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PARTICLE-BASED FLOW CYTOMETRY ASSAY TO DETECT ANTI-ANGIOTENSIN II TYPE I AND TYPE II RECEPTOR ANTIBODIES

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PARTICLE-BASED FLOW CYTOMETRY ASSAY TO DETECT ANTI-ANGIOTENSIN II TYPE I AND TYPE II RECEPTOR ANTIBODIES

By

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ABSTRACT

PARTICLE-BASED FLOW CYTOMETRY ASSAY TO DETECT ANTI-ANGIOTENSIN II TYPE I AND TYPE II RECEPTOR ANTIBODIES

By

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One of the major problems in renal transplantation is organ rejection which can result in allograft loss. To prevent organ rejection, one needs to confirm that the kidney from the donor is compatible with the recipient's immune system. Currently, three major steps are performed to prevent allograft rejection: Human Leukocyte Antigen (HLA) typing, antibody screening, and compatibility testing. Antibody mediated allograft rejection can be caused by HLA antibodies and non-HLA antibodies. Currently screening for non-HLA antibodies is not included in renal transplantation.

One example of a non-HLA antibody that can cause allograft rejection is an anti-angiotensin II receptor type 1 antibody (anti-AT_1). A functional bioassay and enzyme-linked immunosorbent assay (ELISA) have been developed to detect anti-AT_1 and anti-angiotensin II receptor type 2 antibody (anti-AT_2). Using the same principle as in the ELISA, a microbead immunoassay using a flow cytometry based instrument was developed to detect anti-AT_1 and anti-AT_2 antibodies.

The microbead immunoassay was not successfully developed due to the lack of known positive controls for anti-AT_1 and anti-AT_2 antibodies. Another possible issue that binding of the short peptide sequences to the microspheres changed their structure such that immunogenicity was lost.

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INTRODUCTION

In 2005, more than 485,000 Americans were treated for end stage renal disease (ESRD). Diseases listed to cause ESRD were diabetes (36.9 percent (%)), high blood pressure (24.2%), glomerulonephritis (16.2%), cystic kidney (4.6%), other urologic (2.8%), and other unknown causes (17.9%) [1]. Renal transplantation is one of the major treatments for ESRD. One of the major problems in renal transplantation is organ rejection which can result in allograft loss. To prevent organ rejection, one needs to confirm that the kidney from the donor is compatible with the recipient's immune system. Currently, three major steps are performed to confirm the compatibility between the donor and the recipient: Human Leukocyte Antigen (HLA) typing, antibody screening, and compatibility testing. Antibody screening is performed to detect antibodies in the recipient's serum that react with HLA antigens to aid in donor selection. The compatibility testing is performed to detect preformed antibodies in the recipient that are reactive against the donor. Since 1970, a crossmatch is required prior to a renal transplantation [2].

About one-third of acute rejections are antibody-mediated [3]. Antibody-mediated rejection can be caused by HLA antibodies and non-HLA antibodies. Rejection due to HLA antibodies can be minimized by matching HLA antigens, antibody screening and compatibility testing. Currently, screening for non-HLA antibodies is not included in renal transplantation. Some examples of non-HLA antibodies that can cause allograft rejections are anti-phospholipid antibodies, anti-vimentin antibodies, antibodies specific to endothelial antigens, and anti-angiotensin II receptor type 1 (anti-AT_1) antibodies [4-6].

The goal of this research is to develop a screening method to detect anti-angiotensin II receptor type 1 (anti-AT_1) and anti-angiotensin II receptor type 2 (anti-AT_2) antibodies using multiplex immunoassay.

BACKGROUND AND SIGNIFICANCE

Renal transplantation

Renal transplantation is one of the treatments for ESRD. To prevent allograft rejection of the renal transplant, one needs to confirm that the organ from the donor is compatible with the recipient's immune system. Three steps are performed to confirm the compatibility between the donor and the recipient: HLA typing, antibody screening, and compatibility testing.

HLA is the major histocompatibility complex (MHC) located on chromosome 6 in humans. The MHC encodes proteins called MHC molecules or HLA. There are two class of MHC: class I and class II. Three major molecules for MHC class I are: HLA-A, HLA-B, and HLA-C. Three major molecules for MHC class II are: HLA-DR, HLA-DQ, and HLA-DP. MHC class I presents antigens to clusters of differentiation (CD) 8 T cell, and MHC class II presents antigens to CD4 T cells [7].

HLA typing is done by microlymphocytotoxicity test. This test is performed in a special microtest plate in which reagent serum is incubated with isolated lymphocytes. Exogenous complement is added to the mixture and incubated to allow antigen and antibody binding. Complement activation leads to cell death. Eosin dye is added to show lysed lymphocytes. The lysed lymphocytes will take the eosin dye and appear dark under a phase contrast microscope. An estimation of lysed cell count is determined using a phase contrast microscope. Formalin is added as a fixative and to prevent non-specific uptake of the eosin dye by the cells [8].

Antibody screening is performed to detect antibodies in the recipient's serum that react with the HLA antigens present on the donor lymphocytes. Antibody screening assays are similar to the HLA typing, where lymphocytes from an individual whose HLA

types are known are incubated with the recipient's serum [8, 9]. Assays using flow cytometric technology with a panel of microbeads have been utilized for antibody screening. This is done using a mixture of eight microbead groups that have a unique fluorescent property essentially assigning an address. Each microbead group is coated with purified HLA antigens from a single cell line. Recipient sera are added and HLA antibody binds. Bound recipient antibodies are detected with anti-human globulin. Based on the fluorescent signal on the surface of the microbeads and the microbead group identification, HLA specificities can be determined. The use of microbeads coupled with a single cell line of purified HLA antigens allow for a standardized quantity of antigen present in the assay [10]. In a study comparing the microbead assay and the cytotoxicity test, the microbead assay showed a higher sensitivity in the ability to detect HLA antibodies at a higher serum dilution. The study was done on 1,421 sera from patients who are waiting for a kidney transplant or following a kidney transplantation. The microbead array method was able to detect anti-HLA antibodies on 18% of the patients who are waiting for kidney transplant compared to 7% when using the complement dependent cytotoxicity (CDC) method. In addition, the microbead array method was not as labor intensive as the CDC method. Another advantage of using the microbead array method is the ability to determine the HLA antibody specificities [11].

The last step to confirm the compatibility between the donor and the recipient is the compatibility testing. The compatibility testing is performed to detect pre-existing antibodies in the recipient against the donor [8]. The compatibility testing is performed with the CDC method using donor lymphocytes that are incubated with the recipient's serum. The presence of cytotoxic antibodies is determined by looking at the level of

donor cell lysis. If cytotoxic antibodies are determined to be present, renal transplantation is not performed. Compatibility testing using flow cytometric technology can also been used. A titration study on compatibility testing using flow cytometric technology showed a 30 to 250 fold of greater sensitivity [12].

However, there are patients who have allograft rejection without HLA antibodies. A study showed that ten years after renal transplantation, 11% of rejection episodes did not result in the development of anti-HLA antibodies. These patients were screened for anti-HLA antibodies before and after renal transplantation and remains negative. Non-HLA antibody mediated rejection could be a secondary effect from other cellular events [13]. Another study showed that 20% of kidney transplantations performed failed and no anti-HLA antibodies were found post-transplantation. The antibody specificity is not yet resolved but preliminary studies indicate that the antibodies produced were against other antigens [14]. In addition, in some cases of renal transplantation between HLA identical siblings there was still a need for treatment with immunosuppressive drugs to prevent rejection. These studies suggest that graft rejection can be caused by non-HLA antibodies. Since screening for non-HLA antibodies is not included in pre-renal transplantation, one of the causes for failure in kidney transplantation may be due to non-HLA antibodies [15].

Renal transplantation rejection and treatment

There are three types of allograft rejection: hyperacute rejection, acute rejection, and chronic rejection. Hyperacute rejection occurs within hours of transplantation and the transplanted kidney must be removed. Hyperacute rejection is caused by preformed lymphocytotoxic antibodies in the recipient serum directed towards donor antigens [16].

These antibodies should be identified in the antibody screen assay or the compatibility testing [17]. Acute rejection occurs between 4 and 30 days of transplant [16]. About one-third of acute rejection episodes are antibody-mediated [3]. Antibody-mediated rejection can be caused by HLA antibodies and non-HLA antibodies. Chronic rejection occurs more than 3 months after transplantation. In the beginning, chronic rejection may be caused by humoral immune responses since complement, immunoglobulins, and anti-endothelial cell antibodies have been identified in the rejected kidney [18]. However, further testing in later stages of chronic rejection showed the presence of antibody against donor antigens in the recipient serum are decreased or absent [19]. A study using the rat kidney allograft model showed a large numbers of macrophages. In addition, adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) were found on the glomerular capillary endothelium and other renal structures [20]. These adhesion molecules are usually not present in normal renal tissue. This finding indicates that the T cell mediated immune response may be involved in later stages of chronic rejection. Patients with chronic rejection eventually return to dialysis therapy [16].

Rejection due to HLA antibodies can be minimized by matching HLA antigens and modifying the recipients' immune system [21]. Options to prevent and to treat allograft rejection include: a short course of high dose corticosteroids, cytotoxic drugs such as azathioprine, cyclophosphamide and mycophenolate mofetil, or fungal and bacterial derivative such as cyclosporin A, tacrolimus and sirolimus [22, 23].

A short course of high dose corticosteroid is used in the treatment of acute cellular rejection. The effect of corticosteroid is a shift in the leukocyte population where the

number and proportion of neutrophils are increased and T lymphocytes are decreased [24]. In addition, steroids also inhibit antigen stimulated proliferation of T cells by inhibiting interleukin 2 (IL-2). IL-2 is required for the initiation of deoxyribonucleic acid (DNA) synthesis and proliferation of T cells, which is product of cellular immune response in acute cellular rejection [25].

The main effect of cytotoxic drugs such as azathioprine is to interfere with DNA synthesis. Azathioprine is a prodrug that releases 6-mercaptopurine. 6-mercaptopurine is a tissue inhibitor of metalloproteinase which converted to thioguanine nucleotides. Thioguanine derivatives may inhibit purine synthesis which is required for DNA synthesis [3]. Mycophenolate mofetil also inhibit DNA synthesis by blocking de novo synthesis of guanosine monophosphate, a nucleotide. Cyclophosphamide is metabolized to phosphoramide mustard which alkylates DNA and inhibiting DNA replication [22].

Fungal and bacterial derivatives such as cyclosporin A, tacrolimus and sirolimus are immunosuppressive agents that interfere with T cell signaling. Cyclosporin A and tacrolimus block T cell proliferation by inhibiting phosphatase activity of calcium activated enzyme calciuneurin. Activation of calciuneurin allows for clonal expansion of activated T cells. When the enzyme is inhibited, T cells proliferation is inhibited [22]. Sirolimus act by inhibiting IL-2 driven T cell proliferation [3].

Therapies with anti-lymphocyte antibodies can also be used. Some examples are anti-CD 20 which target B cell surface marker CD20 or anti-CD3 which target the T cell surface marker CD3. Removal of preformed or newly developed antibodies can be done by plasma exchange and intravenous immunoglobulin [26]. Plasma exchange has been used to prevent and reverse antibody-mediated rejection [27].

Renin angiotensin aldosterone system

The renin angiotensin aldosterone system (RAAS) regulates renal activity, maintains optimal salt and water balance, and controls tissue growth in the kidney. Renin is an enzyme released by the renal juxtaglomerular cells located in the wall of afferent arteriole. Renin synthesis is influenced by changes in blood pressure, angiotensin II, and the plasma sodium and potassium levels in the body. Renin cleaves the precusor angiotensinogen, which is synthesized by the liver, and forms angiotensin I. Angiotensin I is converted to angiotensin II by angiotensin converting enzyme (ACE). ACE is found in the endothelial cells of the lung, vascular endothelium, and cell membranes of the kidneys, heart, and brain. Angiotensin II binds to angiotensin II receptors found in the vascular endothelium, adrenal, kidney, liver and brain. Binding of the angiotensin II to the angiotensin II receptors initiated the intracellular signaling pathways that mediate vasoconstrictions and aldosterone release. Aldosterone is synthesized by the adrenal gland. Aldosterone binds to the aldosterone receptors in the kidney for reabsorption of water and sodium [28].

Angiotensin II receptors

There are two subtypes of angiotensin II receptors: type 1 (AT_1) and type 2 (AT_2). AT_1 are located in the vasculature, kidney, adrenal gland, heart, liver, and brain. AT_2 are present mainly in the fetus, but in adults they are located in adrenal medulla, uterus, ovary, vascular endothelium, and brain [29-32]. AT_1 and AT_2 are polypeptides containing approximately 360 amino acids that span the cell membrane seven times [33-35]. The gene for AT_1 is located on chromosome 3, and for AT_2 is on the X chromosome [36, 37]. Both receptors are similar in structure, but each has different functions. In

general, AT_2 receptor activation opposes functions mediated by the AT_1 receptor (Table 1) [38].

Table 1. Angiotensin II receptor subtype function.
(Adapted from Brewster and Perazella, 2004).

AT_1 function	AT_2 function
• Systemic and renal vasoconstriction • Increased renal sodium reabsorption • Activation of inflammatory cytokines • Vascular smooth muscle growth • Oxidative stress • Endothelial dysfunction • Increased plasminogen activator inhibitor 1 activity and thrombosis	• Systemic and renal vasodilation • Decreased renal sodium reabsorption • Decreased inflammation • Decreased mitogenesis • Decreased myocyte hyperthrophy • Decreased cardiac fibrosis

Anti-AT_1 antibody

A study was performed on kidney transplant recipients who had allograft rejection, but no anti-HLA antibodies. The study was done over 4 years (2000-2004) and included 278 kidney transplantations, and 119 allograft rejections required treatment. From 119 allograft rejections, 23 were refractory to steroids. Further testing showed that 9 of 23 (39.1%) were due to anti-HLA antibodies, 10 of 23 (43.5%) were due to anti-AT_1 antibodies, and 4 of 23 (17.4%) were not due to anti-HLA antibodies or anti-AT_1 antibodies. The method used to confirm the presence of anti-AT_1 antibodies was a functional bioassay using spontaneously beating cultured neonatal-rat cardiomyocytes that express several G-protein coupled receptors, including AT_1 receptors. The patient sera were added, resulting in a linear increase of cellular activity. To confirm the AT_1 receptor mediated response, immunoglobulin (IgG) stimulated cells (from patient's) were treated with short peptides corresponding to the sequence of the second extracellular loop of the AT_1 receptors. The peptides inhibited the activity from IgG stimulated cells. This

inhibition confirmed that the IgG that bound to the receptors were anti-AT_1 antibodies [39].

Another study observed that patients with hypertension may have autoantibodies against AT_1. Sera from patients with hypertension were screened for autoantibodies against G-protein coupled cardiovascular receptors such as AT_1 and AT_2. The study included 14 patients with malignant essential hypertension (MEH), 12 patients with malignant secondary hypertension (MSH), 11 patients with renovascular non-malignant hypertension (RVH), and 35 subjects that were normotensive healthy blood donors (control). From this population, anti-AT_1 autoantibodies were detected in 14% of MEH, 33% of MSH, 18% of RVH, and 14% of the control population. The presence of anti-AT_1 autoantibodies suggests that it may be involved in the pathogenesis of malignant hypertension. The method used in this study was an enzyme-linked immunosorbent assay (ELISA). Peptide sequences from the AT_1 and AT_2 receptors were used to coat the microtiter plate. The same peptide sequence used in anti-AT_1 antibody study, additional peptide sequences from different parts of AT_1 and AT_2 receptors were also used. Human sera were added and incubated. To detect the presence of the antibody anti-human IgG were added. Antibodies detected in the positive ELISA were purified and confirmed using the functional bioassay. The specificity was anti-AT_1 antibody [40].

Microbead immunoassay

Using the principle of the ELISA described above, my project was to design a microbead immunoassay to allow an easier detection of anti-AT_1 and anti-AT_2 antibodies. The microbead immunoassay uses carboxylated polystyrene microspheres from Luminex. The microspheres size is 5.6 μm. These microspheres are internally dyed with red and infrared fluorophores. The concentrations of red and infrared dyes are different for each group of microspheres. Each different group of microsphere is assigned a specific number that corresponds to a different address based on the intensity of the two dyes. There are up to 100 different microsphere groups available. A different address will demonstrate different output signals during detection. A group of microspheres with the same address will be called a microsphere group. Detection of the different addresses of microsphere groups is done by the a classification laser in the Luminex instrument. The classification laser excites the red and the infrared dyes inside the microspheres and the intensities of the excitation emission are measured by a detector. The classification laser wavelength is 635 nanometer (nm). Classification of different microsphere group using forward and side scatter on the Luminex instrument is in Figure 1.

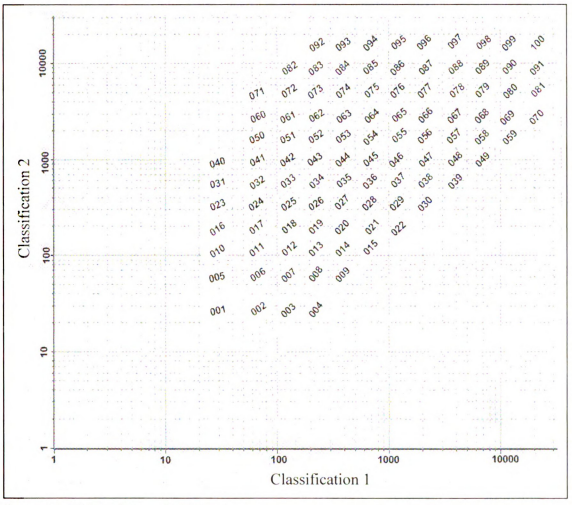

Figure 1: Microspheres classification.
(Adapted from Luminex Corporation, Austin, TX). Classification of 100 Luminex microspheres based on red and infrared dye combination using forward and side scatter on the Luminex instrument.

Microsphere coupling

On the surface of each microsphere carboxyl groups are present that can be covalently coupled with the peptide sequences. The covalent bond will occur between the carboxyl group of the microsphere and the N-terminus of the peptide. Coupling of the peptide is done using 1-Ethyl-3-[3-dimethylaminopropyl] carboiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS). The EDC will react with the

carboxyl group on the microsphere, forming an amine-reactive *O*-acylisourea intermediate. The intermediate may react with an amine on the peptide sequence, yielding a conjugate of the two molecules joined by a stable amide bond. The addition of sulfo-NHS stabilizes the amine-reactive intermediate by converting it to an anime-reactive sulfo-NHS ester (Figure 2) [41, 42].

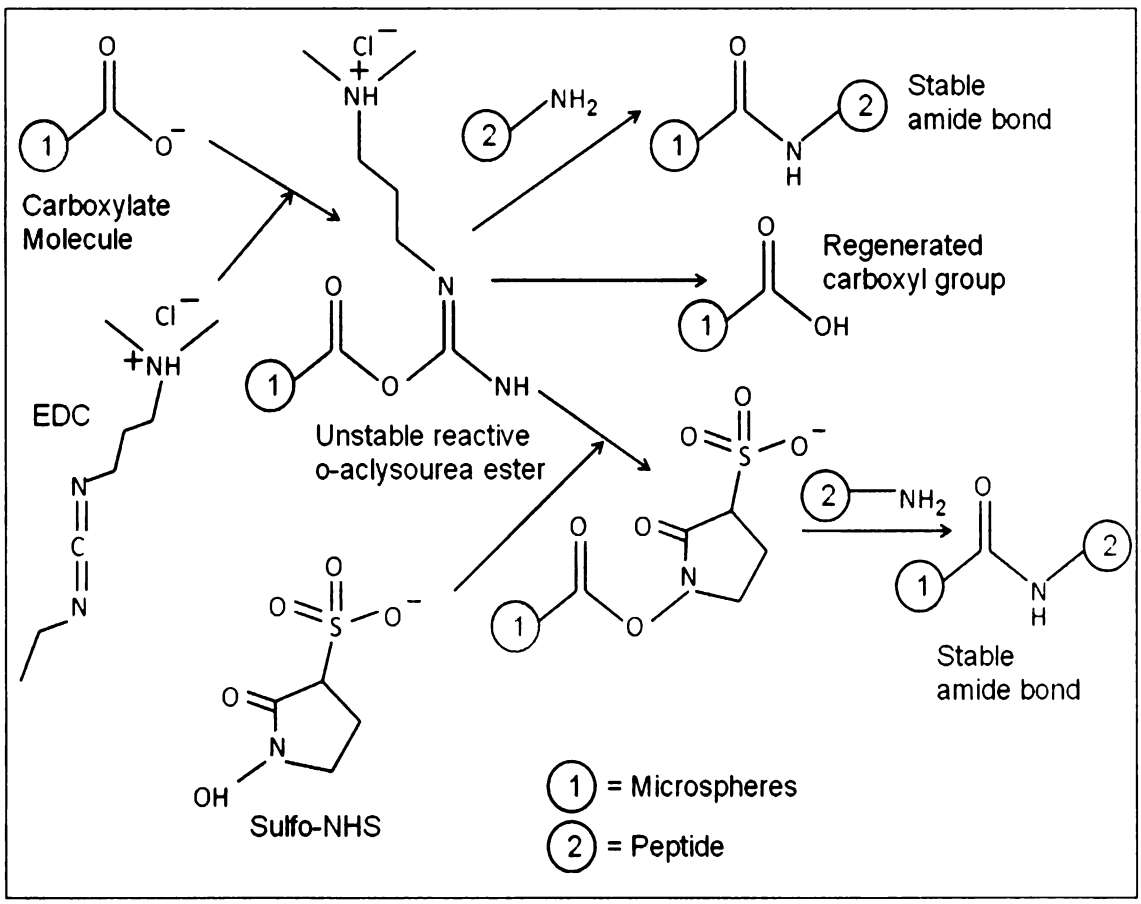

Figure 2. Carboxylated microsphere activation by EDC and Sulfo-NHS. (Adapted from EDC package insert).

The carboxylated microspheres are coupled with a peptide sequence. Each peptide sequence is coupled with one group of microspheres. After the different peptides are coupled with a different microsphere group, the microspheres are blocked with bovine serum albumin (BSA) to prevent non-specific binding. After the blocking step,

primary antibody which is the analyte is added (commercial control or patient's sera). After the addition of primary antibody, the microspheres are washed to remove all of the unbound antibodies. To determine binding of the primary antibody to the peptide sequence on the microspheres, the appropriate secondary antibody to match the primary antibody is added. The secondary antibody is conjugated with phycoerythrin (PE) to allow detection by the reporter laser in the Luminex instrument. The reporter laser wavelength is 532 nm. The emission of the PE is detected by a photomultiplier tube with detection bandwidth of 565-585 nm.

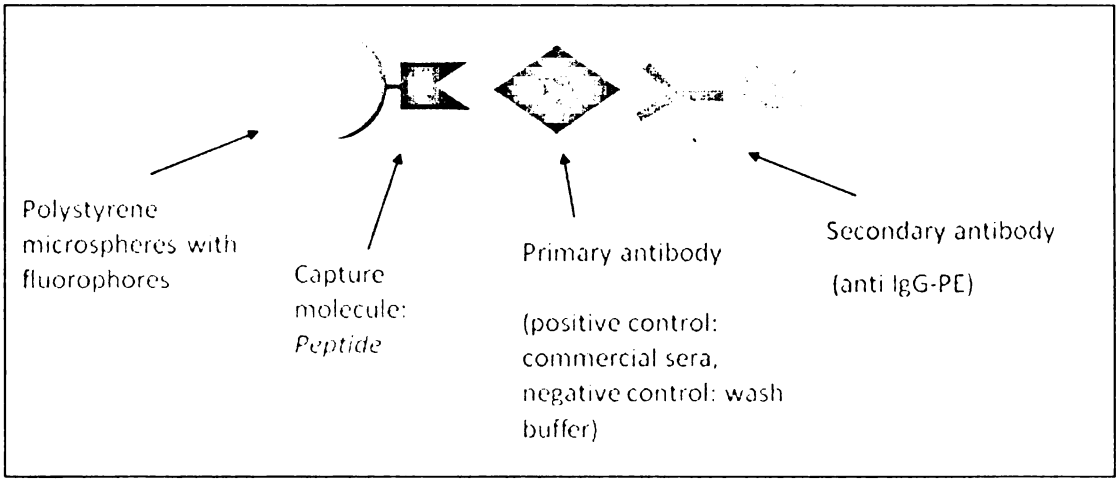

Figure 3. Microbead immunoassay principle.
(Figure adapted and modified from Liquichip® Application Handbook February 2006). Polystyrene microspheres are coupled with peptide sequences from AT_1 and AT_2 receptors. Detection of anti-AT_1 or anti-AT_2 antibody is done by adding secondary antibody with PE.

Luminex instrument

The Luminex instrument is an instrument based on flow cytometry technology. In the Luminex instrument there are two fluidic paths. The first fluidic path includes a syringe driven mechanism that controls the sample intake from the sample container to the cuvette. The advantage of this mechanism is the instrument requires small sample

volume. Sample is acquired using the sample probe from a 96-well microplate and injected into the cuvette. From the cuvette the sample is purged with sheath buffer by the second fluidics path which is driven by positive air pressure and supplies sheath fluid. The second path removes residual sample to minimize carry over. The sample is introduced to the optics path at a reduced rate to ensure that each microsphere is analyzed individually [43].

Project Goal

The goal of this research is to develop a screening method for anti-AT_1 and anti-AT_2 antibodies using microbead immunoassay which utilize a flow cytometry based instrument such as the Luminex that is commonly available in histocompatibility laboratories. The microbead immunoassay will utilize the peptide sequences from the functional bioassay and the ELISA method. The population of interest for this research is renal transplant recipients.

MATERIALS

Materials

All reagents and supplies described, unless otherwise stated were purchased from Michigan State University Stores (East Lansing, MI)

Microspheres

xMAP® Multi-Analyte COOH Microspheres were purchased from Luminex Corporation (Austin, TX). Six microsphere groups were selected based on the manufacturer's recommendation. The microsphere groups selected were: 033, 034, 035, 036, 037, and 038. Catalog numbers for the microspheres can be found in the appendix. On the surface of each microsphere, carboxyl groups were present for covalent coupling with a peptide sequence that is part of either the AT_1 or the AT_2 receptor. The concentration of each microsphere set was 1.25×10^7 microspheres/milliliter (mL).

Peptides

Peptides were synthesized by the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University (East Lansing, MI). The peptide sequences were from Dragun et al. (2005) and Fu et al. (1999). The peptide sequences are parts of the AT_1 and AT_2 receptors. A list of the peptide sequences and its properties are in Table 2.

Table 2. Peptide sequences properties.
Each letter in the peptide sequence represents a single amino acid abbreviation.
Underlined sequences used in both study (Adapted from Dragun et al. and Fu et al.).

Peptide sequence	MW	Reference
IHRNVFFIENTNITVCAFHYESQNSTL	3198.5	AT_1 receptor
AYEIQKNKPRNDD	1590.7	AT_1 receptor
ENTNIT	690.7	AT_1 receptor
AFHYESQ	880.9	AT_1 receptor
ACLSSLPTFYFRDVRTIEYLGVNACI	2952.4	AT_2 receptor

Primary Antibodies

Primary antibodies used in the microbead immunoassay, ELISA, and Dot Blot are listed in Table 3. Catalog numbers for the antibodies are listed in the appendix. These commercial antibodies were used as positive controls.

For screening, 158 samples of human sera and 17 rat sera were used. The human sera consist of renal transplant and preeclampsia patients, and the rat sera were hypertension rat sera. All human samples and rat sera were handled in accordance with the University Committee on Research Involving Human and Animal Subjects.

Table 3. Primary antibodies.

Primary antibody	Source	Stock Concentration
Mouse anti human angiotensin II type 1 receptor (AT_1)	USBio (Swampscott, MA)	0.7 mg/mL
Rabbit anti human angiotensin II type 2 receptor (AT_2)	USBio (Swampscott, MA)	1 mg/mL
Sheep polyclonal to angiotensin type 1 receptor (AT_1)	Abcam (Cambridge, MA)	1 mg/mL
Rabbit polyclonal to angiotensin II type 2 receptor (AT_2)	Abcam (Cambridge, MA)	1 mg/mL

Secondary Antibodies

Secondary antibodies used in the microbead immunoassay, ELISA, and Dot Blot are listed in Table 4. Catalog numbers for the antibodies are listed in the appendix. Antibodies with phycoerythrin (PE) were used in the microbead immunoassay, and antibodies with alkaline phosphatase (AP) were used in the ELISA and Dot Blot.

Table 4. Secondary antibodies.
(PE= phycoerythrin; AP=alkaline phosphatase).

Product	Source	Concentration
Goat anti mouse IgG (PE)	USBio (Swampscott, MA)	0.2 mg/mL
Donkey anti rabbit IgG (PE)	USBio (Swampscott, MA)	0.5 mg/mL
Donkey polyclonal to sheep IgG (PE)	Abcam (Cambridge, MA)	0.50 mg/mL
Rabbit polyclonal to sheep IgG (AP)	Abcam (Cambridge, MA)	1.00 mg/mL
Donkey polyclonal to rabbit IgG (PE)	Abcam (Cambridge, MA)	0.5 mg/mL
Goat polyclonal to rabbit IgG (AP)	Abcam (Cambridge, MA)	1 mg/mL
Sheep polyclonal to rat IgG (AP)	Abcam (Cambridge, MA)	1.00 mg/mL
Goat polyclonal to rat IgG (PE)	Abcam (Cambridge, MA)	0.5 mg/mL
Goat polyclonal to human IgG (PE)	Abcam (Cambridge, MA)	0.5 mg/mL

METHODS

General methods

Peptide reconstitution

Peptide reconstitution method was recommended by the Macromolecular Structure, Sequencing and Synthesis Facility at Michigan State University (East Lansing, MI). Peptide reconstitution was done in 1.5 mL polypropylene microcentrifuge tubes. List of each peptide sequence coupled with the different microsphere group are in Table 5.

Table 5. Peptide sequences and corresponding microsphere group.

Peptide sequence	Peptide ID	Microsphere group
IHRNVFFIENTNITVCAFHYESQNSTL	AT_1-033	033
AYEIQKNKPRNDD	AT_1-034	034
ENTNIT	AT_1-035	035
AFHYESQ	AT_1-036	036
ACLSSLPTFYFRDVRTIEYLGVNACI	AT_2-037	037
None	None	038

A stock solution of each peptide sequences was prepared from the lyophilized peptide. Based on the hydrophobic properties of the peptide sequences, peptide AT_1-033 and peptide AT_2-037 were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) before the addition of distilled water (dH_2O) while peptide AT_1-034, AT_1-035, and AT_1-036 were dissolved in dH_2O only. Table 6 lists the specific amount used to make the stock solution of each peptide sequences. Peptide solutions were aliquoted and stored at -20°C.

Table 6. Peptide sequence reconstitution.

Peptide ID	Peptide (mg)	DMSO (mL)	dH_2O (mL)	Final Concentration
AT_1-033	4	1	3	1 mg/mL
AT_1-034	1	0	1	1 mg/mL
AT_1-035	1	0	1	1 mg/mL
AT_1-036	1	0	1	1 mg/mL
AT_2-037	1.5	0.5	1	1 mg/mL

Peptide coupling with microspheres

The peptide coupling method was adapted from the Luminex Corporation (Austin, TX). Peptide coupling was done in 1 mL polypropylene microcentrifuge tubes (Art Robbins, Sunnyvale, CA).

Microsphere stock solutions were resuspended by vortexing for 10 seconds (sec) followed by sonication for 10 sec. The stock concentration of the microspheres was 1.25 x 10^7 microspheres/mL. 200 microliter (µL) of each microsphere group was aliquoted into a separate 1 mL polypropylene microcentrifuge tube resulting in working concentration of 2.5 x 10^6 microspheres/mL. The microspheres were washed by adding 100 µL of dH_2O, vortexing for 10 sec, sonicating for 10 sec and centrifugation for 8,000 x gravity (g) for two minutes (min). The supernatant was aspirated using a micropipette and discarded after the wash step. The microspheres were resuspended with 80 µL of sodium phosphate monobasic buffer [0.1 molar (M) sodium phosphate monobasic buffer (NaH_2PO_4) pH 6.2] (Sigma-Aldrich, St. Louis, MO) followed by vortexing for 10 sec and sonication for 10 sec. 10 µL of 50 mg/mL sulfo-N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce, Rockford, IL) followed by 10 µL of 50 mg/mL (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Pierce, Rockford, IL) were added to the microspheres. The microspheres were mixed gently by vortexing for 10 sec.

The microspheres were incubated at room temperature for 20 min. During incubation, the microspheres were vortexed at 10 min interval for 10 sec and covered with aluminum foil to prevent photo bleaching.

After incubation, the microspheres were washed twice with 2[N-Morpholino]ethanesulfoic acid (MES) buffer [0.05 M 2[N-Morpholino]ethanesulfoic acid (MES) pH 5.0] (MES buffer #1) (Sigma-Aldrich, St. Louis, MO). The microspheres were washed by adding 250 µL of MES buffer #1 and centrifugation at 8,000 x g for two min. The supernatant was aspirated by a micropipette and discarded after each wash. The microspheres were resuspended in 100 µL of MES buffer #1. A specific amount of each peptide concentration was added to each microsphere set followed by the addition of MES buffer #1 for a total volume of 500 µL. Peptide concentrations used were 125, 100, 50, 25 and 5 µg. For microspheres group 038 only 500 µL of MES buffer #1 were added. Microsphere group 038 serves as a blank. The microspheres were incubated at room temperature for two hours on a rotator. During incubation the microspheres were covered with aluminum foil to prevent photo bleaching.

The microspheres were washed by adding 500 µL of Phosphate Buffer Solution-Tween (PBS-TBN) [PBS 0.138 M NaCl, 0.0027 M KCl, pH 7.4, 0.02% Tween 0.05% sodium azide], and centrifugation for 8,000 x g for two min. The supernatant was aspirated by a micropipette and discarded after each wash. Two more washes were performed by adding 1000 µL of PBS-TBN, and centrifugation for 8,000 x g for two min. The supernatant was aspirated and discarded after each wash. The microspheres were resuspended in 1000 µL of PBS-TBN and stored at 2-8°C in the dark.

Microspheres modification with Adipic Acid Dihydrazide (ADH)

The microsperes modification with ADH method was adapted from Luminex Corporation (Austin, TX). This method was performed to minimize binding issue between the antibody of interest and the peptide sequences, especially with the shorter peptide sequences. The ADH creates a space between the microsphere and the peptide so that immunogenicity of the peptide was not hindered by its binding to the microsphere.

Microsphere stock solutions were resuspended by vortexing for 10 sec and sonication for 10 sec. 40 µL of each microsphere group was aliquoted into separate 1.0 mL polypropylene microcentrifuge tube resulting in working microsphere concentration of 500,000 microspheres. 160µL of dH_2O were added into each microsphere group. The microspheres were centrifuged at 1,000 x g for 2 min. The supernatant was aspirated and discarded. 1 mL of MES buffer [0.1 M MES pH 6.0] (MES buffer #2) was added. The microspheres were vortexed for 10 sec and centrifuged at 1,000 x g for 2 min. The supernatant was aspirated and discarded. The microspheres were resuspended in 1 mL of 35 mg/mL ADH (Sigma-Aldrich, St. Louis, MO) and 200 µL of 200 mg/mL EDC. The microspheres were incubated at room temperature for one hour in the dark on a rotator. After incubation, the microspheres were washed three times with 1 mL of MES buffer [0.1 M MES buffer pH 4.5] (MES buffer #3) and centrifuged at 8,000 x g for 2 min. The ADH-modified microspheres were stored in the dark at 2-8°C.

Peptide coupling to ADH-modified microspheres

After modification of microspheres with ADH, the microspheres were washed with 1 mL of MES buffer #2. Wash step was performed by centrifugation of the microspheres at 8,000 x g for 2 min, aspiration of the supernatant, and mix by vortex and sonication for 10 sec each. A specific amount of peptide concentration was added followed by the addition of MES buffer #2 for a total volume of 500 µL. Peptide amount used were 125, 100, 50, 25 and 5 µg. After the addition of the peptide solution and MES buffer, 50 µL of 200 mg/mL EDC were added. The microspheres were incubated at room temperature on a rotator for two hours in the dark. After incubation, the microspheres were centrifuged at 8,000 x g for 2 min. The supernatant was aspirated and discarded. The microspheres were resuspended in 1 mL of phosphate buffer (PBS) two washes. After the last wash, the microspheres were resuspended in 1 mL of PBS buffer.

Determination of microspheres concentration

The microspheres concentration was calculated after coupling the microspheres with the peptide. Figure 4 lists the formula used to calculate the microsphere concentration.

Microspheres/µL = average x 0.1

Average = the average number of microspheres from 8 large squares on both sides of the hemocytometer.

Figure 4. Microspheres concentration formula.

For each assay, a working peptide coupled microsphere solution was prepared for each microspheres group. The concentration required for a working solution was 5000 microspheres for each microsphere group in 50 µL solution.

Blocking of microspheres with Bovine Serum Albumin (BSA)

A working peptide coupled microsphere solution was prepared for each microsphere group. PBS with 1% weight/volume (w/v) BSA (PBS 1% BSA buffer) (Sigma-Aldrich, St. Louis, MO) was used as blocking solution for the microspheres. The blocking step was performed by the addition of 100 µL PBS 1% BSA buffer into the working microspheres solution followed by centrifugation at 8,000 x g for 2 min. The microspheres were resuspended in 50 µL of PBS 1% BSA buffer.

Microbead immunoassay

After the microspheres were blocked with PBS 1% BSA, 50 µL of primary antibody was added. PBS 1% BSA buffer were used to perform dilutions needed for primary antibody. After primary antibody was added, the microspheres were incubated at room temperature for 60 min on a plate shaker at 120 radius per minute (rpm) in the dark. After incubation, the microspheres were washed twice using 100 µL of PBS 1% BSA buffer. The microspheres were resuspended with 50 µL PBS 1% BSA and mixed. After the last wash, 50 µL of secondary antibody was added and the microspheres were incubated at room temperature for 30 min on a plate shaker at 120 rpm in the dark. Following incubation, the microspheres were washed twice using 100 µL of PBS 1% BSA buffer. The microspheres were transferred to a 96-well microplate for detection on the Luminex instrument (Austin, TX).

Method modifications

Microbead immunoassay using vacuum manifold

The microbead immunoassay method was performed using vacuum manifold. This experiment was performed using microsphere group 033 which was coupled with 125 µg of AT_I-033 peptide, and microsphere group 038 which was not coupled with any peptide. Microsphere group 038 serves as a blank. Each microsphere group was aliquoted into a separate reaction wells. The volume used for primary and secondary antibody was 50 µL. The primary and secondary antibody combinations and concentrations used in this experiment are listed in Table 7.

Table 7. Antibodies in microbead immunoassay (vacuum manifold).
Primary and secondary antibody concentration used in the microbead immunoassay using vacuum manifold. Volume used for each antibody was 50 µL.

Primary Antibody (Concentration)	Mouse anti human AT_I **(70 µg/mL)**	Mouse anti human AT_I **(294 µg/mL)**
Secondary Antibody (Concentration)	Anti mouse IgG- PE **(10 µg/mL)**	Anti mouse IgG- PE **(4 µg/mL)**

Microbead immunoassay using centrifugation

This method modification was performed due to insufficient washing observed during the vacuum manifold method. The microbead immunoassay method was performed using the centrifugation method for the washing step. The wash step was performed with centrifugation at 8,000 x g for 2 min and aspiration of the supernatant using a micropipette. This experiment was performed using microsphere group 033, 034, 035, 036 that were coupled with 125 µg of corresponding peptide, and microsphere group 038 as a blank. Each microsphere group was aliquoted into a separate reaction tube. The volume used for primary and secondary antibody was 50 µL. The primary and secondary antibody combinations and concentrations used in this experiment are listed in Table 8.

Table 8. Antibodies in microbead immunoassay (centrifugation).
Primary and secondary antibody concentration used in the microbead immunoassay using centrifugation. Volume used for each antibody was 50 µL.

Primary Antibody (Concentration)	Mouse anti human AT_I **(420 µg/mL)**
Secondary Antibody (Concentration)	Anti mouse IgG- PE **(4 µg/mL)**

Microbead immunoassay by varying incubation conditions and secondary antibody concentrations

The microbead immunoassay method was performed using the centrifugation method for the wash steps. This experiment was performed to optimize the assay by varying primary and secondary antibody concentration combination. This experiment was performed using microsphere group 033 that was coupled with 125 µg of AT_I-033 peptide, and microsphere group 038 as a blank. During this experiment, each microsphere group was aliquoted into a separate reaction tube. This assay was performed under two different incubation conditions: room temperature and 37°C. The volume used for primary and secondary antibody was 50 µL. The primary and secondary antibody combinations and concentrations used in this experiment are listed in Table 9.

Table 9. Antibodies in microbead immunoassay (modification: incubation, secondary antibody).
Two sets of assay was performed with room temperature incubation and 37°C.

Primary Antibody (Concentration)	Mouse anti human AT_I **(700 µg/mL)**	Mouse anti human AT_I **(700 µg/mL)**
Secondary Antibody (Concentration)	Anti mouse IgG- PE **(4 µg/mL)**	Anti mouse IgG- PE **(40 µg/mL)**

Microbead immunoassay by varying peptide concentrations #1

The microbead immunoassay method was performed using the centrifugation method for the wash steps. This experiment was performed to optimize the assay. This experiment was performed using microsphere group 033 that was coupled with 100, 50,

25, and 5 μg of AT_1-033 peptide, and microsphere group 038 as a blank. During this experiment, each microsphere group was aliquoted into a separate reaction tube. Primary antibody used was 50 μL (70 μg/mL) of mouse anti human AT_1 (USBio, Swampscott, MA). Secondary antibody used was 50 μL (4 μg/mL) of anti mouse IgG- PE (USBio, Swampscott, MA).

Microbead immunoassay by varying peptide concentrations #2

Microbead immunoassay method was performed using the centrifugation method for the wash steps. This experiment was performed using microsphere group 033, 034, 035, and 036 that was coupled with 100, 50, 25, and 5 μg of corresponding peptide, and microsphere group 038 as a blank. During this experiment, each microsphere group was aliquoted into a separate reaction tube. Primary antibody used was 50 μL (4 μg/mL) of sheep polyclonal to angiotensin type 1 receptor (Abcam, Cambridge, MA). Secondary antibody used was 50 μL (5 μg/mL) of anti rabbit IgG- PE (Abcam, Cambridge, MA).

Microbead immunoassay by varying peptide concentrations and primary antibody concentrations

The microbead immunoassay method was performed using the centrifugation method for the wash steps. This experiment was performed to optimize the assay by varying the peptide, the primary antibody and secondary antibody concentration. This experiment was performed using microsphere group 033 that was coupled with 100, 50, 25, and 5 μg of AT_1-033 peptide, and microsphere group 038 as a blank. During this experiment, each microsphere group was aliquoted into a separate reaction tube. Primary antibody used was 50 μL (70 μg/mL and 140 μg/mL) of mouse anti human AT_1 (USBio,

Swampscott, MA). Secondary antibody used was 50 µL (4 µg/mL) of anti mouse IgG-PE (USBio, Swampscott, MA).

Microbead immunoassay by varying primary and secondary antibody concentrations

The microbead immunoassay method was performed using the centrifugation method for the wash steps. This experiment was performed to optimize the assay by varying primary and secondary antibody combination. This experiment was performed using microsphere group 033, 034, 035, 036, and 037 that was coupled with 125 µg of the corresponding peptide, and microsphere group 038 as a blank. During this experiment, each microsphere group was aliquoted into a separate reaction tube. The volume used for primary and secondary antibody was 50 µL. The primary and secondary antibody combinations and concentrations used in this experiment are listed in Table 10.

Table 10. Antibodies in microbead immunoassay (modification: AT_2 secondary antibody)

Anti-AT_1		
Primary Antibody (Concentration)	Sheep polyclonal to angiotensin type 1 receptor (AT_1) **(2 µg/mL)**	
Secondary Antibody (Concentration)	Donkey polyclonal to sheep IgG-PE **(5 µg/mL)**	
Anti-AT_2		
Primary Antibody (Concentration)	Rabbit anti human angiotensin II type receptor **(0.1 µg/mL) (USBio)**	Donkey anti rabbit IgG-PE **(2.5 µg/mL)**
Secondary Antibody (Concentration)	Rabbit polyclonal to angiotensin II type 2 receptor **(0.1 µg/mL) (Abcam)**	Donkey polyclonal to rabbit IgG-PE **(2.5 µg/mL)**

Rat sera screening using microbead immunoassay by centrifugation method

Microbead immunoassay method was performed using the centrifugation method. In this experiment, different microsphere groups are combined prior the addition of

primary antibody. Since known positive control for the anti-AT_1 or anti-AT_2 was not available, screening of hypertension rat sera was performed. This population was used because it was described previously that patients with hypertension may have autoantibodies against anti-AT_1 [39]. This experiment was performed with the peptide coupled microspheres and ADH modified peptide coupled microspheres. Peptide concentrations used was 125 μg. 50 μL of rat sera was used as primary antibody. Secondary antibody used was 50 μL of 4 μg/mL of goat polyclonal to rat IgG-PE (Abcam, Cambridge, MA).

Human sera screening using microbead immunoassay by centrifugation method

Microbead immunoassay method was performed using the centrifugation method. In this experiment, different microsphere groups are combined prior the addition of primary antibody. Since known positive control for the anti-AT_1 or anti-AT_2 was not available, screening of human sera from renal transplant recipient and preeclampsia patients was performed. This population was used because it was described previously that these patients may have the antibody of interest [27, 42]. This experiment was performed with the peptide coupled microspheres and ADH modified peptide coupled microspheres. Peptide concentrations used was 125 μg. 50 μL of human sera was used as primary antibody. Secondary antibody used was 50 μL (4 μg/mL) of goat polyclonal to human IgG-PE (Abcam, Cambridge, MA).

Determination of peptide coupling efficiency

Determination of peptide coupling efficiency was performed by measuring the absorbance of the supernatant of the microspheres at 260 nanometer (nm), 280 nm, 320

nm, and 230 nm using a spectrophotometer (GeneQuant, Amersham Biosciences, Pittsburgh, PA). This method was performed to ensure peptide binding to the microspheres. This procedure was performed using the peptide coupling with microspheres method with some modifications.

Microsphere stock solutions were resuspended by vortexing for 10 sec followed by sonication for 10 sec. The stock concentration of the microspheres was 1.25×10^7 microspheres/mL. 5 µL of each microsphere group was aliquoted into a separate 1 mL polypropylene microcentrifuge tube resulting in working concentration of 62,500 microspheres/mL. 395 µL of dH_2O were added. The microspheres were washed by adding 100 µL of dH_2O, vortexing for 10 sec, sonicating for 10 sec and centrifugation for 8,000 x g for two min. The supernatant was aspirated using a micropipette and discarded after the wash step. The microspheres were resuspended with 80 µL of sodium phosphate monobasic buffer followed by vortexing for 10 sec and sonication for 10 sec. 10 µL of 50 mg/mL Sulfo-NHS followed by 10 µL of 50 mg/mL EDC were added to the microspheres. The microspheres were mixed gently by vortexing for 10 sec. The microspheres were incubated at room temperature for 20 min. During incubation the microspheres were vortexed at ten min interval for 10 sec and covered with aluminum foil to prevent photo bleaching.

Following incubation, the microspheres were washed twice with MES buffer #1. The microspheres were washed by adding 250 µL of MES buffer #1 and centrifugation at 8,000 x g for two min. The supernatant was aspirated by a micropipette and discarded after each wash. The microspheres were resuspended in 100 µL of MES buffer #1. 125 µg of peptide were added, followed by the addition of MES buffer #1 for a total volume

of 500 μL . For microspheres group 038 only 500 μL of MES buffer #1 were added. Microsphere group 038 serves as a blank. 200 μL of the supernatant was aspirated and an absorbance was taken. This step was repeated at 0, 2, 4, 20 and 24 hours. At the end of each reading, the supernatant was returned to each tube and mixed by vortexing for 10 sec. The spectrophotometer was zeroed with MES buffer #1.

This experiment was also performed on ADH-modified microspheres.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA method was performed to check whether the commercial antibodies for the positive control specificity matched the specificity of the peptide sequences without the changing the peptide conformation due to the covalent binding to the microsphere. A plate map of the ELISA is in Table 11. A 96-well plate was coated with 50 µL of 20 µg and 10 µg of peptide solution. The peptide was reconstituted with three different pH: 5.5, 7.0, and 9.6. The plate was incubated at room temperature overnight. After incubation, the peptide solutions were removed and the wells were washed with 200 µL of PBS three times. 200 µL of 3% w/v non-fat dry milk diluted in PBS was added as a blocking reagent. The plate was incubated at room temperature for one hour. After incubation, the plate was washed with PBS three times. 100 µL of primary antibody was added into the appropriate wells with the appropriate dilution. The plate was incubated at room temperature for one hour. After incubation the plate was washed with PBS three times. 100 µL of secondary antibody was added into the wells with the appropriate dilution. The plate was incubated at room temperature for 30 min. After incubation, the plate was washed with PBS three times. An ELISA substrate solution [20 mL of 0.1 M Glycine buffer 1 mM $MgCl_2$ 1 mM $ZnCl_2$ pH 10.4 with 1 tablet of p-nitrophenyl phosphate(pNPP)] (Sigma-Aldrich, St. Louis, MO) was prepared. Each well was developed by adding 100 µL of ELISA substrate solution. The absorbance of the microplate was read on a plate reader at 405 nm. The absorbance from the positive control was compared with the negative control.

Table 11. ELISA plate summary

	1	2	3	4	5	6	7	8	9	10	11	12
A	AT_1-033 20 µg/mL	AT_1-034 20 µg/mL	AT_1-035 20 µg/mL	AT_1-036 20 µg/mL	AT_2-037 20 µg/mL	BSA 5µg/mL	Anti-AT_1 20µg/mL	Anti-AT_1 10µg/mL	Anti-AT_2 20µg/mL	Anti-AT_2 10µg/mL	Anti-AT_1-IgG-AP 20µg/mL	Anti-AT_2-IgG-AP 20µg/mL
B	AT_1-033 10 µg/mL	AT_1-034 10 µg/mL	AT_1-035 10 µg/mL	AT_1-036 10 µg/mL	AT_2-037 10 µg/mL	BSA 5µg/mL	PBS pH 5.5					
C	AT_1-033 20 µg/mL	AT_1-034 20 µg/mL	AT_1-035 20 µg/mL	AT_1-036 20 µg/mL	AT_2-037 20 µg/mL	BSA 5µg/mL	Anti-AT_1 20µg/mL	Anti-AT_1 10µg/mL	Anti-AT_2 20µg/mL	Anti-AT_2 10µg/mL	Anti-AT_1-IgG-AP 20µg/mL	Anti-AT_2-IgG-AP 20µg/mL
D	AT_1-033 10 µg/mL	AT_1-034 10 µg/mL	AT_1-035 10 µg/mL	AT_1-036 10 µg/mL	AT_2-037 10 µg/mL	BSA 5µg/mL	PBS pH 7.0					
E	AT_1-033 20 µg/mL	AT_1-034 20 µg/mL	AT_1-035 20 µg/mL	AT_1-036 20 µg/mL	AT_2-037 20 µg/mL	BSA 5µg/mL	Anti-AT_1 20µg/mL	Anti-AT_1 10µg/mL	Anti-AT_2 20µg/mL	Anti-AT_2 10µg/mL	Anti-AT_1-IgG-AP 20µg/mL	Anti-AT_2-IgG-AP 20µg/mL
F	AT_1-033 10 µg/mL	AT_1-034 10 µg/mL	AT_1-035 10 µg/mL	AT_1-036 10 µg/mL	AT_2-037 10 µg/mL	BSA 5µg/mL	PBS pH 9.6					

The amount of primary antibody used was 100 µL of anti-AT_1 (2 µg/mL) (Abcam, Cambridge, MA) and 100 µL of anti-AT_2 (2 µg/mL) (Abcam, Cambridge, MA). The amount of secondary antibody used was 100 µL of anti-IgG-AP (2 µg/mL) (Abcam, Cambridge, MA).

Dot Blot with non-fat dry milk as blocking agent

The dot blot method was performed to check whether the commercial antibodies for the positive control specificity matched the specificity of the peptide sequences without changing the conformation of the peptide from binding to the microplate or the microsphere. A nylon membrane (Biorad, Hercules,CA) was cut into 7 centimeter (cm) x 1 cm for each assay. The membrane was divided into 7 squares. Peptide solutions with concentration of 20 μg, 5 μL of primary antibody (positive control), and 5 μL of BSA (negative control) were added to the membrane. A general map for the dot blot is in Figure 5.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Peptide AT_1-033	Peptide AT_1-034	Peptide AT_1-035	Peptide AT_1-036	Peptide AT_2-037	BSA	Anti-AT_1	Anti-AT_2

Figure 5. Dot blot map.

Two methods used to apply peptide solutions onto the membrane: with vacuum manifold and without vacuum manifold. The membranes were air dried overnight at room temperature. The membranes were blocked with 3% non-fat dry milk-PBS solution in a 15 mL conical tube on a rotator and incubated at room temperature for one hour. The membranes were washed twice with 0.05% Tween-Phosphate Bigger Solution (Tween-PBS) [PBS 0.138 M NaCl, 0.0027 KCl, pH 7.4, 0.05% Tween] and air dried for 30 minutes. The membranes were washed with 0.3% Tween-PB [PBS 0.138 M NaCl, 0.0027 KCl, pH 7.4, 0.03% Tween] and air dried for 15 minutes. Solutions with the appropriate primary antibodies were prepared in a 15 mL conical tube. The membranes were incubated with the primary antibody solutions and incubated on a rotator for 30 min at room temperature. After incubation, the membranes were washed with 0.05% Tween-

PBS three times and PBS three times. The membranes were air dried for 30 minutes. Solutions with the appropriate secondary antibodies were prepared in a 15 mL conical tube. The membranes were incubated with the secondary antibody solutions and incubated on a rotator for 30 min at room temperature. After incubation, the membranes were washed with 0.05% Tween-PBS three times, followed by 0.05 M Sodium bicarbonate ($NaHCO_3$) buffer [0.05 M $NaHCO_3$ pH 9.5] (Sigma Aldrich, St. Louis, MO). A BCIP/NBT™ substrate system (Sigma Aldrich, St. Louis, MO) was prepared by mixing 1 mL of BCIP/NBT™ substrate in 9 mL of $NaHCO_3$ buffer. The membranes were incubated in the liquid substrate system for 10 min and rinsed with water to stop the reaction. The membranes were air dried.

Dot Blot with Polyethylene Glycol (PEG) as Blocking Agent

The experiment for Dot Blot was repeated with Polyethylene glycol (Sigma Aldrich, St. Louis, MO) as blocking agent. PEG was used as an alternative blocking agent to eliminate any issue with the large molecular weight property of BSA which may interfere with peptide binding. PEG concentrations used were 1%, 3.5%, and 10%.

Data analysis

To determine the presence of anti-AT_1 or anti-AT_2 antibody, fluorescence intensity between the microsphere groups which were coupled with the peptide such as 033, 034, 035, 036, and 037 was compared with the microsphere group 038 which was not coupled with any peptide sequences and serve as a blank. For each run four data sets were analyzed four ways: median fluorescence intensity, trimmed median fluorescence intensity, mean fluorescence intensity, and trimmed mean fluorescence intensity. The

data analysis was calculated by the instrument based on 100 microspheres for each microsphere group. The median was calculated by arranging the values from 100 microspheres from lowest to highest value and looking at the 50^{th} value. The mean was calculated by adding all the values from 100 microspheres and divide that by 100. Trimmed median or trimmed mean were determined by removing the lower and upper five percent of the extreme values.

For each data set, the fluorescent intensity of microsphere group 038 with the analyte (038-positive) is compared with the fluorescent intensity of microsphere group 033, 034, 035, 036, or 037 with the analyte (positive control). The expected difference between 038-positive and the positive control was around 1000 fold.

RESULTS

Microbead immunoassay using vacuum manifold

The results from microbead assay using vacuum manifold are listed in tables 12-15. Table 12 contains the median fluorescence intensity. Table 13 contains the trimmed median fluorescence intensity. Table 14 contains the mean fluorescence intensity. Table 15 contains the trimmed mean fluorescence intensity. The amount of peptide solution used to conjugate with the microspheres was 125 µL (1000 µg/mL).

Table 12. Median fluorescent intensity of microbead immunoassay (vacuum manifold). Primary antibody used 50 µL (70 µg/mL and 294 µg/mL) (USBio). Secondary antibody used 50 µL (10 µg/mL and 5 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Median	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Median
033-AT_1 (AT_1)	**70**	**10**	7	**294**	**5**	11
033-AT_1 (BSA)	0		2	0		2
038 (AT_1)	**70**	**10**	4	**294**	**5**	5
038 (BSA)	0		2	0		2

Table 13. Trimmed median fluorescent intensity of microbead immunoassay (vacuum manifold).
Primary antibody used 50 µL (70 µg/mL and 294 µg/mL). Secondary antibody used 50 µL (10 µg/mL and 5 µg/mL).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Median	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Median
033-AT_1 (AT_1)	**70**	**10**	7	**294**	**5**	11
033-AT_1 (BSA)	0		2	0		2
038 (AT_1)	**70**	**10**	4	**294**	**5**	5
038 (BSA)	0		2	0		2

Table 14. Mean fluorescent intensity of microbead immunoassay (vacuum manifold). Primary antibody used 50 µL (70 µg/mL and 294 µg/mL) (USBio). Secondary antibody used 50 µL (10 µg/mL and 5 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Mean	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Mean
033-AT_1 (AT_1)	**70**	**10**	26.40	**294**	**5**	225.15
033-AT_1 (BSA)	0		3.00	0		4.30
038 (AT_1)	**70**	**10**	7.17	**294**	**5**	20.49
038 (BSA)	0		2.36	0		2.84

Table 15. Trimmed mean fluorescent intensity of microbead immunoassay (vacuum manifold).
Primary antibody used 50 µL (70 µg/mL and 294 µg/mL) (USBio). Secondary antibody used 50 µL (10 µg/mL and 5 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Mean	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Mean
033-AT_1 (AT_1)	**70**	**10**	8.56	**294**	**5**	15.10
033-AT_1 (BSA)	0		2.39	0		2.93
038 (AT_1)	**70**	**10**	4.65	294	**5**	5.90
038 (BSA)	0		2.13	0		2.59

Microbead immunoassay using centrifugation

The median, trimmed median, mean and trimmed mean from microbead assay using centrifugation for the washing steps are listed in Table 16. The amount of peptide solution used to conjugate with the microspheres was 125 µL (1000 µg/mL). The amount of primary antibody used was 50 µL (420 µg/mL) of anti-AT_1. The amount of secondary antibody used was 50 µL (4 µg/mL).

Table 16. Fluorescent intensity for microbead immunoassay (centrifugation). Primary antibody used was 50 μL (420 μg/mL) of anti-AT_1 (USBio). Secondary antibody used was 50 μL (4 μg/mL) (USBio).

Sample	Median	Trimmed Median	Mean	Trimmed Mean
033-AT1 (AT1)	11	11	252.31	31.09
033-AT_1 (BSA)	3	3	9.93	4.57
034-AT_1 (AT1)	11	11	159.75	30.25
034-AT_1 (BSA)	2	2	2.82	2.30
035-AT_1 (AT1)	8	8	64.47	20.92
035-AT_1 (BSA)	2	2	3.03	2.16
036-AT_1 (AT1)	9	9	140.59	22.74
036-AT_1 (BSA)	10	10	14.55	11.29
038 (AT1)	5	5	36.45	7.89
038 (BSA)	2	2	3.17	2.60

Microbead immunoassay by varying incubation conditions and secondary antibody concentrations

The results from microbead immunoassay experiment that vary the incubation conditions along with secondary antibody concentrations are listed in tables 17-20. contains the median fluorescence intensity. Table 18 contains the trimmed median fluorescence intensity. Table 19 contains the mean fluorescence intensity. Table 20 contains the trimmed mean fluorescence intensity. The amount of peptide solution used to conjugate with the microspheres was 125 μL (1000 μg/mL).

Table 17. Median fluorescent intensity for microbead immunoassay (modification: incubation, secondary antibody).
Secondary antibody used was 50 µL (4 µg/ml and 40 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Median	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Median
Room temperature incubation						
033-AT$_1$ (AT$_1$)	700	**4**	9	700	**40**	200
033-AT$_1$ (BSA)	0		3	0		17
038 (AT$_1$)	700		41.5	700		247
038 (BSA)	0		2	0		5
37 °C incubation						
033-AT$_1$ (AT$_1$)	700	**4**	24	700	**40**	287.5
033-AT$_1$ (BSA)	0		4	0		22
038 (AT$_1$)	700		12	700		94
038 (BSA)	0		1	0		3

Table 18. Trimmed median fluorescent intensity for microbead immunoassay (modification: incubation, secondary antibody).
Secondary antibody used was 50 µL (4 µg/ml and 40 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Median	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Median
Room temperature incubation						
033-AT$_1$ (AT$_1$)	700	**4**	9	700	**40**	200
033-AT$_1$ (BSA)	0		3	0		17
038 (AT$_1$)	700		41.5	700		247
038 (BSA)	0		2	0		5
37 °C incubation						
033-AT$_1$ (AT$_1$)	700	**4**	24	700	**40**	287.5
033-AT$_1$ (BSA)	0		4	0		22
038 (AT$_1$)	700		12	700		93
038 (BSA)	0		1	0		3

Table 19. Mean fluorescent intensity for microbead immunoassay (modification: incubation, secondary antibody).
Secondary antibody used was 50 µL (4 µg/ml and 40 µg/mL) (USBio).

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Mean	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Mean
Room temperature incubation						
033-AT_1 (AT_1)	700	**4**	341.36	700	**40**	1500.75
033-AT_1 (BSA)	0		6.05	0		110.61
038 (AT_1)	700		728.63	700		2954.76
038 (BSA)	0		7.29	0		29.77
37 °C incubation						
033-AT_1 (AT_1)	700	**4**	369.55	700	**40**	1601.52
033-AT_1 (BSA)	0		15.19	0		126.41
038 (AT_1)	700		275.40	700		380.06
038 (BSA)	0		2.67	0		92.87

Table 20. Trimmed mean fluorescent intensity microbead immunoassay (modification: incubation, secondary antibody).
Secondary antibody used was 50 µL (4 µg/ml and 40 µg/mL)(USBio)

Sample	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Mean	Primary Antibody (µg/mL)	Secondary Antibody (µg/mL)	Trimmed Mean
Room temperature incubation						
033-AT_1 (AT_1)	700	**4**	59.43	700	**40**	641.21
033-AT_1 (BSA)	0		3.48	0		26.83
038 (AT_1)	700		322.29	700		2290.64
038 (BSA)	0		2.66	0		7.66
37 °C incubation						
033-AT_1 (AT_1)	700	**4**	52.8	700	**40**	735.84
033-AT_1 (BSA)	0		6.43	0		57.71
038 (AT_1)	700		20.65	700		131.5
038 (BSA)	0		2.28	0		641.21

Microbead immunoassay by varying peptide concentrations #1

The median, trimmed median, mean and trimmed mean from microbead immunoassay by varying peptide concentrations #1 are listed in Table 21. The peptide solution concentrations used for coupling were 100, 50, 25 and 5 μL (1000 μg/mL). The amount of primary antibody used was 50 μL (70 μg/mL) anti-AT_1 (USBio). The amount of secondary antibody used was 50 μL (4 μg/mL) (USBio).

Table 21. Fluorescent intensity for microbead immunoassay (modification: peptide concentrations).
Peptide solution concentrations used for coupling were 100, 50, 25 and 5 μL (1000 μg/mL).

Sample	**Peptide (μg)**	Median	Trimmed Median	Mean	Trimmed Mean
033-AT_1 (AT_1)	**100**	4	4	10.12	5.10
033-AT_1 (BSA)		2	2	4.43	3.21
033-AT_1 (AT_1)	**50**	4	4	28.25	6.23
033-AT_1 (BSA)		3	3	4.75	3.47
033-AT_1 (AT_1)	**25**	4	4	68.42	6.68
033-AT_1 (BSA)		3	3	5.29	3.60
033-AT_1 (AT_1)	**5**	4	4	196.37	9.37
033-AT_1 (BSA)		2	2	3.93	3.27
038 (AT_1)	**0**	4	4	147.46	6.00
038 (BSA)		2	2	3.11	2.46

Microbead immunoassay by varying peptide concentrations #2

The median, trimmed median, mean and trimmed mean for microbead immunoassay by varying peptide concentrations #2 are listed in Table 22. The peptide solution concentrations used for coupling were 100, 50, 25 and 5 μg. The amount of primary antibody used was 50 μL (4 μg/mL) anti-AT_1. The amount of secondary antibody used was 50 μL (5 μg/mL).

Table 22. Fluorescent intensity for microbead immunoassay (modification: peptide concentrations, microsphere groups).
Peptide solution concentrations used for coupling were 100, 50, 25 and 5 μL (1000 μg/mL). Microsphere group 033, 034, 035, 036, 037 and 038 were used.

Sample	**Peptide (μg)**	Median	Trimmed Median	Mean	Trimmed Mean
033-AT_1 (AT_1)	**100**	7	7	19.21	9.78
033-AT_1 (BSA)		10	10	20.64	14.61
033-AT_1 (AT_1)	**50**	7	7	11.61	8.22
033-AT_1 (BSA)		10	10	23.16	14.36
033-AT_1 (AT_1)	**25**	10	10	83.81	12.77
033-AT_1 (BSA)		8	8	17.28	11.22
033-AT_1 (AT_1)	**5**	6	6	23.62	8.28
033-AT_1 (BSA)		8	8	16.75	10.39
034-AT_1 (AT_1)	**100**	13	13	186.42	18.93
034-AT_1 (BSA)		6	6	16.31	8.82
034-AT_1 (AT_1)	**50**	7	7	19.85	9.60
034-AT_1 (BSA)		5	5	13.82	8.24
034-AT_1 (AT_1)	**25**	7	7	60.96	8.20
034-AT_1 (BSA)		5	5	9.78	8.02
034-AT_1 (AT_1)	**5**	9	9	28.63	10.92
034-AT_1 (BSA)		6	6	10.61	8.62
035-AT_1 (AT_1)	**100**	11	11	39.98	13.17
035-AT_1 (BSA)		6	6	9.82	8.00
035-AT_1 (AT_1)	**50**	10	10	30.88	13.07
035-AT_1 (BSA)		6	6	10.05	8.17
035-AT_1 (AT_1)	**25**	12	12	66.68	17.16
035-AT_1 (BSA)		7	7	10.82	8.96
035-AT_1 (AT_1)	**5**	13	13	127.46	15.90
035-AT_1 (BSA)		6	6	9.88	8.46
036-AT_1 (AT_1)	**100**	9	9	52.74	11.30
036-AT_1 (BSA)		10	10	21.29	14.73
036-AT_1 (AT_1)	**50**	7	7	25.28	8.05
036-AT_1 (BSA)		5	5	8.10	7.02
036-AT_1 (AT_1)	**25**	9	9	19.25	10.64
036-AT_1 (BSA)		6	6	10.41	8.72
036-AT_1 (AT_1)	**5**	15	15	189.50	24.96
036-AT_1 (BSA)		6	6	12.60	8.76
038 (AT_1)	**0**	8	8	33.42	9.87
038 (BSA)		6	6	11.24	8.49

Microbead immunoassay by varying peptide concentrations and primary antibody concentrations

The median, trimmed median, mean and trimmed mean for microbead immunoassay by varying peptide concentrations and primary antibody concentrations are listed in Table 23. The peptide solution concentration used for coupling was 100, 50, 25 and 5 μL (1000 μg/mL). The amount of primary antibody used was 50 μL (70 μg/mL and 140 μg/mL) of anti- AT_1 (USBio). The amount of secondary antibody used was 50 μL (4 μg/mL) (USBio).

Table 23. Fluorescent intensity for microbead immunoassay (modification: peptide concentrations, primary antibody).
Primary antibody used was 50 μL (70 μg/mL and 140 μg/mL). The peptide amount for coupling were 100, 50, 25, and 5 μL (1000 μg/mL).

Sample	**Peptide (μg)**	**Primary Antibody (μg/mL)**	Median	Trimmed Median	Mean	Trimmed Mean
033-AT_1 (AT_1)	**100**	**70**	4	4	31.81	4.93
033-AT_1 (AT_1)		**140**	5	5	19.64	8.02
033-AT_1 (BSA)		**0**	3	3	5.64	4.46
033-AT_1 (AT_1)	**50**	**70**	4	4	38.99	6.16
033-AT_1 (AT_1)		**140**	4	4	60.56	6.31
033-AT_1 (BSA)		**0**	3	3	8.08	4.36
033-AT_1 (AT_1)	**125**	**70**	4	4	28.37	5.79
033-AT_1 (AT_1)		**140**	5	5	150.93	7.58
033-AT_1 (BSA)		**0**	3	3	6.04	4.91
033-AT_1 (AT_1)	**5**	**70**	4	4	74.92	5.97
033-AT_1 (AT_1)		**140**	4	4	81.46	5.77
033-AT_1 (BSA)		**0**	3	3	4.90	3.94
038 (AT_1)	**0**	**70**	7	7	187.09	22.48
038 (AT_1)		**140**	4	4	178.75	6.63
038 (BSA)		**0**	2	2	3.10	2.51

Microbead immunoassay by varying primary antibody source for anti-AT_2

The median, trimmed median, mean and trimmed mean from microbead immunoassay by varying secondary antibody concentrations are listed in Table 24. The

peptide solution concentration used for coupling was 125 µL (1000 µg/mL). For anti-AT_1, primary antibody used was 50 µL (2 µg/mL) of anti-AT_1 (USBio). For anti-AT_2 primary antibody used was 50 µL (0.1 µg/mL) of anti-AT_2 by two different manufacturers: USBio and Abcam. Secondary antibody used was 50 µL (4 µg/mL).

Table 24. Fluorescent intensity for microbead immunoassay (modification: primary and secondary antibody).
Primary antibody used was 50 µL (2 µg/mL) for anti-AT_1 (USBio); 50 µL (0.1 µg/mL) of anti-AT_2 from USBio and Abcam. Secondary antibody for anti-AT_1 used was 50 µL (5 µg/mL); Secondary antibody for anti-AT_2 antibody used was 50 µL (2.5 µg/mL).

Sample	Primary Antibody (µg/mL)	Median	Trimmed Median	Mean	Trimmed Mean
033-AT_1 (AT_1)	2	4	4	14.59	4.92
033-AT_1 (BSA)	0	5	5	10.58	6.58
034-AT_1 (AT_1)	2	4	4	6.08	4.91
034-AT_1 (BSA)	0	3	3	3.89	3.46
035-AT_1 (AT_1)	2	4	4	6.08	4.56
035-AT_1 (BSA)	0	3	3	5.04	4.37
036-AT_1 (AT_1)	2	4	4	16.25	4.71
036-AT_1 (BSA)	0	3	3	4.84	3.89
037-AT_2 (AT_2) **(USBio)**	0.1	4	4	5.67	4.83
037-AT_2 (AT_2) **(Abcam)**	0.1	4	4	5.84	5.12
037-AT_2 (BSA) **(USbio)**	0	4	4	5.75	4.93
037-AT_2 (BSA) **(Abcam)**	0	4	4	5.40	4.52
038 (AT_1)	2	4	4	7.07	4.63
038 (AT_2) **(USBio)**	0.1	5	5	6.05	5.35
038 (AT_2) **(Abcam)**	0.1	3	3	4.41	3.90
038 (BSA)	0	2	2	2.94	2.67
038 (BSA) **(USBio)**	0	3	3	6.96	3.65
038 (BSA) **(Abcam)**	0	2.5	2.5	3.19	2.92

Rat sera screening using microbead immunoassay by centrifugation method

The results from rat sera screening using microbead immunoassay by centrifugation method are listed in tables 25-28. Table 25 contains the median fluorescence intensity. Table 26 contains the trimmed median fluorescence intensity. Table 27 contains the mean fluorescence intensity. Table 28 contains the trimmed mean fluorescence intensity. In this experiment, different microsphere groups were combined prior the addition of primary antibody. The peptide solution concentrations used to couple with the microspheres was 125 µL (1000 µg/mL). The amount of rat sera used was 50 µL of undiluted rat sera. The amount of secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Table 25. Median fluorescent intensity of rat sera screening.
The rat sera screening was performed using the microbead immunoassay by centrifugation method. Rat sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	12.5	8	8	11	20	5
1	206.5	11	8	9	21.5	9
2	21	10	9	9	20	8
3	52.5	12	12	13	17	13
4	24	10	10	7	4	9
5	11	6	8	8	11.5	8
6	35	11	10	8	22	13
7	21	8	9	9	27	9.5
8	41	12	10	9	24	9
9	224	9	7	6	9	6
10	376.5	18	18	16	54	20
11	36	9	9.5	8	8.5	7
12	25	8	8	8	19	7
13	32	10	10	8	14.5	7
14	35	21	20	20	42	18
15	43	7	9	10	25	9
16	126	25	25	21	22	27
17	21	6	9	7	1	8

Table 26. Trimmed median fluorescent intensity of rat sera screening.
The rat sera screening was performed using the microbead immunoassay by centrifugation method. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	12.5	8	8	11	20	5
1	206.5	11	8	9	21.5	9
2	21	10	9	9	20	8
3	52.5	12	12	13	17	13
4	24	10	10	7	4	9
5	11	6	8	8	11.5	8
6	35	11	10	8	22	13
7	21	8	9	9	27	9.5
8	41	12	10	9	24	9
9	224	9	7	6	9	6
10	376.5	18	18	16	54	20
11	36	9	9.5	8	8.5	7
12	25	8	8	8	19	7
13	32	10	10	8	14.5	7
14	35	21	20	20	42	18
15	43	7	9	10	25	9
16	126	25	25	21	22	27
17	21	6	9	7	1	8

Table 27. Mean fluorescent intensity of rat sera screening.
The rat sera screening was performed using the microbead immunoassay by centrifugation method. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	40.62	18.22	18.38	18.39	45.78	16.15
1	701.02	46.14	51.16	20.90	145.66	27.07
2	117.05	22.17	18.80	27.14	88.10	36.91
3	142.20	23.26	26.52	20.97	46.72	24.10
4	60.35	17.29	18.01	20.24	14.03	20.78
5	36.07	13.75	22.47	14.75	17.29	24.25
6	129.47	22.96	21.29	20.18	99.03	26.32
7	120.96	20.19	24.97	22.90	96.44	24.29
8	170.15	24.18	28.68	27.87	88.50	19.40
9	599.77	12.97	12.90	13.74	235.97	41.01
10	1326.80	68.96	83.19	31.07	874.78	63.59
11	132.61	16.33	22.01	37.41	39.67	17.11
12	62.83	17.14	33.32	21.89	54.59	19.41
13	96.62	19.78	25.50	25.00	35.46	33.27
14	95.23	28.45	31.19	29.10	88.07	31.00
15	137.27	23.80	21.51	19.97	33.84	25.17
16	341.45	30.96	34.23	36.00	170.34	45.36
17	59.08	16.55	19.00	18.39	57.70	19.64

Table 28. Trimmed mean fluorescent intensity of rat sera screening.
The rat sera screening was performed using the microbead immunoassay by centrifugation method. Rat sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	23.89	13.59	15.38	15.16	31.17	12.16
1	478.28	16.22	15.01	15.17	66.76	15.36
2	59.56	15.40	14.09	14.94	51.81	13.37
3	93.11	16.94	18.54	17.48	30.45	18.03
4	42.14	14.17	14.27	13.35	12.70	14.01
5	28.60	11.47	12.61	12.27	17.29	12.55
6	72.44	16.72	16.16	15.55	62.90	17.65
7	40.49	15.36	15.54	15.09	71.89	15.41
8	95.61	18.37	16.86	16.61	43.24	16.77
9	416.66	10.88	10.49	10.31	155.18	9.47
10	843.83	23.73	25.09	22.85	580.76	29.55
11	62.97	13.94	14.13	14.37	25.32	13.02
12	43.07	14.37	14.01	14.46	39.77	13.87
13	57.85	15.68	15.82	15.67	22.64	15.19
14	66.75	23.40	24.09	24.06	66.46	24.13
15	99.03	14.29	14.93	15.90	26.87	15.55
16	235.00	26.81	27.94	24.57	101.88	29.70
17	42.89	13.12	14.29	14.31	35.78	14.86

The results from rat sera screening using microbead immunoassay by centrifugation method and ADH modified microspheres are listed in tables 29-32. Table 29 contains the median fluorescence intensity. Table 30 contains the trimmed median fluorescence intensity. Table 31 contains the mean fluorescence intensity. Table 32 contains the trimmed mean fluorescence intensity. In this experiment, different microsphere groups were combined prior the addition of primary antibody. The peptide solution concentrations used to couple with the ADH modified microspheres was 125 µL (1000 µg/mL). The amount of rat sera used was 50 µL of undiluted rat sera. The amount of secondary antibody used was 50 µL (4 µg/mL) (Abcam). Due to limited amount of sample, sample #9 and #11 were not used for this experiment.

Table 29. Median fluorescent intensity of rat sera screening (ADH microspheres). The rat sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	14	7.5	8	6	12	12
1	8.5	11	5.5	8	10	8
2	6	4.5	5.5	7	7	6.5
3	9	5.5	11	7	10	10
4	10	9	10	8.5	6	10
5	8.5	9	7	4.5	11	9
6	7.5	10	8	16	8	13.5
7	7	5	8	11.5	8	10
8	14	11	6	8	10	7.5
10	13.5	10	4	12	15	10
12	10	8	9	8	9.5	8.5
13	8	8	9	9	4	9
14	22	27.5	28	29	30	27
15	10	8	8	14.5	10	12
16	26	29	25	22.5	26.5	31
17	12.5	9	9	7.5	8	8

Table 30. Trimmed median fluorescent intensity of rat sera screening (ADH microspheres).
The rat sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	14	7.5	8	6	12	12
1	8.5	11	5.5	8	10	8
2	6	4.5	5.5	7	7	6.5
3	9	5.5	11	7	10	10
4	10	9	10	8.5	6	10
5	8.5	9	7	4.5	11	9
6	7.5	10	8	16	8	13.5
7	7	5	8	11.5	8	10
8	14	11	6	8	10	7.5
10	13.5	10	4	12	15	10
12	10	8	9	8	9.5	8.5
13	8	8	9	9	4	9
14	22	27.5	28	29	30	27
15	10	8	8	14.5	10	12
16	26	29	25	22.5	26.5	31
17	12.5	9	9	7.5	8	8

Table 31. Mean fluorescent intensity of rat sera screening (ADH microspheres). The rat sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	19.13	10.22	12.01	11.79	15.29	17.76
1	13.21	14.23	12.22	13.06	13.91	12.22
2	20.33	11.14	12.04	12.60	12.67	15.19
3	15.16	12.72	14.60	13.78	15.64	15.13
4	21.69	16.82	15.74	14.09	14.39	15.28
5	15.72	12.42	11.97	11.39	16.11	17.22
6	13.28	14.58	23.89	21.31	17.89	24.91
7	13.08	11.78	13.33	14.38	13.58	14.29
8	16.90	13.78	13.43	15.53	19.47	12.40
10	19.99	16.39	12.82	12.90	16.48	13.57
12	14.87	21.96	12.73	21.66	14.70	13.77
13	16.67	15.94	14.17	14.08	12.04	14.39
14	26.71	29.71	31.30	31.41	33.63	29.46
15	14.04	14.29	13.20	19.80	19.72	16.25
16	27.57	29.22	29.34	24.73	31.79	33.23
17	26.63	12.56	13.40	14.02	11.36	14.29

Table 32. Trimmed mean fluorescent intensity of rat sera screening (ADH microspheres). The rat sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Rat sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	16.37	9.19	10.39	10.05	14.32	13.16
1	11.03	12.83	10.21	11.44	11.82	10.10
2	11.98	9.44	10.63	11.36	10.92	11.14
3	13.16	11.01	13.36	11.76	14.14	13.97
4	14.41	13.91	14.46	12.43	11.23	13.67
5	12.92	10.97	10.61	9.45	14.50	12.43
6	11.48	12.69	11.92	18.92	13.97	16.25
7	10.76	10.06	11.91	13.35	10.21	12.78
8	14.76	12.22	11.89	13.82	15.26	11.21
10	15.76	15.03	11.09	11.95	15.22	12.50
12	12.19	12.73	11.29	10.97	13.05	11.78
13	12.23	10.90	10.96	12.80	8.88	12.55
14	24.49	28.30	29.50	30.54	31.91	28.27
15	12.60	12.73	11.86	15.62	13.59	14.87
16	25.82	28.47	28.12	23.57	28.04	32.15
17	16.47	11.55	11.47	12.23	10.00	12.36

Human sera using microbead immunoassay by centrifugation method

The results from human sera screening are listed in tables 33-36. There were 158 samples of human sera used. The results listed here are representative of 158 of the human samples. The microbead immunoassay by centrifugation method was used. Table 33 contains the median fluorescence intensity. Table 34 contains the trimmed median fluorescence intensity. Table 35 contains the mean fluorescence intensity. Table 36 contains the trimmed mean fluorescence intensity. In this experiment, different microsphere groups were combined prior the addition of primary antibody. The peptide solution concentrations used to couple with the microspheres was 125 μL (1000 μg/mL). The amount of human sera used was 50 μL. The amount of secondary antibody used was 50 μL (4 μg/mL) (Abcam).

Table 33. Median fluorescent intensity of human sera screening.
The human sera screening was performed using the microbead immunoassay by centrifugation method. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	5	3	2	3	2	4
1	5	4	3	3	2	3
2	6	4	4	2	4	3
3	3	3	3	4	6	4
4	4	3	2	4	6	5
5	5	3	4	4	5.5	3
6	5	3	2	3	4	4
7	5	3	3	4	5.5	3
8	5	3	3	4	3	4
9	6	3	3	4	4	4
10	5	5	2	5	5	3

Table 34. Trimmed median fluorescence intensity of human sera screening.
The human sera screening was performed using the microbead immunoassay by centrifugation method. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	5	3	2	3	2	4
1	5	4	3	3	2	3
2	6	4	4	2	4	3
3	3	3	3	4	6	4
4	4	3	2	4	6	5
5	5	3	4	4	5.5	3
6	5	3	2	3	4	4
7	5	3	3	4	5.5	3
8	5	3	3	4	3	4
9	6	3	3	4	4	4
10	5	5	2	5	5	3

Table 35. Mean fluorescence intensity of human sera screening.
The human sera screening was performed using the microbead immunoassay by centrifugation method. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	10.33	5.74	4.36	4.84	11.00	5.48
1	10.68	5.34	4.61	4.99	5.88	4.99
2	8.60	5.73	4.86	4.39	6.24	4.57
3	9.34	5.80	5.22	5.69	7.95	6.48
4	8.06	5.62	4.92	6.56	8.31	7.30
5	9.52	5.85	6.80	6.61	8.53	11.12
6	13.42	6.16	6.01	5.76	9.82	6.64
7	8.18	5.94	6.94	7.27	9.08	6.31
8	8.76	5.02	5.31	5.58	9.61	7.02
9	10.66	6.43	6.41	5.90	6.21	7.40
10	8.21	5.78	5.23	12.48	7.42	5.94

Table 36. Trimmed mean fluorescence intensity of human sera screening.
The human sera screening was performed using the microbead immunoassay by centrifugation method. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	5.54	4.40	3.68	4.37	4.13	4.87
1	6.21	4.86	4.01	4.44	3.78	4.42
2	6.82	5.07	4.34	3.81	5.40	3.88
3	5.80	4.52	4.62	4.87	6.47	5.32
4	6.56	5.01	4.29	5.58	6.78	6.45
5	7.27	5.18	6.07	5.62	7.16	4.95
6	6.64	5.50	5.25	5.01	7.02	5.94
7	6.49	5.33	6.09	5.36	6.60	5.67
8	5.68	4.60	4.75	5.08	5.24	4.94
9	7.02	5.63	4.92	5.19	5.29	5.46
10	6.50	5.15	4.65	5.95	6.70	5.21

The results from human sera screening using the ADH modified microspheres are listed in tables 37-40. The microbead immunoassay by centrifugation method was used. Table 37 contains the median fluorescence intensity. Table 38 contains the trimmed median fluorescence intensity. Table 39 contains the mean fluorescence intensity. Table 40 contains the trimmed mean fluorescence intensity. In this experiment, different microsphere groups were combined prior the addition of primary antibody. The peptide solution concentrations used to couple with the ADH modified microspheres was 125 µL (1000 µg/mL). The amount of human sera used was 50 µL. The amount of secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Table 37. Median fluorescent intensity of human sera screening (ADH microspheres). The human sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	16	25	5	13	45	18
1	4	4	4	4	5	3
2	4	5	3	4	5	5
3	5	4	4	4	4	4
4	3	4	3	4	1	4
5	4	3	4	4	2	4
6	3	3	3	2	3.5	3
7	4	4	2.5	4	5	5
8	4	3	2	4	3	3
9	6	4	4	4	4	6
10	4	4	3	4	4	4

Table 38. Trimmed median fluorescence intensity of human sera screening (ADH microspheres).
The human sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	16	25	5	13	45	18
1	4	4	4	4	5	3
2	4	5	3	4	5	5
3	5	4	4	4	4	4
4	3	4	3	4	1	4
5	4	3	4	4	2	4
6	3	3	3	2	3.5	3
7	4	4	2.5	4	5	5
8	4	3	2	4	3	3
9	6	4	4	4	4	6
10	4	4	3	4	4	4

Table 39. Mean fluorescence intensity of human sera screening (ADH microspheres).
The human sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Human sera used was 50 µL, and secondary antibody used was 50 µL (4 µg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	25.22	30.17	5.36	13.61	56.44	32.48
1	4.98	5.09	4.54	4.88	10.23	4.63
2	6.41	5.81	4.66	5.70	6.84	5.85
3	9.92	4.45	4.79	7.26	5.54	4.83
4	4.43	6.12	4.21	4.42	4.33	5.14
5	5.82	5.21	5.55	5.38	6.81	5.54
6	4.81	4.68	4.50	4.30	5.15	6.57
7	5.03	4.94	4.91	4.36	5.68	5.38
8	4.48	4.01	3.79	4.89	4.82	4.53
9	12.07	6.53	6.14	6.44	7.45	8.14
10	5.13	6.54	5.51	4.61	4.79	4.74

Table 40. Trimmed mean fluorescence intensity of human sera screening (ADH microspheres).
The human sera screening was performed using the microbead immunoassay by centrifugation method and ADH modified microspheres. Human sera used was 50 μL, and secondary antibody used was 50 μL (4 μg/mL) (Abcam).

Sample	Microsphere group					
	AT_1-033	AT_1-034	AT_1-035	AT_1-036	AT_2-037	038
Negative	17.77	27.27	5.13	13.20	51.73	18.76
1	4.26	4.54	4.21	4.39	5.03	4.19
2	5.68	5.13	4.09	5.16	5.91	5.38
3	5.13	4.08	4.31	4.72	4.92	4.50
4	3.88	5.03	3.86	4.03	3.77	4.56
5	5.31	4.82	4.96	5.05	3.96	5.02
6	4.39	4.03	4.10	3.88	4.37	4.01
7	4.70	4.47	3.85	4.15	5.32	5.13
8	3.99	3.69	3.44	4.60	4.40	3.83
9	8.30	5.63	5.56	5.37	5.69	6.36
10	4.57	4.62	3.66	4.20	4.22	4.39

In addition to microbead immunoassay experiments, other experiments such as determination of peptide coupling efficiency, the ELISA and the dot blot were perfomed. These experiments were performed to aid in the development of the microbead immunoassay.

Determination of peptide coupling efficiency

The results from the determination of peptide coupling efficiency by measuring the absorbance at 230 nm over time are listed in Table 41. Initial peptide solution concentration used for coupling was 125 µL (1000µg/mL).

Table 41. Peptide absorbance at 230 nm.
Initial peptide solution concentration was 125 µL (1000µg/mL).

Peptide ID	Absorbance at 230 nm				
	0 hours	2 hours	4 hours	20 hours	24 hours
AT_1- 033	1.756	1.698	1.683	1.633	1.613
AT_1- 034	0.959	0.910	0.892	0.882	0.880
AT_1- 035	0.183	0.167	0.154	0.139	0.129
AT_1- 036	1.403	1.274	1.261	1.255	1.239
AT_2- 037	0.909	0.772	0.768	0.763	0.758
Blank (MES buffer)	0.066	0.093	0.067	0.059	0.057

The results from the determination of peptide coupling efficiency by measuring the absorbance at 260 nm over time are listed in Table 42.

Table 42. Peptide absorbance at 260 nm.
Initial peptide solution concentration was 125 µL (1000µg/mL).

Peptide ID	Absorbance at 260 nm				
	0 hours	2 hours	4 hours	20 hours	24 hours
AT_1- 033	0.049	0.059	0.053	0.051	0.018
AT_1- 034	0.095	0.096	0.084	0.082	0.079
AT_1- 035	0.011	0.024	0.011	0.000	0.002
AT_1- 036	0.208	0.195	0.183	0.177	0.163
AT_2- 037	0.077	0.045	0.042	0.047	0.042
Blank (MES buffer)	0.030	0.061	0.041	0.034	0.031

The results from the determination of peptide coupling efficiency by measuring the absorbance at 280 nm over time are listed in Table 43.

Table 43. Peptide absorbance at 280 nm.
Initial peptide amount was 125 µL (1000µg/mL).

Peptide ID	Absorbance at 280 nm				
	0 hours	2 hours	4 hours	20 hours	24 hours
AT_1- 033	0.036	0.046	0.041	0.038	0.005
AT_1- 034	0.173	0.165	0.155	0.151	0.151
AT_1- 035	0.007	0.019	0.007	-0.004	0.001
AT_1- 036	0.255	0.237	0.225	0.221	0.202
AT_2- 037	0.074	0.039	0.036	0.040	0.040
Blank (MES buffer)	0.021	0.050	0.030	0.023	0.018

The results from the determination of peptide coupling efficiency by measuring the absorbance at 320 nm over time are listed in Table 44.

Table 44. Peptide absorbance at 320 nm.
Initial peptide amount was 125 µL (1000µg/mL).

Peptide ID	Absorbance at 320 nm				
	0 hours	2 hours	4 hours	20 hours	24 hours
AT_1- 033	0.009	0.019	0.013	0.020	-0.007
AT_1- 034	-0.009	0.000	-0.011	-0.009	-0.006
AT_1- 035	0.005	0.004	-0.008	-0.014	-0.014
AT_1- 036	0.010	0.019	0.006	0.005	-0.011
AT_2- 037	0.020	0.001	-0.001	0.006	0.002
Blank (MES buffer)	-0.009	0.10	-0.006	-0.11	-0.016

ELISA

The absorbance reading at 405 nm from the ELISA is in Table 45.

Table 45. ELISA result based on absorbance at 405 nm. Row A-B was performed with pH 5.5, row C-D was performed with pH 7.0, and row E-F was performed with pH 9.6. Primary Antibody 100 µL (2 µg/mL)of anti-AT_1 (Abcam, Cambridge, MA), 100 µL (2 µg/mL) of anti-AT_1 (Abcam, Cambridge, MA). Secondary Antibody 100 µL (2 µg/mL) of anti-IgG-AP µg/mL (Abcam, Cambridge, MA). * is the negative control column.

	1	**2**	**3**	**4**	**5**	**6**	**7***	**8**	**9**	**10**	**11**	**12**
Coat →	Peptide AT_1-033	Peptide AT_1-034	Peptide AT_1-035	Peptide AT_1-035	Peptide AT_1-036	Peptide AT_1-037	BSA 5 µg/mL	Anti-AT_1 20 µg/mL	Anti-AT_1 10 µg/mL	Anti-AT_2 20 µg/mL	Anti-AT_1-IgG-AP 20µg/mL	Anti-AT_2-IgG-AP 20µg/mL
A	1.892 20 µg/mL	2.218 20 µg/mL	2.094 20 µg/mL	2.386 20 µg/mL	1.851 20 µg/mL	1.639 20 µg/mL	3.614	3.905	3.372	1.654	3.639	3.660
B	2.768 10 µg/mL	2.697 10 µg/mL	2.854 10 µg/mL	2.705 10 µg/mL	1.687 10 µg/mL	2.150 10 µg/mL						
C	2.210 20 µg/mL	2.239 20 µg/mL	3.054 20 µg/mL	2.084 20 µg/mL	2.109 20 µg/mL	2.152 20 µg/mL	3.783	3.663	3.783	3.728	3.630	3.561
D	2.673 10 µg/mL	2.730 10 µg/mL	2.598 10 µg/mL	2.959 10 µg/mL	2.898 10 µg/mL	2.850 10 µg/mL						
E	3.055 20 µg/mL	3.153 20 µg/mL	2.945 20 µg/mL	3.318 20 µg/mL	2.944 20 µg/mL	2.407 20 µg/mL	3.686	3.693	3.712	3.443	3.734	3.807
F	3.014 10 µg/mL	2.969 10 µg/mL	2.979 10 µg/mL	3.339 10 µg/mL	2.826 10 µg/mL	2.610 10 µg/mL						

Dot Blot with non-fat dry milk as blocking agent

This method used non- fat dry milk as blocking agent. A general map for this dot blot is in Figure 6. The result for dot blot without vacuum method is in Figure 7 and the dot blot with the vacuum method is in Figure 8. The peptide concentration solution used was 10 μL (1000 μg/mL). The amount of BSA solution used was 20 μL (20 μg/mL). The amount of anti-AT_1 and anti-AT_2 amount used were 5 μL (μg/mL). The amount of primary antibody used for AT_1 was 10 mL (2 μg/mL) and for AT_2 was 10 mL (0.1 μg/mL). The amount of secondary antibody used for anti-IgG-AT_1-AP was 10 mL (1 μg/ml), and for anti-IgG-AT_2-AP was 10 mL (1 μg/mL).

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8
Peptide AT_1-033	Peptide AT_1-034	Peptide AT_1-035	Peptide AT_1-036	Peptide AT_2-037	BSA	Anti-AT_1	Anti-AT_2

Figure 6. Dot blot map with 3% non-fat dry milk.

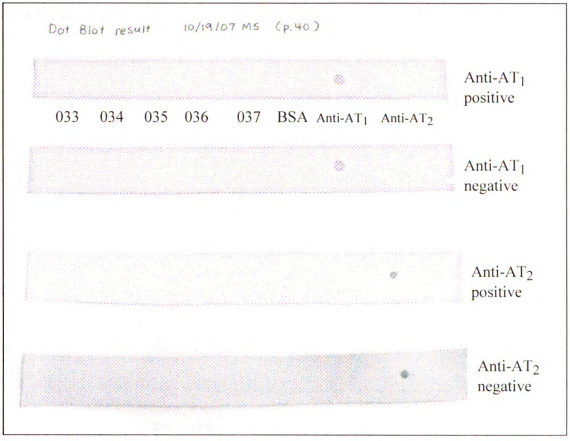

Figure 7. Dot Blot with 3% non-fat dry milk (without vacuum method). Primary antibody used for AT_1 was 10 mL (2 µg/mL) and for AT_2 was 10 mL (0.1 µg/mL). Secondary antibody for anti-IgG-AT_1-AP was 10 mL (1 µg/ml), and for anti-IgG-AT_2-AP was 10 mL (1 µg/mL).

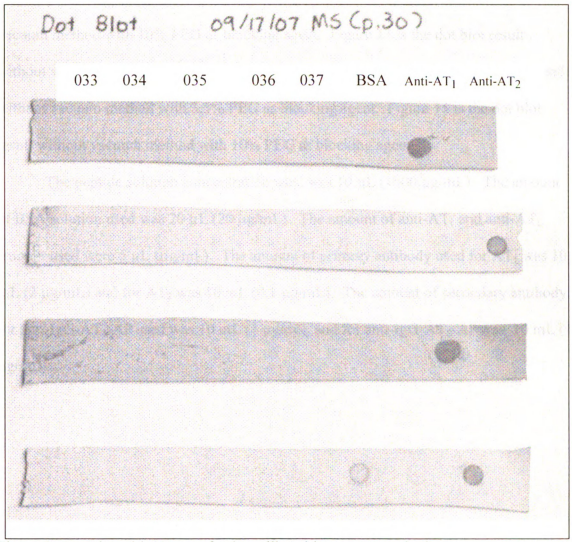

Figure 8. Dot Blot with 3% non-fat dry milk (with vacuum method).
Primary antibody used for AT_1 was 10 mL (2 µg/mL) and for AT_2 was 10 mL (0.1 µg/mL). Secondary antibody for anti-IgG-AT_1-AP was 10 mL (1 µg/ml), and for anti-IgG-AT_2-AP was 10 mL (1 µg/mL) (handwritten).

Dot Blot with PEG milk as blocking agent

This method used PEG as blocking agent. PEG concentrations used were 1%, 3.5%, and 10%. Figure 9 is the map for the dot blot with PEG as blocking agent. The dot blot without vacuum method result is in figures 10-12 and the dot blot with the vacuum method is in figures 13-15. Figure 10 is the dot blot result without vacuum method with 1% PEG as blocking agent. Figure 11 is the dot blot result without vacuum

method with 3.5% PEG as blocking agent. Figure 12 is the dot blot result without vacuum method with 10% PEG as blocking agent. Figure 13 is the dot blot result without vacuum method with 1% PEG as blocking agent. Figure 14 is the dot blot result without vacuum method with 3.5% PEG as blocking agent. Figure 15 is the dot blot result without vacuum method with 10% PEG as blocking agent.

The peptide solution concentration used was 10 μL (1000 μg/mL). The amount of BSA solution used was 20 μL (20 μg/mL). The amount of anti-AT_1 and anti-AT_2 amount used were 5 μL (μg/mL). The amount of primary antibody used for AT_1 was 10 mL (2 μg/mL) and for AT_2 was 10 mL (0.1 μg/mL). The amount of secondary antibody for anti-IgG-AT_1-AP used was 10 mL (1 μg/ml), and for anti-IgG-AT_2-AP was 10 mL (1 μg/mL).

Lane								
1	2	3	4	5	6	7	8	9
blank	Peptide AT_1-033	Peptide AT_1-034	Peptide AT_1-035	Peptide AT_1-036	blank	BSA	blank	Anti-AT_1

Figure 9. Dot blot map with PEG.

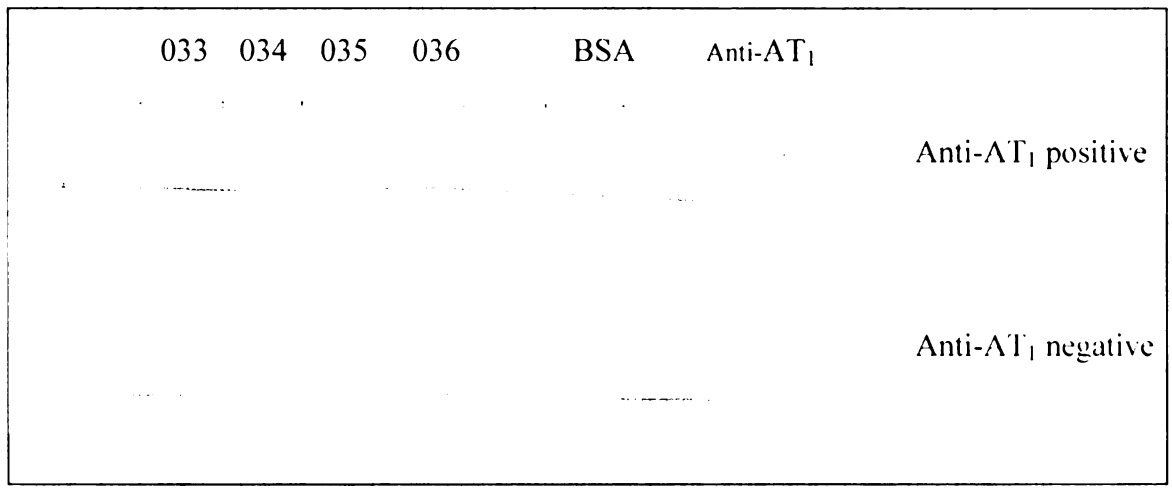

Figure 10. Dot Blot with 1% PEG as a blocking solution (without vacuum method).

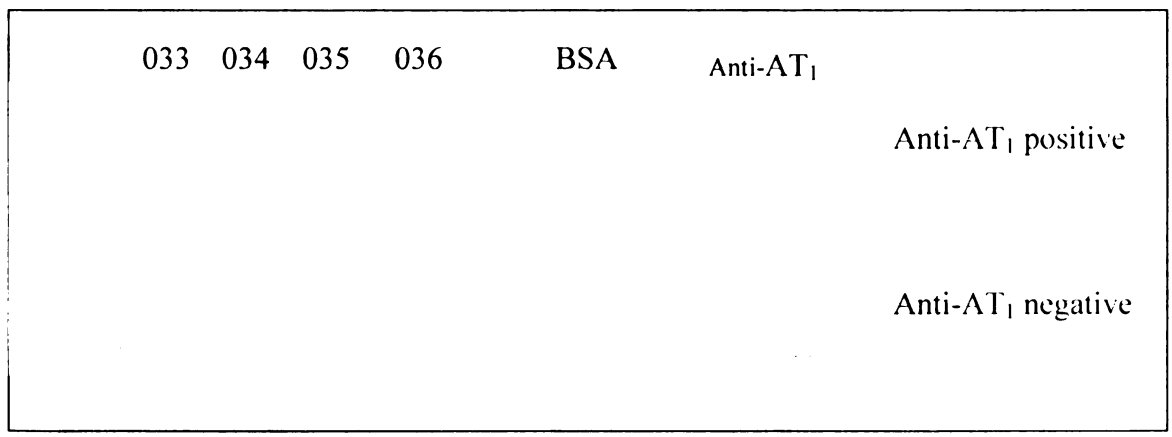

Figure 11. Dot Blot with 3.5% PEG as a blocking solution (without vacuum method).

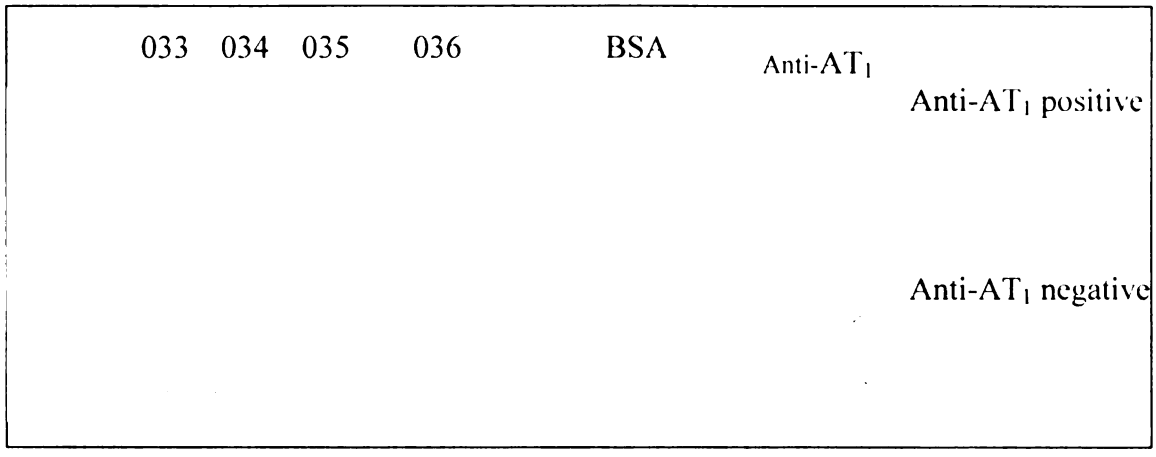

Figure 12. Dot Blot with 10% PEG as a blocking solution (without vacuum method).

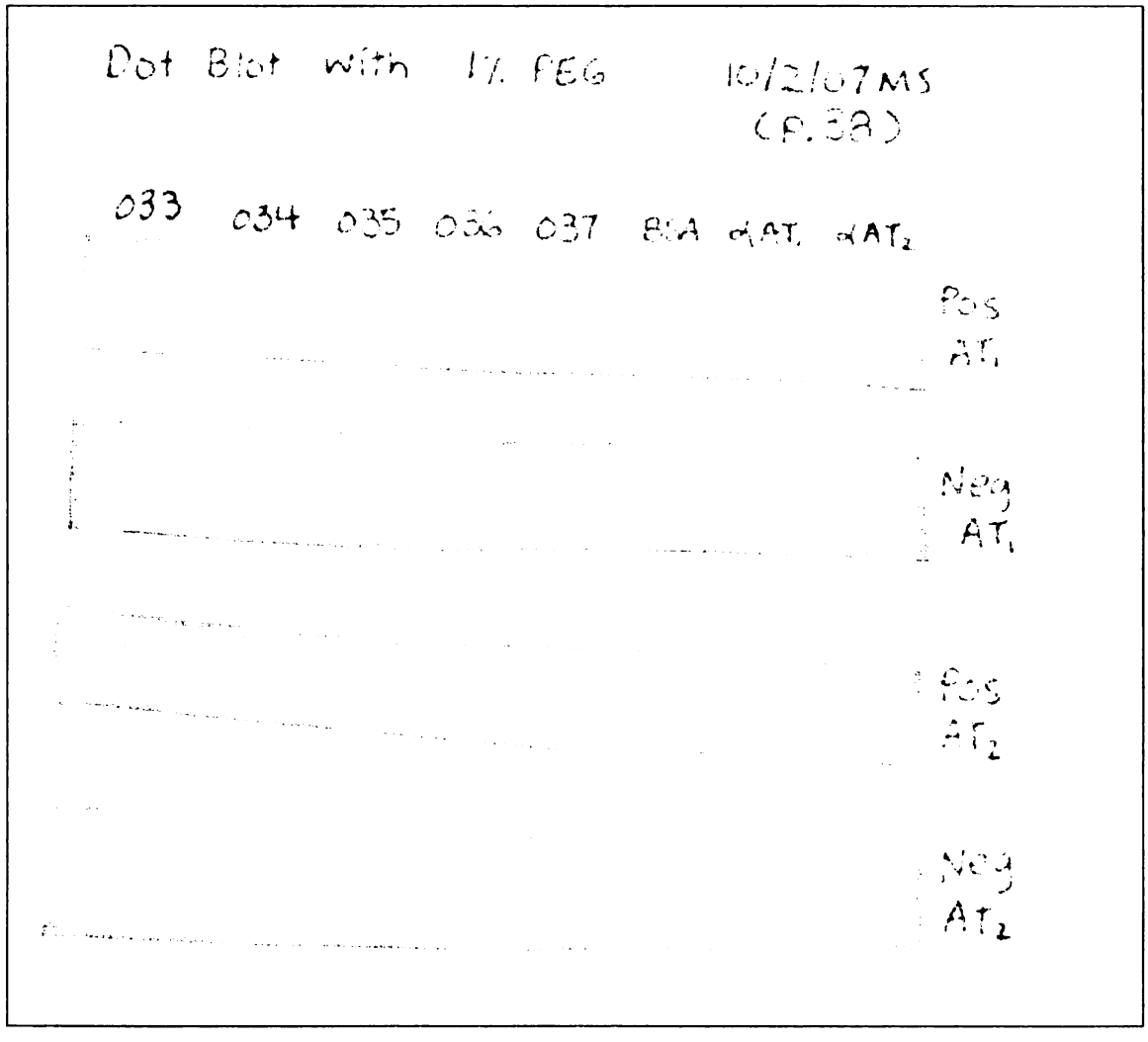

Figure 13. Dot Blot with 1% PEG as a blocking solution (with vacuum method) (handwritten)

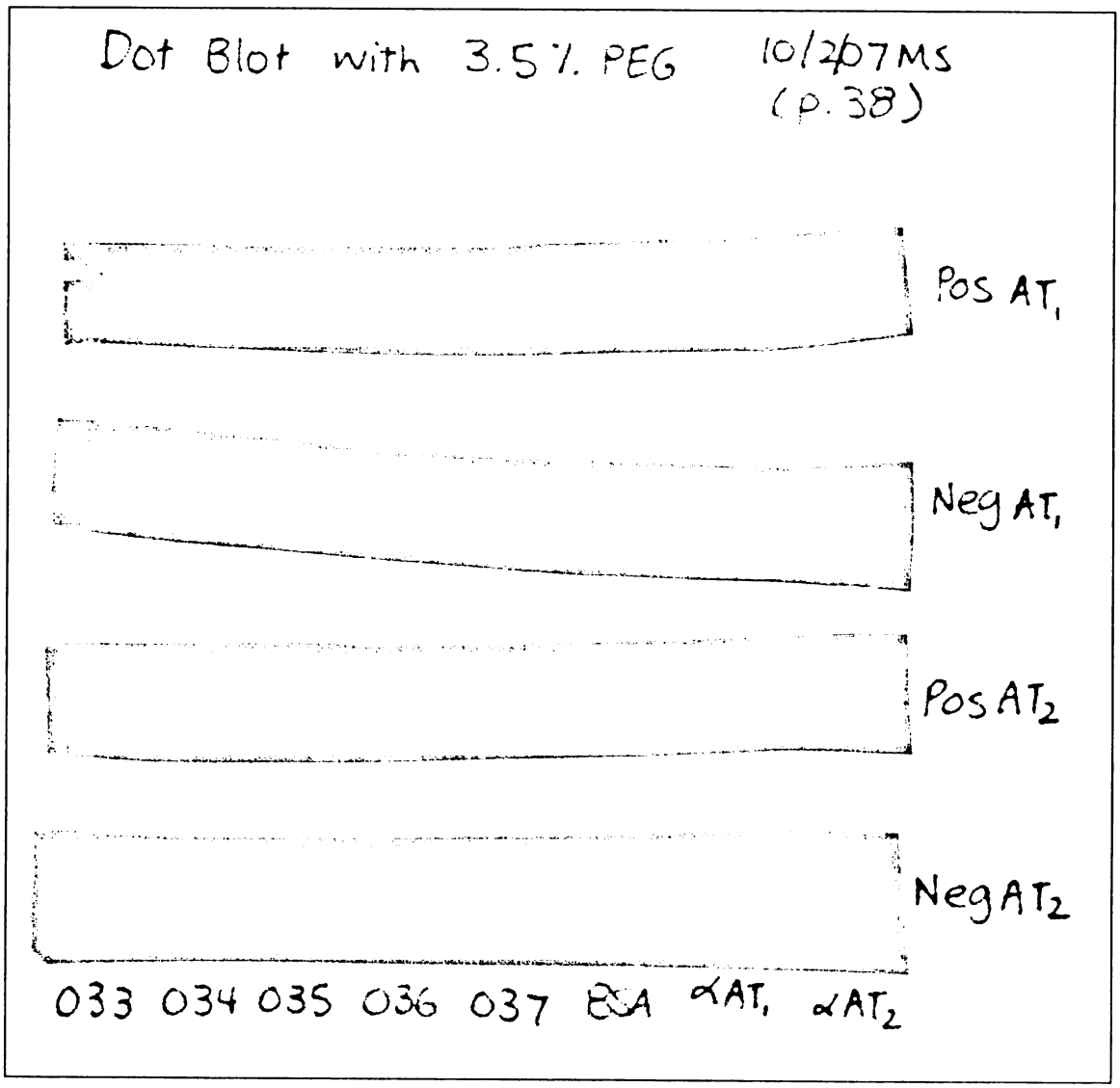

Figure 14. Dot Blot with 3.5% PEG as a blocking solution (with vacuum method) (handwritten).

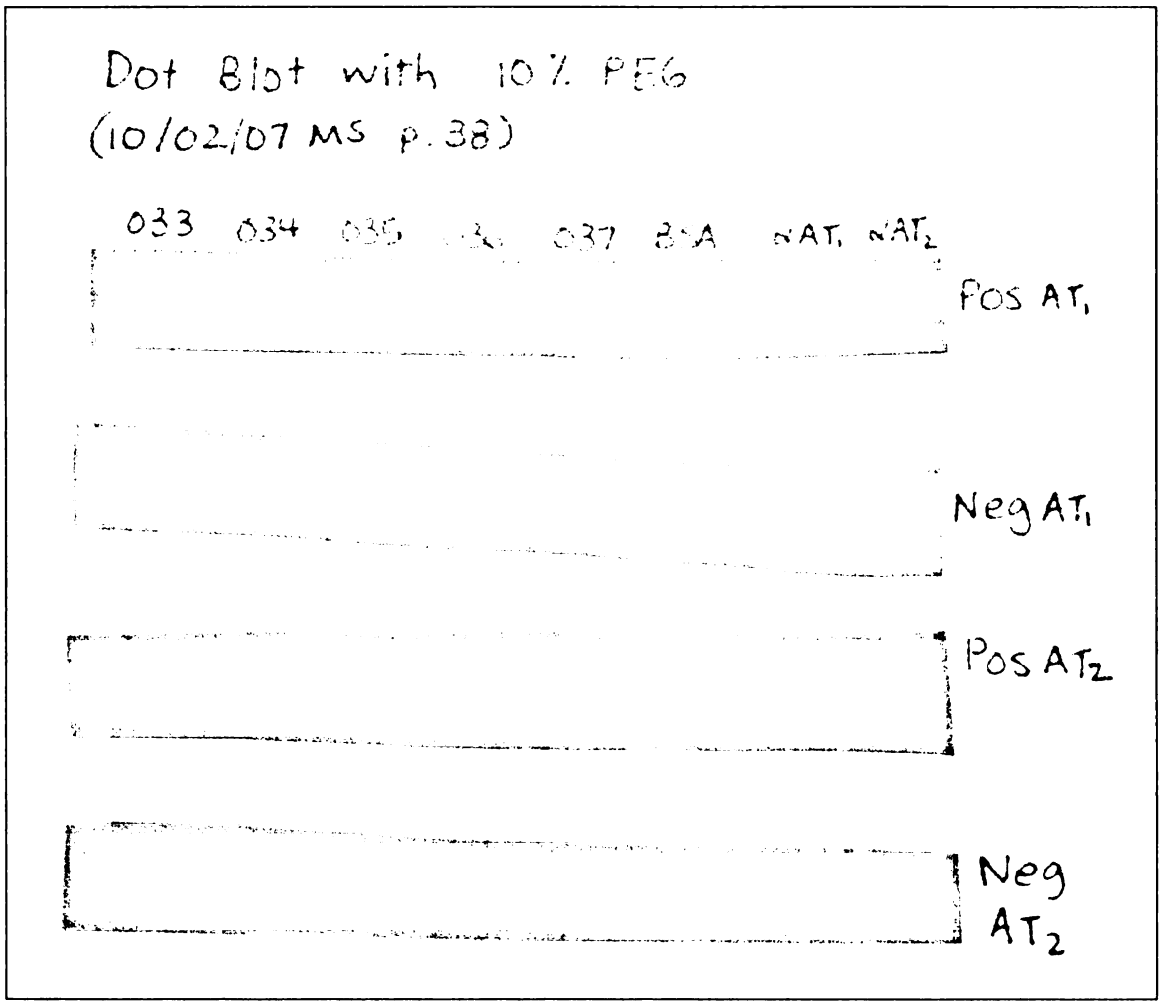

Figure 15. Dot Blot with 10% PEG as a blocking solution (with vacuum method) (handwritten).

DISCUSSION

The goal of this research was to develop a screening method for anti-AT_1 and anti-AT_2 antibodies using microbead immunoassay.

Microbead immunoassay

Microbead immunoassay using vacuum manifold

The microbead immunoassay using vacuum manifold method was adapted from the Luminex Corporation (Austin, TX). In this method, a commercial anti-AT_1 antibody was used. Sample 033-AT_1 (AT_1) was the positive control for microsphere group 033 coupled with peptide AT_1-033. Sample 038 (AT_1) was the blank for the assay. To determine the presence of anti-AT_1 or anti-AT_2 the fluorescence intensity between 033-AT_1 (AT_1) and 038 (AT_1) was compared. From the median fluorescence intensity between the 033-AT_1 (AT_1) and 038 (AT_1) (Table 12), it showed that there was an increase in the fluorescence intensity however the increase was lower than 1000 fold. The trimmed median fluorescence intensity result (Table 13) was identical as the median fluorescence intensity. The mean fluorescence intensity result (Table 14), showed a higher increase in fluorescence intensity signal, however, when the outliers were taken out, which was shown with the trimmed mean fluorescence intensity (Table 15), the signal increase was still not as great as expected. One possible cause for the low signal was that there was a low amount of primary antibody bound to the peptide on the microsphere resulting in low amount of secondary antibody bound shown by the low fluorescence intensity. To solve this problem, the amount of the primary antibody was increased. When the amount of primary antibody was increased, the difference was still not as great as expected.

One issue found while performing the microbead immunoassay with vacuum manifold was the inconsistency of the aspiration of the wash buffer between wells. To ensure that the microspheres were well equally washed, the washing step, where it was aspirated with the vacuum manifold, was replaced with a centrifugation method where the microspheres were centrifugated, and the supernatant aspirated manually using a micropipette.

Microbead immunoassay using centrifugation

In this method, the amount of primary antibody was increased almost double from the vacuum manifold method. The secondary antibody concentration used was slightly lower than previous experiment. Similar results (Table 16) were produced in this experiment; there was an increase in fluorescence intensity but not as great as expected.

Microbead immunoassay by varying incubation conditions and secondary antibody concentrations

To optimize antigen antibody binding, two sets of incubation conditions were performed: 37°C and room temperature. In addition, different combinations of secondary antibody concentrations were used. By comparing the fluorescence intensity between 033-AT_1 positive and 038 positive (Tables 17-20) at room temperature incubation, it showed that when the secondary antibody concentration was increased, a higher background shown by the increase signal for 038 positive was produced. In addition, the change in incubation condition to 37°C also produced a higher background.

A possible cause, discussed previously, could be insufficient antibody binding to the peptide on the microsphere and resulting in a low signal. Due to the limit of primary antibody concentration available, one can decrease the amount of the peptide bound to the microspheres and therefore decrease the amount of primary antibody required.

Microbead immunoassay by varying peptide concentration #1 and #2

Experiments done up to this point were only using microsphere group 033 which was coupled with peptide AT_1-033. Peptide AT_1-033 was chosen because it was the longer peptide and contains peptide AT_1-035 and AT_1-036. The result from varying the peptide concentration (Table 21) showed the same trend as previous results, where there was a slight increase of signal for the positive control.

One possible cause was the specificity of the primary antibody used could be directed to other parts of the AT_1 receptor. The AT_1 receptor consists of polypeptides containing approximatey 360 amino acids that span the cell membrane 7 times [33-35]. The epitope for the antibody used for the experiments may not correspond to the peptide sequence AT_1-033. To address this, an experiment with the other peptide sequences was performed. In addition, a different manufacturer for the primary and secondary antibody was used. The result (Table 22) showed similar trend as previous result where there was a slight increase of signal for the positive control. In addition, as the peptide concentration decrease, the background signal for the negative control of each microsphere group increased. This showed that when there was less peptide bound to the microsphere, there was an increase in non-specific binding.

Microbead immunoassay by varying peptide concentrations and primary antibody concentrations

To optimize antigen antibody binding, different peptide concentrations and primary antibody concentrations were used to obtain the correct antigen antibody ratio. The result in Table 23 showed that as the peptide concentration decreased, the background signal was increased. This showed that there was an increase in non-specific binding.

Microbead immunoassay by varying primary and secondary antibody concentrations

This experiment was performed with other peptide sequences to address the specificity issue. In addition, different combinations of primary and secondary antibody concentrations were used. The result (Table 24) showed similar trend as previous results where there was a slight increase of signal for the positive control.

Problem solving

The problem with the specificity of the commercial antibody used was not solved by using the other peptide sequences. An attempt to obtain positive control used in the ELISA and the functional bioassay method was made and failed. A search for anti-AT_1 and anti-AT_2 commercial antibodies that have the specificity matching the peptide sequences was done and the antibodies of interest were not found. The commercial antibodies for AT_1 and AT_2 receptor that were available were directed to other parts of the receptor that were not used in the ELISA or the bioassay studies.

Since the search for a commercial primary antibody or a known positive primary antibody failed, 17 samples of sera from hypertension rat were used with the microbead assay method. This sample population was used because it was described previously that patients with hypertension may have autoantibodies against anti-AT_1 [39].

Another problem with the antibody specificity issue could be caused by the peptide sequences. When the peptide sequences were coupled to the microspheres, it may change the immunogenicity of the peptide and result in no binding to the primary antibody. In addition, the blocking step with BSA may also hide the peptide and therefore inhibit binding to the primary antibody. This issue may occur with the shorter peptide sequences AT_1-035 and AT_1-036. One solution suggested was to add an ADH

molecule to the microsphere prior to the addition of the peptide sequence. This method was performed using the ADH modified microspheres and the peptide coupling to the ADH modified microspheres. The next experiment with the rat sera screening was performed with the microspheres coupled with the peptide and the ADH modified microspheres coupled with the peptide.

Rat sera screening using the microbead immunoassay by centrifugation

Rat sera screening using the microbead immunoassay by centrifugation was performed on 17 samples. By comparing the median fluorescence intensity (Table 25) between the negative control and the sample, there were a few that showed a greater increase in fluorescence intensity. The same trend was also observed in the trimmed median, mean and trimmed mean results (Tables 26-28).

When the samples were screened using the ADH modified microspheres the fluorescence intensity between the negative control and the sample were about the same (tables 29-32). This showed that ADH modified microspheres did not work. Further consultation on the ADH modified microspheres method with the manufacturer showed that the method was performed incorrectly. By lowering the amount of microspheres used, the amount of ADH and other reagents in the method should have been decreased proportionally. Initially, the manufacturer did not recommend decreasing the ADH and other reagents proportionally. However, further discussion with the manufacturer after the project was completed suggests that the ADH and other reagents should have been decreased proportionally.

Another possible solution was to screen human sera from renal transplantation population. This population was used because the availability of human sera from the

MSU Immunohematology and Serology Laboratory and the presence of anti-AT_1 antibody have been described previously [27]. In addition, human sera from preeclampsia patients were used since the presence of anti-AT_1 autoantibody has been described [42].

Human sera screening using microbead immunoassay by centrifugation

The human sera screening using the microbead immunoassay by centrifugation was performed using the microsphere coupled with the peptide and the ADH modified microspheres coupled with the peptide. 158 human sera were used in this experiment. The median fluorescence intensity (Table 33) between the negative control and the sample showed very small or no difference. The same trend was also observed in the trimmed median, mean and trimmed mean results (Tables 34-36). This showed that there was no antibody present or the assay was still not working.

When the samples were screened using the ADH modified microspheres the fluorescence intensity between the negative control and the sample were about the same (Tables 37-40). The same method was performed on this set of samples; therefore the failed result could be due to incorrect method used.

One possibility discussed previously was regarding the amount of the peptide sequence on the microsphere. To determine if the peptide coupling was successful, one can measure the absorbance of the supernatant of the microsphere solution. As the peptide bound with the microsphere, the amount of the peptide in the supernatant should decrease.

Determination of peptide coupling efficiency

The absorbance was taken using four different wavelengths. Based on the absorbance, the absorbance that showed the greatest decrease over time was used to determine the concentration. From the results in tables 41-44, the wavelength of choice to determine the decrease in peptide concentration was 230 nm. Using the initial concentration and the initial absorbance for each peptide sequence, the molar absorptivity for each peptide sequence was calculated. The molar absorptivity was calculated based on Beer's Law (Figure 16).

Figure 16. Beer's Law formula

$$A = \varepsilon b c$$

A= absorbance
ε = molar absorptivity
b = path length of the sample (1 cm)
c = concentration (M)

Using the molar absorptivity for each peptide sequence, the concentration of each peptide over time was calculated and shown in Table 46.

Table 46. Peptide concentration based on absorbance at 230 nm

Peptide ID	Concentration (μg/mL)				
	0 hours	2 hours	4 hours	20 hours	24 hours
AT_1- 033	125	120.7	119.6	115.9	114.4
AT_1- 034	125	118.2	115.6	114.2	113.9
AT_1- 035	125	108.2	94.5	78.8	68.3
AT_1- 036	125	112.9	111.7	111.2	109.7
AT_2- 037	125	104.7	104.1	103.4	102.7

The result (Table 46) showed a decrease in peptide concentration over time which indicates binding of the peptide to the microsphere. In addition, incubation past two hours did not increase the efficiency of the peptide binding to the microsphere.

Another solution proposed to solve the antibody specificity issue was the change in the conformation of the peptide during the coupling reaction to the microspheres. Two methods were performed to overcome this problem: the ELISA method and the Dot Blot method.

ELISA

The result (Table 45) showed small difference in absorbance between each wells. This showed either no peptide binding to the microplate or the specificity of the primary antibody used was incorrect.

Dot Blot with non-fat dry milk as blocking agent

The dot blot result with non-fat dry milk with the vacuum method (Figure 8) showed that there was no binding between the peptide and the primary antibody. One possible problem with the vacuum method was the possibility of the peptide solution to be filtered out from the membrane. To overcome this problem, another dot blot with non-fat dry milk without the vacuum method was performed. The result (Figure 7) still showed no binding between the peptide and the primary antibody.

Dot Blot with PEG as blocking agent

One issue with using non-fat dry milk as a blocking agent was that since it has a large molecular weight, it may cover the peptide sequence bound to the membrane. To address this problem, PEG which has lower molecular weight was used as the blocking agent. This method was performed with the vacuum method (Figures 13-15) and the result showed that there was no binding between the peptide and the primary antibody used. In addition, the positive control (primary antibody and secondary antibody only) color was less compare to the dot blot that used non-fat dry milk as blocking agent. This

indicates that PEG did not block as well and therefore there was an increase in non specific binding. In the method without the vacuum (Figures 10-12), there was also no binding between the peptide and the primary antibody.

CONCLUSION

The development of the microbead immunoassay to detect anti-AT_1 and anti-AT_2 was not successfully developed. Two possible sources of the problem: the peptide sequences and the primary antibody.

The peptide sequences were coupled with the microspheres. The peptide efficiency experiment showed that there was peptide bound to the microsphere. However, this may create an issue with the immunogenicity of the peptide to bind the antibody of interest. An attempt to omit the coupling step that may hinder the peptide's immunogenicity was done by performing the ELISA method and the dot blot method.

Another factor was the primary antibody, which is a known positive control to the anti-AT_1 or anti-AT_2 that will react with the peptide sequences used in this assay. The peptide sequences used in this research were selected from the ELISA and the bioassay method. The commercial antibodies available for purchase directed to AT_1 and AT_2 receptors were directed to a different part of the receptor than the ELISA or the bioassay studies. If a known positive controls were available, it is possible that anti-AT_1 or anti-AT_2 could be detected. In addition, performing the ADH modified microspheres coupling method with the appropriate reagent amount may also solve the problem with the short peptide sequences.

RECOMMENDATION

Major problem with the development of this assay was the availability of the positive control for the anti-AT_1 and anti-AT_2. If a known positive control available, I would recommend to do the dot blot assay to check the binding ability of the antibody to the peptide sequences used in the assay.

The result from the rat sera screening showed that there were a few samples showing a good separation between the positive and the negative control. With the availability of more hypertension rat sera, I would recommend repeating the assay. In addition, varying peptide concentrations, secondary antibody concentration, and rat sera volume used may help in the development of this assay.

APPENDIX

Table 47. Microspheres catalog number

Microsphere ID	Catalog #
033	L100-C033-01
034	L100-C034-01
035	L100-C035-01
036	L100-C036-01
037	L100-C037-01
038	L100-C038-01

Table 48. Antibodies catalog number

Product	Source	Catalog #
Mouse anti human angiotensin II type 1 receptor (AT_1)	USBio	A2295-07
Goat anti mouse IgG (PE)	USBio	I1904-74X
Rabbit anti human angiotensin II type receptor (AT_2)	USBio	A2295-075
Donkey anti rabbit IgG H&L (PE)	USBio	I1903-13B
Sheep polyclonal to angiotensin factor 1 receptor	Abcam	Ab31667
Donkey polyclonal to sheep IgG H&L (PE)	Abcam	Ab7009
Rabbit polyclonal to sheep IgG H&L (AP)	Abcam	Ab6748
Rabbit polyclonal to angiotensin II type 2 receptor	Abcam	Ab31210
Donkey polyclonal to rabbit IgG (PE)	Abcam	Ab7007
Goat polyclonal to rabbit IgG (AP)	Abcam	Ab6722
Sheep polyclonal to rat IgG H&L (AP)	Abcam	Ab6853
Goat polyclonal to rat IgG H&L (PE)	Abcam	Ab7010
Goat polyclonal to human IgG H&L (PE)	Abcam	Ab7006

Table 49. Reagents catalog number

Product	Source	Catalog #
ADH (Adipic acid dihydrazide)	Sigma	A0638
BCIP/NBT Liquid (Dot Blot substrate)	Sigma	B1911
BSA (Bovine serum albumin)	Sigma	A3803-10g
DMSO (Dimethyl sulfoxide)	Sigma	D2650
Dry milk (non fat)	Carnation	
EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride)	Pierce	22980
Glycine	Sigma	G7126
$MgCl_2$ (Magnesium Chloride)	JT Baker	2444-01
Sulfo-NHS (Sulfo-N-hydroxysulfosuccinimide)	Pierce	24510
MES (2[N-Morpholino]ethanesulfoic acid)	Sigma	M-2933
Tween-20 (CAS 9005-64-5)	USB	20605
NaN_3 (Sodium Azide)	Sigma	S2002
NaCl (Sodium chloride)	JT Baker	3624-05
NaOH (Sodium hydroxide)	JT Baker	3722-05
NaH_2PO_4 (Sodium phosphate monobasic, anhydrous)	Sigma	S-5011-5006
$NaHCO_3$ (Sodium hydrogencarbonate)	JT Baker	3506-05
NaOH (Sodium hydroxide)	JT Baker	3722-05
KCl (Potassium chloride)	Colombus Chem. Industries	14673
KH_2PO_4 (Potassium phosphate)	Sigma	P5379-5006
PEG (Polyethylene glycol MW 1,500)	Fluka	73034
pNPP (p-nitrophenyl phosphate) tablets	Sigma	N2765
$ZnCl_2$ (Zinc chloride)	Sigma	Z4875

Table 50. Consumables

Product	Source	Catalog #
1.2 µm PVDF filter micro titer plates	Millipore	MABVN1250
1.5 ml tube	Eppendorf	022364111
1.0 ml microcentrifuges tube	Art Robbins Instruments	16674-2
15 ml conical polypropylene tube	Corning	430052
50 ml conical polypropylene tube	Corning	430290
250 ml filter system, vacuum	Nalgene	126-0045
96-wells, 250 µl, V-bottom microplate	Whatman	7701-1250

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