunits thereof, immobilized onto the surface of the substrate. In embodiment, the polypeptides are immobilized on the substrate surface to form a microarray. In another embodiment, the substrate includes at least one nanoparticle, with the polypeptides being immobilized on the surface of the nanoparticle.

The present invention also provides a kit for diagnosis of *T. cruzi* infection that includes an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2 and/or 4, in which the polypeptides are immobilized onto a surface of the substrate. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The kit also includes packaging materials and instructions for use. Optionally, the kit may include at least one antigenic *T. cruzi* polypeptide identified by the screening method described above and immobilized onto the surface of the substrate. The kit may be formulated for medical or veterinary use.

The present invention also provides a diagnostic method for obtaining information about a known or suspected *T. cruzi* infection in a mammal, or for determining whether a mammal is or has been infected by *T. cruzi*. Execution of the method involves obtaining a biological sample from the mammal, contacting the biological sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2, and/or 4, or antigenic analogs or subunits thereof, and evaluating the presence, absence, intensity or pattern of interaction of components of the biological sample with the antigenic *T. cruzi* polypeptides. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. Optionally, an antigenic *T. cruzi* polypeptide identified

according to the screening method described herein, or antigenic analogs or subunits thereof, can be included in the plurality of antigenic *T. cruzi* polypeptides. In a preferred embodiment, the biological sample is contacted with an article that includes a substrate and a plurality of individually
5 addressable antigenic *T. cruzi* polypeptides immobilized onto a surface of the substrate. Information that can be obtained according to the method includes, for example, the presence or absence of *T. cruzi* infection, the identity of the infective strain, the length of the infection, the stage of the infection, whether the infection is still present or the mammal has been cured, the vaccination
10 status of the mammal, the success of treatment, or any combination thereof. The method can, for example, be a serodiagnostic method, wherein the biological sample component that interacts with an antigenic *T. cruzi* polypeptide is an antibody from the mammal. Alternatively, the method may be embodied by a cellular assay method where the biological sample component
15 that interacts with an antigenic *T. cruzi* polypeptide is T cell from the mammal. Like all diagnostic methods described herein, the method can be implemented as a multiplexed assay in which the biological sample is contacted simultaneously with the plurality of antigenic *T. cruzi* polypeptides. The biological sample can, for example, be obtained from a person suspected of
20 having or being exposed to disease, or obtained from an actual or potential blood donor or transplant donor. Alternatively, the biological sample is obtained from a pooled blood product supply intended for use in transfusions or research.

Also provided by the invention is a method for detecting a *T. cruzi*
25 infection, particularly a maternally transmitted *T. cruzi* infection, in an infant born to a mother who is known to have, or suspected of having, a *T. cruzi* infection. A biological sample is obtained from the infant and contacted with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof. The biological sample is preferably a
30 bodily fluid, more preferably blood, plasma or serum. The timing for obtaining the sample from the infant is important, as enough time should have elapsed after the birth such that antibodies produced by the infant can be detected. The sample can be obtained from the infant at about two or three months after birth

but is preferably obtained about 4, 5 or 6 months after birth, or later.

Preferably, at least one polypeptide is selected from the polypeptides listed in Table 1, Table 2 and/or Table 4. The presence, absence, intensity or pattern of interaction of components of the biological sample, particularly antibodies, with the antigenic *T. cruzi* polypeptides is evaluated to determine whether the infant exhibits an antibody response that exceeds background levels.

The method optionally further includes comparing the infant's antibody response to the plurality of antigenic *T. cruzi* polypeptides with the antibody response of the infant's mother to the same or similar panel of *T. cruzi* polypeptides. Comparison with the mother's antibody response is especially useful when the infant's antibody response is higher than background level. A biological sample is obtained from the infant's mother and contacted with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof. The biological sample of the mother can be obtained prior to birth, during birth, or after birth. The presence, absence, intensity or pattern of interaction of components of the mother's biological sample with the antigenic *T. cruzi* polypeptides is compared to the presence, absence, intensity or pattern of interaction of components of the infant's biological sample with the antigenic *T. cruzi* polypeptides, to determine whether the infant's antibody response differs from the mother's antibody response. A difference in antibody responses, where the infant's response is above background levels, indicates that the infant may have a *T. cruzi* infection. Similar antibody responses for mother and infant indicate that maternal antibodies may still be present in the infant's bodily fluids. In that event, the comparison is optionally repeated using a biological sample can be obtained from the infant at a later date.

Additionally or alternatively, the method further optionally includes comparing the infant's antibody response to the plurality of antigenic *T. cruzi* polypeptides with the infant's antibody response to the same or similar panel of *T. cruzi* polypeptides as measured earlier, i.e., shortly after birth. Shortly after birth, the infant's antibody response is expected to mirror the mother's antibody response, reflecting the presence of maternal antibodies in the infant's bodily fluids. A biological sample is obtained from the infant shortly after birth,

contacted with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, and the presence, absence, intensity or pattern of interaction of components of the infant's earlier biological sample with the antigenic *T. cruzi* polypeptides is compared to the
5 presence, absence, intensity or pattern of interaction of components of the infant's later biological sample, or of the mother's biological sample, or both, with the antigenic *T. cruzi* polypeptides, to determine whether the infant's later antibody response differs from the mother's antibody response, wherein a difference in antibody responses indicates that the infant may have a *T. cruzi*
10 infection.

In instances wherein the method identifies an infant having or suspected of having a *T. cruzi* infection, the method further optionally includes treating the infant for a *T. cruzi* infection, for example by administering a therapeutic agent to the infant.

15 In another aspect, the present invention provides a method for detecting contamination of a blood product supply with *T. cruzi*. The method of detecting contamination includes selecting a sample from the blood supply, contacting the sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides selected from the polypeptides identified in Table 1, 2 and/or 4, or
20 antigenic analogs or subunits thereof, and evaluating the presence, absence, intensity or pattern of interaction of components of the sample with the antigenic *T. cruzi* polypeptides to determine whether the blood supply is contaminated with *T. cruzi*. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4,
25 with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. Optionally, an antigenic *T. cruzi* polypeptide identified according to the screening method described herein, or antigenic analogs or subunits thereof, can be included in the plurality of antigenic *T. cruzi* polypeptides. In a preferred embodiment, the
30 blood supply sample is contacted with an article that includes a substrate and a plurality of individually addressable antigenic *T. cruzi* polypeptides immobilized onto a surface of the substrate.

Blood products that can be tested include whole blood, a blood product, or a blood fraction. For example, a cellular blood component, a liquid blood component, a blood protein, or mixtures thereof, or a red blood cell concentrate, a leukocyte concentrate, a platelet concentrate, plasma, serum, a clotting factor,
5 an enzymes, albumin, plasminogen, or a immunoglobulin, or mixtures of thereof, can be tested for contamination according to the method.

The method of detecting contamination can be a serodiagnostic method, wherein the sample component that interacts with an antigenic *T. cruzi* polypeptide is an antibody. Alternatively, the method can take the form of a
10 cellular assay method, wherein the sample component that interacts with an antigenic *T. cruzi* polypeptide is T cell.

In yet another aspect, the present invention provides a multicomponent vaccine. In one embodiment, the vaccine includes a plurality of immunogenic *T. cruzi* polypeptides selected from the *T. cruzi* polypeptides listed in Table 1, 2,
15 and/or 4, or immunogenic subunits or analogs thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The multicomponent polypeptide vaccine optionally includes at least one
20 immunogenic *T. cruzi* polypeptide identified according to the screening method described herein, or immunogenic subunit or analog thereof. In another embodiment, the multicomponent vaccine includes one or more polynucleotides operably encoding a plurality of immunogenic *T. cruzi* polypeptides selected from the *T. cruzi* polypeptides listed in Table 1, 2, and/or 4 or immunogenic
25 subunits or analogs thereof. In some embodiments, some or all of the polypeptides are selected from the polypeptides listed in Table 2 and/or Table 4, with the proviso that at least one of the polypeptides selected from Table 2 and/or Table 4 is a polypeptide that is not listed in Table 1. The multicomponent polynucleotide vaccine optionally includes a polynucleotide
30 operably encoding a polypeptide identified according to the screening method, or immunogenic subunit or analog thereof. The multicomponent vaccine may be a therapeutic or prophylactic vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a pictorial overview of the bio-plex array analysis method; A) shows the protein-antibody-microsphere complex used by the bio-plex method, B) shows multiple complexes in the well of a microplate substrate, and C) shows laser excitation of the complexes as they flow through a flow cytometer.

Figure 2 provides a pictorial overview of the Gateway[®] cloning method used to provide an expression vector used for the preparation of *T. cruzi* polypeptide antigens in one embodiment of the invention.

Figure 3 shows assay development using varicella voster (VV)-ovalbumin sera; A, BioPlex assay; B, ELISA assay.

Figure 4 shows testing of protein pools for antigenic potential using A, negative control sera; B, very low positive sera; C, borderline positive sera; and D, strong positive sera. From left to right, in each panel at each of the sera dilutions, the tested samples are: lysate control, ovalbumin, pool 1C, pool 2A, pool 2I, pool 3A, pool 3K and pool 6. Pool 3K reacted with antibodies from infected individuals and was a candidate for further testing.

Figure 5 shows testing of the component proteins of pool 3K for antigenic potential using A, negative control sera; B, very low positive sera; C, borderline positive sera; and D, strong positive sera. From left to right, in each panel at each of the sera dilutions, the tested samples are: lysate control, ovalbumin, protein 3K-1, 3K-2, 3K-3, 3K-4, 3K-5 and 3K-6, and pool 3K. Proteins 3K-1, 3K-2, 3K-3 and 3K-5 demonstrated varying degrees of reactivity to antibodies in sera from infected individuals.

Figure 6 shows testing of four different serum samples using a panel of serodiagnostic proteins; A, strong seropositive serum; B, Subject 58: T cell reactive/seronegative serum; C, Subject 44: T cell non-reactive/seronegative serum; D, Subject 60: T cell non-reactive/seronegative. From left to right, in each panel at each of the sera dilutions, the test proteins are: lysate control, ovalbumin, protein 3K-1, 3K-3, 1A-1 and 4A-3, and pool 3K. Subject 58, declared seronegative by standard serological assay but exhibiting T cell reactivity to *T. cruzi* antigens, is of particular interest because antibodies are

detected that recognize the recombinant *T. cruzi* antigens but not the parasite lysate.

Figure 7 is a schematic showing a screening process for the high-throughput selection of diagnostic proteins for detection of *T. cruzi* infection.

5 Figure 8 shows an SDS-PAGE gel of production of pooled protein. Sets of 6-8 genes were moved in pools from pDONR entry plasmids into pDEST-PTD4 via a Gateway LR reaction (Invitrogen) and the resulting plasmids transformed into BL21(DE3)pLysS cells for protein production. Recombinant HIS-tagged proteins were purified on Co+2 affinity resin and the bound proteins
10 analyzed by SDS-PAGE. Protein pools depicted in lanes 1-5 were generated from the pooling of 8 genes, while lanes 6, 7 and 8 were derived from 7, 6, and 6 genes respectively. Lane "S" contained molecular weight standards (BenchMark Prestained Protein Standard; Invitrogen). Overall, approximately 80% of genes yielded proteins when expressed as pools.

15 Figure 9 shows reactivity of a representative set of proteins tested with individual sera. A selection of 29 individual recombinant proteins was tested for the ability to bind IgGs present in the sera of 54 subjects. The sera are grouped as "uniformly positive" (reactive on all three conventional serological tests and a commercial assay kit), "inconclusive" (negative on at least one
20 conventional serologic tests), "negative by conventional tests" (negative by all three conventional tests), and "known negative" (from residents of North America with very low chance of being infected based upon residency and travel history). Recombinant ovalbumin and *T. cruzi* lysate –coated beads were used as negative and positive controls, respectively. Horizontal bars in each box
25 indicate mean fluorescence intensity (MFI) on a scale from 0 to 30,000 arbitrary light units. A number of the recombinant proteins either failed to discriminate between uniformly positive and known negative sera sets (e.g. 3, 16, 17, 22, 26, 27, 28) or showed no reaction with either set (e.g. 10). In contrast a number of proteins detected nearly the entire uniformly positive group, as well as some in
30 the inconclusive and conventional negative groups but none in the known negative set (e.g. 4, 11, 19).

Figure 10 shows stability of serological responses over time. The MFI of sera to a panel of 16 recombinant proteins (top 16 in Table 2), GFP negative

control protein and *T. cruzi* lysate for a total of 18 measurements (bars) are shown for each serum. A) Reactivity of a set of 8 known negative sera. B) Stability of unique pattern of antigen activity for 6 seropositive subjects assayed at 4 time points over 12-21 months. Arrows in lower right panel (RD 07) indicate that detection of protein “8” (paraflagellar rod protein) which distinguishes the pattern of reactivity of serum RD07 from that of the similar RD09.

Figure 11 shows the effect of benznidazole treatment on serological responses in chronically infected subjects. The MFI of sera to a panel of 16 recombinant proteins (top 16 in Table 2), GFP negative control protein and *T. cruzi* lysate for a total of 18 measurements (bars) are shown for each serum. A) Change in pattern of reactivity in 4 subjects over 36 months post-benznidazole treatment, measured using both the multiplex serologic assay (left) and conventional serology (right). B) Benznidazole-treated subjects exhibiting no evidence of change in multiplex assay for 24 months post-treatment (left; PP044) and or only changes in reactivity to selected recombinant proteins (right; PP024).

Figure 12 shows the pattern of antigen-specific antibody responses in chronic Chagas disease over time (A, 0 months; B, 12 months) and among different individuals.

Figure 13 shows the effects of benznidazole treatment over time on antibody titers to recombinant proteins; A, untreated; B, treated.

Figure 14 shows the effect of treatment with benznidazole on *T. cruzi*-specific memory T cell responses in chronic Chagas disease subjects. The frequency of IFN-(gamma)-producing T cells specific for *T. cruzi* significantly decreased in the benznidazole-treated patient group vs non-treated, at 12 months after follow-up. ELISPOT responses became negative in 11/25 (44%), 6 patients at 12 months post-treatment and 5 patients at 24 months post treatment.

Figure 15 shows the antibody profiles of four mothers (A, B, C, D) with chronic Chagas disease and their infants at a time point relatively soon after birth, and again later when the infant is 6-7 months old.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

In one aspect, the present invention is directed to the detection of *T. cruzi* infection in a mammal, particularly a human. A plurality of *T. cruzi* polypeptides, or subunits or analogs thereof, that are detectable by antibodies
5 present in a bodily fluid, such as blood, plasma or serum, of at least some individuals that are infected with *T. cruzi* are included in a multicomponent panel for use in a diagnostic assay, which may be a serodiagnostic assay or a cellular assay. The term "serodiagnostic" is used because the assay is typically performed on a blood component such as whole blood, plasma or serum, but it
10 should be understood that any bodily fluid that may provide evidence of an immune response to *T. cruzi* can be assayed using the serodiagnostic test of the invention.

The panel components are contacted with a bodily fluid of an individual such as blood, plasma, serum, urine, saliva or tears and the like, and the
15 presence of absence of evidence of an immune response to *T. cruzi* in the individual is evaluated. The body fluid that is tested can be that of an individual patient to be screened, or it can be a body fluid that is part of a blood or plasma supply, for example, pooled or unpooled, that is available for transfusion and/or research.

20 An immune response indicative of *T. cruzi* infection may be evidenced by the binding of antibodies in the biological fluid to panel components. The panel components can likewise be used to assess the presence of a T cell response in the subject.

In a preferred embodiment, the diagnostic test is highly specific for *T. cruzi*
25 infection and sufficiently sensitive to detect infection in subjects considered negative with conventional serological assays based on *T. cruzi* lysates due to a poor or inconsistent B cell response to infection. Optionally the test can include, as specificity controls, polypeptide antigens that are recognized when other infections are present.

30 The diagnostic test can detect the presence or absence of *T. cruzi* infection. In some embodiments, and depending on the antigenic polypeptides selected for including in the multicomponent panel, the pattern of antigen recognition may provide additional information such as the stage of infection or

the severity of disease. The antigen recognition pattern may also be useful to discriminate among patients with active or latent infections, and those who have been cured or vaccinated.

The multicomponent diagnostic assay (also referred to herein as a
5 multiplexed assay) has advantages over conventional serodiagnostic methods. For example, the multiplexed assay of the invention consistently detects infection, whereas conventional assays are plagued by high failure rates and inconsistent performance. The Examples below show that infected subjects produce individual patterns of antibody responses that differ from one another,
10 rendering serodiagnostics based on a single antigen a less effective diagnostic than a multiplexed assay. There are numerous examples of the failure of conventional serology to detect infection, and parasitological tests are also unreliable. Individuals who are seropositive in the multiplex assay of the invention are likely to be infected with *T. cruzi*, particularly if they exhibit
15 antibodies to at least 4, more preferably 6, and even more preferably 8 different recombinant *T. cruzi* proteins, and/or were born in endemic areas and/or have evidence of heart disease. Such individuals are likely to be infected with *T. cruzi* even if they exhibit negative results with conventional serologic assays.

Additionally, the multiplexed serodiagnostic assay of the invention
20 provides a better measure of the efficacy and effectiveness of therapeutic treatment than conventional serological or parasitological assays. Most subjects are negative by parasitological assays prior to treatment and remain positive by conventional serology for long periods of time after treatment. However a multiplexed assay using a selected set of recombinant proteins as described
25 herein can detect changes in antibody levels upon completion of treatment. The use of multiple targets allows serologic changes to be detected following treatment when similar changes are not consistently observed using conventional serologic tests.

Also, the *T. cruzi* proteins identified herein for use in the multiplexed
30 assays of the invention (both serodiagnostic and cellular) are expected to be effective diagnostics for different *T. cruzi* strains in different regions, since many of them are unique to *T. cruzi* and/or highly abundant proteins, such as

"housekeeping" proteins, that are expected to show little variation among the different strains.

The invention is applicable to human disease but also has veterinary applications. For example, a diagnostic assay developed according to the
5 invention can be used to diagnose *T. cruzi* infection in farm animals or pets, such as dogs.

Antigenic polypeptides

A *T. cruzi* polypeptide, or subunit or analog thereof, that is suitable for
10 inclusion in the panel is one that reacts to antibodies in the sera of individuals infected with *T. cruzi*. Such a polypeptide is referred to herein as an antigenic polypeptide or a polypeptide antigen.

A preferred antigenic polypeptide, or antigenic subunit or analog thereof, is one that detectably binds antibodies in a bodily fluid of a subject who
15 is known to be infected or to have been infected by *T. cruzi*, but whose bodily fluid is seronegative when assayed by conventional means. A bodily fluid that is seronegative when assayed by conventional means is one that, for example, does not show a positive reaction (antibody binding) when exposed to antigens from either whole or semi-purified parasite lysates, for example those from
20 epimastigotes, in conventional diagnostic tests. A subject who shows evidence of *T. cruzi* infection using, for example, a T cell assay, polymerase chain reaction (PCR), hemoculture, or xenodiagnostic techniques, is considered to known to be infected or to have been infected by *T. cruzi*, even if the subject shows a negative response to a conventional serodiagnostic test.

25 Another preferred polypeptide, or subunit or analog thereof, is one that detectably binds antibodies in a bodily fluid of a subject who is seropositive when assayed by conventional means, regardless of whether the polypeptide also exhibits detectable binding to antibodies in a bodily fluid of a subject who is known to be infected or to have been infected by *T. cruzi*, but whose bodily
30 fluid is seronegative when assayed by conventional means.

The antigenic *T. cruzi* polypeptides, and antigenic subunits and analogs thereof, bind antibodies in a bodily fluid of a subject, such as blood, plasma or sera, thereby providing evidence of exposure to *T. cruzi*. These antigenic

polypeptides, and antigenic subunits and analogs thereof, may also be immunogenic; i.e., they may also, when delivered to a subject in an appropriate manner, cause an immune response (either humoral or cellular or both) in the subject. Immunogenic *T. cruzi* polypeptides, as well as immunogenic subunits and analogs thereof, are therefore expected to be useful in vaccines, as described below.

It should be understood that the term "polypeptide" as used herein refers to a polymer of amino acids and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, and protein are included within the definition of polypeptide.

An antigenic *T. cruzi* polypeptide according to the invention is not limited to a naturally occurring antigenic *T. cruzi* polypeptide; it can include an antigenic subunit or antigenic analog of a *T. cruzi* polypeptide. Likewise the antigenic polypeptide can be a multivalent construct that includes epitopes obtained from different antigenic polypeptides of *T. cruzi*. An antigenic analog of an antigenic *T. cruzi* polypeptide is a polypeptide having one or more amino acid substitutions, insertions, or deletions relative to an antigenic *T. cruzi* polypeptide, such that antigenicity is not entirely eliminated. Substitutes for an amino acid are preferably conservative and are selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and *vice versa* to maintain a positive charge; Glu for Asp and *vice versa* to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH₂. Antigenic subunits of an antigenic *T. cruzi* polypeptide are antigenic *T. cruzi* polypeptides that are truncated at either or both of the N-terminus or C-terminus, without eliminating their ability to detect serum antibodies against *T. cruzi*. Preferably, an antigenic subunit contains an epitope recognized by a host B cell or T cell. Fragments of an antigenic *T. cruzi* protein

contain at least about eight amino acids, preferably at least about 12 amino acids, more preferably at least about 20 amino acids.

Examples of antigenic *T. cruzi* polypeptides suitable for inclusion in the multicomponent panel of the invention are listed in Tables 1, 2 and 4 in the Examples, below. The "Gene ID Numbers" represent gene numbers assigned by annotators of the *T. cruzi* genome and are accessed via the *T. cruzi* genome database on the worldwide web at "TcruziDB.org".

Furthermore, as described below, the present invention also includes a method for identifying additional antigenic polypeptides indicative of *T. cruzi* infection. The use of the additional *T. cruzi* polypeptides thus identified, or antigenic subunit or analog thereof, alone or in combination with each other, with the antigenic *T. cruzi* polypeptides of Table 1, 2 and 4, and/or with other known antigens, in diagnostic and therapeutic applications relating to *T. cruzi* infection as described is also envisioned. It should be understood that the antigenic *T. cruzi* polypeptides described herein or identified using the screening method described herein are generally useful in any of diagnostic and/or therapeutic applications relating to *T. cruzi* infection.

Antigenic polypeptides used in the multicomponent panel of the invention preferably include polypeptides that are abundant during the two stages (amastigote and trypomastigote) that are prevalent in the life cycle of the parasite in mammals. In a mammalian host, *T. cruzi* cycles between a dividing intracellular stage (the amastigote) and a non-replicative extracellular trypomastigote form which circulates in the blood. The presence of two developmental stages of *T. cruzi* in mammalian hosts provides two anatomically and (to some degree) antigenically distinct targets of immune detection - the trypomastigotes in the bloodstream and the amastigotes in the cytoplasm of infected cells. The intracellular location of amastigotes of *T. cruzi* has long been considered a "hiding place" for the parasite wherein it is not susceptible to immune recognition and control. Notably, most current serological tests for *T. cruzi* are based upon antigens from epimastigotes, the form of *T. cruzi* present in insects but not humans. Thus, in a preferred embodiment, an antigenic polypeptide for use in a *T. cruzi* diagnostic test or vaccine according to the invention can be one that is expressed by *T. cruzi* in the extracellular

(trypomastigote) stage, in the intracellular (amastigote) stage, or during both stages of the life cycle.

Diagnostic method

5 The diagnostic of the invention utilizes a multicomponent panel to assess the presence of an immune response (e.g., the presence of antibodies or reactive T cells) in the subject to multiple antigenic *T. cruzi* polypeptides, or antigenic subunits or analogs thereof. The panel may contain a number of antigenic *T. cruzi* polypeptides, or antigenic subunits or analogs thereof,
10 wherein said number is between 5 and 50 or even more, depending on the embodiment and the intended application. For example, the panel may contain 5, 8, 10, 12, 15, 18, 20, 25, 30, 40 or more antigenic *T. cruzi* polypeptides. A typical multicomponent panel may contain 10 to 20 antigenic *T. cruzi* polypeptides. Preferably, some or all of the antigenic *T. cruzi* polypeptides used
15 in the multicomponent panel are selected from those listed in one or more of Tables 1, 2 or 4. Conveniently, the *T. cruzi* polypeptides that are used in the multicomponent diagnostic test can be recombinant polypeptides; however they can be naturally occurring polypeptides or polypeptides that have been chemically or enzymatically synthesized, as well.

20 In one embodiment, the diagnostic test takes the form of a serodiagnostic assay, which detects a humoral (antibody) immune response in the subject. The binding of an antibody that is present in a biological fluid, such as a serum antibody, to any of the various components of the panel is determined. The threshold for a diagnosis of *T. cruzi* infection can be readily
25 determined by the scientist, medical personnel, or clinician, for example based upon the response of known infected and control sera to the particular panel being used. For example, diagnosis criteria can be based on the number of "hits" (i.e., positive binding events) or they can represent a more quantitative determination based, for example, on the intensity of binding and optional
30 subtraction of background. As an illustrative example, the multicomponent panel could contain 15 to 20 antigenic polypeptides, or antigenic analogs or subunits thereof, and a positive diagnosis could be interpreted as, say, 5 or more positive responses. Optionally, the serodiagnostic test could be further refined

to set quantitative cutoffs for positive and negative based upon the background response to each individual panel component. So, for example, the response to each polypeptide could be set to be >2 standard deviations above the response of "pooled normal," sera and an individual would have to have responses to a
5 minimum of 5 out of 20 polypeptides.

The serodiagnostic assay of the invention can take any convenient form. For example, standard immunoassays such as indirect immunofluorescence assays (IFA), enzyme-linked immunosorbent assay (ELISA),
radioimmunoassay (RIA), fluorescent bead technology and Western blots can
10 be employed. Detection can be by way of an enzyme label, radiolabel, chemical label, fluorescent label, chemiluminescent label, a change in spectroscopic or electrical property, and the like.

In another embodiment, the diagnostic method can take the form of a cellular assay. In this embodiment, a multicomponent panel of antigenic *T. cruzi*
15 polypeptides as described herein is used to assess T cell responses in a mammalian subject, thereby providing another method for evaluating the presence or absence (or stage, etc.) of *T. cruzi* infection. Individuals are known who are serologically negative (based upon conventional tests) but who have T cells reactive with parasite antigens (usually a lysate of trypomastigotes and
20 epimastigotes - but in some cases also against specific *T. cruzi* polypeptides). This suggests that T cell responses may be a sensitive way to assess infection, or to determine the stage of infection or exposure.

Recombinant antigenic *T. cruzi* polypeptides can be readily produced, for example, as histidine-tagged polypeptides. These His-tagged polypeptides
25 can be purified onto a nickel-coated substrate, then added to a blood fraction comprising peripheral blood lymphocytes (e.g., a peripheral blood mononuclear cell, PBMC, fraction). The ability of the T cells to make IFN-gamma is then assessed, for example using an ELISPOT assay (e.g., Laucella et al., J Infect Dis. 2004 Mar 1;189(5):909-18). As another example, antigenic *T. cruzi*
30 polypeptides, or antigenic analogs or subunits thereof, can be bound to major histocompatibility complex (MHC) tetramers and presented to T cells, for example in a composition of peripheral blood lymphocytes, in a microarray format. In this assay, smaller polypeptides, for example antigenic peptide

subunits of antigenic *T. cruzi* polypeptides described herein, are preferred as they are more readily bound to the MHC tetramers and recognized by the T cells. Antigenic subunits of antigenic *T. cruzi* polypeptides can be predicted using various computer algorithms, and are amenable to chemical synthesis.

- 5 Binding of T cells to the spots containing MHC-polypeptide complexes indicates recognition and hence *T. cruzi* infection. See, for example, Stone et al. (Proc. Nat'l. Acad. Sci. USA, 2005, 102:3744) and Soen et al. (PLoS. Biol, 2003, 1:429) for a description of the general technique.

In a multiplexed assay, multiple analytes are simultaneously measured.

- 10 Each polypeptide antigen is positioned such that it is individually addressable. For example, the polypeptide antigens can be immobilized in a substrate. In a preferred embodiment, the multiplexed serodiagnostic assay of the invention is performed using a bioassay such as the Luminex system (Luminex Corporation, Austin, TX). The Luminex system, which utilizes fluorescently labeled
15 microspheres, allows up to 100 analytes to be simultaneously measured in a single microplate well, using very small sample volumes. However, other multiplex platforms such as protein microarrays can also be used, and the invention is not intended to be limited by the type of multiplex platform selected.

- 20 The panel components can be assembled on any convenient substrate, for example on a microtiter plate, on beads, or in a microarray on a microchip. A microarray format is advantageous because it is inexpensive and easy to read using a standard fluorescence microscope. In this format, one might just use the total number of spots (proteins) positive for each test patient to make a positive
25 or negative diagnosis. In addition, the diagnostic test of the invention is well-suited to adaptation for use with commercially available high-throughput devices and immunoassay protocols, for example those available from Abbott Laboratories and Applied Biosystems, Inc. The serodiagnostic assay can also take the form of an immunochromatographic test, in the form of a test strip
30 loaded with the panel components. The bodily fluid can be wicked up onto the test strip and the binding pattern of antibodies from the fluid can be evaluated.

Detection of T. cruzi infection in infants

In another aspect, the invention provides method for determining whether an infant has a *T. cruzi* infection. In situations where where the infant's mother is infected with *T. cruzi*, the method facilitates early detection of a
5 maternally transmitted infection. A maternally transmitted infection can be transmitted prior to or during birth (a congenital infection), or it may be transmitted after birth, as through breastfeeding.

At birth and for a period shortly thereafter, the antibody response of an infant born to an infected mother mirrors the antibody response of the infant's
10 infected mother, reflecting the presence of maternal antibodies in the baby's fluids. With time, however, if the infant is infected, the infant will begin producing its own antibodies, and the pattern of response will begin to differentiate from that of the mother. Eventually, typically by about six months after birth, the antibody response of the infant will either diminish to near
15 background levels (if the infant is not infected), or will appear distinct from that of the mother, indicating possible infection.

The method for detecting *T. cruzi* infection in an infant, particularly an infant born to a mother with a known or suspected *T. cruzi* infection, therefore includes analyzing at least one biological sample obtained from the infant.
20 Preferably the biological sample is a body fluid such as blood, plasma or serum. The sample is obtained at a time after birth by which the infant's antibody response to the antigenic *T. cruzi* polypeptides, if the infant is infected, is detectably different from the mother's antibody response. Preferably the sample is obtained from the infant at about 6 months of age, but the sample can be
25 obtained earlier, for example at about 5 months, 4 months, 3 months or 2 months. Likewise, the sample can be obtained later since after 6 months the baby is expected to be producing its own antibodies at a detectable level. Analysis is preferably performed using the multiplexed assay of the invention.

An infant that exhibits a background level antibody response to *T. cruzi*
30 antigens in the multiplexed assay is unlikely to be infected with *T. cruzi*. However, an antibody response that exceeds background levels indicates possible infection. Optionally, the method therefore also includes administering a therapeutic agent an infant suspected of having a *T. cruzi* infection.

In a preferred embodiment of the method, the infant's antibody response to the *T. cruzi* antigen panel is first analyzed shortly after birth. When a neonate's antibody response is measured shortly after birth (preferably no later than one month after birth, more preferably no later than two months after birth), the neonate's antibody response will parallel that of its mother, due to the presence of maternal antibodies. Optionally, the mother's antibody response to the antigen panel is thus analyzed. The infant's antibody response at the later time point (when its own antibodies have begun to be produced) is compared to the antibody response of the mother, and/or to its own antibody response at a time shortly after birth. Comparison of the antibody response of the later infant sample with the antibody response of the earlier neonate sample and/or with the antibody response of the mother (preferably using a sample obtained from the mother at about the same time as the sample or samples are obtained from the infant, although the sample from the mother can be obtained at any convenient time as it is expected to be fairly stable) is preferred, as it facilitates the determination as to whether the infant's own antibody response is sufficiently different from the mother's to support the diagnosis of *T. cruzi* infection.

It should be understood that in this method, as in all methods involving the use of the multiplexed assay of the invention, the panel of serodiagnostic targets can include any antigenic *T. cruzi* polypeptide described herein, or subsequently discovered using the screening assay of the invention. Preferably, multiple *T. cruzi* polypeptide antigens are selected from those listed in Tables 1, 2 and/or 4 and used to assess the infant's antibody response in a multiplexed assay.

It should also be noted that the method of detecting *T. cruzi* infection in an infant according to the invention can take the form of either a serodiagnostic method, wherein the sample components that interact with an antigenic *T. cruzi* polypeptides are antibodies, or a cellular assay method, and wherein the sample components that interact with the antigenic *T. cruzi* polypeptides are T cells.

Blood supply screening

The diagnostic test of the invention can be used to detect the presence of *T. cruzi* infection in blood and blood products or fractions include whole blood

as well as such as cellular blood components, including red blood cell concentrates, leukocyte concentrates, and platelet concentrates and extracts; liquid blood components such as plasma and serum; and blood proteins such as clotting factors, enzymes, albumin, plasminogen, and immunoglobulins, or mixtures of cellular, protein and/or liquid blood components. Details regarding the make-up of blood, the usefulness of blood transfusions, cell-types found in blood and proteins found in blood are set forth in U.S. Pat. No. 5,232,844. Techniques regarding blood plasma fractionation are generally well known to those of ordinary skill in the art and an excellent survey of blood fractionation also appears in Kirk-Othmer's Encyclopedia of Chemical Technology, Third Edition, Interscience Publishers, Volume 4.

A sample is contacted with a multicomponent panel of the invention, and a positive or negative response is detected as described above for clinical use of the assay in patients suspected of having *T. cruzi* infection. Advantageously, the diagnostic test is readily automated, for example using microchip technology, for the processing of large numbers of samples.

Prophylactic and therapeutic immunization

In another aspect, the present invention is directed to both prophylactic and therapeutic immunization against *T. cruzi* infection and the chronic disease state, known as Chagas disease, that often eventually follows initial *T. cruzi* infection. Antigenic *T. cruzi* polypeptides described herein, or identified using a screening method described herein, may be immunogenic. That is, they may elicit a humoral (B cell) response and/or a cell-mediated immune response (i.e., a "T cell" response) in the subject. A cell-mediated response can involve the mobilization helper T cells, cytotoxic T-lymphocytes (CTLs), or both. Preferably, an immunogenic polypeptide elicits one or more of an antibody-mediated response, a CD4⁺ Th1-mediated response (Th1: type 1 helper T cell), and a CD8⁺ T cell response. Therapeutic administration of the polynucleotide or polypeptide vaccine to infected subjects is expected to be effective to delay or prevent the progression of the *T. cruzi* infection to a chronic disease state, and also to arrest or cure the chronic disease state that follows *T. cruzi* infection. Prophylactic administration of the polynucleotide or polypeptide vaccine to

uninfected subjects is expected to be effective to reduce either or both if the morbidity and mortality associated with infection by *T. cruzi*. Further, if an uninfected, vaccinated subject is subsequently infected with *T. cruzi*, the vaccine is effective to prevent progression of the initial infection to a chronic disease state. As discussed in more detail hereinbelow, the vaccine can contain or encode a single immunogenic polypeptide or multiple immunogenic polypeptides. Methods for identifying nucleotide sequences encoding such polypeptides from a *T. cruzi* genomic library using, for example, expression library immunization (ELI) or DNA microarray analysis are described below.

Advantages of a genetic vaccine

The choice of polynucleotide delivery as an immunization technique offers several advantages over other vaccine or antigen delivery systems. Vaccines containing genetic material are favored over traditional vaccines because of the ease of construction and production of the vectors, the potential for modification of the sequences by site-directed mutagenesis to enhance the antigenic potency of the individual epitopes or to abolish epitopes that may trigger unwanted response, in the case of DNA vaccines, the stability of DNA, the lack of the dangers associated with live and attenuated vaccines, their ability to induce both humoral and cell mediated immunity and, in particular, CD8⁺ T cell responses, and the persistence of the immune responses. Successful induction of humoral and/or cellular immune responses to plasmid-encoded antigens using various routes of gene delivery have been shown to provide partial or complete protection against numerous infectious agents including influenza virus, bovine herpes virus I, human hepatitis B virus, human immunodeficiency virus-1, as well as parasitic protozoans like *Plasmodium* and *Leishmania* (Donnelly et al., Ann. Rev. Immunol. 15:617-648, 1997). Representative papers describing the use of DNA vaccines in humans and primates include Endresz et al. (Vaccine 17:50-58, 1999), McCluskie et al. (Mol. Med. 5:287-300, 1999), Wang et al. (Infect. Immun. 66:4193-202, 1998), Le Borgne et al. (Virology 240:304-315, 1998), Tacket et al. (Vaccine 17:2826-9, 1999), Jones et al. (Vaccine 17:3065-71, 1999) and Wang et al. (Science

282(5388):476-80, 1998). The ability to enhance the immune response by the co-delivery of genes encoding cytokines is also well-established.

Polynucleotide vaccine

5 The polynucleotide vaccine of the invention includes at least one, preferably at least two, nucleotide coding regions, each coding region encoding an immunogenic polypeptide component from *T. cruzi* as identified herein and/or using the screening method described herein. When it contains two or more nucleotide coding regions, the polynucleotide vaccine is referred to herein
10 as a "multicomponent" polynucleotide vaccine. It is desirable to minimize the number of different immunogenic polypeptides encoded by the nucleotide coding regions in the polynucleotide vaccine; however, it is nonetheless contemplated that a polynucleotide vaccine that generates the highest level of protection will encode 10 or more immunogenic polypeptides.

15 The polynucleotide vaccine can contain DNA, RNA, a modified nucleic acid, or any combination thereof. Preferably, the vaccine comprises one or more cloning or expression vectors; more preferably, the vaccine comprises a plurality of expression vectors each capable of autonomous expression of a nucleotide coding region in a mammalian cell to produce at least one
20 immunogenic polypeptide or cytokine, as further described below. An expression vector preferably includes a eukaryotic promoter sequence, more preferably the nucleotide sequence of a strong eukaryotic promoter, operably linked to one or more coding regions. A promoter is a DNA fragment that acts as a regulatory signal and binds RNA polymerase in a cell to initiate
25 transcription of a downstream (3' direction) coding sequence; transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. A promoter is "operably linked" to a nucleic acid sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The invention is not limited by the use of any particular
30 eukaryotic promoter, and a wide variety are known; preferably, however, the expression vector contains a CMV or RSV promoter. The promoter can be, but need not be, heterologous with respect to the host cell. The promoter used is preferably a constitutive promoter.

A vector useful in the present invention can be circular or linear, single-stranded or double stranded and can be a plasmid, cosmid, or episome but is preferably a plasmid. In a preferred embodiment, each nucleotide coding region (whether it encodes an immunogenic polypeptide or a cytokine) is on a separate
5 vector; however, it is to be understood that one or more coding regions can be present on a single vector, and these coding regions can be under the control of a single or multiple promoters.

There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccines. Preferred embodiments of
10 the polynucleotide vaccine of the invention employ constructs using the plasmids VR1012 (Vical Inc., San Diego CA), pCMVI.UBF3/2 (S. Johnston, University of Texas) or pcDNA3.1 (InVitrogen Corporation, Carlsbad, CA) as the vector. Plasmids VR1012 and pCMVI.UBF3/2 are particularly preferred. In addition, the vector construct can contain immunostimulatory sequences
15 (ISS), such as unmethylated dCpG motifs, that stimulate the animal's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences encoding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-12 (IL-12) and co-stimulatory molecules such B7-1, B7-2, CD40. The cytokines can be used in
20 various combinations to fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to control or eliminate the *T. cruzi* infection.

The polynucleotide vaccine can also encode a fusion product containing the antigenic polypeptide and a molecule, such as CTLA-4, that directs the
25 fusion product to antigen-presenting cells inside the host. Plasmid DNA can also be delivered using attenuated bacteria as delivery system, a method that is suitable for DNA vaccines that are administered orally. Bacteria are transformed with an independently replicating plasmid, which becomes released into the host cell cytoplasm following the death of the attenuated bacterium in
30 the host cell.

An alternative approach to delivering the polynucleotide to an animal involves the use of a viral or bacterial vector. Examples of suitable viral vectors include adenovirus, polio virus, pox viruses such as vaccinia, canary pox, and

fowl pox, herpes viruses, including catfish herpes virus, adenovirus-associated vector, and retroviruses. Exemplary bacterial vectors include attenuated forms of *Salmonella*, *Shigella*, *Edwardsiella ictaluri*, *Yersinia ruckerii*, and *Listeria monocytogenes*. Preferably, the polynucleotide is a vector, such as a plasmid,
5 that is capable of autologous expression of the nucleotide sequence encoding the immunogenic polypeptide.

Preferably, the polynucleotide vaccine further includes at least one nucleotide coding region encoding a cytokine. Preferred cytokines include interleukin-12 (IL-12), granulocyte-macrophage colony-stimulating factor (GM-
10 CSF), interleukin-6 (IL-6), interleukin-18 (IL-18), γ -interferon, α,β -interferons, and chemokines. Especially preferred cytokines include IL-12 and GM-CSF.

Plasmids and other delivery systems are made using techniques well-known in the art of molecular biology. The invention should be understood as including methods of making and using the polynucleotide vaccine.

15

Polypeptide vaccine

The polypeptide vaccine of the invention includes at least one, preferably at least two, immunogenic polypeptides from *T. cruzi* as described herein and/or as identified using the screening method described herein. As
20 with the polynucleotide vaccine, it is desirable to minimize the number of different immunogenic polypeptides supplied in the vaccine; however, it is nonetheless contemplated that a polypeptide vaccine that generates the highest level of protection will contain 10 or more immunogenic polypeptides.

Because a CD8⁺ T cell response cannot normally be directly triggered
25 by the administration of a conventional protein subunit vaccine, the immunogenic polypeptides contained in the polypeptide vaccine preferably include one or more membrane transporting sequences (MTS) fused to their N-terminus or C-terminus or both. A membrane transporting sequence allows for transport of the immunogenic polypeptide across a lipid bilayer, allowing it to
30 be delivered to the inside of a mammalian cell. In a particularly preferred embodiment, the immunogenic polypeptides are shocked with urea, as described further in Example VIII, prior to administration as a vaccine. From there, portions of the polypeptide can be degraded in the proteasome, and the

resulting peptides can be displayed as class I MHC-peptide complexes on the cell surface. In this way, a polypeptide vaccine can stimulate a CD8+ T cell immune response. In another preferred embodiment, the immunogenic polypeptides are attached to nanoparticles and administered to a subject (e.g.,
5 Plebanski et al., J. Immunol. 2004, 173:3148; Plebanski et al., Vaccine, 2004, 23:258). A polypeptide vaccine of the invention is optionally adjuvanted using any convenient and effective adjuvant, as known to one of skill in the art.

The invention should be understood as including methods of making and using the polypeptide vaccine.

10

Pharmaceutical compositions

The polynucleotide and polypeptide vaccines of the invention are readily formulated as pharmaceutical compositions for veterinary or human use. The pharmaceutical composition optionally includes excipients or diluents that are
15 pharmaceutically acceptable as carriers and compatible with the genetic material. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like
20 and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, salts, and/or adjuvants which enhance the effectiveness of the immune-stimulating composition. Methods of making and using such pharmaceutical compositions are also included in the invention.

25

Administration of the polynucleotide vaccine

The polynucleotide vaccine of the invention can be administered to the mammal using any convenient method, such as intramuscular injection, topical or transdermal application to the mammal's skin, or use of a gene gun, wherein
30 particles coated with the polynucleotide vaccine are shot into the mammal's skin. The amount of polynucleotide administered to the mammal is affected by the nature, size and disease state of the mammal as well as the delivery method; for example, typically less DNA is required for gene gun administration than for

intramuscular injection. Further, if a polynucleotide encoding a cytokine is co-delivered with nucleotide coding regions encoding the immunogenic polypeptide from *T. cruzi*, the amount of polynucleotide encoding the immunogenic polypeptide from *T. cruzi* in the vaccine is optionally reduced.

5 Hundreds of publications have now reported the efficacy of DNA vaccines in small and large animal models of infectious diseases, cancer and autoimmune diseases (Donnelly et al., Rev. Immunol. 15:617, 1997). Vaccine dosages for humans can be readily extended from the murine models by one skilled in the art of genetic immunization, and a substantial literature on genetic
10 immunization of humans is now available to the skilled practitioner. For example, Wang et al. (Science 282:476-480, 1998) vaccinated humans with plasmid DNA encoding a malaria protein, and the same group has developed a plan for manufacturing and testing the efficacy of a multigene *Plasmodium falciparum* liver-stage DNA vaccine in humans (Hoffman et al., Immunol. Cell
15 Biol. 75:376, 1997). In general, the polynucleotide vaccine of the invention is administered in dosages that contain the smallest amount of polynucleotide necessary for effective immunization. It is typically administered to human subjects in dosages containing about 20 µg to about 2500 µg plasmid DNA; in some instances 500 µg or more of plasmid DNA may be indicated. Typically
20 the vaccine is administered in two or more injections at time intervals, for example at four week intervals.

Administration of the polypeptide vaccine.

 Like the polynucleotide vaccine, the polypeptide vaccine can be
25 administered to the mammal using any convenient method, such as intramuscular or intraperitoneal injection, topical administration, oral or intranasal administration, inhalation, perfusion and the like. The amount of polypeptide administered to the mammal is affected by the nature, size and disease state of the mammal, as well as by the delivery method. Intraperitoneal
30 injection of 25 to 50 µg of polypeptide containing a membrane transducing sequence has been shown to result in import of the protein into nearly 100% of murine blood and spleen cells within 20 minutes (Schwarze et al., Science 285:1569-1572, 1999) and the sensitization of cytotoxic T cells (Schutze-

Redelmeier et al., J. Immunol. 157:650-655, 1996). Useful dosages of the polypeptide vaccine for humans can be readily determined by evaluating its activity *in vivo* activity in mice.

5 *Administration of a combination of polynucleotide vaccine and polypeptide vaccine.*

The invention contemplates administration of both a polynucleotide vaccine and a polypeptide vaccine to a mammal in a serial protocol. For example, a plasmid-based DNA vaccine may be administered to a mammal to
10 “prime” the immune system, followed by the one or more administrations of a polypeptide vaccine or a viral vaccine (e.g., vaccinia vector carrying the genes that encode the immunogenic polypeptides and, optionally, cytokines) to further stimulate the mammal’s immune system. The order of administration of the different types of vaccines, and the nature of the vaccines administered in any
15 given dose (e.g., polypeptide vaccine, plasmid vaccine, viral vector vaccine) can be readily determined by one of skill in the art to invoke the most effective immune response in the mammal.

Screening method for identification of antigenic T. cruzi polypeptides

20 In another aspect, the invention provides high-throughput method to screen putative *T. cruzi* polypeptides for diagnostic potential. The antigenic polypeptides thus identified can be incorporated into a diagnostic test for *T. cruzi* as described herein.

T. cruzi polypeptides that are preferred candidates for screening, either
25 individually or as part of a pool, have one or more of the following characteristics or features. The *T. cruzi* polypeptides may be abundant in the trpomastigote and/or amastigote stages of the *T. cruzi* life cycle in mammals, as described in more detail above. Additionally or alternatively, the *T. cruzi* polypeptides may be, or may be likely to be, surface-associated or secreted.
30 Surface associated-antigenic polypeptides include, for example, *T. cruzi* proteins that are anchored to the plasma membrane by glycosylphosphatidylinositols, or GPIs, and those that have transmembrane domains or are otherwise embedded in the plasma membrane. This property

can be evaluated, for example, by analyzing the polypeptide sequence for the presence of an N-terminal leader sequence which directs the polypeptide to the cell membrane; by analyzing the polypeptide sequence for the presence of a known GPI sequence that facilitates attachment of the polypeptide to the cell surface; and/or by analyzing the polypeptide sequence for the presence of a transmembrane domain. Another preferred feature is that the polypeptide is unique to *T. cruzi* and not expressed in other organisms, including other kinetoplastids. This can be determined by performing BLAST searches of GenBank entries for other organisms and/or comparative genomics with *T. brucei* and *Leishmania major*. This feature enhances the specificity of the diagnostic test.

Another preferred feature is that the *T. cruzi* polypeptide be one that is less likely than others to be highly variant. For example, members of large gene families that appear to undergo rearrangements that create new variants are generally not preferred. However, pools of large gene family members (such as the trans-sialidase family, the Mucin-associated surface protein (MASP) family, and other smaller families of genes can be cloned and tested using degenerate primers. In that case, rather than a bead or a spot in the diagnostic test containing only one gene family member, it may have ten or hundreds, thereby circumventing the problem of recombination and variation in these families, and providing a better representation of the family than a single (possibly variant) protein.

The screening method involves providing two substrates that include a plurality of individually addressable candidate antigens derived from *T. cruzi*, in which the antigens present on both substrates are substantially the same. A substrate, as defined herein, is a surface of unreactive material that can be used to contain the individually addressable candidate antigens in isolation from one another. For example, a multi-welled array system such as a 96 well microplate is a substrate useful in the method of screening for serodiagnostic *T. cruzi* antigens. Individually addressed candidate antigens refers to potentially serodiagnostic *T. cruzi* antigens that have been positioned and/or labeled in such a way that differing antigens can be discretely identified using methods known to those skilled in the art. For example, antigens obtained directly or indirectly

from *T. cruzi*, labeled with a fluorescent label with a different wavelength sensitivity from other fluorescent labels used with other antigens and positioned within a specific well or set of wells on a multi-welled array system, are individually addressed candidate antigens.

5 Candidate antigens immobilized on the first substrate are contacted with a body fluid from an organism known to be positive for *T. cruzi* infection based on a detection method such as a T cell assay, polymerase chain reaction (PCR), hemoculture or xenodiagnostic techniques. The organism is preferably a mammal, more preferably a dog or a human. Preferably, the organism exhibits
10 negative serology when tested for *T. cruzi* infection utilize conventional serodiagnostic tests that rely on antigens from either whole to semi-purified parasite lysates, for example from epimastigotes, that react with anti-*T. cruzi* IgG antibodies.

 Candidate antigens immobilized on the first substrate are contacted with
15 the second substrate with a body fluid from an organism known or reasonably believed to be unexposed to *T. cruzi* infection. The second substrate serves as a control. The organism does not exhibit a strong positive serological signal indicating infection by *T. cruzi*. Preferably, the organism shows no evidence of *T. cruzi* infection by any other diagnostic test as well. Optionally, the screening
20 method includes testing of additional substrates using body fluids that are strongly, weakly and/or borderline seropositive using conventional tests for *T. cruzi*, as described in more detail below.

 The body fluid may be any fluid found within the body of an organism that is capable of containing components of *T. cruzi* or immune system
25 components prepared in response to exposure to *T. cruzi*. For example, an immune system component may be an antibody that specifically binds to a *T. cruzi* antigen. Such body fluids include, for example, blood, plasma, serum, urine, saliva, tears, lymphatic fluid, and the like.

 The organism itself may be any organism that can be infected by *T.*
30 *cruzi*, including vector organisms. For example, organisms may include insect vectors of Chagas disease belonging to the Hemiptera order, Reduviidae family, and Triatominae subfamily. The organism can also be a vertebrate reservoir of *T. cruzi* infection. Mammals are most susceptible to infection with *T. cruzi*,

with approximately 150 species known to serve as reservoirs. Birds, amphibians, and reptiles are naturally resistant to infection. In the domestic cycle, frequently infected mammals, besides humans, are dogs, cats, mice, rats, guinea pigs, and rabbits. Pigs, goats, cattle, and horses can be infected by *T. cruzi*, but generally only manifest transitory parasitemia. Humans are a preferred organism due to the importance of diagnosing *T. cruzi* infection in humans.

Antigens that exhibit binding to antibodies present in the bodily fluid contacted with the first substrate but little or no binding to antibodies present in the control bodily fluid contacted with the second substrate are identified as antigenic *T. cruzi* polypeptides for use in the multicomponent diagnostic assay. The binding of an antigen to an antibody can be detected by various means known to those skilled in the art. For example, the association may be detected using flow cytometry, or by enzyme immunoassay (EIA) or enzyme-linked immunoassay (ELISA). Preferably, the association of one or more antibodies with multiple antigens is detected using a multiplex analysis system such as the Bio-Plex multiplex analysis system commercially available from, for example, Bio-Rad[®] Laboratories (Hercules, CA).

The Bio-Plex suspension array system is a biomarker assay system that includes a flow-based 96-well fluorescent microplate assay reader integrated with specialized software, automated validation and calibration protocols, and assay kits. The multiplex analysis system utilizes up to 100 fluorescent color-coded bead sets, each of which can be conjugated with a different specific antigen. The term "multiplexing" refers to the ability to analyze many different antigens essentially simultaneously. To perform a multiplexed assay, sample and reporter antibodies are allowed to react with the conjugated bead mixture in microplate wells. The constituents of each well are drawn up into the flow-based Bio-Plex array reader, which identifies each specific reaction based on bead color and quantitates it. The magnitude of the reaction is measured using fluorescently labeled reporter antibodies specific for each antibody that may associate with the antigen being tested.

The Bio-Plex suspension array system uses a liquid suspension array of about 100 sets of micrometer-sized beads, each internally dyed with different

ratios of two spectrally distinct fluorophores to assign it a unique spectral address. The overall operation of the Bio-Plex array system is illustrated in FIGs 1A through 1C. As shown in FIG 1A, polypeptide antigen 10 is bound to a microsphere bead 20 by, for example, a histidine tag. The polypeptide antigen 10 is then contacted with a sample of sera containing an antibody; for example, an anti-*T. cruzi* antibody 30. This antibody, in turn, is contacted with a fluorescently labeled reporter antibody 40 to form a microsphere-antigen-antibody complex 50. As shown in FIG 1B, since the microsphere beads 20 provide a large variety of different colors, and the microsphere beads 20 were earlier attached only to specific polypeptide antigens 10, a number of microsphere-antigen-antibody complexes 50 may be present in a microplate well 60. The complexes 50 are then run through a flow cytometry apparatus 70 that includes a classifying laser 90 and a reporting laser 80. The reporting laser 80 determines the amount of a particular antigen present, based on the amount of fluorescently labeled reporter antibody 40. The classifying laser 90, on the other hand, determines the frequency of fluorescence provided by the microsphere bead 20, and based on this frequency, the identity of the polypeptide antigen 10 can be determined.

In the embodiment used in this invention, the Bio-Plex assay utilizes dyed beads containing nickel to capture the His-tagged *T. cruzi* polypeptides produced in the host bacterial cells. Each spectrally addressed bead captures a different protein. The protein-conjugated beads are allowed to react with a sample, and biomolecules in the sample (typically antibodies) bind to the bound protein antigens as further described in the Examples.

It should be understood that the invention is not limited to multiplexing as employed in the Bio-Plex assay; other multiplexed approaches can readily be used. For example, protein arrays can be placed on a matrix, and the response to the individual proteins on the solid-phase array can be assayed.

An important advantage of using a multiplexed method is that a plurality of serodiagnostic antigens may be identified during a single run of the analysis. For example, serological responses to as many as a 100 individual proteins can be screened at one time, and the pattern of responsiveness to all 100, or any subset thereof, can be used to make or assist in making a diagnosis. Tests in

current use employ crude antigen preparations from *T. cruzi* itself (an undefined composition thus not very reproducible), or utilize an individual recombinant protein (i.e., a single target thus not very robust) or a string of three portions of three different proteins combined in one synthetic protein. In the latter case,
5 there is more than one target, but still the response to only a single entity is measured, thus the assay lacks robustness.

Optionally, the method of the invention can utilize more than two substrates that include a plurality of individually addressed candidate antigens. These additional substrates can be used, for example, to evaluate the body fluids
10 from organisms classified as providing a very high positive sera response, intermediate positive sera response, very low positive sera response or a borderline positive sera response. When used to supplement data obtained on sera from control (uninfected) organisms and organisms that are or have been infected but show little or no positive serological response, these substrates can
15 provide additional information on candidate *T. cruzi* antigens that may be used as serodiagnostic antigens. Measuring the pattern of responses to many antigens is also useful for evaluating other aspects of the *T. cruzi* infection such as the stage of the disease, its severity, or the particular strain of *T. cruzi* involved.

20 The method of screening for serodiagnostic *T. cruzi* antigens can also be done at the less specific level of an antigen pool, rather than specific antigens. An antigen pool, as defined herein, is a plurality of antigens in a mixture. Antigen pools may be used either as a means of evaluating a wide number of antigens more rapidly, as a means of evaluating mixtures of antigens for
25 possible interactions, or simply out of necessity when the identify of specific antigens is not known. The method of screening antigen pools can be done either as an independent analysis method, or it may be an optional preliminary step to the screening of individual *T. cruzi* antigens for potential as serodiagnostic antigens. In either case, the method of analyzing antigen pools
30 includes providing two substrates that include a plurality of individually addressable antigen pools derived from *T. cruzi*, where the antigen pools present on the two substrates are substantially the same. The first substrate is then contacted with a body fluid from an organism known to be serologically

positive for *T. cruzi* infection, while the second substrate is then contacted with a body fluid from an organism known to be not serologically positive for *T. cruzi* infection. Antigen pools that associate with an antibody present in the body fluid from an organism known to be serologically positive but that are
5 absent or present to a lesser degree in the body fluid of an organism known to be not serologically positive for *T. cruzi* infection are thereby identified as serodiagnostic antigen pools that may be used by themselves or further evaluated to determine that specific antigens involved.

The candidate antigens derived from *T. cruzi* that are screened by the
10 method of the invention can include antigens formed from polypeptides, polysaccharides, polynucleotides, or other substances present in *T. cruzi* that are capable of being specifically bound by antibodies. As polypeptides are known by those skilled in the art to be the most common and diverse antigens, the method of the invention preferably identifies polypeptide antigens. Polypeptide
15 antigens can be obtained directly from *T. cruzi* using biochemical separation technology, particularly protein purification methods. More preferably, the polypeptide antigens are obtained using recombinant DNA technology. Using recombinant DNA technology, nucleotide sequences from *T. cruzi* are inserted into a host organism where they are used to direct product of a polypeptide that
20 may contain one or more antigens that may be detected by the screening method.

The smallest useful peptide sequence contemplated to provide an antigen is generally on the order of about 6 amino acids in length. Thus, this size will generally correspond to the smallest polypeptide antigens that are
25 screened for by the method of the invention. It is proposed that short peptides that incorporate a species-specific amino acid sequence will provide advantages in certain circumstances, for example, in the preparation of vaccines or for use in methods of detecting *T. cruzi*. Exemplary advantages of shorter peptides include the ease of preparation and purification, and the relatively low cost and
30 improved reproducibility of production. However, the size of polypeptide antigens may be significantly larger. Longer polypeptide antigens identified by the method may be on the order of 15 to 50 amino acids in length, or may represent an entire protein, including modified proteins such as fusion proteins.

Preparation of the polypeptide antigens will generally include the use of an expression vector. An expression vector is a cloning vector that contains the necessary regulatory sequences to allow transcription and translation of a cloned gene or genes. An expression vector preferably includes a promoter sequence
5 operably linked to one or more coding regions. A promoter is a DNA fragment that acts as a regulatory signal and binds RNA polymerase in a cell to initiate transcription of a downstream (3' direction) coding sequence; transcription is the formation of an RNA chain in accordance with the genetic information contained in the DNA. A promoter is "operably linked" to a nucleic acid
10 sequence if it is does, or can be used to, control or regulate transcription of that nucleic acid sequence. The invention is not limited by the use of any particular promoter. A wide variety of promoters are known by those skilled in the art.

A vector useful in the present invention can be circular or linear, single-stranded or double stranded and can be a plasmid, cosmid, or episome but is
15 preferably a plasmid. In a preferred embodiment, each nucleotide coding region encoding an antigenic polypeptide is on a separate vector; however, it is to be understood that one or more coding regions can be present on a single vector, and these coding regions can be under the control of a single or multiple promoters.

20 There are numerous expression vectors known to those of ordinary skill in the art useful for the production of polypeptide antigens. A preferred expression vector is an expression vector formed using the Gateway[®] cloning method. The Gateway[®] cloning method is a universal cloning technique that allows transfer of DNA fragments between different cloning g vectors while
25 maintaining the reading frame, and has effectively replaced the use of restriction endonucleases and ligases. The steps involved in the Gateway cloning method are shown in FIG 2. First, a gene is selected for cloning. For the present invention, this would be a gene selected from *T. cruzi*. The gene is then provided with primers and amplified using PCR technology with the help of an
30 attB tagged primer pair, as readily practiced by those skilled in the art. The PCR fragment then combined with a donor vector (pDONR[™]) that includes attP sites to provide an entry clone, using the BP reaction. An integration reaction between the attB and the attP sites combines the PCR fragment with

the donor vector. The resulting entry clone contains the gene of interest flanked by attL sites. The LR reaction is then used to combine the entry clone with a destination vector to produce an expression vector. In the LR reaction, a recombination reaction is used to link the entry clone with the destination vector (pDEST™) using the attL and attR sites and a clonase enzyme. The attL sites are already found in the entry clone, while the destination vector includes the attR sites. The LR reaction is carried out to transfer the sequence of interest into one or more destination vectors in simultaneous reactions, making the technology high throughput. For example, as this method allows multiple genes to be transferred to one or more vectors in one experiment, this method readily allows multiple antigenic polypeptides to be prepared that can then be screened by the method of the invention, potentially revealing a plurality of serodiagnostic antigens.

The *T. cruzi* genes are cloned into expression vectors, as described, which are then expressed in a host cell, such as a bacterial cell, yeast cell, insect cell, protozoan cell, or mammalian cell. A preferred host cell is a bacterial cell, for example an *E. coli* cell. Another preferred cell is a protozoan cell, more preferably a kinetoplastid cell, most preferably a *Crithidia* cell (U.S. Pat. No. 6,368,827; April 9, 2002). Proteins are isolated from the host cell, purified, and analyzed. Preferably, the proteins are purified onto beads that are then used in a Bio-Plex assay, as described. Various serum samples (e.g., negative, mildly positive, strongly positive) can be efficiently screened for reactivity with a large number of gene products to identify those gene products that are associated with *T. cruzi* infection, for example those indicative of the existence of and, optionally, the extent and/or stage of *T. cruzi* infection. Typically, the identified antigens elicit an antibody response *T. cruzi* in a mammal. The genes and gene products thus identified are useful in diagnostic assays for *T. cruzi*.

The Examples that follow provide representative data from a screening of more than 350 *T. cruzi* gene products. Following FIGs 4-6 in the Examples, a procedure including a preliminary screening of antigen pools, followed by screening for specific antigens, is described. Four different substrates were used to provide data for the reactivity of the antigens in sera with various levels of reactivity to *T. cruzi* antigens. One pool, labeled "3K" on FIG 4D, was

selected for further analysis because it showed good reactivity with the positive sera. The "3K" pool was broken down into individual constituent gene products, and four of the gene products (antigens) were identified as potential candidates for diagnostic use. The "top" antigens identified this way can be
5 combined into a single, robust diagnostic assay for *T. cruzi*; see, for instance, Example 9. Examples of gene products identified in accordance with the invention are also described.

The method of screening is also capable of identifying antigens that do not consistently elicit a strong B cell response. The majority of conventional
10 and commercially available serological methods for diagnosis and blood screening of *T. cruzi* infection utilize either crude or semi-purified parasite lysates typically from epimastigotes. However the complex nature of molecules in these lysates creates a test that routinely gives false positive diagnosis. Research to improve serological diagnosis techniques has focused on the
15 identification, characterization and cloning of particular *T. cruzi* antigens that elicit a strong B cell response. Experiments have demonstrated that some individuals declared negative by current serological tests in fact respond to parasite lysate by producing IFN-gamma in ELISPOT assays. These individuals therefore have T cells that have been exposed to parasite antigen but
20 have a poor B cell antibody response to the antigens in the serological tests that use parasite lysate. The present invention is capable of detecting components present in the sera of such individuals, as shown in FIG 6.

It is to be understood that other screening methods are applicable to the identification of antigenic *T. cruzi* polypeptides to be included in the
25 multicomponent panel for the diagnostic test. For example, U.S. Pat. No. 6,875,584, issued April 5, 2005, describes screening methods that can be used to identify additional antigenic *T. cruzi* polypeptides for use in a diagnostic test and/or as vaccine components. As another example, a nickel-coated substrate, such as a nanoparticle array, can be used to immobilize His-tagged candidate
30 antigens which can then be contacted with serum or other blood product (in the case of the serodiagnostic test) or MHC-peptide complexes (in the case of the cellular test) to detect evidence of an immune response in the serum.

It is to be understood that any of the diagnostic, therapeutic or laboratory methods described herein can be performed with one or more protein antigens as set forth in Table 1, Table 3 and/or Table 4 herein; or as elsewhere described in the specification.

5 The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

10 EXAMPLES

The majority of current serological tests for *T. cruzi* infection utilize whole to semi-purified parasite lysates and are often inconclusive or result in false positives. Recent studies have identified individuals who are seronegative for *T. cruzi* infection by standard tests but are positive by PCR (Salomone et. al. 15 *Emerg. Infect. Disease*, 2003, 9:1558) or have demonstrable cellular immune responses to *T. cruzi*. With respect to the latter, our lab has recently demonstrated that some individuals declared negative by current serological tests in fact have demonstrable T cell responses to parasite lysate as seen in ELISPOT assays. These individuals therefore have T cells which have been 20 exposed to parasite antigen but have a poor B cell antibody response to the mix of antigens in the serological test. It is apparent that the use of lysates is a poor test for *T. cruzi* infection and we expect that screening with multiple recombinant proteins will be able to reduce the number of false positives, and more importantly false negatives.

25 We have therefore developed a high-throughput method to screen large numbers of recombinantly expressed *T. cruzi* proteins for their serodiagnosis potential. Specifically, we combined a set of putative *T. cruzi* genes cloned into the Gateway System™ with the BioPlex LiquiChip bead technology to screen large numbers of recombinantly expressed proteins for their antigenicity using
30 only a small volume of sample (<100μl). So far, we have produced 34 pools of approximately 10 proteins each and screened them for antigenicity. From the preliminary testing, 11 pools were found to bind readily detectable amounts of antibodies in the sera of *T. cruzi*-infected subjects. These pools were then

broken down and each gene was expressed individually and tested. From these 81 genes we have been able to define more than 15 proteins with serodiagnostic potential.

Our method utilizes a blind screening process that has identified several
5 known antigens as well as previously unidentified antigenic proteins from within pools containing multiple non-antigenic proteins. The use of the BioPlex technology is not limited to antigen screening but its full potential may be realized as a novel method of blood donor screening. The highly antigenic proteins we discovered, and expect to continue to discover, with this method
10 can be used to create a highly sensitive and specific test for *T. cruzi* infection.

Example 1: Buffer and Medium Preparation

A variety of buffers were used in the Bio-Plex multiplex analysis. The
15 buffers were prepared as follows. To prepare 1 liter of PBS/BSA (10 mM NaH_2PO_4 , 150 mM NaCl, and 0.1% (w/v) BSA), 8.77 g NaCl (MW 58.44 g/mol) and 1.4 g $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ (MW 137.99 g/mol) were dissolved in 900 ml H_2O and the pH was adjusted to 7.4 using NaOH. Then, dissolve 1 gram of BSA and adjust the volume to 1 liter. Before use, filter the buffer using a 0.45
20 μM filter. Sodium Azide should be added to 0.5% when storing the PBS/BSA buffer for long term. Azide should not be used with Carboxy Beads.

To prepare 1 liter of coupling buffer (50 mM MES), 11.67 g MES (MW 233.2 g/mol) was dissolved in 900 ml H_2O and the pH was adjusted to 5.0 using NaOH. The volume was then adjusted to 1 liter using additional H_2O . Before
25 use, the buffer should be filtered using a 0.45 μM filter.

To prepare 1 liter of activation buffer (100 mM NaH_2PO_4), 13.80 g $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ (MW 137.99 g/mol) was dissolved in 900 ml H_2O and the pH was adjusted to 6.3 using NaOH. The volume was then adjusted to 1 liter using additional H_2O . Before use, the buffer should be filtered using a 0.45 μM filter.
30

To prepare Buffer Z, 8 M urea, 20 mM Hepes, and 100 mM NaCl are combined and dissolved in deionized water to form a solution. The pH of the solution is adjusted to 8.0, and the solution is filtered through a 0.45 μM filter and stored at room temperature. Imidazole (the side chain molecule in

histidine) is added to Buffer Z at varying concentrations to either prevent the cobalt resin from binding non-specifically to something other than the histidine tag, or to out-competing the binding of the histidine tag and thus causing the protein to elute off the resin.

- 5 To prepare LB (*Luria-Bertani*) Medium, 10 g tryptone, 5 g yeast extract, and 10 g. NaCl were dissolved in 1 L deionized water and autoclaved for 25 minutes. For plates, 15 grams of agarose were also dissolved into the water prior to autoclaving.

10 Example 2: Production of Protein Pools or Individual Proteins

To provide a large set of *T. cruzi* proteins, over 350 proteins in pools of approximately 10 proteins each were prepared. The proteins were prepared using the Gateway® universal cloning technique developed by Invitrogen™.

- 15 The procedure can be carried out by cloning a pool of several genes together, which results in a pool of proteins, or by cloning an individual gene, resulting in the preparation of an individual protein. For preparation of an individual protein, a gene that codes for a desired *T. cruzi* protein is first selected for cloning. This gene is amplified from *T. cruzi* genomic DNA using gene specific
20 primers flanked by lambda phage recombination sites, attB1 (5') and attB2 (3') and polymerase chain reaction. Gel purification of the att-flanked PCR produced was carried out by separating the PCR reaction product on a 1% agarose gel using electrophoresis. The particular gene is identified by comparison with a DNA standard containing bands of known size. The band of
25 the gene of interest is cut out of the gel and purified using Sigma-Aldrich's GenElute Minus EtBr Spin Columns (Catalog No. 5-6501).

- The Gateway® BP reaction is then used to insert the att-flanked *T. cruzi* gene fragment with a pDONR™201 vector (Catalog No. 11798-014, Invitrogen Corp., Carlsbad, CA). The BP reaction is conducted by adding the 5 µl of gel-
30 purified attB-flanked PCR product (40-100 fmoles), 1 µl of the pDONR™201 vector (supercoiled, 150 ng/µl), and 2 µl 5x BP Clonase Reaction Buffer (Catalog No. 11789-013) to obtain a final volume of 8 µl. The BP Clonase™

enzyme mix (Catalog No. 11789-013, Invitrogen Corp., Carlsbad, CA) is mixed gently, and then 2 µl of the enzyme mix was added to the BP reaction mixture and mixed well. The reaction was then incubated at (room temperature) 25° C overnight. Next, 1 µl of Proteinase K solution (Catalog No. 11789-013, 5 Invitrogen Corp., Carlsbad, CA 2 µg/µl) was added, and the mixture was allowed to incubate for 10 minutes at 37° C. Five microliters of the BP reaction are transformed by heat shock into chemical competent DH5α cells and grown up overnight at 37°C shaking at 280 RPM in 5 mL of LB with 50 mg/L kanamycin to select for pDONR201-transformed cells. The plasmid is then 10 purified from the culture using a QIAprep Spin Miniprep Kit (Catalog No. 27106, Qiagen Inc., Valencia ,CA).

For the next step of protein production, the Gateway LR[®] recombination reaction was used to insert the gene of interest in pDONR201 into a destination vector to provide the final expression clone. The destination vector in this case 15 is a modified version of Invitrogen's pRSET (Catalog No. V351-20), called pDEST-PTD4. First, the pDEST-PTD4 was linearized by restriction digest of a novel site (PvuII) within the cell death cassette. The linearized plasmid was purified using QIAquick Gel Extraction Kit (Catalog No. 28207, Qiagen Inc., Valencia ,CA). The LR reaction between the gene of interest in the 20 pDONR[™]201 vector and the desired pDEST-PTD4 expression vector was then set up. First, 300 ng of the pDONR entry clone (prepared above), 300 ng of linearized pDEST-PTD4 (Invitrogen Corp., Carlsbad, CA), and 2 µl LR Clonase Reaction Buffer (Catalog No. 11791-019, Invitrogen Corp., Carlsbad, CA), 2 µl LR Clonase Enzyme Mix, and deionized water are combined to 25 obtain a final volume of 10 µl and mixed thoroughly by flicking the tube. The reaction was then incubated overnight at 25° C. Next, 2 µl proteinase K solution (2 µg/µl) was added and the mix was allowed to incubate for 10 minutes at 37° C. DH5α cells were then transformed by heat shock with 6 µl of LR reaction products, and plated onto LB agar plates containing 150 mg/L 30 ampicillin and incubated overnight at 37° C to select for ampicillin-resistant expression clones.

Next, all of the colonies were scraped clean with a clean sterile spatula, and used to inoculate a tube of 5 mL LB containing 150 mg/L ampicillin, and

grown overnight at 37° C, 280 RPM. The pDEST-PTD4 containing the gene of interest is purified from the culture using a QIAprep Spin Miniprep Kit (Catalog No. 27106, Qiagen Inc., Valencia ,CA). The miniprep preparation should contain copies of each gene of the pool from the desired pDEST vector. Three
5 microliters of purified pDEST-PTD4 containing the gene of interest was then transformed into BL21(DE3)pLysS chemical competent cells. The culture was then directly inoculated into 10 ml LB/ ampicillin (Amp)/chloramphenicol (CAM) (100 mg/L)/(34 mg/L) and grown overnight, shaking at 37° C at 280 RPM.

10 On the fifth day, a 10 ml starter culture was inoculated into 500 ml LB/Amp/CAM and grown to an OD600 of 0.4. Protein expression was then induced with 0.3 mM concentration of IPTG (isopropyl-β-D-thiogalactopyranoside), using 150 µl of 1M IPTG in 500 ml culture. The cells were spun down at 5,000 rpm for 8 minutes and 10 mL Buffer Z (8M urea, 20
15 mM Hepes, 100 mM NaCl) containing 15 mM imidazole was added. The cells were then sonicated three times for 25 seconds at an amplitude of 40. The samples were spun down at 13,000 rpm for 10 minutes and the supernatant is combined with 1 ml settled BD TALON™ Metal Affinity Resin (BD Biosciences Clontech, Catalog No 635502) and rocked overnight at 4° C.

20 The resin/cell lysate slurry is then placed into an empty 0.8x4cm chromatography column and the resin bed is allowed to settle. The liquid was allowed to run through and the resin bed was washed with 10 bed volumes (10 mL) of Buffer Z containing 15 mM imidazole. Once the 10 mL wash has run through, the His-tag protein was eluted with 3 bed volumes (3 mL) of Buffer Z
25 containing 250 mM imidazole. The resulting sample contained the purified protein of interest. The sample was then desalted into Buffer Z (without imidazole) using a PD-10 desalting column (Amersham Biosciences, Catalog No.17-0851-01). The resulting imidazole-free sample is quantified and diluted to a concentration of 10 µg/mL which is ready to be used to bind to Bio-Plex
30 beads for testing.

Example 3: Preparation of Bio-Plex Beads

LiquiChip™ Ni-NTA beads (Qiagen Inc., Valencia, CA) were used to bind His-tagged purified proteins in the Bio-Plex assay, but had to be prepared before use. First, the protein samples were desalted into Buffer Z that does not contain Imidazole using Amersham PD-10 desalting columns (Amersham Biosciences Corp, Piscataway, NJ). The protein was then quantified using a BCA assay and diluted to a concentration of 10 µg/ml with Buffer Z. The LiquiChip™ Ni-NTA Bead stock was then vortexed for 30 seconds at full speed. Next, 50 µl of bead suspension was pipetted out and placed into a 1.5 ml microcentrifuge tube. His-tagged protein dilution (50 µl) was then added to the 50 µl LiquiChip™ Bead suspension. The beads were then incubated at 4° C in the dark from at least 4 hours to overnight. Buffer (900 µl PBS/BSA (10 mM NaH₂PO₄, 150 mM NaCl, 0.1% BSA pH 7.4)) was then added to the protein-coupled LiquiChip™ Bead suspension, adding 0.5% azide as a preservative.

Example 4: Preparation of Positive Controls

Positive and negative controls were used in the Bio-Plex analysis of *T. cruzi* antigens. The positive control consists of proteins from a *T. cruzi* lysate coupled to LiquiChip™ Carboxy Beads. The beads thus contain a mix of *T. cruzi* proteins bound to their surface, and function as a general antigen mix. The LiquiChip™ Carboxy Beads bind to the proteins in a random manner, forming covalent bonds to amine groups in lysine side chains. The first step in the preparation of positive controls was the activation of Carboxy Beads using EDC/NHS. First, approximately 10 mg each of EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (Fluka catalog No. 03449)) and NHS (N-hydroxysulfosuccinimide (Fluka catalog No. 56485)) were weighed into two microcentrifuge tubes. The LiquiChip™ CarboxyBead suspension (1 ml) was then centrifuged for 5 minutes at 10,000 rpm in a microcentrifuge. The supernatant was removed with a 200 µl pipette and discarded. The beads were then washed twice by adding 80 µl of activation buffer and centrifuged for 5

minutes at 10,000 rpm. The supernatant was then carefully removed.

Activation buffer (80 µl) was then added to the bead pellet at the bottom of the tube. The pellet should not be resuspended. The pellet in activation buffer was then vortexed for at least 2 minutes. De-ionized water was then added to the weighed EDC and NHS aliquots to provide solutions with a concentration of 50 mg/ml. NHS solution (10 µl) and EDC solution (10 µl) were then added to the bead suspension, which was then incubated for 20 minutes in the dark. Finally, the beads were centrifuged for 5 minutes at 10,000 rpm, after which the supernatant was removed and discarded.

10 The activated beads were then coupled to the *T. cruzi* lysate. First, the *T. cruzi* pellet was freeze/thawed about 5 times. Insoluble particles were removed by centrifugation. The protein stock was then diluted with coupling buffer to a concentration of 100 µg/ml and a volume of 500 µl. Any foreign protein, azide, glycine, Tris, or other reagent containing primary amine groups present in the protein preparation should be removed by dialysis or gel
15 filtration. Coupling buffer (500 µl) was then added to the beads, which were then resuspended by vortexing. The beads were then washed twice by adding 500 µl of coupling buffer, centrifuging for 5 minutes at 10,000 rpm, removing the supernatant, and then repeating the process. Diluted protein solution (500
20 µl), prepared earlier, was then added. Next, the tube containing the activated beads and the protein solution was gently agitated on a shaker for 2 hours in the dark at room temperature. The beads were then washed twice with PBS/BSA buffer. The beads were then resuspended in 500 µl PBS/BSA, and 0.5% azide was added as a preservative. The bead number was then adjusted to provide the
25 desired concentration per microliter.

Example 5: Bio-Plex Analysis of Proteins

At the start of the analysis, a dilution series of the serum to be tested was prepared on a Millipore 96 well filtration plate. The BioPlex Bead/Protein preparation, prepared according to Example 3, was then added to the wells on a Millipore 96 well filtration plate. When preparing beads according to the normal protocol, 10 µl of bead suspension is sufficient to make a useful data

point. However when testing beads in which multiple proteins are bound to an individual bead, it may be necessary to combine the beads into a single tube and distribute them to wells so that enough of each bead is present in a given well to give an accurate data point. Controls are preferably included for each sample
5 (sera/protein) being analyzed. For example, the Bio-Plex analysis for *T. cruzi* antigens included a bead coated with ovalbumin (OVA) as a negative control and with *T. cruzi* lysate as a positive control.

To prepare for the Bio-Plex analysis, 30 μ l of PBS/BSA buffer and 10 μ l of an individual bead suspension (or a predetermined volume containing
10 multiple beads each with different proteins bound) were added to the Millipore 96 well filtration plate. The filtration plate was then placed on the vacuum manifold and the sample liquid was pulled through the plate. Next, 50 μ l of PBS/BSA and 50 μ l of serum dilution were added. The beads were then incubated for 1 hour at room temperature while being shaken on a plate shaker.
15 Each well was then washed four times with 200 μ l PBS/BSA to remove any unbound IgG antibodies from the well. PBS/BSA buffer (90 μ l) was then added to each well and beads that had settled to the bottom of the filtration plate well due to washing were resuspended. An aliquot (10 μ l) of the secondary reporter molecule was then added. This provided a 1:30 dilution (0.5 mg/ml) of
20 antibody. A higher dilution may be used, but a 1:30 dilution makes sure that secondary antibody is not limited by residual unbound IgG. The solution was then incubated for 1 hour at room temperature while being shaken.

The assay solution was then drawn into the Bio-Plex array reader, which illuminates and reads the sample. When a red diode "classification" laser (635
25 nm) in the Bio-Plex array reader illuminates a dyed bead, the bead's fluorescent signature identifies it as a member of one of the 100 possible sets. Bio-Plex Manager software correlates each bead set to the assay reagent that has been coupled to it. In this way the Bio-Plex system can distinguish between the different assays combined within a single microplate well. A green "reporter"
30 laser (532 nm) in the array reader simultaneously excites a fluorescent reporter tag (phycoerythrin, or PE) bound to the detection antibody used in the assay. The amount of green fluorescence is proportional to the amount of analyte captured in the immunoassay. Extrapolating to a standard curve allowed

quantitation of the analyte in each sample: The results for specific proteins are described in Example 8, and shown in FIGs 4-6.

5 Example 6: Bio-Plex Assay of VV-Ovalbumin Sera

Ovalbumin (OVA) chosen as the protein antigen to develop the BioPlex method. Mice were infected with Vaccinia virus (VV) containing the OVA gene in order to raise serum antibodies to the protein. Sera was collected at 7
10 days post infection, followed by a boost and an additional sera collection 7 more days later. OVA protein was expressed in *E. coli* and purified using a His-tag and bound to BioPlex beads via a Ni-NTA residue and adsorbed to an ELISA plate for analysis. The sera was diluted and tested using the BioPlex Assay described in Example 5. The results are shown in FIG 3A. The results obtained
15 were very comparable to those obtained using ELISA, as described in Example 7.

Example 7: ELISA Assay of VV-Ovalbumin Sera

20 A comparison assay on the ovalbumin of mice infected with Vaccinia virus was run using the ELISA (Enzyme-linked Immunosorbent Assay) method. First, a 96-well polystyrene Immunolon microtiter plate (Dynex Technologies, Chantilly, Va.) was coated with 100 µl of 10 µg/ml ovalbumin (OVA) in PBS overnight at 4° C or 2 hours at 37° C. The wells were then washed three times
25 with PBS-T (PBS-Tween 20 buffer) and then blocked with 1% BSA for 2 hours. Serum dilutions were then added to each well and the wells were incubated for 2 hours at room temperature or overnight at 4° C. After incubation, the wells were washed five times with PBST. Biotinylated secondary mouse antibody (1:100 dilution) was then added and the wells were allowed to set for 1 hour at
30 room temperature. The wells were then washed again for five times with PBST. Horseradish peroxidase-conjugated streptavidin was then added for 30 minutes at room temperature at a 1:100 dilution. The wells were then washed again five times with PBST. Finally, a developing reagent (2,2'-azido-di-[3-

ethylbenzthiazoline sulfonate], ABTS) was added. The results of the ELISA assay of ovalbumin sera are shown in FIG 3B.

Example 8: Bio-Plex Assay Results for Pooled and Specific Proteins

5 Using the method of protein production described in Example 2, over 350 proteins in pools of approximately 10 proteins each were prepared. Each of the pools were screened for antigenicity using the Bio-Plex technology, as described in Example 5. From the preliminary testing, 11 pools were found to bind readily detectable amounts of antibodies in the sera of *T. cruzi*-infected
10 subjects. These pools were then broken down and each gene was expressed individually and tested. From the over 80 genes expressed, 15 proteins have been confirmed as having serodiagnostic potential.

 A Hemagen[®] Diagnostics Chagas Disease Test Kit (Hemagen Diagnostics, Inc., Columbia, MD) was used to evaluate and confirm the
15 presence of anti-*T. cruzi* antibodies in sera from areas of active transmission in Argentina. Sera from non-endemic uninfected in-house sera served as the negative controls. Sera from 4 individuals from areas of active transmission, all of which have tested seronegative using standard assays but 1 of which tests positive for T cell-reactivity to *T. cruzi*, were used for the very low positive
20 control. Sera from 5 individuals that were borderline positive/negative using standard serological assays were used to make up the borderline positive control and sera from 7 individuals that were consistently seropositive using standard serological assays make up the strong positive control.

 Genes of interest were first cloned into the Gateway holding vectors
25 (pDONR[™] vector) and archived as single vectors or are placed into pools. Pools of genes in pDONR[™] vectors can be moved simultaneously into either DNA vaccination vectors or protein expression vectors without the loss of individual genes in the pool. The resulting pools were expressed in *E. coli* strain BL21(DE3)pLysS cells, minimizing the possible toxic effects of
30 individual genes. The protein pools are purified and tested using the Bio-Plex bead technology for antigenicity. The results from analysis of the protein pools using the Bio-Plex analysis method are shown in FIGs 4A-2D. The headings in the figures indicate the type of sera being tested, based on the four categories

(negative, very low positive, borderline positive, and strong positive) resulting from the evaluation using the Hemagen[®] Test Kit. As indicated by the arrow, FIG 4D demonstrates a pool that shows high fluorescence, and hence contains a high level of protein that binds to *T. cruzi*-specific antibodies.

5 Once a pool of proteins was identified using the Bio-Plex screening method as having possible antigenic properties, the individual genes in the pool were examined and tested to find which ones provided reactive antigens. The genes were first moved individually from the pDONR holding vector into an expression vector, followed by expression, purification and testing. Those
10 proteins that exhibit binding to antibodies in infected individuals were then retested for confirmation and identified. The results of screening the pools for individual proteins is shown in FIGs 5A-5D. The arrow in FIG 5D shows a particular protein that reacted strongly with anti-*T. cruzi* antibodies present in strong positive sera.

15 From the proteins that were screened, many that showed antigenic activity were proteins that had been previously characterized as *T. cruzi* antigens. This provides a level of proof to the capacity of this technique to discover single antigens in pools. Selected ribosomal proteins, ubiquitin, calcium binding proteins, and paraflagellar rod proteins have all been described
20 previously as being possible targets for serological diagnosis of *T. cruzi* infection. A list of the individual proteins identified as *T. cruzi* antigens using the Bio-Plex screening method are shown below in Table 1. The "Gene ID numbers" represent gene numbers assigned by annotators of the *T. cruzi* genome and are accessed via the *T. cruzi* genome database on the worldwide
25 web at "TcruziDB.org."

Table 1

Assay Protein ID	Protein	T. Cruzi database accession numbers	Gene ID numbers
1a-1	Tc beta-tubulin	Tc00.1047053506563.40	6998.t00004
1a-5	Tc alpha tubulin	Tc00.1047053411235.9	11788.t00001
1c-3	60S ribosomal protein L2, putative	Tc00.1047053508299.60	5568.t00006
2b-3	hypothetical protein, conserved	Tc00.1047053506529.460	6986.t00046
2c-1	cytochrome C oxidase subunit IV, putative	Tc00.1047053506529.360	6986.t00036
2c-9	hypothetical protein	Tc00.1047053506529.610	6986.t00061
2i-1	hypothetical protein, conserved	Tc00.1047053510887.50	6003.t00005
3d-3	iron superoxide dismutase, putative	Tc00.1047053509775.40	5781.t00004
3d-4	trans-splicing factor, putative	Tc00.1047053503583.40	4650.t00004
3j-1	60S ribosomal protein L28, putative	Tc00.1047053506297.270	6890.t00027
3k-1	glycosomal phosphoenolpyruvate carboxykinase, putative (Phosphoenolpyruvate Carboxykinase (Pepck))	Tc00.1047053508441.20	7730.t00002
3k-2	ubiquitin-fusion protein, putative (polyubiquitin / ribosomal protein CEP52)	none	7355.t00001
3k-3	60S acidic ribosomal subunit protein, putative (Calmodulin-ubiquitin associated protein CUB2.8)	Tc00.1047053508355.250	7695.t00025
3k-5	ef-hand protein 5, putative	Tc00.1047053506391.30	6925.t00003
4a-3	paraflagellar rod protein 3	Tc00.1047053509617.20	8152.t00002
B1	axoneme central apparatus protein, putative	Tc00.1047053510955.40	8553.t00004
B2	serine carboxypeptidase (CBP1), putative	Tc00.1047053509695.220	8171.t00022
B5	aminopeptidase, putative	Tc00.1047053511289.30	8647.t00003
B7	elongation factor-1 gamma, putative	Tc00.1047053510163.20	8322.t00002
B8	hypothetical protein, conserved	Tc00.1047053506531.20	6987.t00002
D3	hypothetical protein, conserved	Tc00.1047053506489.30	6967.t00003

Research to improve serological diagnosis techniques has focused on the identification, characterization and cloning of particular *T. cruzi* antigens that elicit a strong B cell response. The use of *T. cruzi* specific antigens in a serological test gives a high level of specificity to a serological test, eliminating the problems that arise due to cross-reactivity to a parasite lysate. However using only a single antigen may not be sensitive enough to detect all individuals that are infected, and thus the use of multiple antigens is preferred. Recent

evidence demonstrates that some individuals declared negative by current serological tests in fact respond to parasite lysate by producing IFN- γ in ELISPOT assays. These individuals therefore have T cells that have been exposed to parasite antigen, but have a poor B cell antibody response to the antigens in the serological tests that use parasite lysate. The ability to evaluate the T cell reactivity of individual proteins to sera from various subjects using the Bio-Plex analysis is shown in FIG 6A – 6D.

Example 9: High throughput selection of effective serodiagnostics for *T. cruzi* infection and multiplex diagnostic for Chagas disease

As noted above, diagnosis of *T. cruzi* infection by direct pathogen detection is complicated by the low parasite burden in subjects persistently infected with this agent of human Chagas disease. In this study, we sought to improve upon current diagnostics for *T. cruzi* infection by screening for diagnostic candidates that displayed the ability to detect infection in subjects that went undetected or gave discordant results using other conventional serologic tests. We screened more than 400 recombinant proteins of *T. cruzi*, including randomly selected and those known to be highly expressed in the parasite stages present in mammalian hosts, for the ability to detect anti-parasite antibodies in the sera of subjects with confirmed or suspected *T. cruzi* infection. A set of 16 protein groups were then incorporated into a multiplex bead array format which detected 100% of more than 100 confirmed positive sera and also documented consistent, strong and broad responses in samples undetected or discordant using conventional serologic tests. Each serum had a distinct but highly stable reaction pattern. The end result was thus the identification of a panel of recombinant proteins that more reliably detects *T. cruzi* infection than do a combination of existing conventional tests. Additionally, we show that a multiplex assay utilizing this diagnostic panel has utility in monitoring drug treatment efficacy in chronic Chagas disease. These results substantially extend the variety and quality of diagnostic targets for Chagas disease and offer a useful tool for determining treatment success or failure.

Methods

Parasites, gene selection and cloning. *T. cruzi* epimastigotes of the Sylvio, CL Brenner, Brazil, CL, Tulahuen, M83, M91 and Chapulin isolates were maintained in logarithmic phase growth (Kirchhoff et al., 1984, J Immunol 133: 2731-2735) and used as a source of genomic DNA. Primer sets incorporating lambda phage recombination sites flanking the 18-21 base gene-specific sequence (excluding both start and stop codons), were designed for each gene of interest, the genes cloned by PCR from the pooled DNA of the 8 *T. cruzi* isolates, and Gateway adapted gene product inserted into the pDONR-201 plasmid (Invitrogen, Carlsbad, CA). To speed the cloning process while improving our chances of cloning full-length non-mutated genes, at least 10 clones positive for the appropriate sized insert were pooled for each gene. *T. cruzi* lysate from Brazil strain amastigote and trypomastigotes was prepared as previously described (Laucella et al., 2004, J Infectious Diseases 189:909-918).

pDEST-PTD construction. The pDEST-PTD protein expression vector was created from pTAT-HA (Nagahara et al., 1998, Nat Med 4: 1449-1452) by replacing the BamHI-flanked TAT sequence with a BamHI-flanked PTD-4 encoding sequence (Ho et al., 2001, Cancer Res 61: 474-477), followed by Gateway (Invitrogen)- adaptation of the plasmid utilizing the NcoI and XhoI cloning sites. The Gateway cloning cassette was PCR amplified from pDEST-YFP (gift from Dr. Boris Striepen, University of Georgia, GA).

Protein production and purification. Genes in pDONR plasmids were transferred to pDEST-PTD4 via a Gateway LR reaction and the proteins expressed in BL21(DE3)pLysS cells were extracted by sonication in 8M urea, 20mM HEPES, 100mM NaCl, pH 8.0 containing 15mM imidazole. The lysate was then applied to TALON Metal Affinity Resin (BD Biosciences Clontech, Palo Alto, CA) and bound protein was eluted with 250mM imidazole. Imidazole was removed on PD-10 desalting columns (GE Healthcare, Piscataway, NJ) and protein concentration was estimated using a modified Bradford assay. Proteins were diluted to 10µg/mL (in 8M urea) and stored in 1mL aliquots at -20C until use.

Human sera. Sera were obtained from individuals living in areas of Santiago del Estero, Argentina endemic for *Trypanosoma cruzi* and were

analyzed using conventional serologic tests (e.g. immunofluorescence assay (IFI), hemagglutination (HAI), and ELISA) performed at the Diagnostic Department of the Instituto Nacional de Parasitología "Dr. Mario Fatale Chabén" and in our laboratory by a commercial ELISA serodiagnostic kit (Hemagen Diagnostics, Columbia, MD). The latter assay was carried out as per the manufacturer's instructions with a positive response defined as 10% above the cutoff (0.250+mean of negative control absorbencies). Three serum pools were created: a "sero-negative" pool consists of 4 sera negative on all assays; a "borderline positive" pool made up of 5 sera with a response at or just above the equivocal zone of the Hemagen test (between cutoff and below cutoff + 10%); a "strong positive" pool containing 7 sera that gave unequivocally positive responses on all tests. True negative controls were obtained from volunteer donors who were not from endemic areas. Sera used for subsequent analysis of individual proteins were obtained from *T. cruzi*-infected adult volunteers aged 29 to 61 recruited through the Chagas Disease Section of the Cardiology Department, Hospital Interzonal General de Agudos "Eva Perón", Buenos Aires, Argentina and infection status was determined serologically as described above. In some cases, subjects treated by a 30 day course of benznidazole as previously described (Viotti et al., 2006, Ann Intern Med 144: 724-734) donated serum samples prior to treatment and at regular intervals following treatment. The protocols were approved by the IRBs of the University of Georgia and the Hospital Interzonal General de Agudos "Eva Perón" and signed informed consent was obtained from all individuals prior to inclusion in the study.

Multiplex assay. Recombinant proteins were attached to Liquichip™ Ni-NTA beads (Qiagen) or Beadlyte Nickel Beads (Upstate Biotechnology) by overnight incubation at 4°C in the dark. The sets of distinct addressable beads, each with a different protein attached, were pooled in equal volumes along with positive and negative control beads, consisting respectively of Liquichip™ Carboxy Beads (Qiagen) coupled to *T. cruzi* lysate and Liquichip™ Ni-NTA beads coated with recombinant HIS-tagged green fluorescent protein (GFP). Sera at 1:500 dilutions were added and the multiplex assays conducted using standard procedures (Waterboer et al., 2006, J Immunol Methods 309: 200-204). Antibody binding to individual beads was detected with goat anti-human

IgG conjugated to phycoerythrin (Jackson ImmunoResearch, West Grove, PA) and quantified on a BioPlex Suspension Array System (BioRad).

Statistical analysis. Serum samples were assayed in duplicate and the weighted mean fluorescence intensity (MFI) was calculated for a minimum of 30 beads per determination. The ratio of the specific MFI for each antigen to the MFI of the negative control (GFP- or OVA-coupled) protein was then calculated for each serum and antigen in the assay. Values above the mean plus 4 standard deviations of a minimum of sixteen true negative sera run in the same assay, and individually determined for each antigen, were considered positive.

Results

As part of a vaccine discovery effort, nearly 1500 genes from *T. cruzi* have been cloned into Gateway entry vector plasmids that allow them to be easily moved into a range of other plasmids. Genes were selected for cloning using a variety of criteria, initially including known expression in *T. cruzi* lifecycle stages that are present throughout infection in mammals (e.g. trypomastigotes and amastigotes), high likelihood of being surface expressed or secreted and expected presence in the genome at low copy number. With the completion of the *T. cruzi* genome sequencing project (El-Sayed et al., 2005, Science 309: 409-415) and whole organism proteome analysis (Atwood et al., 2005, Science 309: 473-476) the additional criterion of being relatively high in abundance in the proteomes of trypomastigotes and amastigotes was added as a basis for selection. Recombinant proteins produced in *E. coli* had N-terminal tags carrying the 6XHis-, PTD (Ho et al., 2001, Cancer Res 61: 474-477) and HA- tags for purification, protein translocation, and identification, respectively were captured by Ni-coupled Luminex beads for use in a multiplex bead array assay.

Selection of the diagnostic panel. The initial selection screen (Figure 7) used approximately 420 proteins produced in pools of 8-10 proteins each. Production of pooled proteins was accomplished by moving sets of genes in batch into the PTD-4 expression plasmid and was confirmed by SDS-PAGE analysis (Figure 8). In addition to the individual or pooled recombinant *T. cruzi*

proteins, each screening experiment included negative control recombinant protein (ovalbumin or GFP) expressed from the PTD-4 plasmid as well as a lysate of trypomastigotes and amastigotes of *T. cruzi* that had been chemically coupled to BioPlex beads.

5 To screen the pooled proteins we also took a pooling approach by assembling sera from subjects with and without documented infection with *T. cruzi*. Screening of 51 protein pools revealed 21 pools that were reactive with one or more of the serum pools 1-3 (Figure 7). Reactive pools were then broken down into their individual constituent proteins; a total of 140 proteins were
10 successfully expressed and individually rescreened with the serum pools, ultimately resulting in the selection of 55 proteins with serodiagnostic potential (Figure 7 and Table 2). An additional 22 proteins that were either identified as high-abundance proteins using proteome analysis (Atwood et al., 2005, Science 309: 473-476) and/or as being unique to *T. cruzi* (and thus not encoded in the *T.*
15 *brucei* or *Leishmania major* genomes) were then screened using the pooled sera, and 4 of these 22 were found to be reactive with one or more serum pools. Of the resulting 59 candidate proteins recognized by antibodies in the serum of *T. cruzi*-infected subjects, a substantial number were subsequently excluded from further testing either because they exhibited significant reactivity with sera from
20 the true negative pool, or because they interfered with other beads in the multibead assays, perhaps because of protein-protein interactions. Preference was also given to *T. cruzi* proteins that detected antibodies in sera from the “borderline” pools. Ultimately 39 proteins (in bold and italics in Table 2) were selected for extensive further testing with a wider array of individual subject
25 sera.

Table 2: The 59 candidate diagnostic proteins screened independently with individual (non-pooled) sera.

Gene Id	Gene name(s)	Notes	% reactive with 121 known positive sera
Tc00.1047053506391.10, and Tc00.1047053509233.180	calmodulin and ATPase beta subunit	high abundance	32.23%
Tc00.1047053507029.30	heat shock 70 kDa protein, mitochondrial precursor, putative	high abundance	52.89%
Tc00.1047053510955.40	axoneme central apparatus protein, putative		42.15%
Tc00.1047053511215.119	69 kDa parafflagellar rod protein, putative		23.97%
Tc00.1047053511271.10	dispersed gene family 1 fragment 4	unique to <i>T. cruzi</i>	5.08%
Tc00.1047053506529.610	hypothetical protein		17.27%
Tc00.1047053506391.30	EF-hand protein 5		2.48%
Tc00.1047053506635.130	hypothetical protein, conserved	high abundance	68.60%
Tc00.1047053511265.10	dispersed gene family 1 fragment 5	unique to <i>T. cruzi</i>	8.62%
Tc00.1047053511289.30	aminopapilidase, putative		11.57%
Tc00.1047053506195.110	malate dehydrogenase, putative	high abundance	24.79%
Tc00.1047053508461.140	poly(A)-binding protein	high abundance	34.17%
Tc00.1047053508441.20	glycosomal phosphoenolpyruvate carboxykinase, putative	high abundance	59.29%
Tc00.1047053508355.250	60S acidic ribosomal subunit protein, putative	high abundance	75.21%
Tc00.1047053511633.79	microtubule-associated protein homolog	high abundance	74.38%
Tc00.1047053510433.20, and Tc00.1047053504277.11, and Tc00.1047053504157.130	ToIT proteins	unique to <i>T. cruzi</i>	74.38%
Tc00.1047053411235.9	alpha tubulin		
Tc00.1047053510877.30	hypothetical protein, conserved		
Tc00.1047053509695.220	serine carboxypeptidase (CBP1), putative		
Tc00.1047053510887.50	hypothetical protein, conserved		
Tc00.1047053509141.40	hypothetical protein, conserved		
Tc00.1047053506247.220	histidine ammonia-lyase		
Tc00.1047053509995.10	60S ribosomal protein L4, putative		
Tc00.1047053504163.50	fructose-bisphosphate aldolase, glycosomal, putative		
Tc00.1047053507089.270	dihydrolipoyl dehydrogenase, putative		
Tc00.1047053511019.90	iron superoxide dismutase, putative		
Tc00.1047053509017.20	hypothetical protein, conserved		
Tc00.1047053506529.360	cytochrome C oxidase subunit IV, putative		
Tc00.1047053510187.50	tyrosine aminotransferase, putative		
Tc00.1047053505989.110	hypothetical protein, conserved		
Tc00.1047053508209.140	protein disulfide isomerase, putative		
Tc00.1047053506531.20	hypothetical protein, conserved		
Tc00.1047053504153.280	hypothetical protein, conserved		
Tc00.1047053509233.180	ATPase beta subunit, putative		
Tc00.1047053506563.40	beta tubulin		
Tc00.1047053506459.290	elongation factor-1 gamma, putative		
Tc00.1047053508707.200	nucleoside diphosphate kinase, putative		
Tc00.1047053506529.460	hypothetical protein, conserved		
Tc00.1047053506297.270	60S ribosomal protein L26, putative		
Tc00.1047053511527.34	60S ribosomal protein L2, putative		
Tc00.1047053507483.4	polyubiquitin, putative		
Tc00.1047053509053.70	p22 protein precursor, putative		
Tc00.1047053506585.40	glucose-regulated protein 78, putative		
Tc00.1047053511185	dispersed gene family 1 fragment 8		
Tc00.1047053511589.130	14-3-3 protein, putative		
Tc00.1047053511167.90	14-3-3 protein, putative		
Tc00.1047053507241.30	arginine kinase, putative		
Tc00.1047053510579.70	nascent polypeptide associated complex subunit, putative		
Tc00.1047053506925.300	cyclophilin a		
Tc00.1047053509775.40	iron superoxide dismutase, putative		
Tc00.1047053503583.40	trans-splicing factor, putative		
Tc00.1047053510099.120	d-isomer specific 2-hydroxyacid dehydrogenase-protein, putative		
Tc00.1047053507093.300	hypothetical protein, conserved		
Tc00.1047053508479.340	succinyl-CoA synthetase alpha subunit, putative		
Tc00.1047053509815.120	dispersed gene family 1 fragment 9		
Tc00.1047053511727.270	RNA-binding protein, putative		
Tc00.1047053503781.80	universal minicircle sequence binding protein (UMSBP), putative		
Tc00.1047053506201.39	translation elongation factor 1-beta, putative		
Tc00.1047053506845.20	hypothetical protein		

5 Note: Tc00 numbers indicate closest homologue(s) present in the *T. cruzi* CL
 Brener sequence database (Tcruzidb.org) based upon sequencing of the genes
 (for top 16) or predicted based upon primer sequences used in cloning. Because
 some primers for PCR cloning were designed prior to the release of the *T. cruzi*
 10 CL Brener sequence and the cloning involved the pooling of multiple clone
 derived from the PCR of a mixture of *T. cruzi* strains (see Material and
 Methods), some proteins were derived from mixtures of genes (e.g. numbers 1
 and 16) and/or had a percent sequence identity <100% relative to the CL Brener
 strain (range 94.7 to 100%). In some cases (e.g. # 5 and 9) genes > 2 kb in
 15 length were cloned in ~2 kb fragments in order to facilitate cloning and protein
 production. Items listed in bold type were selected for screening using >100
 individual sera. Items underlined were selected to be part of the final 16 set
 bead array for screening of discordant sera or sera from subjects post-treatment
 with benznidazole.

Although the Luminex bead array technology theoretically accommodates up to 100 distinct, addressable beads in a single well – and thus the ability to assay up to 100 individual proteins - at the time of this work only 17 distinct beads were available with the ability to capture his-tagged proteins.

5 Thus our goal in the second part of the screen was to identify a set of the 16 best *T. cruzi* proteins (allowing a bead for a control non-*T. cruzi* protein). The 39 candidate diagnostic proteins were tested in sets of 8-15, with each protein on a separate bead and with a negative control bead (HIS-tagged ovalbumin (OVA)) and a positive control bead (*T. cruzi* lysate) included in each assay sample.

10 Between 38 and 48 individual sera from endemic subjects were used to test each protein. These sera are grouped as “uniformly positive” (reactive on all conventional serological tests), “inconclusive” (positive on at least one, but not all, conventional serologic tests), and “negative by conventional tests”, and “known negative” (from residents of North America). Figure 9 shows a

15 representative set of 29 proteins tested with 54 individual sera and indicates the range of reactivities of both sera and proteins. In addition to providing the basis on which to select the top proteins, this analysis also revealed that among the 30 sera that were inconclusive or negative on conventional tests, nearly half (14 of 30) had substantial reactivity to 3 or more recombinant *T. cruzi* proteins but not

20 with the control OVA protein.

Following repeated screening, 16 proteins were selected to be part of the diagnostic panel (underlined in Table 2). DNA sequencing and mass spectrometric analysis confirmed the identity of each gene and protein and determined that one of the preparations contained two distinct proteins

25 (Calmodulin and an ATPase) and a second contained a mixture of related TolT proteins. This protein set was then used to screen a larger set of sera, most from chronically infected subjects living in Buenos Aires, and the percentage of these proteins reactive with 121 sera from well-characterized subjects was determined (Table 2). A serum was determined to be positive for any particular test

30 antigen if the average luminescence (MFI) was >4 standard deviations above that of a set of true negative sera run in the same assay. Across all experiments, for the 19 true negative sera assayed multiple times (142 sample runs tested on 16 protein preparations for a total of 2272 determinations), none had S.D. >4

and only 17 of the 2272 determinations were >3 S.D. above the average negative serum values (and 9 of these 17 were from one serum sample reactive with the same antigen in multiple tests). Thus this was a highly stringent cutoff. Sera from all 121 of the confirmed chronically infected subjects reacted with at least 1 of the 16 recombinant protein preparations at the >4 S.D. cutoff and all but 7 reacted with >1 protein. As shown in Table 2, 6 of the 16 of the antigens each detected >50% of the sera and 3 antigens approached a 75% detection rate. Of the 121 sera tested, 118 would have been detected as positive using only 4 of the antigens and 100% would be detected using as few as 7 antigens.

10 *Borderline samples.* We then used our 16 bead multiplex test to attempt to resolve questionable infection status in subjects due to discordant results on conventional tests (Table 3). In this analysis, a cutoff for reactivity for each protein in the panel was set at the MFI plus 4 SD above the mean of a set of 16 negative sera. For comparison, the result of multiplex analysis of a pool of
15 strongly positive sera assayed on different days is also shown. The strong positive serum pool showed excellent cross-assay consistency with 11 of the 16 protein preparations positive on each of 8 assays and consistent negative reactivity with 3 of 16. Antibodies to the remaining 2 proteins were also detected but at a lower level that sometimes fell below the strict cutoff of 4 S.D.
20 above the mean. The sera classified as “conventional seronegative with no other evidence of infection” broke into 2 groups based upon the results of the multiplex test. Eight of the 16 failed to react with any of the 16 protein panel (although several reacted with the *T. cruzi* lysate) while the remaining 8 reacted with from 2-4 proteins. A similar nearly 50/50 split was observed in the group
25 of 12 conventional seronegatives who were born in an endemic region, and in 5 individuals who had cardiopathologies consistent with Chagas disease. Lastly, testing in the multiplex assay of sera classified as “positive discordant” (based upon reactivity on 2 of the 3 conventional serologic tests but negative on the 3rd test) confirmed the positive diagnosis in all 7 cases with reactivity evident on 2-
30 6 recombinant proteins by each serum. Without a clear gold standard diagnostic it is not possible certify on a case-by-case basis that the multiplex assay more accurately detects infection than does conventional serology - particularly in cases where there is reactivity to only 1 or 2 proteins and near the

>4 S.D. cutoff. And while the birth place and presence of heart disease may support a positive diagnostic test, these criteria do not appear to distinguish between those likely to have reactivity with one or more recombinant proteins in the selected panel and those who do not react. However it is clear that conventional serological tests fail to detect a substantial number of individuals, many with antibodies to multiple *T. cruzi* antigens. It is noteworthy that screening of sera with a parasite lysate also routinely fails to detect sera that exhibit reactivity to multiple recombinant *T. cruzi* proteins. The set of 4 most frequently recognized proteins detected all 7 of the discordant positive samples as well as 13 of the 15 discordant negative or negative samples that reacted with at least 1 protein. Expanding the panel to the 7 proteins that detected all of the seropositive samples (see above) allowed us to detect all of these 15 questionable “negative” samples.

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Sera	Serology	HAI	ELISA	IFI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	lysate	#>4 SD
Pooled sera from strongly reactive																						
Fc383	neg	nr	nr	nr	0.41	-0.71	-0.16	-1.50	0.43	0.90	-1.22	0.47	-0.35	0.11	-0.45	0.73	0.65	1.61	0.78	-0.51	4.90	0
Fc323	neg	nr	nr	nr	-1.07	-0.50	0.42	-1.94	-1.81	-1.37	-1.26	-0.49	0.16	-0.50	-0.84	-0.71	-1.32	-0.58	-0.36	2.89	-0.73	0
Fc438	neg	nr	nr	nr	-1.13	-0.32	0.41	0.52	-0.61	0.93	-0.12	-0.08	-1.15	0.15	-0.79	-0.88	-1.13	0.51	0.32	-0.77	-0.33	0
Fc410	neg	nr	nr	nr	0.45	-0.11	0.66	-1.81	1.02	-0.68	-1.16	1.21	-0.25	-0.37	-0.97	-0.43	-0.86	0.97	0.99	1.08	-1.16	0
Fc411	neg	nr	nr	nr	0.03	0.40	0.42	-0.21	0.05	2.88	0.13	0.44	1.84	-0.29	1.01	-0.07	2.96	0.67	1.65	0.55	9.09	0
Fc453	neg	nr	0.024	nr	1.53	0.59	-0.68	1.51	1.24	-0.22	0.58	0.00	0.45	0.34	0.85	0.61	-0.24	-0.48	-0.06	2.09	0.10	0
Fc408	discord	32	nr	nr	0.55	-0.11	-0.18	0.39	0.94	-0.38	0.40	1.72	-0.04	0.02	-0.45	0.12	0.99	0.46	2.08	1.16	1.93	0
Fc409	discord	32	nr	nr	-0.09	1.42	1.61	0.65	-0.64	2.35	0.09	1.59	0.55	0.37	2.36	1.47	3.14	0.20	1.96	2.81	0.35	0
Fc437	neg	nr	nr	nr	-0.09	2.96	1.90	1.69	0.55	nd	0.07	1.73	-0.78	-1.02	1.13	1.11	4.05	2.29	2.73	0.91	-0.24	2
Fc436	neg	nr	nr	nr	1.26	5.11	1.41	-0.10	0.28	2.57	0.07	1.40	0.59	2.05	-0.81	4.33	3.05	1.30	1.40	3.10	0.58	2
Fc415	neg	nr	nr	nr	1.70	5.04	4.12	0.22	1.20	0.61	0.17	1.92	2.27	2.01	1.69	1.24	1.42	2.71	2.84	2.38	-0.27	2
Fc324	neg	nr	nr	nr	-0.50	0.03	0.62	0.18	-0.69	0.50	-0.11	-0.84	-1.66	2.55	-0.79	-0.35	-0.64	5.32	-0.76	-0.54	-0.24	2
Fc426	discord	nr	0.088	nr	-1.39	2.45	2.01	0.77	-2.06	-0.89	0.08	2.24	-0.81	-1.42	-1.15	0.98	2.19	4.32	4.55	0.07	-0.06	2
Fc320	neg	nr	nr	nr	-0.79	0.76	5.19	-1.84	0.92	1.56	-1.67	-0.16	2.41	-0.49	3.86	1.28	4.75	0.63	5.05	17.92	3.63	4
Fc434	neg	nr	0.038	nr	8.46	1.78	6.78	-0.43	7.10	2.04	-0.73	2.82	3.18	4.67	1.82	1.58	2.69	0.48	1.91	3.56	0.82	4
Fc439	neg	nr	nr	nr	-1.31	-0.84	6.29	4.65	1.18	-0.51	0.95	0.88	-1.09	0.17	-0.44	-0.97	0.80	4.76	1.23	1.18	0.43	4
no other evidence																						
Fc423	neg	nr	0.023	nr	-2.05	0.25	0.27	1.03	-1.72	-0.21	0.95	-1.14	-0.72	-0.86	-0.84	2.02	-1.87	0.25	-1.16	-0.98	-0.91	0
Fc444	neg	nr	0.059	nr	-1.44	-0.91	-1.90	0.76	-1.24	-1.68	-1.55	-0.96	-1.01	-0.75</								

Sera judged cumulatively as "seronegative" based upon conventional serology were grouped into negative but "no other evidence" of exposure (16 sera), those "born in an endemic area" (12), those with evidence of "heart disease" consistent with Chagas disease(5) and compared to (top) pools of strongly positive sera (high reactivity in all serological tests) and to (bottom) sera from subjects who were negative on at least one of the three conventional serologic tests (discordant positive). Reactivity in the conventional serological tests (HAI, ELISA and IFI) and the summary consensus of these tests (neg = below cut-off for all three tests; discord = positive on one of the three tests; discord + = positive on 2 of the 3 tests), as well as reactivity to the 16 recombinant protein sets and the *T. cruzi* lysate are shown. Cutoffs for a positive ELISA is an O.D.> 0.200 and for IFA and HAI is a dilution >1/32 (a reaction at 1/16 is considered "reactive but negative" and < 1/16 non-reactive (nr)). The metric for reactivity of each serum for each protein is expressed as the number of standard deviations that the ratio of the MFI for *T. cruzi* protein to the MFI for GFP was above the average ratios of sixteen true negative sera run in the same assay. Values >4 S.D. above this "background" reactivity are considered reactive and are highlighted. The total number of reactive recombinant proteins for each serum is indicated in the right-most column. nd = not determined (insufficient numbers of beads detected in this sample)

Monitoring treatment efficacy. There is a pressing need for a means to assess treatment efficacy in Chagas disease so we next used the multiplex assay to monitor changes in serology over time in subjects treated with benznidazole (BZ). Representative data from a set of 16 non-endemic normals (Figure 10A) demonstrates the background level of detection of responses in uninfected individuals, displayed as the MFI for each protein. To establish the stability of serological responses over time in the absence of treatment, serial serum samples were obtained from chronically infected, seropositive subjects, all without clinical disease; a representative set of 6 subjects screened at 4 times points for up to 21 months is shown in Figure 10B. Each subject exhibits a distinct pattern of serological responses and both the pattern and the potency of those responses are remarkably stable over time. In contrast, a representative set of 4 (from a total of 38) subjects followed for up to 36 months after treatment with BZ shows that some subjects exhibit a post-treatment decrease in the strength of responses to most *T. cruzi* antigens tested (Figure 11A). In many cases this fall is evident by 2 months post-treatment (e.g. PP001, PP115, PP164) and is followed by a transient increase at 6 months. Interestingly, this early drop in antibody levels following treatment is also sometimes evident, although less consistently so, with conventional serological tests, particularly with indirect hemagglutination (Figure 11A). Subject PP117 has borderline positive

serology in both the multiplex and the conventional serologic assays, and is representative of a case in which documenting changes following treatment would be difficult. Figure 11A). Figure 11B presents 2 other patterns of responses following treatment. PP044 shows essentially no change in the pattern or potency of antibody responses up to 24 months post-treatment. Subject PP024 is similar in that responses to the several prominently detected proteins are relatively stable over time. However the MFI reading for numerous other antigens falls consistently over the 24 month monitoring period. Thus, although it might take more time and additional assays to determine treatment efficacy in these two subjects, a preliminary assessment would be that treatment failed in the case of PP044 but was successful for subject PP024.

Discussion

The poor quality of diagnostics for *T. cruzi* infection is a major impediment to coping with a disease that affects as many as 20 million people. Without quality diagnostics, the statistic of the disease burden is at best a guess, the ability to conclusively identify who should be treated, or should be allowed to donate blood or tissues is greatly compromised and the effectiveness of interventions to limit transmission or drugs to treat those infected is impossible to determine with any certainty.

In the early stages of *T. cruzi* infection, parasites can often be detected in blood. However, as immunity develops, even amplification techniques such as xenodiagnosis, hemaculture, and PCR, despite being repeated multiple times, routinely fail to detect infection (Castro et al., 2002, Parasitol Res 88: 894-900; Picka et al., 2007, Braz J Infect Dis 11: 226-233; Salomone et al., 2000, Am J Cardiol 85: 1274-1276; Duarte et al., 2006, Rev Soc Bras Med Trop 39: 385-387). Consequently, determination of infection status is largely dependent on the consensus results of multiple tests with different formats (e.g. ELISA, indirect fluorescent antibody, indirect hemagglutination, complement fixation). However the unreliability of these tests is well documented (Pirard et al., 2005, Transfusion 45: 554-561; Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Avila et al., 1993, J Clin Microbiol 31: 2421-2426; Castro et al., 2002, Parasitol Res 88: 894-900; Caballero et al., 2007, Clin Vaccine Immunol.

14:1045-1049; Silveira-Lacerda et al., 2004, Vox Sang 87: 204-207; Wincker et al., 1994, Am J Trop Med Hyg 51: 771-777; Gutierrez et al., 2004, Parasitology 129: 439-444; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka et al., 2007, Braz J Infect Dis 11: 226-233; Zarate-Blades et al., 2007, Diagn Microbiol Infect Dis 57: 229-232). Many of these tests, including one recently licensed by the United States Food and Drug Administration for use as a blood screening test in the U.S. (Tobler et al., 2007, Transfusion 47: 90-96), use crude or semi-purified parasite preparations derived from parasite stages present in the insect vector but not in infected humans. Recently a number of recombinant parasite proteins or peptides have also come into limited use for diagnosis (da Silveira et al., 2001, Trends Parasitol 17: 286-291; Chang et al., 2006, Transfusion 46: 1737-1744; Kirchhoff et al., 1984, J Immunol 133: 2731-2735; Laucella et al., 2004, J Infectious Diseases 189:909-918).

A subject whose serum is consistently positive on multiple of the currently used tests is relatively easily determined to be infected. But the infection status of individuals positive on only one test (as in blood bank screening) is unclear and detection of parasites in subjects who are negative using multiple conventional serologic tests (Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Gutierrez et al., 2004, Parasitology 129: 439-444; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka, et al., 2007, Braz J Infect Dis 11: 226-233; Wincker et al., 1994, FEMS Microbiol Lett 124: 419-423) or who are positive by alternative but not widely available serological tests (Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; Zarate-Blades et al., 2007, Diagn Microbiol Infect Dis 57: 229-232) is not uncommon.

Furthermore, currently available tests are inadequate for monitoring treatment efficacy (Sanchez Negrette et al., 2008, Clin Vaccine Immunol 15: 297-302; Bahia-Oliveira et al., 2000, J Infect Dis 182: 634-638; Solari et al., 2001, J Antimicrob Chemother 48: 515-519) and thus may also give inaccurate measurements of the effectiveness of other interventions.

With these deficits in mind, we set out to identify parasite proteins that would more effectively detect *T. cruzi* infection and provide a tool for monitoring changes in infection status over time. Development of a repository of nearly 1500 *T. cruzi* genes cloned into Gateway entry vectors provided a

relatively straightforward approach to producing a large number and diversity of *T. cruzi* proteins appropriate for high-throughput screening of diagnostics. Adding the targeted approach of selecting proteins documented for high level expression in trypomastigote and amastigote stages of *T. cruzi* allowed us to
5 also focus on the proteins that would be predicted to elicit the strongest antibody response in infected humans. The Luminex-based multiplex bead array system permitted us to screen many proteins simultaneously with very low requirements for serum. The production of histidine-tagged proteins also made it relatively uncomplicated to attach the recombinant proteins to Luminex
10 beads. This latter point is not trivial as the proteins could be coupled to the assay beads directly from the denaturing urea-based lysis buffer without the requirement of movement to a non-denaturing buffer, wherein many of the proteins precipitated. The strong response detected using proteins prepared in this way suggests either that natively folded proteins are not required for the
15 detection of these antibodies or that re-folding of the proteins attached to the Luminex beads during buffer exchange resulted in the formation of native conformational epitopes.

In addition to its utility for screening of a large number of proteins, the Luminex system also excels as a platform for multiplex analysis of antibodies to
20 a relatively large set of targets. We were restricted in this work by the number of Luminex bead sets manufactured with Ni²⁺ and thus sought to identify a maximum of 16 independent *T. cruzi* proteins that gave informative results from a large set of human sera. The ultimate panel selected by the screen included at least one protein previously identified as a potential diagnostic, the
25 mitochondrial HSP-70 (Krautz et al., 1998, Am J Trop Med Hyg 58: 137-143). It is possible that other proteins revealed in our screen have been studied previously. However since the identity of some of these previously assayed proteins is somewhat cryptic (da Silveira et al., 2001, Trends Parasitol 17: 286-291) and few have been associated with annotated genes in the sequenced *T.*
30 *cruzi* genome, this is difficult to determine. Also, over half of the antigens selected in our screen were among the 50 most abundant proteins in the trypomastigote and amastigote proteomes (Atwood et al., 2005, Science 309: 473-476). Two hypothetical proteins and 2 proteins unique to *T. cruzi* among

the sequenced kinetoplastids, including 2 fragments from the very large and multicopy dispersed gene family protein, were among the proteins selected. Proteins that are unique to *T. cruzi* could be particularly useful in a serological screen as they are absent from *Leishmania*, one of the potentially confounding infections in terms of diagnosis of *T. cruzi*. However the dispersed gene family fragments were among the worst performers in the large scale screen – with only 5-9% of all confirmed positive sera having detectable antibodies to these. Similarly, other gene family proteins, including trans-sialidases, mucins and mucin-associated proteins (MASPS) were part of the screen but failed to make even the initial selection cuts in our assays, presumably because only a small fraction of their diversity would be represented in the recombinant proteins screened.

A multiplex approach like the Luminex also provided a more detailed examination of responses than is possible using a single target consisting of either an individual protein or a protein/peptide mixture. Each individual was seen clearly to have a distinct pattern of responses to the protein panel and that this pattern was impressively stable over time (several years). This is both interesting scientifically and serves as further validation of the quality and consistency of the data generated using this multiplex methodology. This heterogeneity of responses to pathogens among individuals appears to be more the norm than the exception, as similar results have been reported for individuals infected or immunized with viral (vaccinia), bacterial (*Francisella tularensis*) and protozoal (*Plasmodium falciparum*) pathogens (Davies et al., 2007, Proteomics 7: 1678-1686; Sundaresh et al., 2006, Bioinformatics 22: 1760-1766; Sundaresh et al., 2007, Bioinformatics 23: i508-518). Thus serodiagnostics in general are likely to need to move toward multiplex assays, as single antigens that are recognized by all individuals infected by any pathogen appear to be rare (Davies et al., 2007, Proteomics 7: 1678-1686).

The ability to simultaneously and independently assess antibody responses to multiple targets was instrumental to our success in addressing the issues of the detection of serological responses in subjects who are negative by conventional serology and the relatively rapid detection of changes in selected responses following drug treatment. The multiplex assay detected 100% of 121

samples consistently positive by conventional serology, and 100% of samples positive on 2 out of 3 conventional tests. In addition, however, we also detected antibodies specific for one or more recombinant proteins in 18 of 33 subjects judged as negative by conventional serology. Other investigators have

5 documented cases of conventional seronegative subjects being seropositive on alternative tests or even parasite positive (Salomone et al., 2003, Emerg Infect Dis 9: 1558-1562; Caballero et al., 2007, Clin Vaccine Immunol. 14:1045-1049; Gutierrez et al., 2004, Parasitology 129: 439-44; Marcon et al., 2002, Diagn Microbiol Infect Dis 43: 39-43; Picka et al., 2007, Braz J Infect Dis 11: 226-

10 233; Zarate-Blades et al., 2007, Diagn Microbiol Infect Dis 57: 229-232; Wincker et al., 1994, FEMS Microbiol Lett 124: 419-423) although these previous reports of “infected seronegatives” have been somewhat anecdotal – presumably because investigators rarely screen for parasites in seronegative subjects. However in some studies parasite-positive conventional seronegatives

15 are very well documented. For example Picka et al. (Braz J Infect Dis 11: 226-233, 2007) reported on one subject who was negative by up to 5 replicates of 4 different conventional serological tests yet was positive by a combined hemaculture-PCR approach. The multiple examples of failed conventional serology to detect infection in combination with the well-documented

20 unreliability of parasitological tests, supports the conclusion that individuals who are seropositive in our multiplex assay are likely to be infected with *T. cruzi*. This conclusion is further supported by on-going studies demonstrating *T. cruzi* –specific T cell responses in subjects who are negative by conventional serology but positive in our multiplex assays. Without more sensitive

25 parasitological tests we cannot conclusively determine if the subjects who are negative by conventional serology but positive in our multiplex assay are infected or possibly “exposed” but not still infected with *T. cruzi*. And without additional extensive validation, we cannot exclude the possibility that other infections or immunological conditions resulted in some of the multiplex

30 positive responses, although standard clinical analysis failed to detect other complicating infections in these subjects. However, especially for subjects who have antibodies to up to 8 different recombinant *T. cruzi* proteins and were born in endemic areas and/or have evidence of heart disease, it is reasonable to

conclude that they are indeed infected with *T. cruzi* despite their negative results with conventional serologic assays. Overall these studies support the already documented conclusion that current serological tests can misdiagnose infection – perhaps to a significant extent.

5 A second issue we addressed using the multiplex serological assay for *T. cruzi* infection was that of efficacy of therapeutic treatment. Because most subjects are negative by parasitological assays prior to treatment (making a negative result after treatment uninformative) and remain positive by conventional serology for extensive periods of time after treatment, assessing
10 whether treatment actually achieved cure) has been problematic. When combined with other evidence of treatment failures and the adverse effects of the drugs, the absence of a method to detect treatment efficacy has resulted in a very low rate of treatment in chronic Chagas disease. This absence of a reliable and timely test for treatment efficacy is also a major impediment to the
15 development and testing of new drugs – an area that has been at a virtual standstill for decades.

 - Herein we show that the multiplex assay using the selected set of recombinant proteins can detect significant changes in antibody levels, in some cases as early as the first post-treatment assay point (2 months post-treatment
20 completion). These changes are not evident in all cases – an outcome that is not surprising given that treatment failure is common (Viotti et al., 1994, Am Heart J 127: 151-162). Our ability to assess responses to multiple targets on an individual basis appears to be crucial to the success of detection of serologic changes following treatment, as similar changes are not consistently observed
25 using conventional serologic tests. Previous studies have suggested that various recombinant antigens may provide better assessment of treatment efficacy relative to conventional serology (Sanchez Negrette et al., 2008, Clin Vaccine Immunol 15: 297-302; Sosa Estani et al., 1998, Am J Trop Med Hyg 59: 526-529).

30 In conclusion, we define a set of diagnostic targets and an assay approach that we believe is a significant improvement upon current diagnostic tests for *T. cruzi* infection both for more consistently detecting infection and for assessing the effectiveness of treatment. Additional validation of these targets

and the general methodology will require analysis of a larger set of subjects, a process that is currently on-going. Herein we have also not addressed the question of whether the antigens we identify would be useful throughout the wide endemic range for *T. cruzi*. Heterogeneity among different parasites strains in distinct regions could present a challenge. However here again this is a concern that a multiplex assay might rather easily address – it seems unlikely that all 16 proteins in our pool, most of which are abundant housekeeping proteins, would vary substantially among parasites in various regions. The problem of infection confirmation by detection of parasites or parasite products is likely to continue to be a roadblock to full acceptance of the results of this test, or any other, when they conflict with conventional serologic tests – despite the proven inadequacy of these “standard” tests. A downside of the Luminex system for multiplex analysis is the reagent expense as well as the requirement for specialized equipment to “read” the results. However, other multiplex platforms such as protein microarrays could be more cost conservative and require less infrastructure (Davies et al., 2007, Proteomics 7: 1678-1686; Kartalov et al., 2006, Biotechniques 40: 85-90). Also, our results suggest that the number of proteins in the analysis could be reduced without substantial loss of sensitivity and the possibility exists for additional improvements in sensitivity by the inclusion of *T. cruzi* proteins previously validated by others, or that could be detected in additional screens like that described herein. At a minimum, these results begin to lay the groundwork for the removal of one of the major impediments to the development and effective implementation of treatments for *T. cruzi* infection.

25

Example 10. Preliminary Panel of Serodiagnostic Proteins

In a preliminary study that eventually resulted in the serodiagnostic proteins described in Example 9, 53 diagnostic proteins was selected (Table 4) from a group of 59 candidate proteins identified through screening with serum pools and individual sera. The preliminary serodiagnostic panel selected for further study consisted of the top 16 proteins in Table 4.

30

Table 4. Protein antigens

Current panel constituents	Published as antigens?	Common Name	Annotated gene or Tc00 id of closest homolog
1		hypothetical protein	Tc00.1047053508767.10
2		hypothetical protein, conserved	Tc00.1047053506635.130
3	1,3 (ribosomal P protein, TcP0?)	60S acidic ribosomal subunit protein, putative	Tc00.1047053508355.250
4	1-2 (flagellar CaBP, 1F8)	flagellar calcium-binding protein, putative	Tc00.1047053507491.151
5	1,3 (MAP)	microtubule-associated protein, putative	Tc00.1047053511633.79
6	1 (heat shock)	heat shock 70 kDa protein, mitochondrial precursor, putative	Tc00.1047053507029.30
7	1 (paraflagellar assoc. prot)	69 kDa paraflagellar rod protein, putative	Tc00.1047053511215.119
8		EF-hand protein 5	Tc00.1047053506391.30
9		aminopeptidase, putative	Tc00.1047053511289.30
10		axoneme central apparatus protein, putative	Tc00.1047053510955.40
11		hypothetical protein	Tc00.1047053506529.610
12		glycosomal phosphoenolpyruvate carboxykinase, putative	Tc00.1047053508441.20
13		dispersed gene family protein 1 fragment	Tc00.1047053511271.10
14		malate dehydrogenase, putative	Tc00.1047053506195.110
15		dispersed gene family protein 1 fragment	Tc00.1047053511265.10
16		poly(A)-binding protein	Tc00.1047053508461.140

Understudy - ranked by approximate potential

17	1 (cytoskeleton assoc?)	beta tubulin	Tc00.1047053506563.40
18	1 (cytoskeleton assoc?)	alpha tubulin	Tc00.1047053411235.9
19	1 (ribosomal prot)	60S ribosomal protein L28, putative	Tc00.1047053506297.270
20	1 (ribosomal prot)	polyubiquitin (pseudogene), putative	Tc00.1047053507483.4
21		iron superoxide dismutase, putative	Tc00.1047053511019.90
22		iron superoxide dismutase, putative	Tc00.1047053509775.40
23		elongation factor-1 gamma, putative	Tc00.1047053506459.290
24		hypothetical protein, to be annotated	Tc00.1047053507515.4
25		dispersed gene family protein 1 fragment	Tc00.1047053509815.120
26		hypothetical protein, conserved	Tc00.1047053504153.280
27		p22 protein precursor, putative	Tc00.1047053509053.70
28		25 kDa translation elongation factor 1-beta, putative	Tc00.1047053506201.39
29		universal minicircle sequence binding protein (UMSBP), putative	Tc00.1047053503781.80
30		hypothetical protein, conserved	Tc00.1047053510877.30
31		d-isomer specific 2-hydroxyacid dehydrogenase-protein, putative	Tc00.1047053510099.120
32		RNA-binding protein, putative	Tc00.1047053511727.270
33		stress-induced protein sti1, putative	Tc00.1047053506321.290
34		glutamyl carboxypeptidase, putative	Tc00.1047053510837.20
35	1 (trans-sialidase)	chunk of conserved hypothetical protein	Tc00.1047053509099.160
36		centrin, putative	Tc00.1047053506559.380
37		possible salivary proline-rich protein rp15	Tc00.1047053506835.110
38		tuzin	Tc00.1047053507485.140
39	1 (trans-sialidase)	chunk of putative trans-sialidase	Tc00.1047053507997.14
40	4 (cruzipain)	cysteine peptidase, putative	Tc00.1047053507603.270
41	1 (heat shock)	Tc 85 kDa antigen with homology to heat shock proteins	Tc00.1047053509643.130
42	1 (heat shock)	Tc hsp 70	Tc00.1047053511211.170
43		serine carboxypeptidase (CBP1), putative	Tc00.1047053509695.220
44		hypothetical protein, conserved	Tc00.1047053510887.50
45		hypothetical protein, conserved	Tc00.1047053509141.40
46		dihydrolipoyl dehydrogenase, putative	Tc00.1047053507089.270
47		hypothetical protein, conserved	Tc00.1047053506529.460
48		trans-splicing factor, putative	Tc00.1047053503583.40
49		GTP-binding nuclear protein rtb2, putative	Tc00.1047053503539.30
50		14-3-3 protein, putative	Tc00.1047053511167.90
51		nascent polypeptide associated complex subunit, putative	Tc00.1047053510579.70
52		hypothetical protein, conserved	Tc00.1047053507093.300
53		succinyl-CoA synthetase alpha subunit, putative	Tc00.1047053508479.340

1 da Silveira, et al., TRENDS in Parasitology Vol.17 No.6 June 2001, p.286-291

2 Umezawa, et al., TRANSFUSION Volume 43, Jan. 2003, p. 91-97

3 Umezawa, et al., JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 449-452

4 Martinez, et al., INFECTION AND IMMUNITY, Nov. 1991, p. 4275-4277

- 5 Figure 12 shows the pattern of responses of 11 subjects to 11 antigens and 2 controls (ovalbumin as the negative control, and *T. cruzi* lysate as a positive control) at the time of the first bleed (Figure 12A) and 12 months later (Figure 12B). Three aspects are worthy of mention. First, the pattern of responses is unique in each individual; no one appears to respond similarly.
- 10 Second, the pattern of responses is stable over the 12 month period. This is a strong indicator that the assay is indeed reproducibly detecting a persistent

response. This is also important with respect to using the test to monitor changes after therapy. Third, no single recombinant protein (or even combination of 3 or 4 proteins) detects all responders. This result validates the need for a multicomponent test for *T. cruzi* infection.

5

Example 11. Monitoring treatment for Chagas disease

Benzindazole is the primary compound used for treatment of Chagas disease, although there is controversy about its efficacy in treating chronically
10 infected subjects, such as those who have been infected >20 years). The left side of Figure 13 below shows consistent serologic responses at 0 and 12 months in the absence of treatment in 4 subjects. The right side shows 6 treated subjects, treated at time 0 and reassayed at 12 and 24 months post-treatment. Changes in serology are obvious in 3 of the treated subjects at 12 months. The
15 other 3 subjects have little to no change, even at 24 months. It is worth noting that conventional serology conducted on these same sera showed inconsistent or no changes. Furthermore, the percentage of individuals showing serological changes consistent with cure is similar to that reported in other like studies but using much longer follow-ups. E.g., a recent study by Fabbro et al. (Rev Soc
20 Bras Med Trop. 2007 Jan-Feb;40(1):1-10), reported a treatment efficacy rate of 35-63% based upon conventional serology but required an average 16 year follow-up to see this change. Moreover, nearly 100% of those showing changes in serology also showed changes in T cell responses (Figure 14; note decreasing to undetectable responses within 12-25 months). Similar changes were not seen
25 in untreated subjects or in treated subjects who failed to exhibit serological changes. Overall we conclude that this multiplex assay has the capability to relatively rapidly detect treatment success or failure – especially when coupled with assays of T cell reactivity.

30

Example 12. Maternal/neonate diagnostics

The unique pattern of responses in each individual may have utility with respect to monitoring congenital infection, which is currently very difficult. If the pattern of the serologic response in mother and newborn is similar, then we would suspect that the infant's serologic response is a result of maternally derived antibodies rather than to antibodies produced by the infant. However if the patterns are different, then we would suspect that the child is infected and therefore should be treated.

Four separate pairs of mother and newborn were evaluated. Serum antibody titers against individual recombinant *T. cruzi* proteins were determined for both the mother and the infant at a time point relatively soon after birth (within weeks), and again approximately 6 months later. For all pairs, the infant's pattern of response shortly after birth resembles the mother's pattern of response, indicating the presence of maternal antibodies (Figure 15). At the later time point, two of the infants (Figure 15B, and D) show a pattern of response differs from the mother's, indicating that these infants may be infected and producing their own antibodies. Another infant (Figure 15A) shows a response that is near background levels, suggesting that the infant is likely not infected. Circles over the individual measurements indicate statistically significant responses.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. An article comprising:
a substrate comprising a surface; and
5 a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, immobilized on said substrate surface; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1.
10
2. The article of claim 1 wherein the polypeptides are immobilized on the substrate surface to form a microarray.
3. The article of claim 1 wherein the substrate comprises at least one
15 nanoparticle, and wherein the polypeptides are immobilized on the surface of the nanoparticle.
4. A kit for diagnosis of *T. cruzi* infection comprising:
an article according to claim 1; and
20 packaging materials and instructions for use.
5. The kit of claim 4 formulated for medical or veterinary use.
6. A method for obtaining information about a known or suspected *T. cruzi*
25 infection in a mammal, or for determining whether a mammal is or has been infected by *T. cruzi*, said method comprising:
obtaining a biological sample from the mammal;
contacting the biological sample with a plurality of individually
addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits
30 thereof; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1; and

evaluating the presence, absence, intensity or pattern of interaction of components of the biological sample with the antigenic *T. cruzi* polypeptides to determine the presence or absence of *T. cruzi* infection, the identity of the infective strain, the length of the infection, the stage of the infection, whether
5 the infection is still present or the mammal has been cured, the vaccination status of the mammal, the success of treatment, or any combination thereof.

7. The method of claim 6 wherein the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof, is
10 immobilized on a substrate surface.

8. The method of claim 6 wherein the mammal is a human or a dog.

9. The method of claim 6 wherein the biological sample comprises a body
15 fluid comprising an antibody.

10. The method of claim 9 wherein the body fluid comprises blood, plasma or serum.

20 11. The method of claim 6 wherein the biological sample comprises a mononuclear blood cell.

12. The method of claim 6 wherein the biological sample comprises a peripheral blood mononuclear cell (PBMC) fraction of blood from the mammal.
25

13. The method of claim 6 wherein the method is a serodiagnostic method, and wherein the biological sample component that interacts with the antigenic *T. cruzi* polypeptide is an antibody from the mammal.

30 14. The method of claim 6 wherein the method is a cellular assay method, and wherein the biological sample component that interacts with the antigenic *T. cruzi* polypeptide is T cell from the mammal.

15. The method of claim 6 wherein the method comprises a multiplexed assay wherein the biological sample is contacted simultaneously with the plurality of antigenic *T. cruzi* polypeptides.
- 5 16. The method of claim 6 wherein the biological sample is obtained from an actual or potential blood donor or transplant donor.
17. The method of claim 6 wherein the biological sample is obtained from a pooled blood product supply intended for use in transfusions or research.
- 10 18. A method for detecting contamination of a blood product supply with *T. cruzi*, the method comprising:
- selecting a sample from the blood supply;
- contacting the sample with a plurality of individually addressable
- 15 antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; wherein at least one polypeptide is selected from the polypeptides listed in Table 2 or Table 4, with the proviso that the at least one polypeptide is not listed in Table 1; and
- evaluating the presence, absence, intensity or pattern of interaction of
- 20 components of the sample with the antigenic *T. cruzi* polypeptides to determine whether the blood supply is contaminated with *T. cruzi*.
19. The method of claim 18 wherein the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits
- 25 thereof, is immobilized on a substrate surface.
20. The method of claim 18 wherein the blood product supply comprises whole blood, a blood product, or a blood fraction.
- 30 21. The method of claim 18 wherein the blood product supply comprises a cellular blood component, a liquid blood component, a blood protein, or mixtures thereof.

22. The method of claim 18 wherein the blood product supply comprises a red blood cell concentrate, a leukocyte concentrate, a platelet concentrate, plasma, serum, a clotting factor, an enzymes, albumin, plasminogen, or a immunoglobulin, or mixtures of thereof.

5

23. The method of claim 18 wherein the method is a serodiagnostic method, and wherein the sample component that interacts with the antigenic *T. cruzi* polypeptide is an antibody.

10 24. A method for determining whether an infant has a *T. cruzi* infection, said method comprising:

obtaining a biological sample from an infant born to a mother known to have or suspected of having a *T. cruzi* infection, wherein said biological sample is obtained later than about 3 months after birth of the infant;

15 contacting the infant's biological sample with a plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; and

evaluating the presence, absence, intensity or pattern of interaction of components of the biological sample with the antigenic *T. cruzi* polypeptides to determine whether the infant exhibits an antibody response that exceeds background levels.

25. The method of claim 24 further comprising:
obtaining a biological sample from the infant's mother;
25 contacting the mother's biological sample with the plurality of individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or subunits thereof; and

comparing the presence, absence, intensity or pattern of interaction of components of the mother's biological sample with the antigenic *T. cruzi* polypeptides, to the presence, absence, intensity or pattern of interaction of
30 components of the infant's biological sample with the antigenic *T. cruzi* polypeptides, to determine whether the infant's antibody response differs from

the mother's antibody response, wherein a difference in antibody responses indicates that the infant may have a *T. cruzi* infection.

26. The method of claim 24 wherein at least one polypeptide is selected
5 from the polypeptides listed in Table 1, Table 2 or Table 4

27. The method of claim 24 further comprising:
obtaining an earlier biological sample from the infant shortly after birth;
contacting the infant's earlier biological sample with the plurality of
10 individually addressable antigenic *T. cruzi* polypeptides, or antigenic analogs or
subunits thereof; and
comparing the presence, absence, intensity or pattern of interaction of
components of the infant's earlier biological sample with the antigenic *T. cruzi*
polypeptides, to the presence, absence, intensity or pattern of interaction of
15 components of the infant's later biological sample, or of the mother's biological
sample, or both, with the antigenic *T. cruzi* polypeptides, to determine whether
the infant's later antibody response differs from the mother's antibody response,
wherein a difference in antibody responses indicates that the infant may have a
T. cruzi infection.

20

28. The method of claim 24 wherein the biological sample comprises a body
fluid comprising an antibody.

29. The method of claim 24 wherein the body fluid comprises blood, plasma
25 or serum.

30. The method of claim 25 further comprising administering a therapeutic
agent to the infant to treat a *T. cruzi* infection.

Fig. 1A

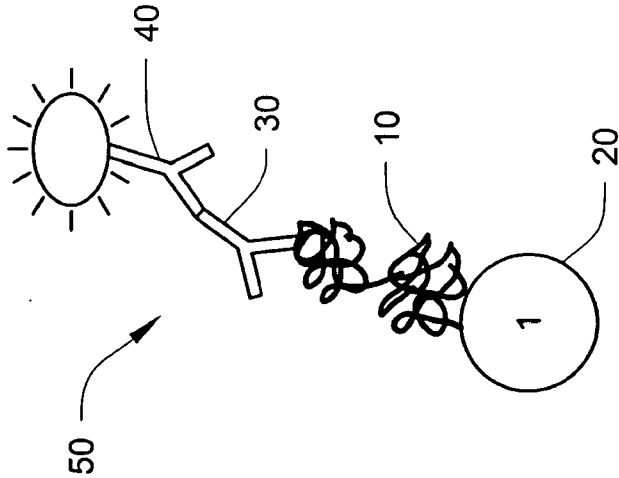


Fig. 1B

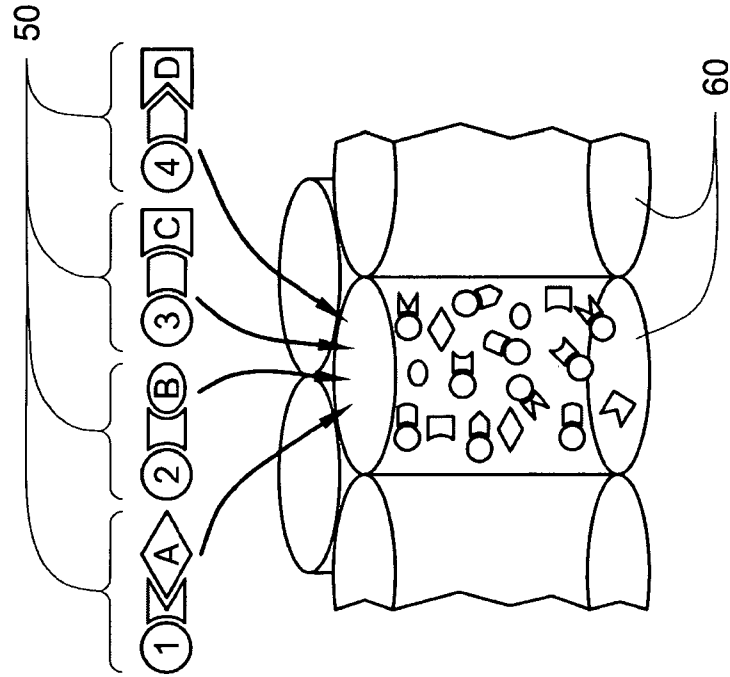
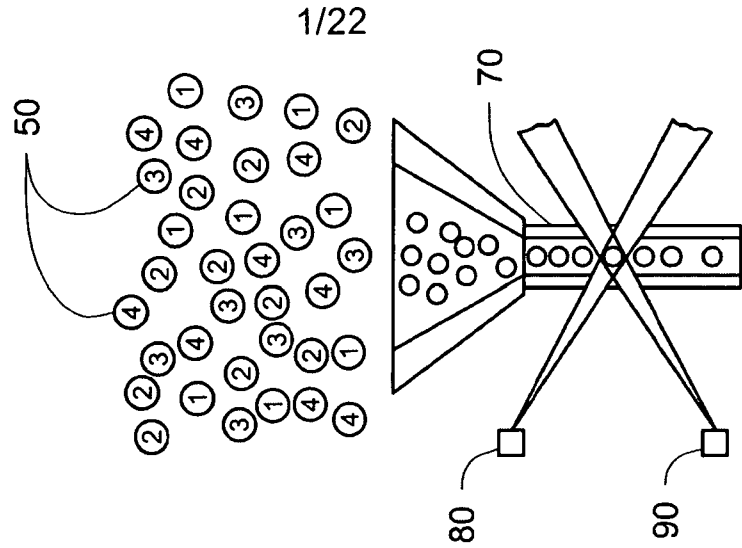
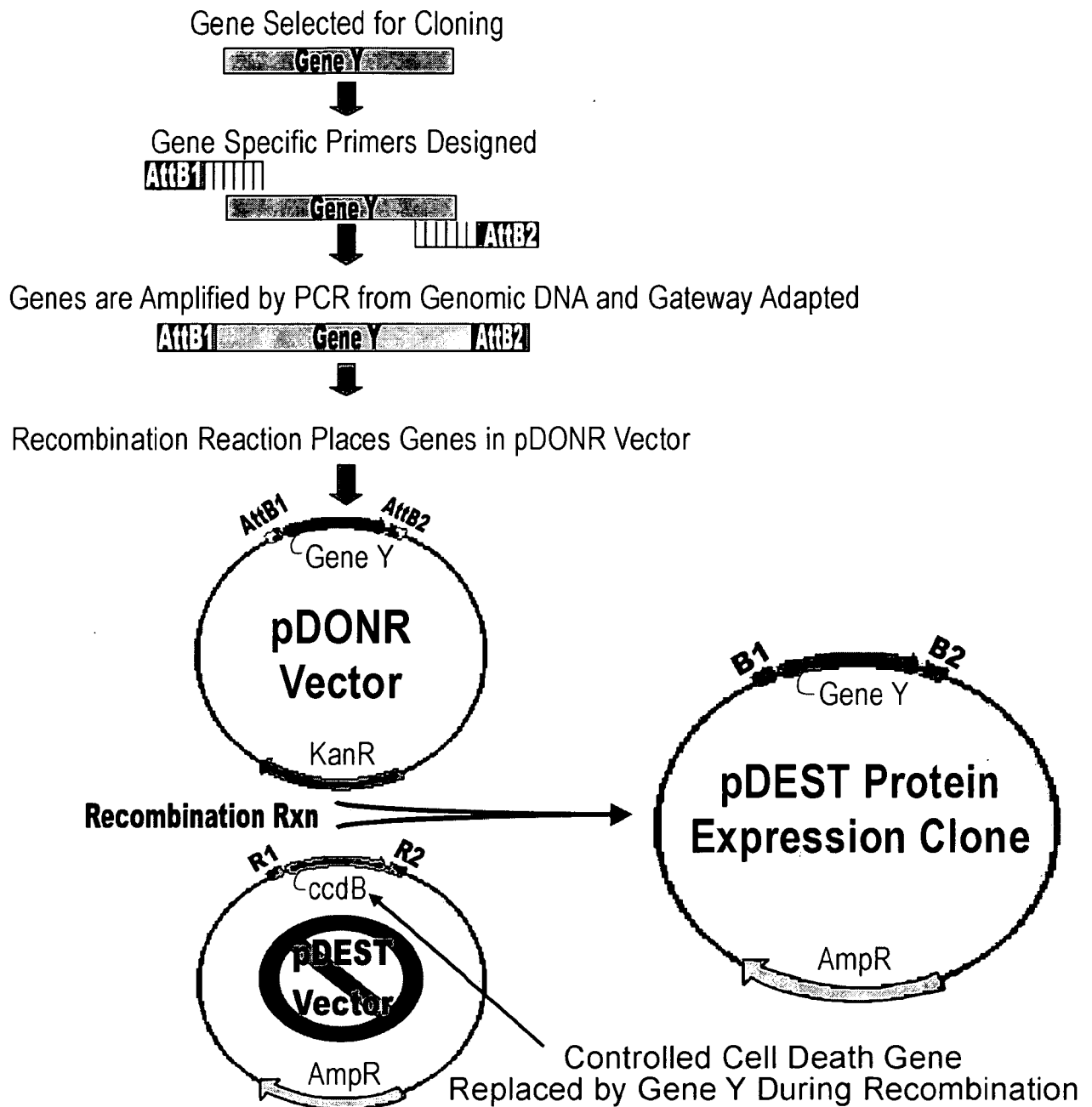


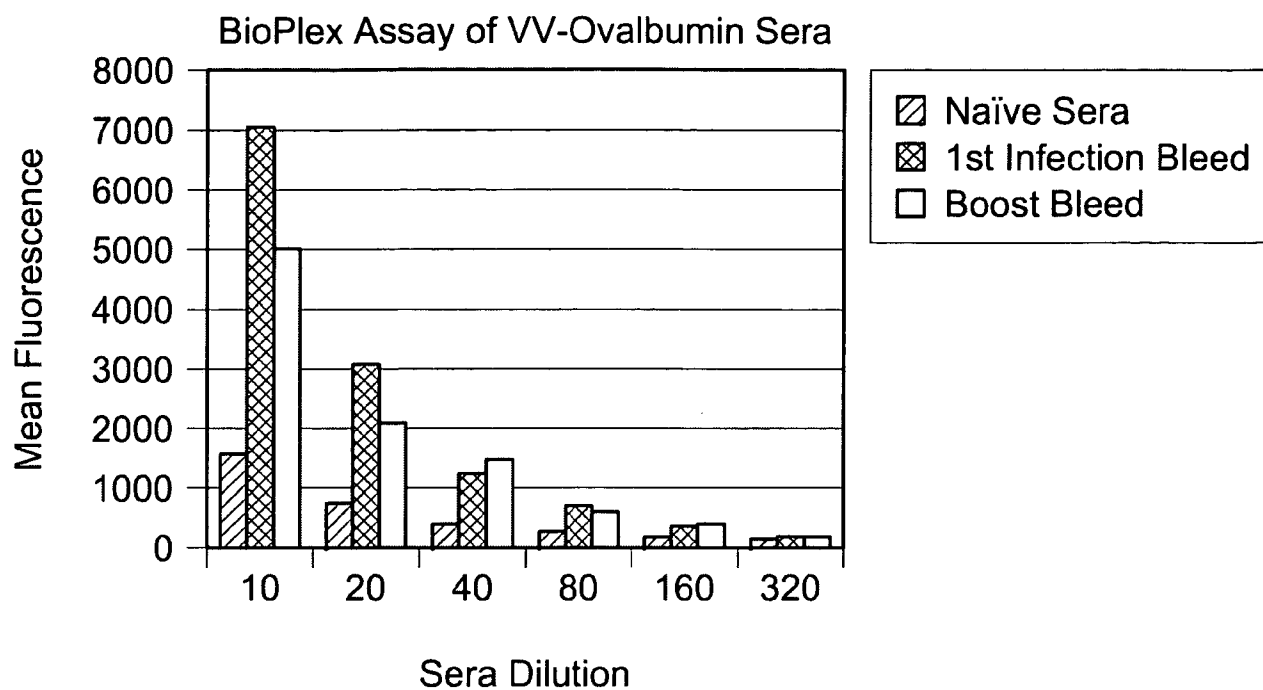
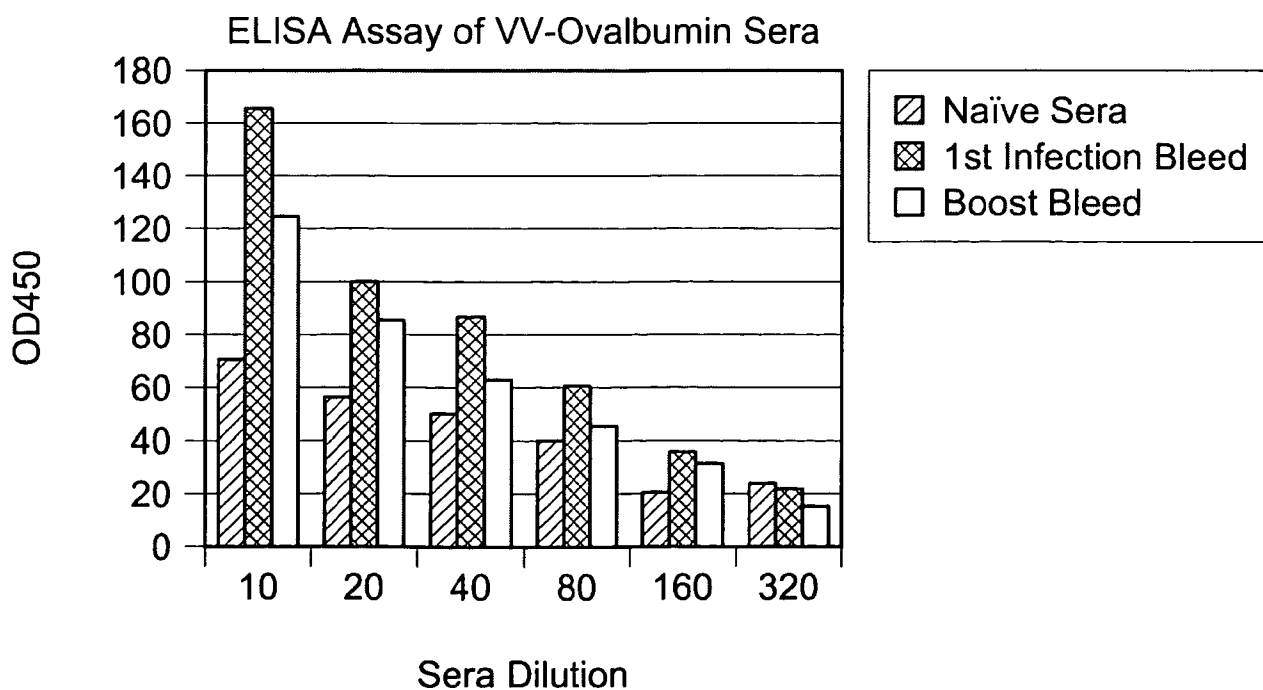
Fig. 1C



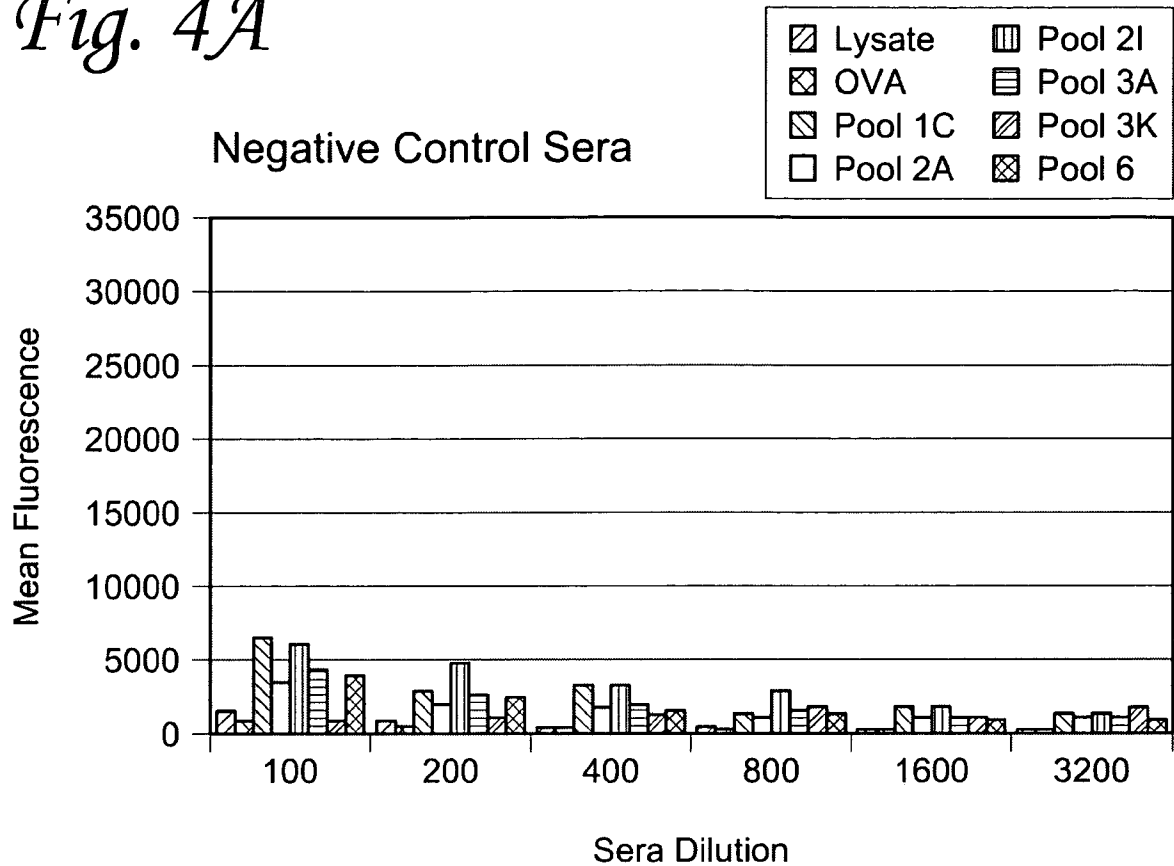
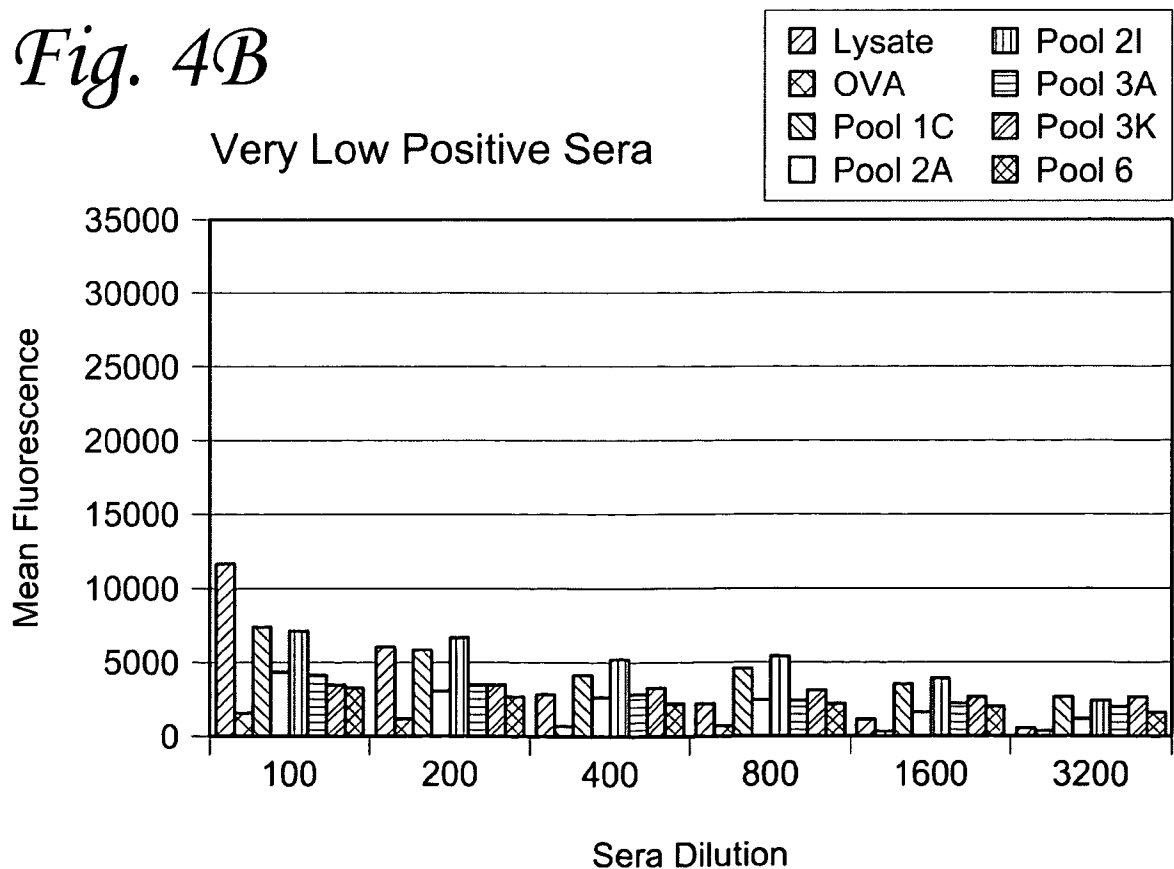
2/22

*Fig. 2*

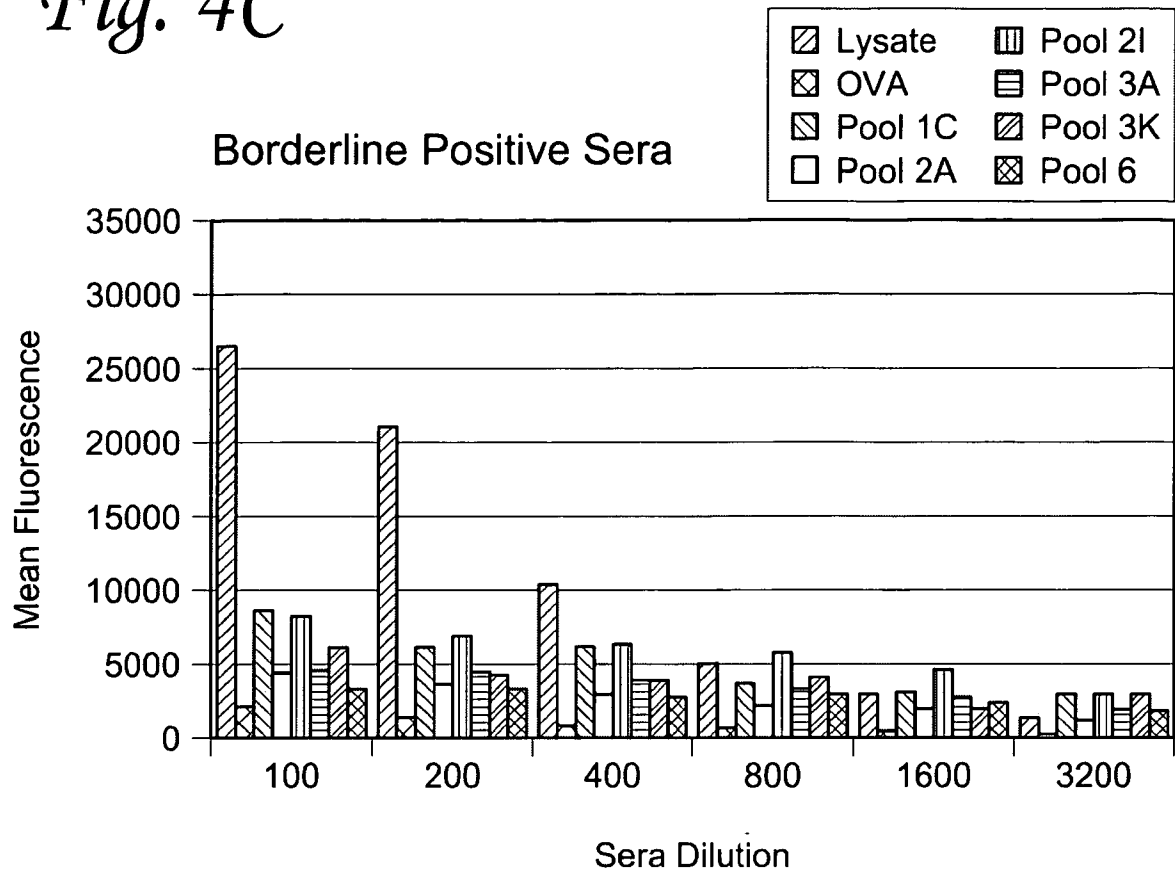
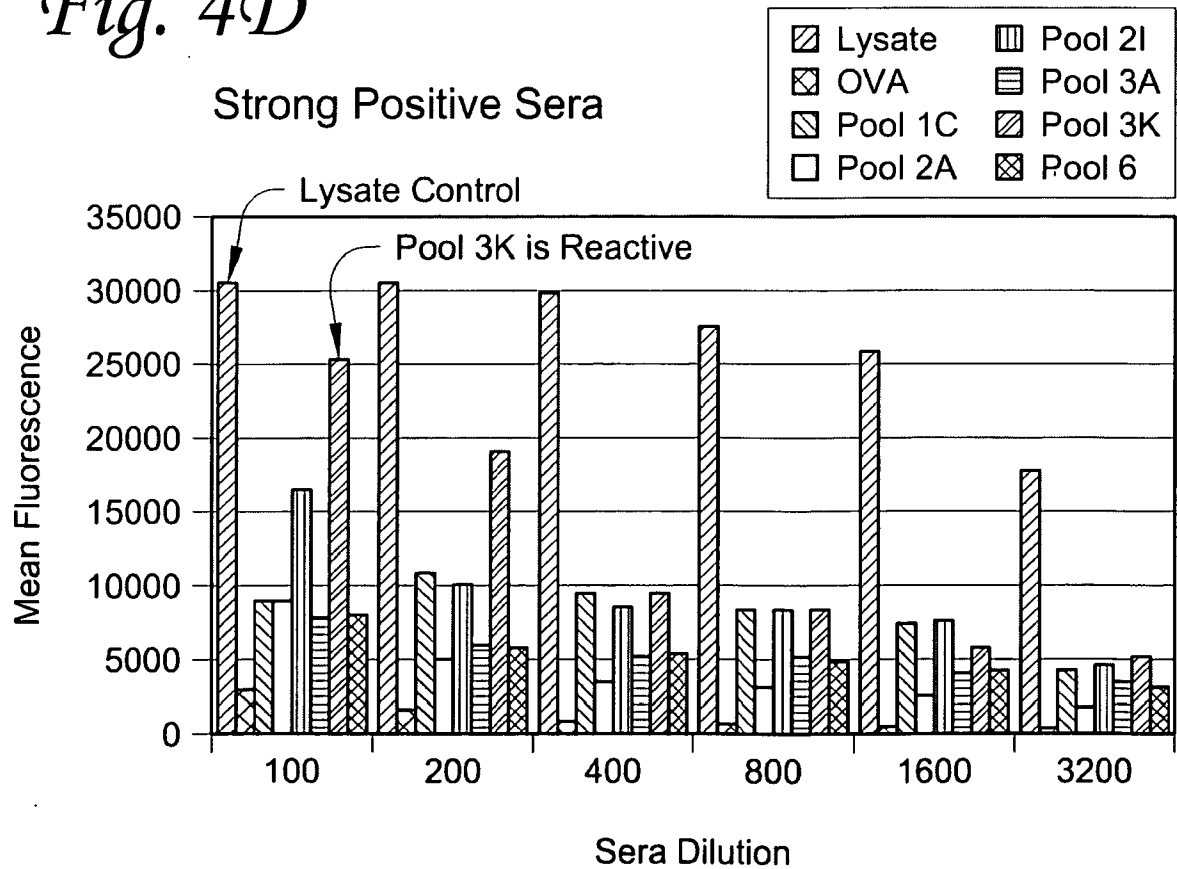
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Fig. 3A*Fig. 3B*

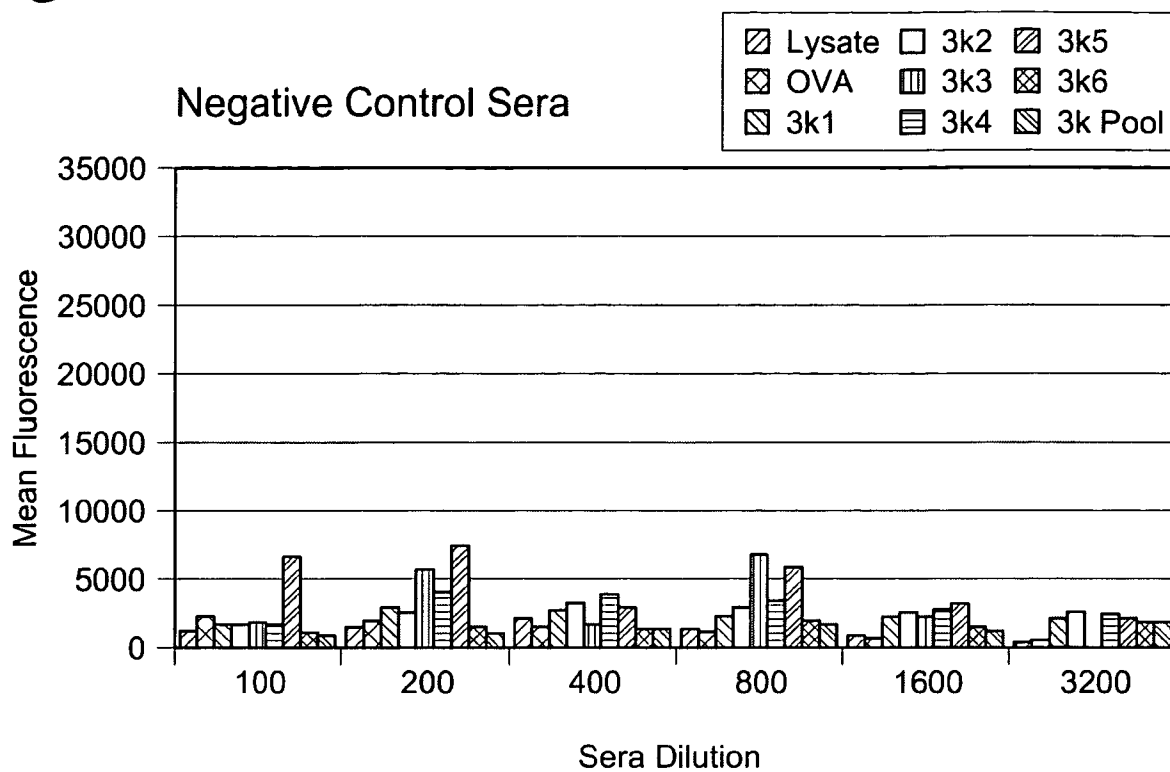
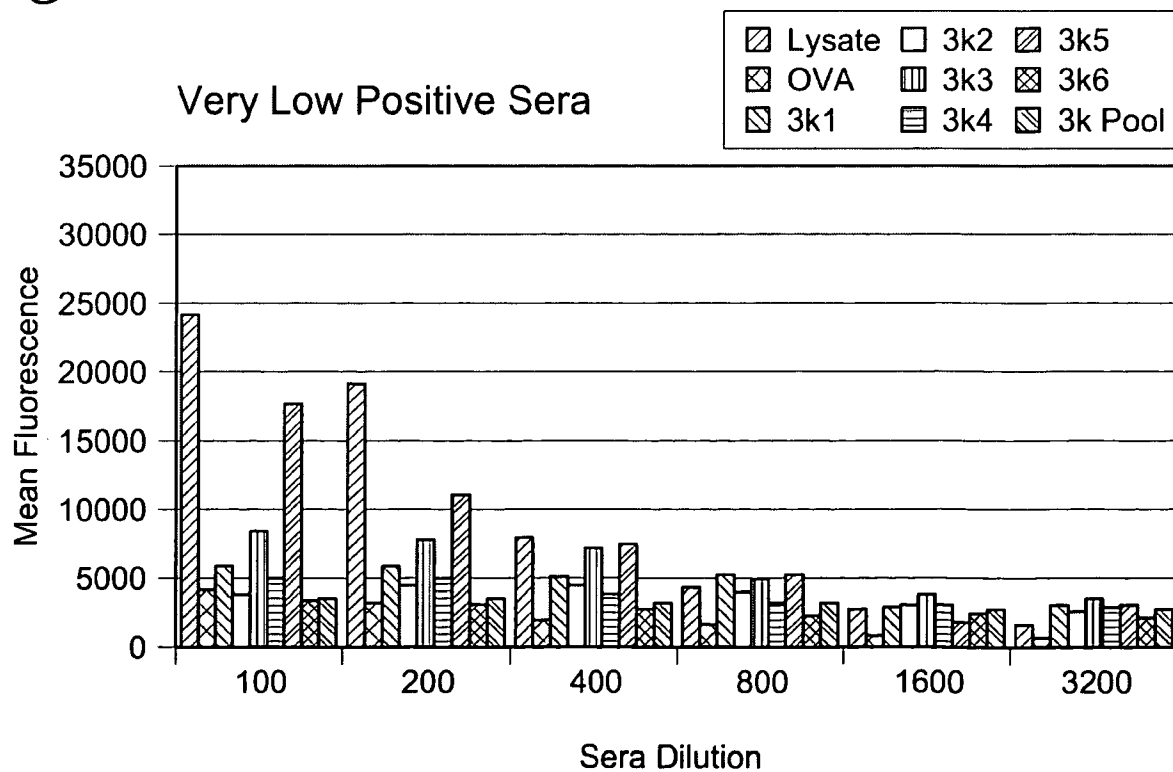
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Fig. 4A*Fig. 4B*

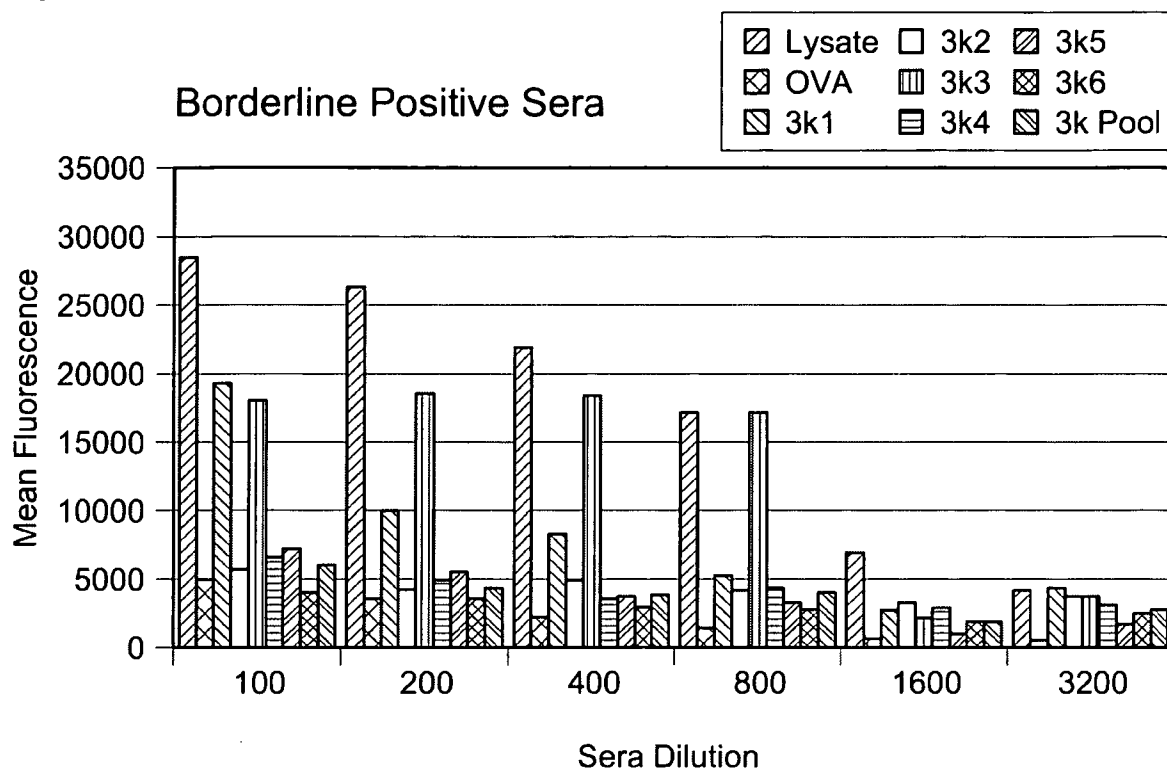
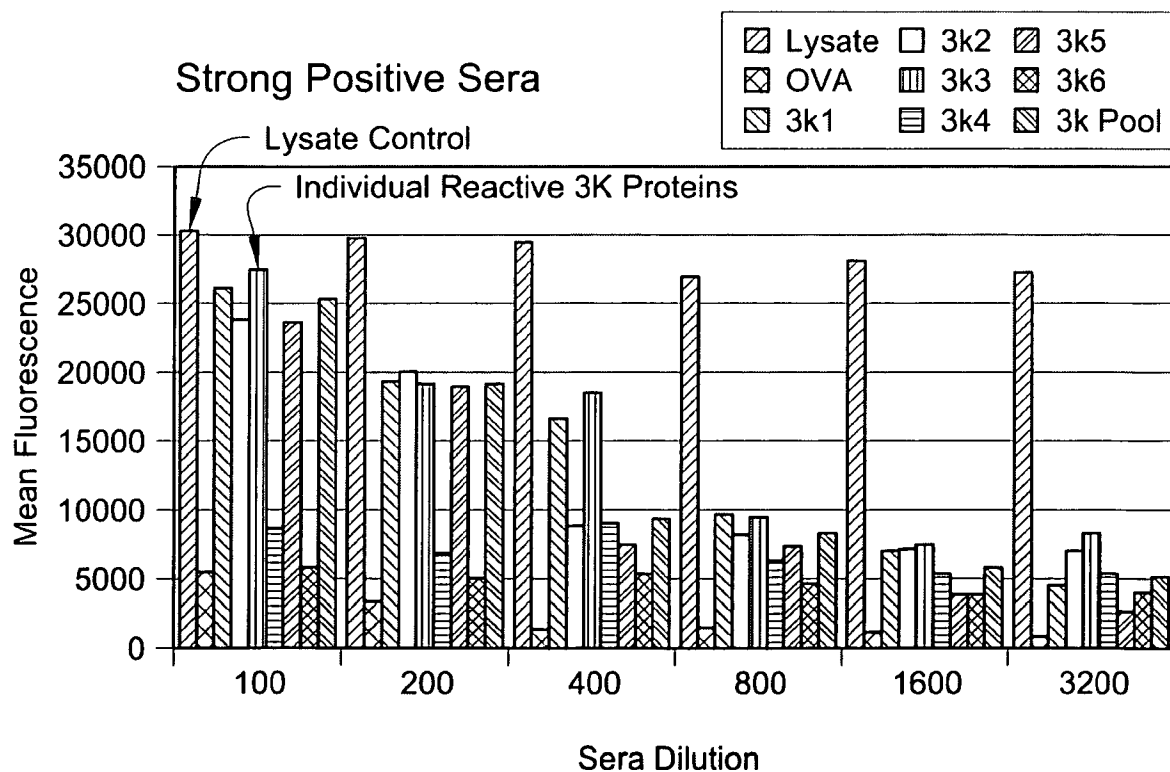
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Fig. 4C*Fig. 4D*

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Fig. 5A*Fig. 5B*

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Fig. 5C*Fig. 5D*

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Fig. 6A

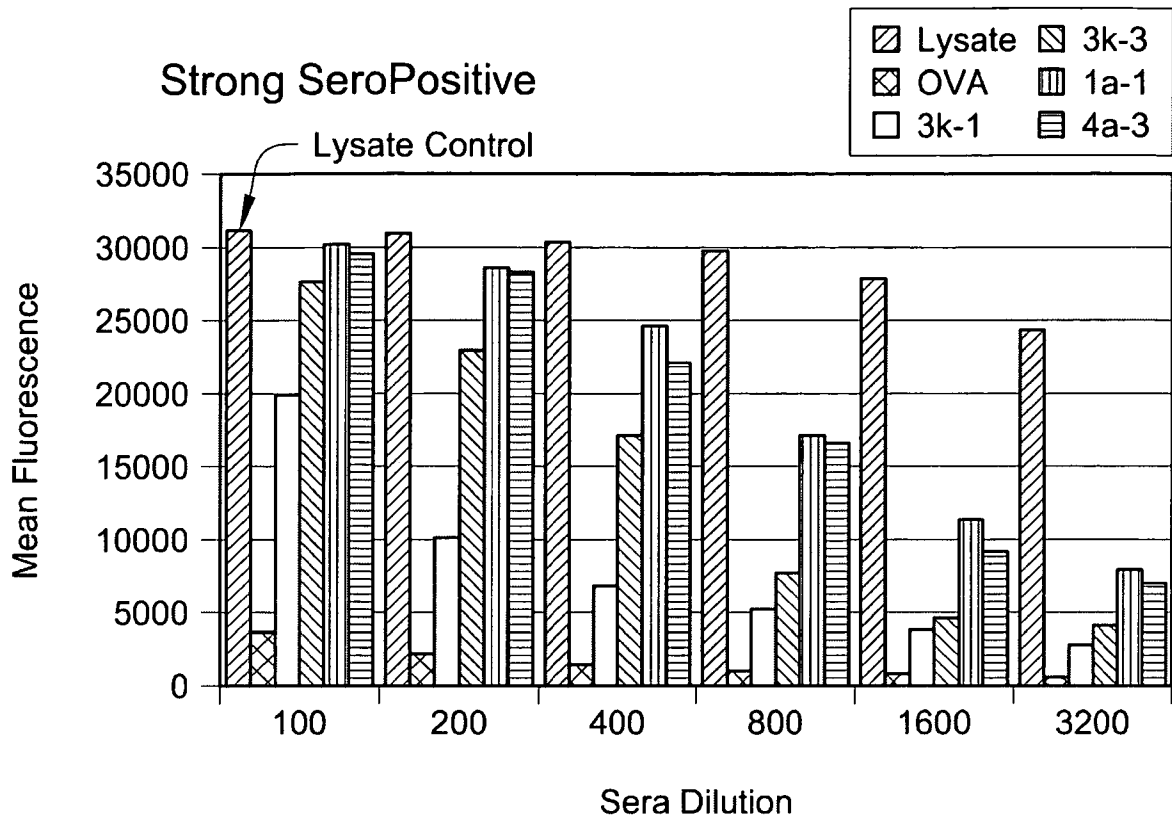
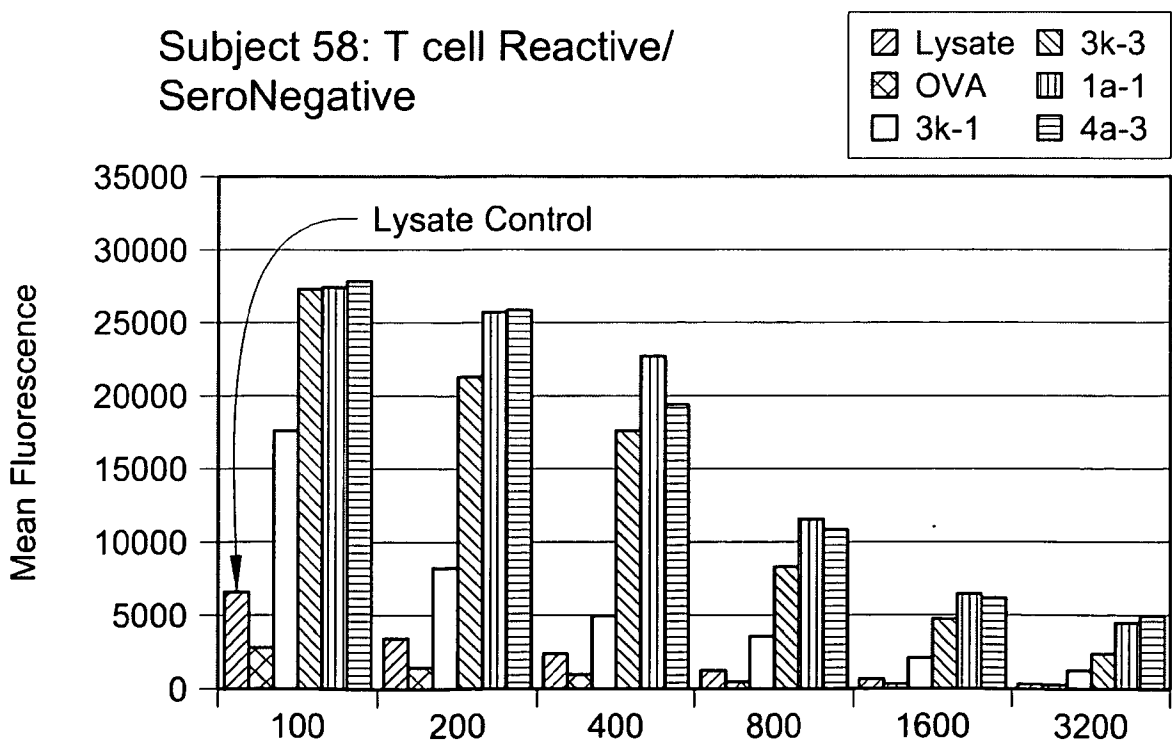
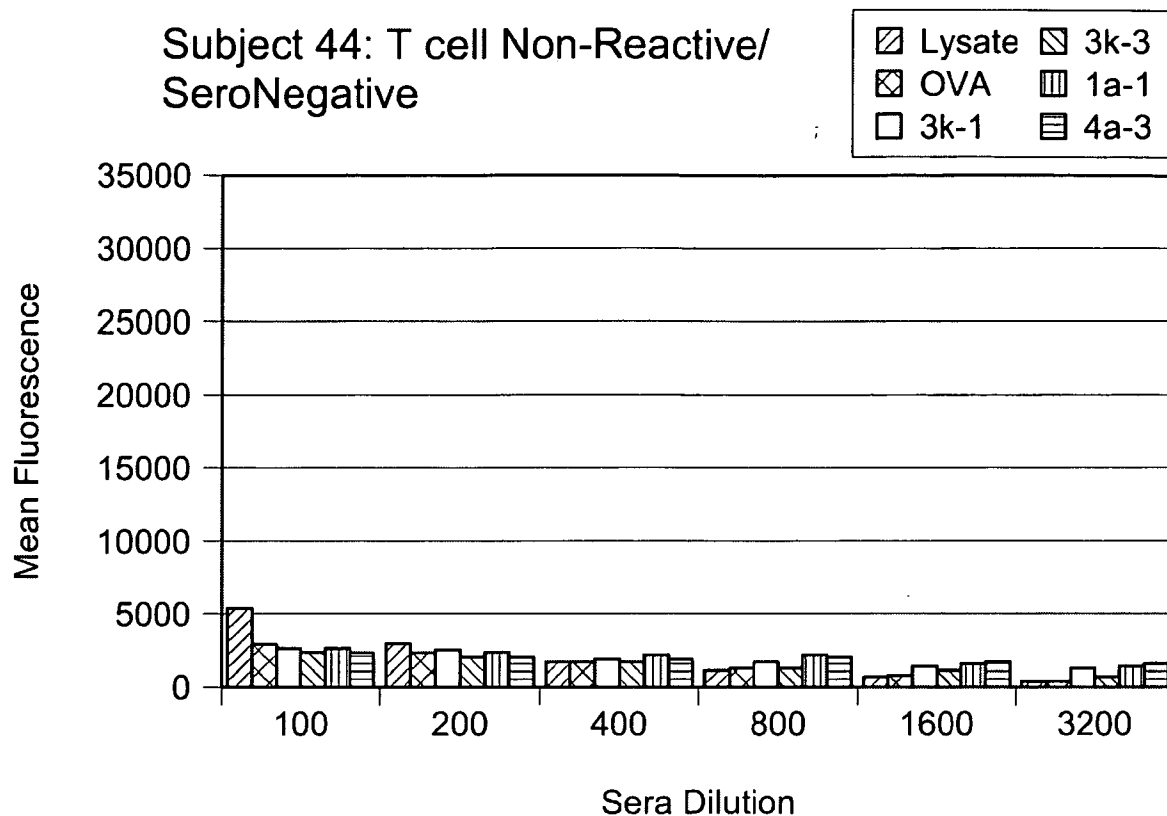
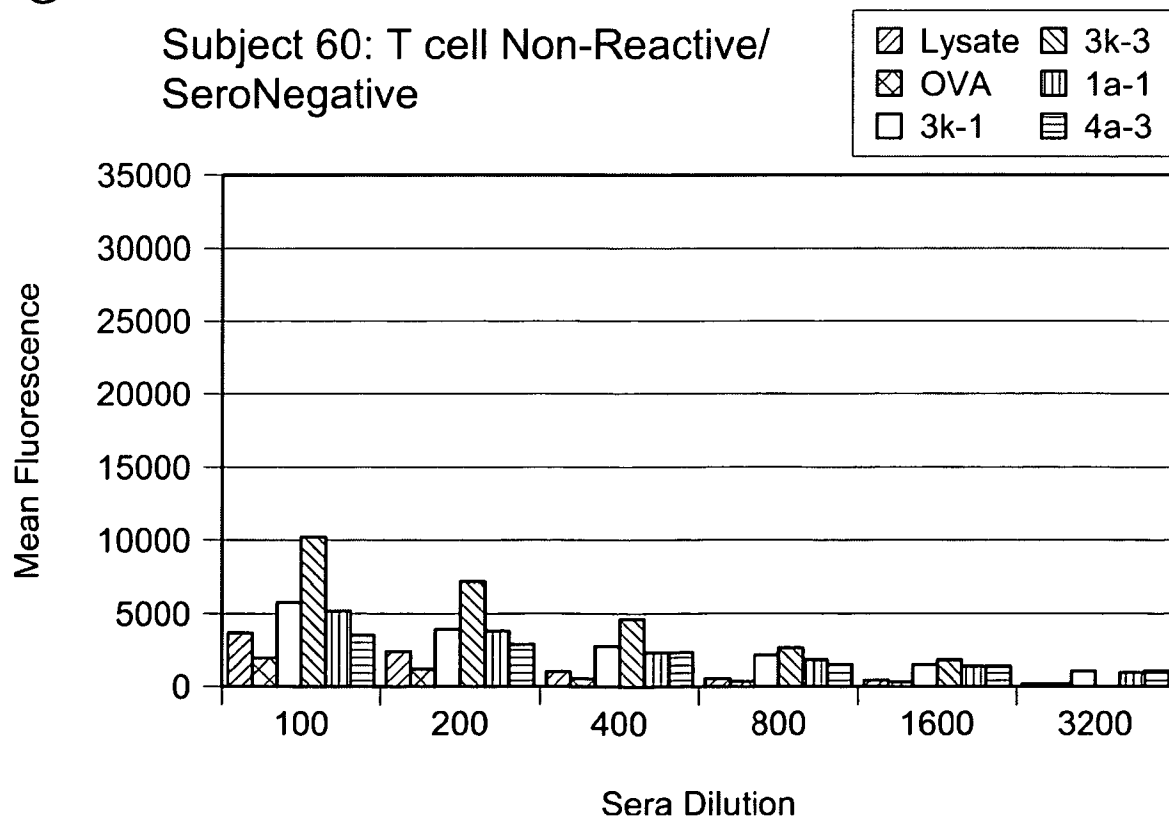


Fig. 6B



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Fig. 6C*Fig. 6D*

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Selection of Diagnostic Proteins

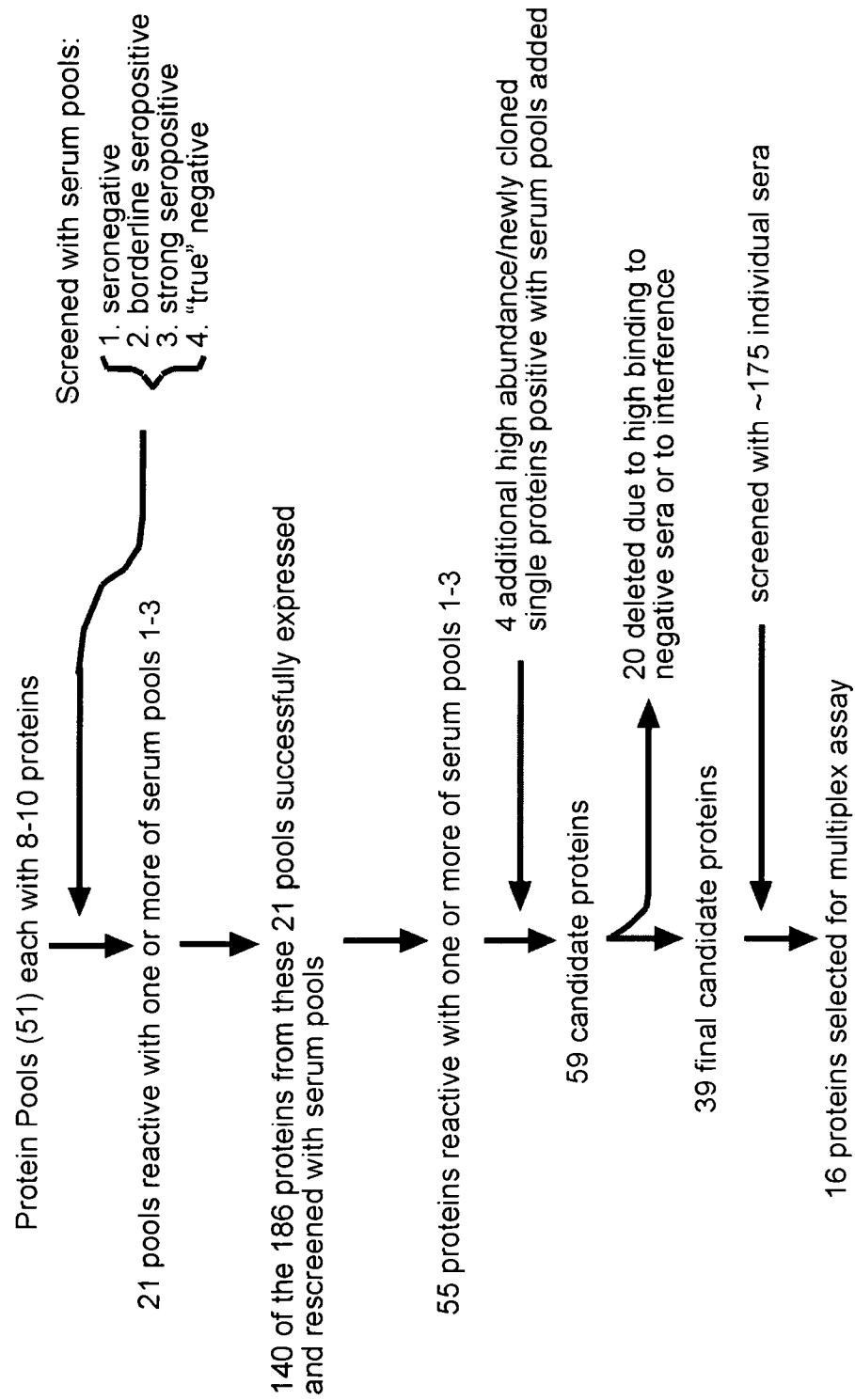
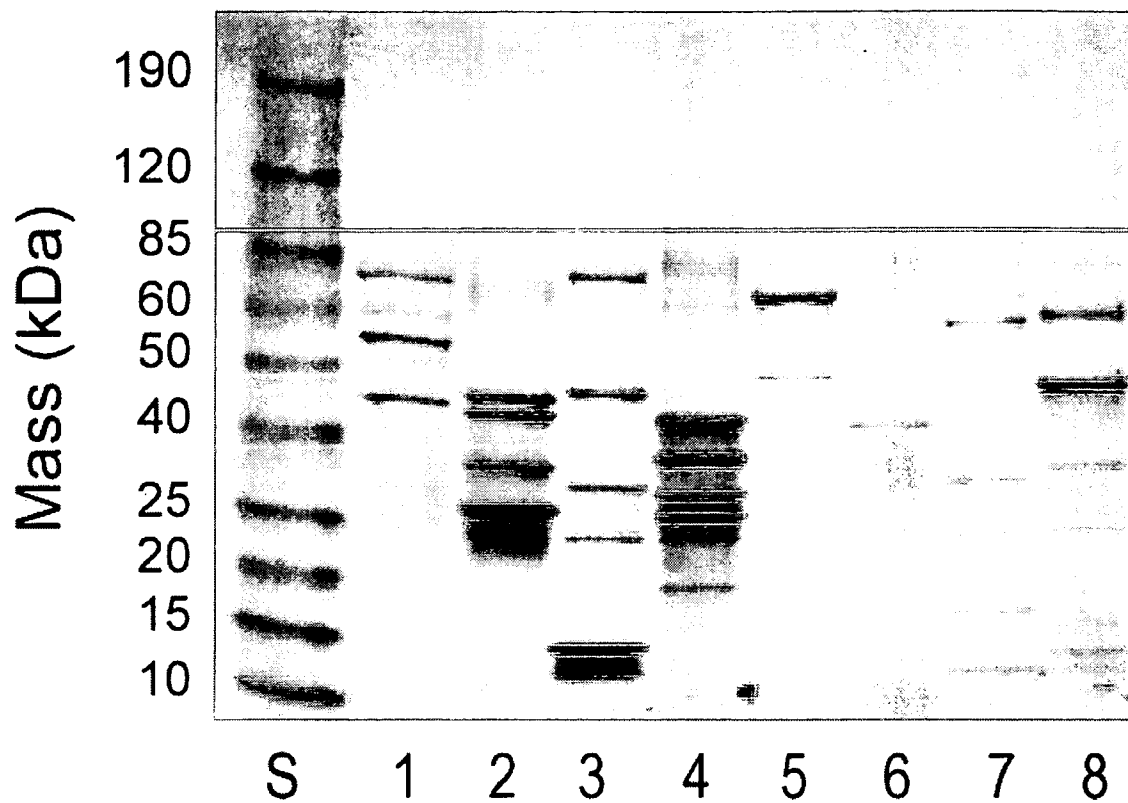


Fig. 7

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*Fig. 8*

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MFI

Fig. 9

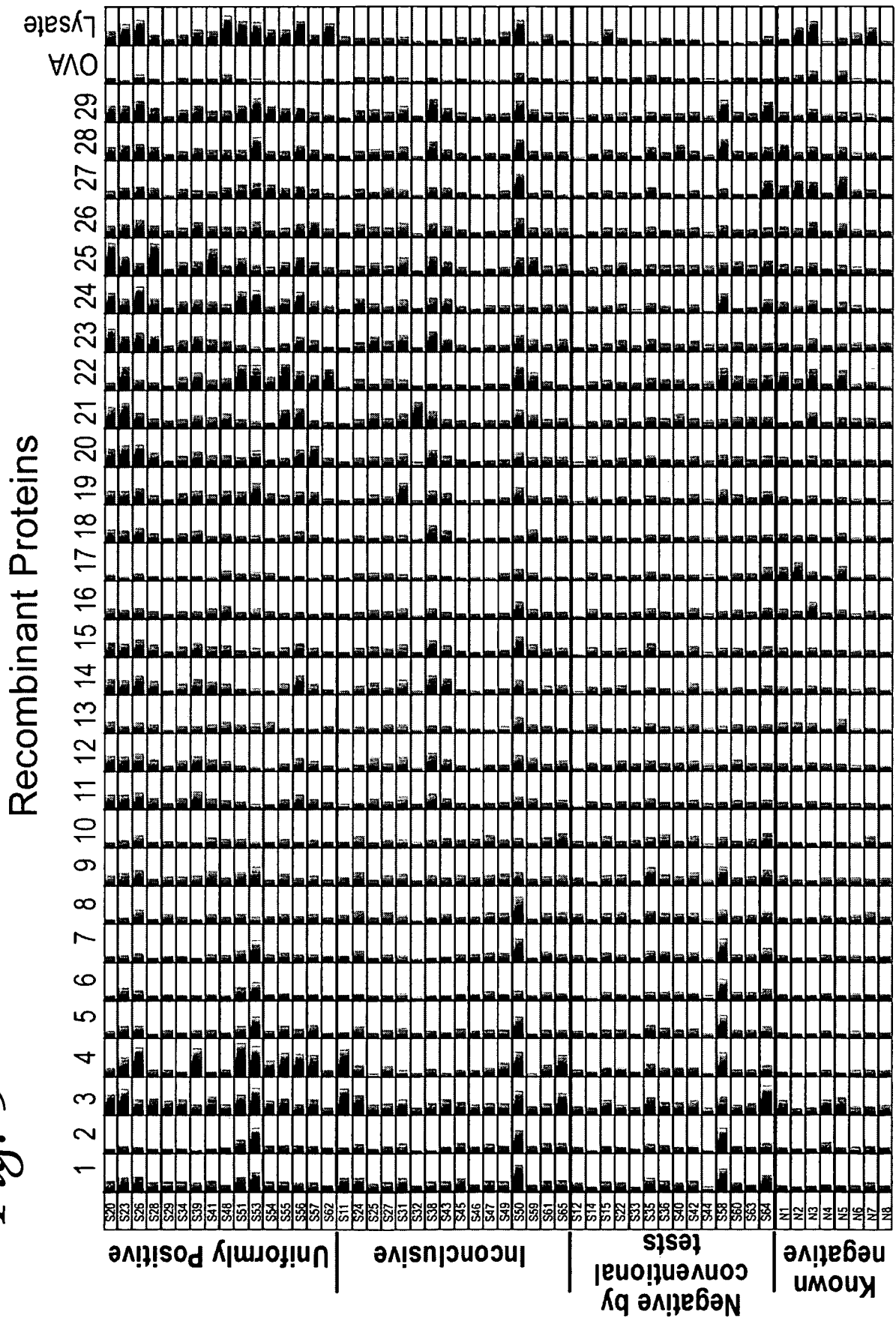
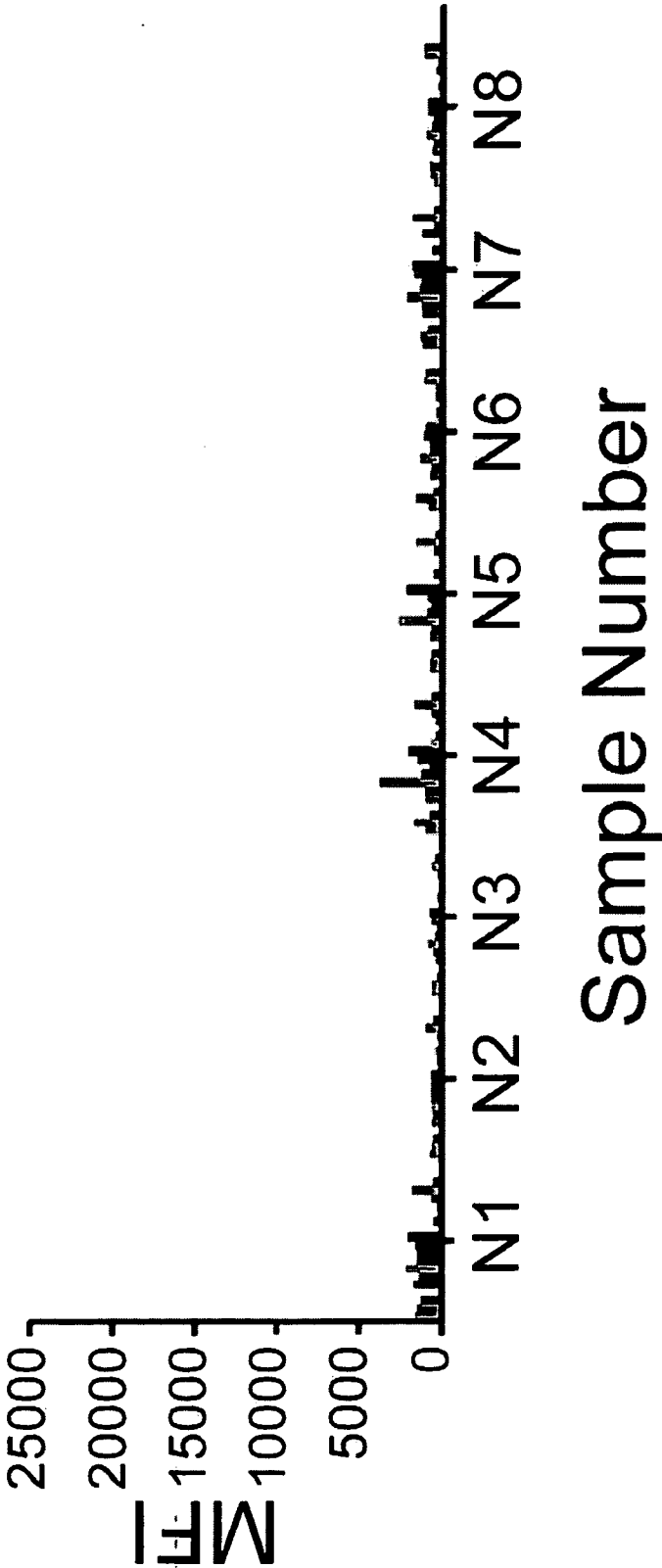
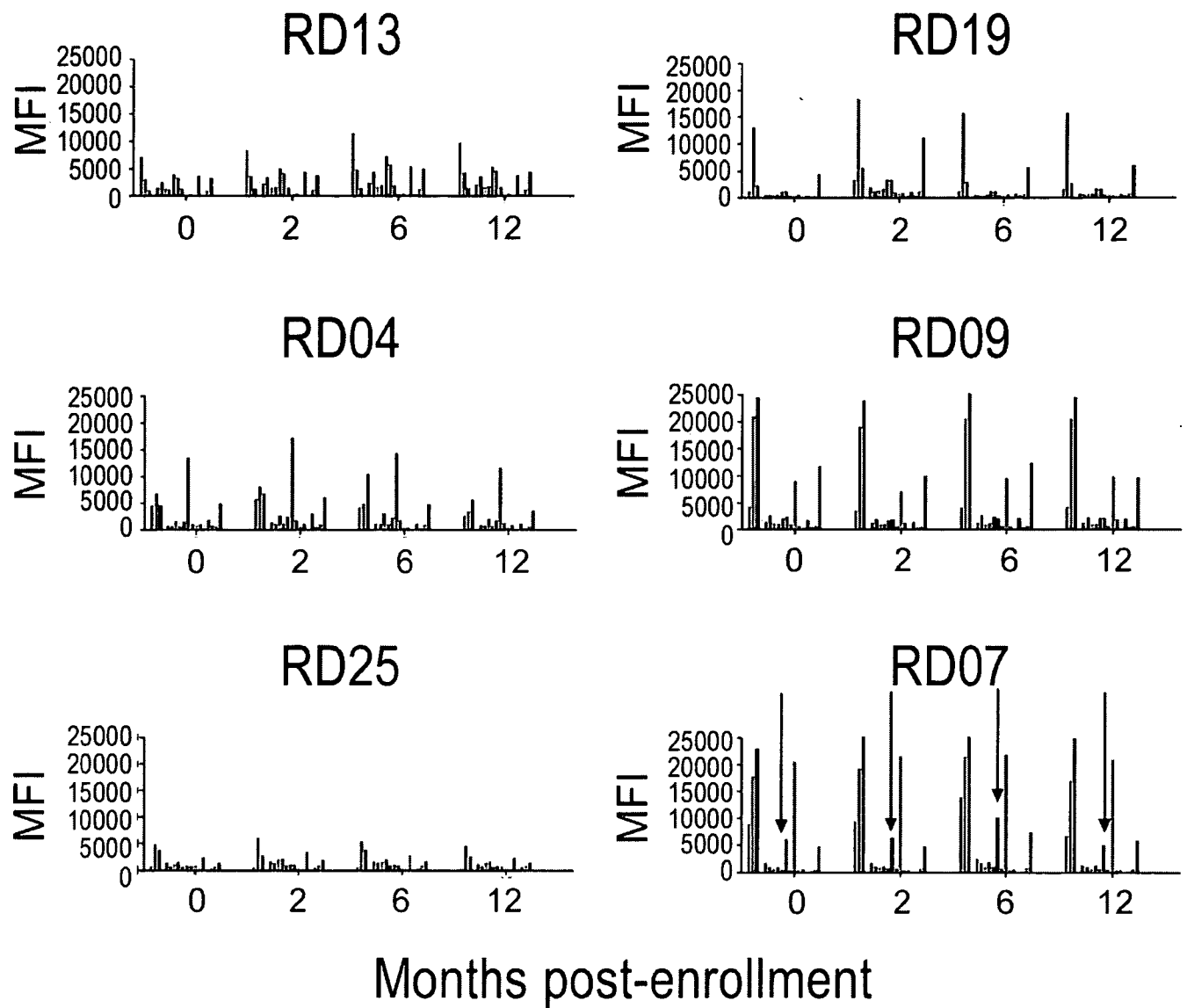


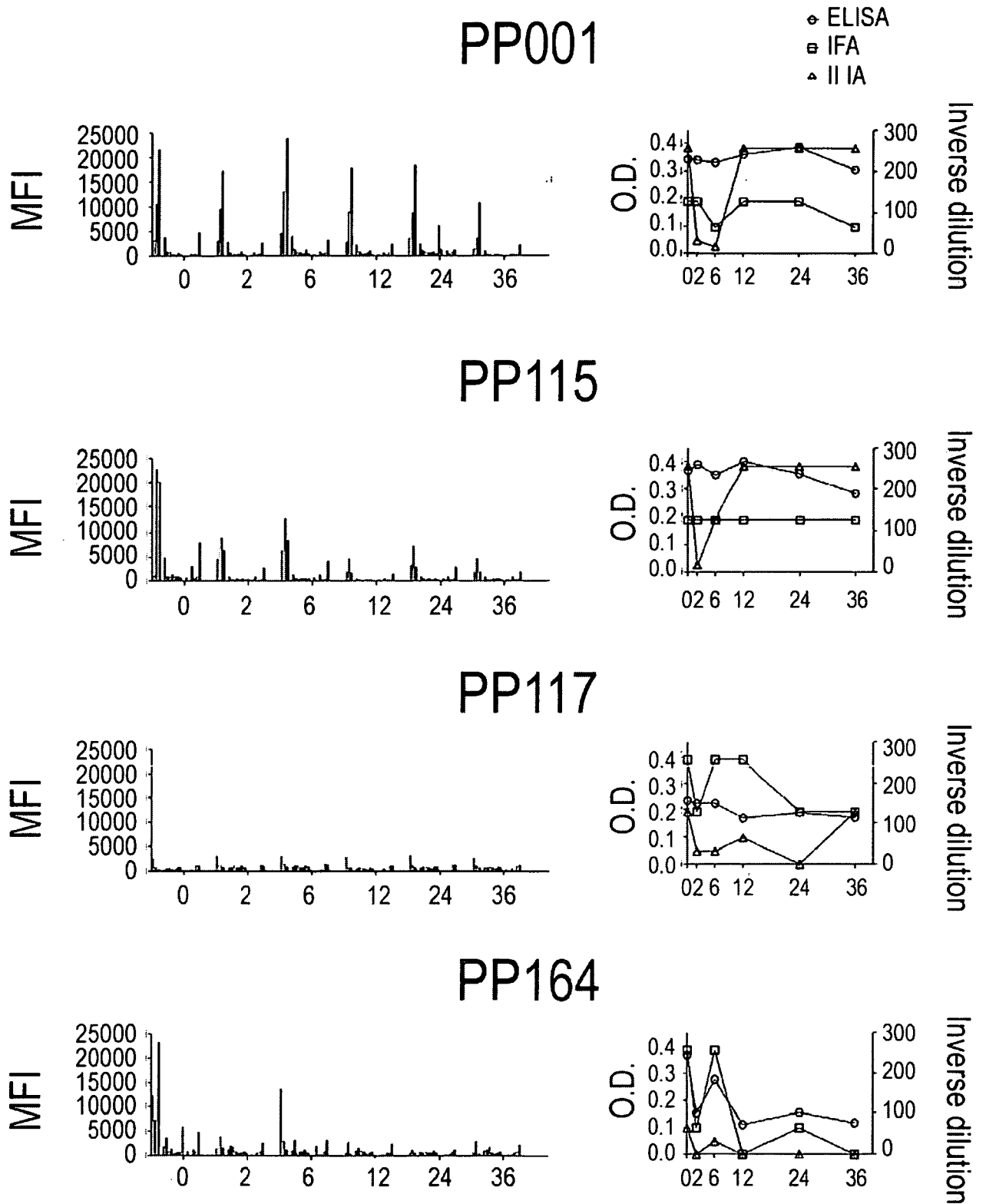
Fig. 10A



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*Fig. 10B*

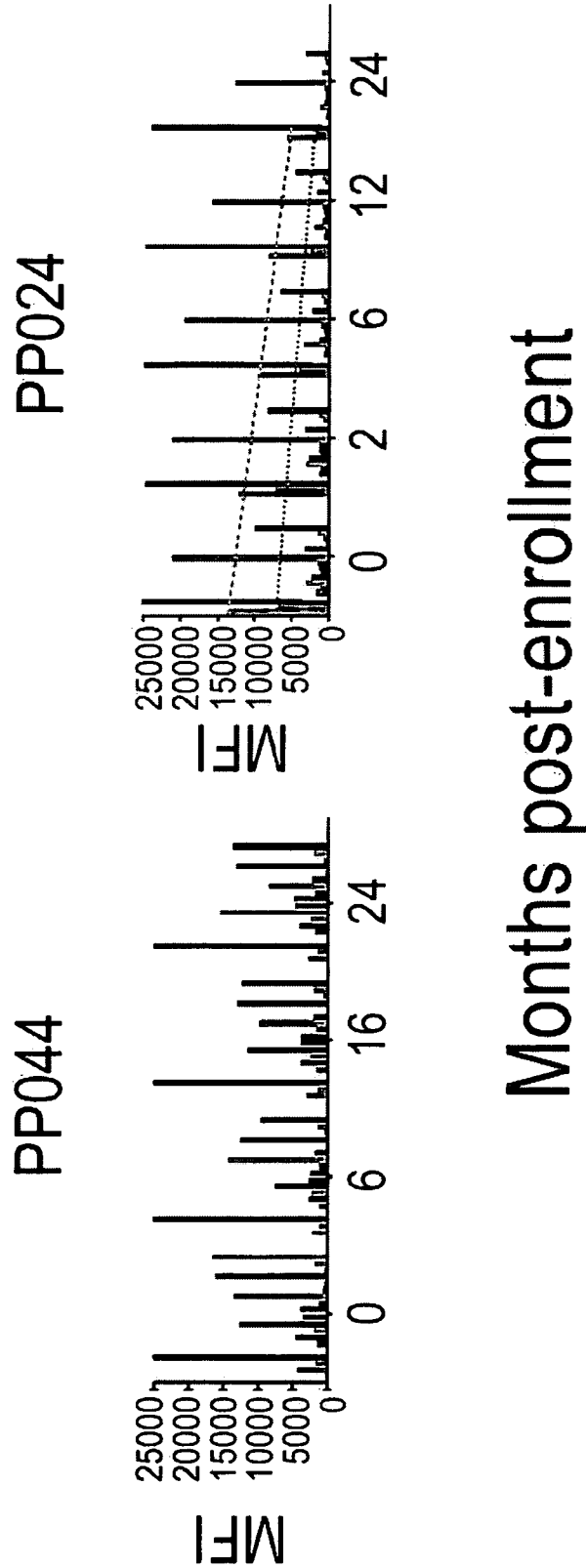
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Months post-enrollment

Fig. 11A

Fig. 11B



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Fig. 12A

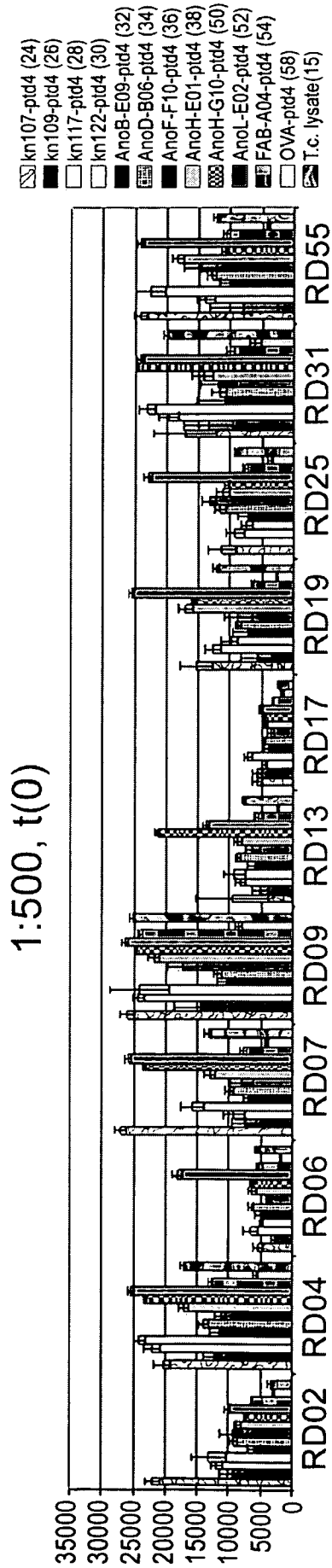
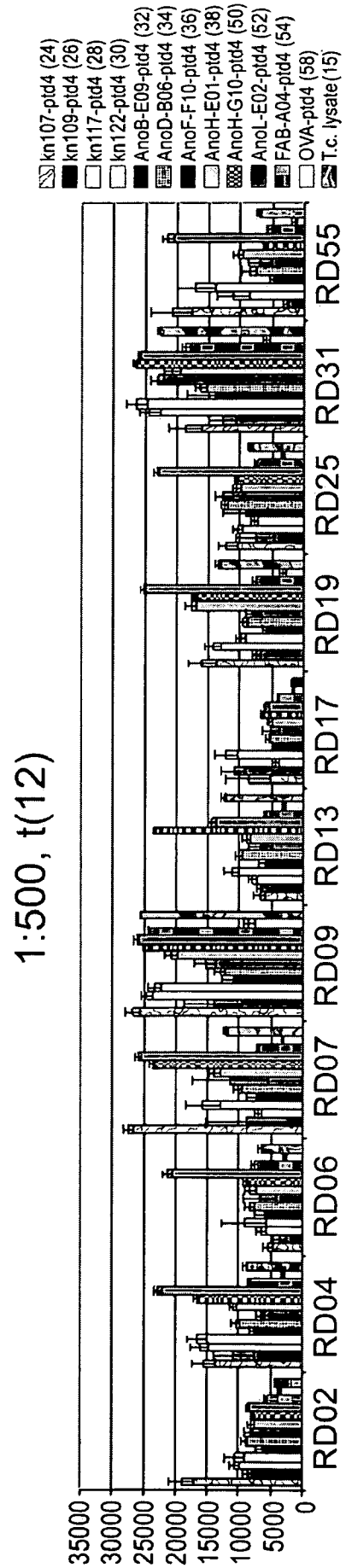


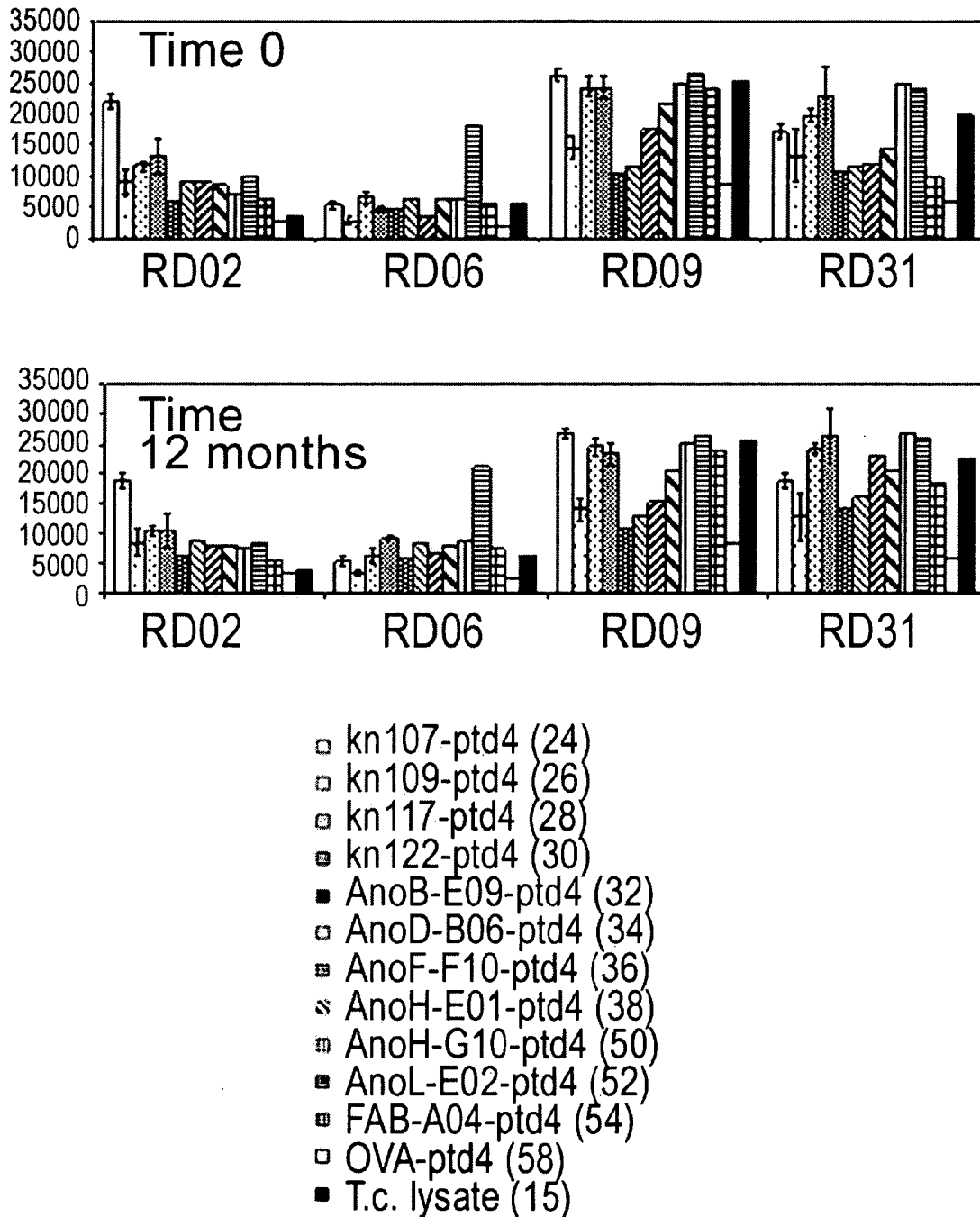
Fig. 12B



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Fig. 13A

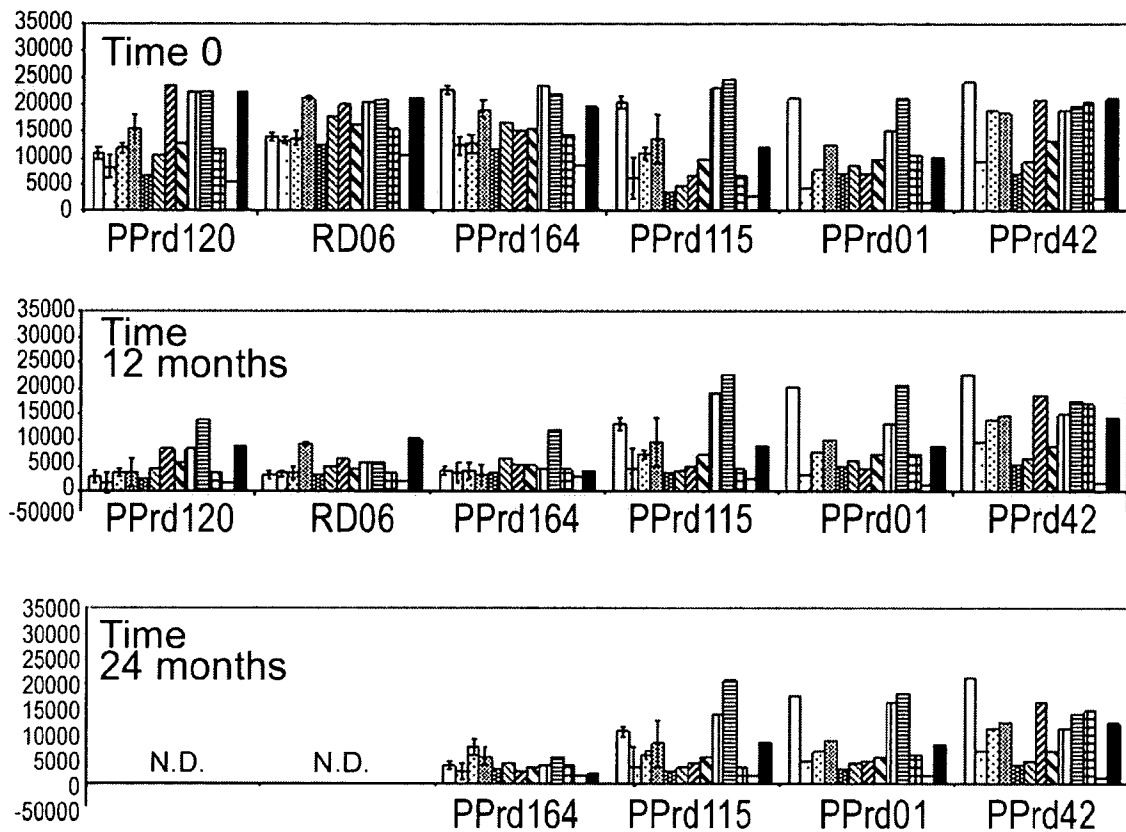
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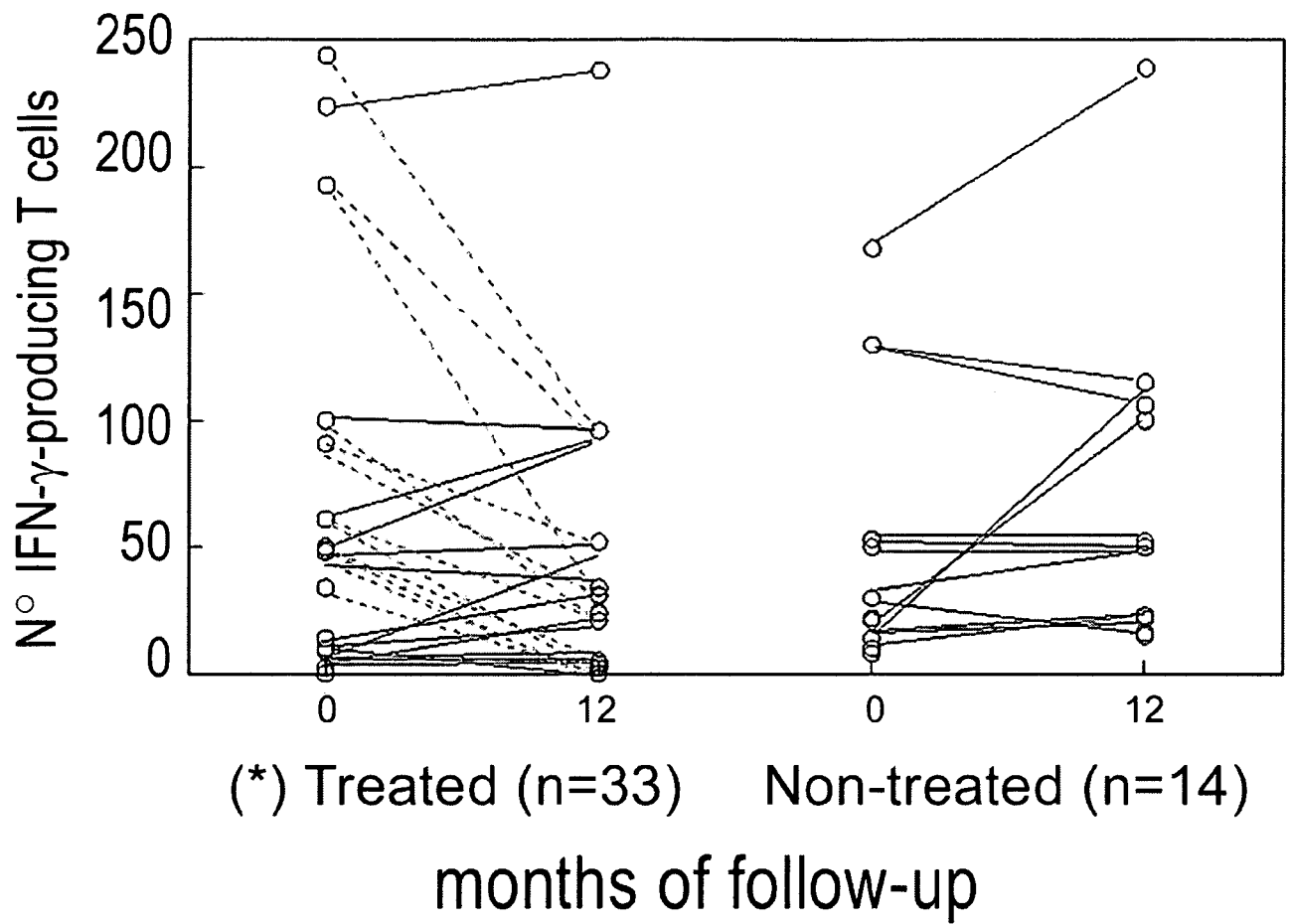
Fig. 13B

treated

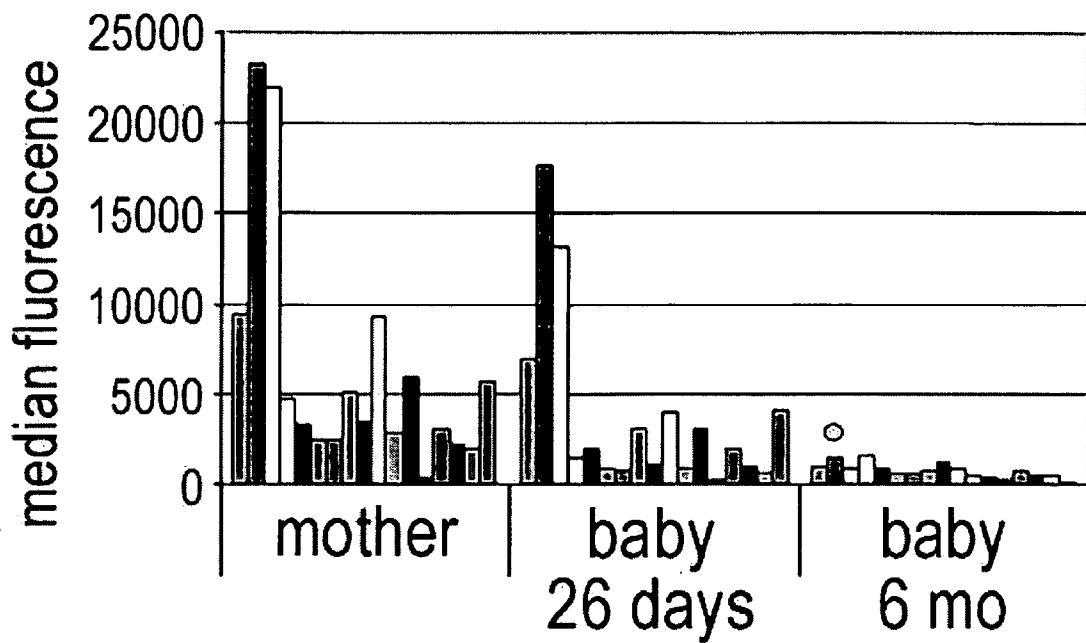
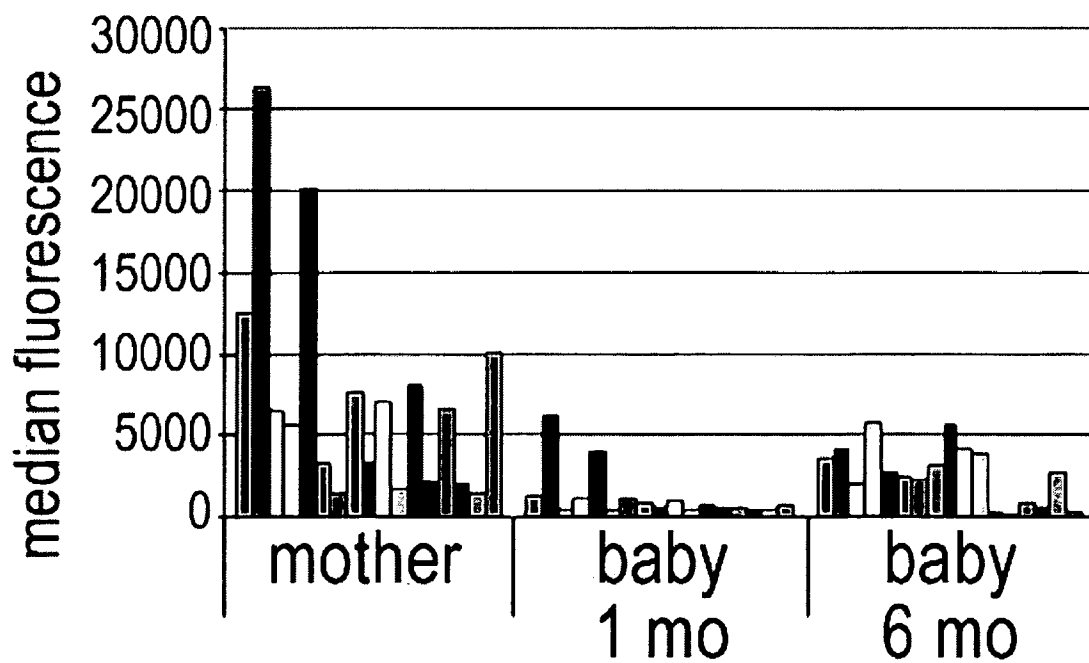


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- kn117-ptd4 (28)
- ▨ kn122-ptd4 (30)
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- ▨ AnoD-B06-ptd4 (34)
- ▨ AnoF-F10-ptd4 (36)
- ▨ AnoH-E01-ptd4 (38)
- ▨ AnoH-G10-ptd4 (50)
- ▨ AnoL-E02-ptd4 (52)
- ▨ FAB-A04-ptd4 (54)
- OVA-ptd4 (58)
- T.c. lysate (15)

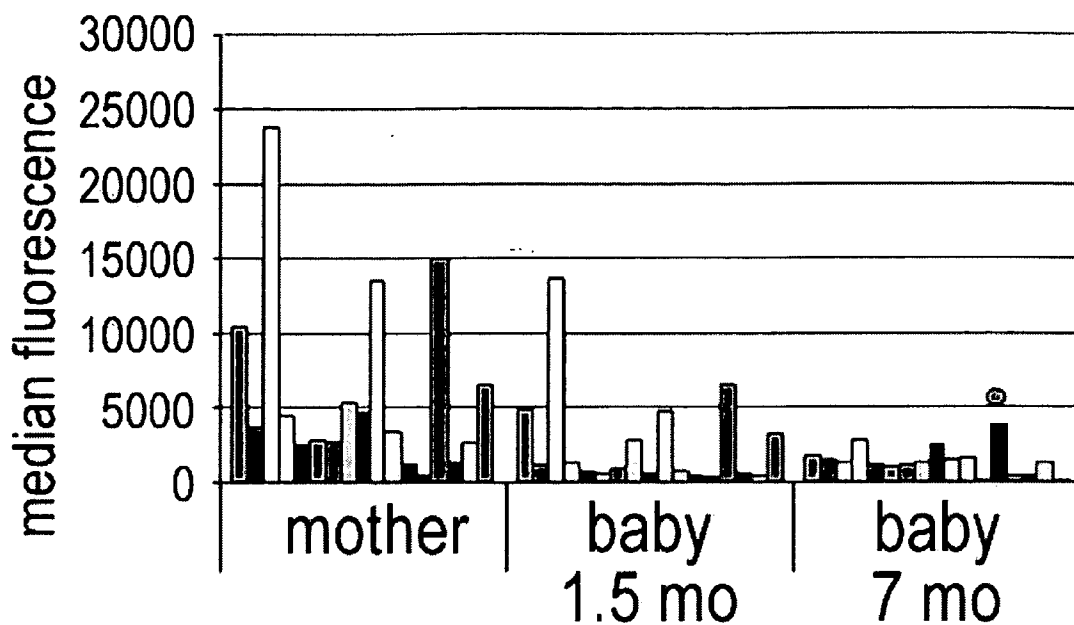
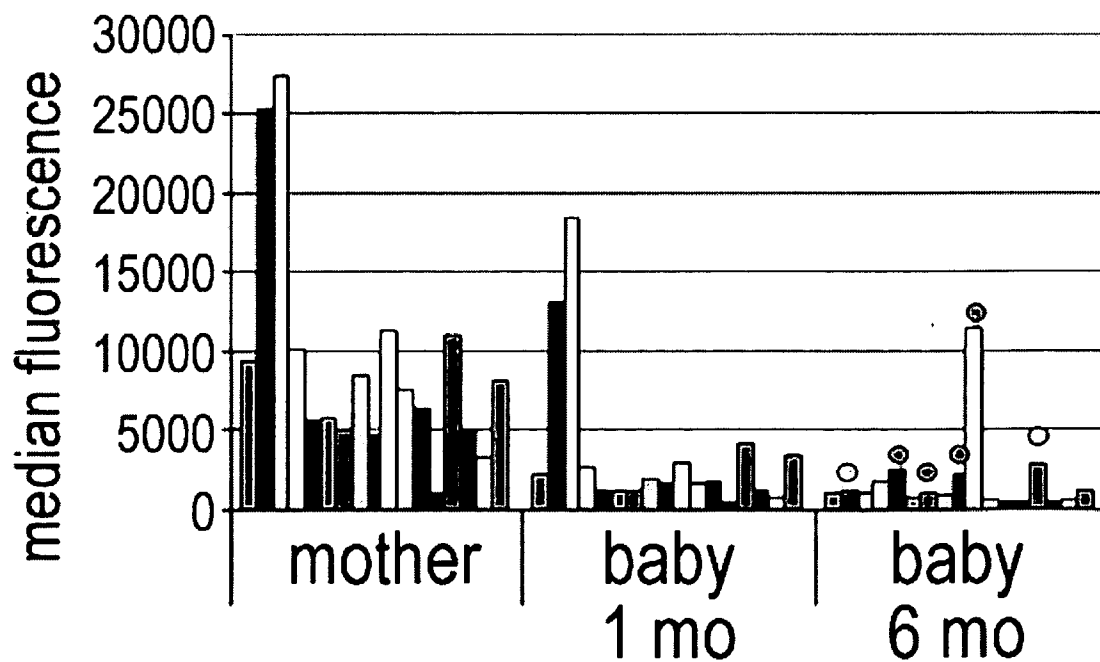
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*Fig. 14*

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Fig. 15A*Fig. 15B*

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Fig. 15C*Fig. 15D*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/09174

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00 (2008.04)

USPC - 424/191.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 424/191.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 424/191.1;435/7.22;436/86Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST (PGPB,USPT,USOC,EPAB,JPAB) Google (Patents, Scholar, and Web)
Search Terms Used: trypanosoma cruzi (polypeptide OR peptide) substrate (human OR dog) therapeutic agent microtubule.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6,419,933 B1 (REED et al.) 16 July 2002 (16.07.2002), entire document, especially:	24, 25, 27-29
-----	Abstract; col. 2, ln 16-29; col. 5, ln19-25; col. 9, ln 59-64	-----
Y		1-23, 26, 30
Y	US 2006/0228300 A1 (CHANG et al.) 12 October 2006 (12.10.2006), para [0082], [0111], [0160], [0161], [0215], [0418]	1-23, 26, 30
A	US 2005/0158347 A1 (TARLETON et al.) 21 July 2005 (21.07.2005)	1-30
A	US 2004/0241729 A1 (LIEW) 2 December 2004 (02.12.2004)	1-30
A	US 2005/0244505 A1 (HIGBEE et al.) 3 November 2005 (03.11.2005)	1-30



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

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"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

19 October 2008 (19.10.2008)

Date of mailing of the international search report

04 NOV 2008

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