Comparison of two Elisa-based Angiotensin II -type-1 receptor (AT_1R) autoantibody detection assays using an antibody cross-reactivity check approach

Adak N. Karamafrooz[1], Robert Carroll[2], Dave Lowe[1], MichaelTrinh[1], Rui Pei[1], Julie Nguyen[1]
1. Research and Development, Thermo Fisher Scientific, West Hills, CA, United States. 2. Health Sciences, University of South Australia, Adelaide, SA, Australia.

Abstract

Elevation of autoantibodies against Angiotensin II Type 1 receptor (AT_1R) in human sera has been associated with several disorders, including pre-eclampsia and renal-allograph rejection, therefore, an accurate and reliable measurement of those antibodies is crucial. The enzyme-linked immunosorbent assay (ELISA) is the primary method for measuring AT1R-specific antibodies (AT1R-Ab) levels. We have performed a cross-reactivity study comparing a commercial AT_1R antibody detection kit (EIA AT1RX, CellTrend) and a research-developed ELISA assay to demonstrate the accuracy and sensitivity of the ELISA as a detection platform for AT1R-specific antibodies.

Introduction

Rapid and accurate methods for the detection of human leukocyte antigens (HLA) and non-HLA antigens associated with the pathogenesis of hyperacute, acute, and chronic allograft rejection are clinically important for the successful management of long-term allograft transplantations. Among non-HLA antigens, antibodies against AT_1R have been extensively investigated and shown to be associated with vascular inflammation, leading to refractory vascular rejection and eventual allograft loss, enhancing the mortality risk in kidney transplant patients. In the current study, we verified the sensitivity of a commercial AT_1R-Ab detection kit (CellTrend) as well as a purified AT_1R mutant using AT_1R-specific antibodies that were eluted from live cells expressing AT_1R antigen. Furthermore, we discovered that certain reagents are incompatible with the commercial AT1R kit, resulting in a loss of detection signal.

Materials and methods

Transfection and expression of AT_1R: T-rex 293 adherent cells were maintained in DMEM high glucose supplemented with 10% FBS, 1% penicillin-streptomycin, and glutamax at 37ºC and 5% CO2 in a humidified incubator. T-Rex 293 cells were stably transfected with AT_1R wild type (AT_1R-WT) or AT_1R constitutively active mutant using a Neon electroporation system**.** AT_1R surface expression was confirmed using flow cytometry and AT_1R-specific antibodies.

Purification of AT_1R using affinity chromatography: The purification was performed using affinity chromatography based on *Wingler et al.,* 2019 with minor adjustments. AT_1R was eluted using 0.1 M glycine (pH 2.0-2.8) and neutralized with 1M Tris (pH 9.5). The eluted fraction was buffer exchanged with HEPES buffer (500 mM NaCl, 0.01% MNG, 0.004% (w/v) CHS, protease inhibitor, pH 7.5) and analyzed using BCA assay, western blot, and LC-MS mass spectrometry protein sequencing.

Preparation of ELISA tray for detection of anti-AT_1R autoantibodies: a) Stably transfected cells with AT_1R construct were seeded on Poly-D-Lysine-coated 96 well plates, induced 24 hours later using doxycycline, and fixed with %4 formaldehyde. **b)** The affinity-purified AT_1R antigen was supplemented with protease inhibitor and coated overnight at 4ºC with shaking on the Maleimide-activated ELISA plates and then blocked with ELISA blocking buffer for 1 hour and washed three times before testing.

Antibody adsorption with crossmatch cells and elution (AXE procedure): T-rex293 cells expressing AT_1R were harvested 48 hours post-induction. Cell pellets were incubated with human serum positive for AT_1R-Ab for 30 minutes at 37ºC with agitation. The supernatant was then retrieved, and the pellets were washed several times before antibody elution using elution buffer (Glycine buffer pH 3.0, $MgCl_2$) and neutralized using Tris buffer (pH 8.0). The serum-derived antibodies were tested on the AT_1R ELISA plates.

Adsorb out™ bead (AOB) treatment: Adsorb Out™ (One Lambda) microparticles are used to reduce the background reactivity due to nonspecific binding. Briefly, test serum and AOB were dispensed in varying dilutions, and incubated for 30 minutes at room temperature with agitation. Following centrifugation, the supernatant was transferred into a new tube without disturbing the pelleted beads. The sera were then transferred to the ELISA tray for further processing.

Results

Figure 1: Effect of serially diluted Adsorb Out bead (AOB) on standards & controls of the *CellTrend* AT_1R-Ab Assay kit (lot 64). Serially diluted AOB was added to the kit's standard sample, positive and negative controls, or diluent buffer (DB). When used at high concentrations, AOB would interfere with the detection signal. At higher dilutions (10^{-7}), the inhibitory effect is diminished, and the signal is restored.

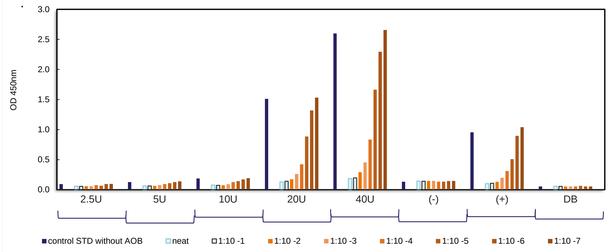


Figure 2: effect of BSA on the *CellTrend* AT_1R kit's detection signal. a). BSA, as the major component of the AOB buffer, impairs the detection signal in the positive control of the AT_1R Kit, while other components do not interfere with the signal detection. **b)**: The presence of BSA, even at very low concentrations, affects the signal detection on the CT Elisa plate. Samples are titrated with varying BSA concentrations prior to testing on the ELISA tray.

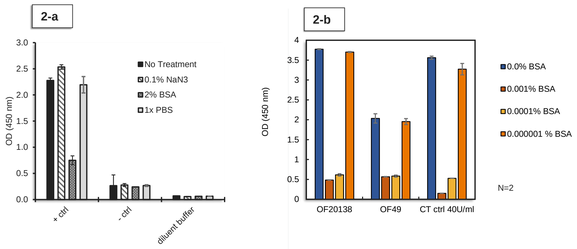


Figure 3: Cell-based ELISA tray: Standards and controls from *CellTrend* AT_1R kit as well as patients' sera reacted to the ELISA tray Coated with T-Rex 293 Cells expressing AT_1R active mutant, confirming the specificity of AT_1R antigen present on the cell surface.

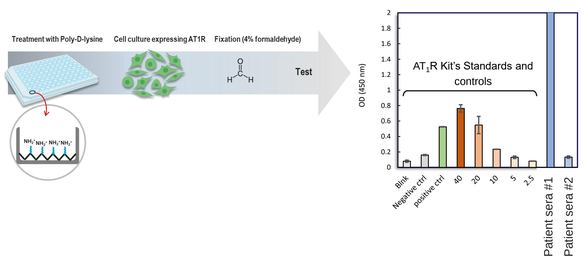


Figure 4: Purified AT1R-coated ELISA tray. Affinity-purified AT_1R consecutively active mutant is coated on an ELISA tray. *CellTrend* standards **(4-a)** as well as human serum positive and negative for AT_1R-Ab **(4-b)** are tested on the AT1R-coated ELISA tray.

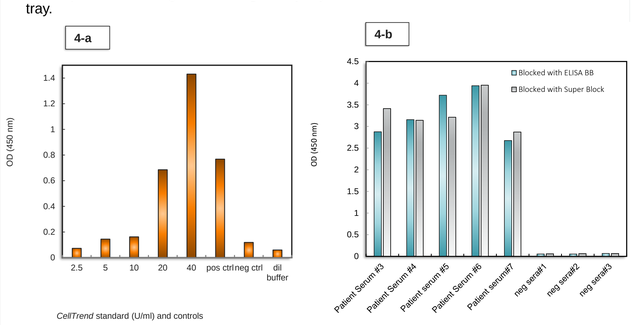
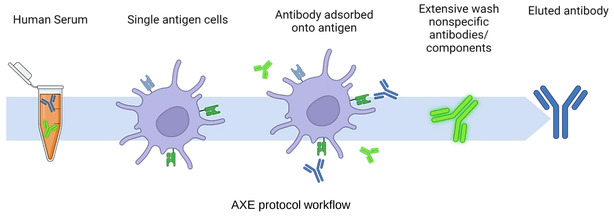
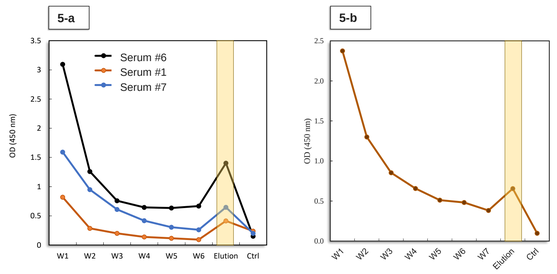


Figure 5: Cross-reaction of the eluted AT_1R-specific antibodies with the ELISA trays. Using AXE method (Ref.1), eluted antibodies from patient serum positive for AT_1R, which had been captured on AT_1R-expressing cells, cross-reacted with both the AT_1R ELISA tray from the CellTrend kit **(5-a)** and the purified AT_1R coated Elisa plate **(5-b)**.





Conclusions

- There have been studies that show pretreatment of the samples with Adsorb out™ bead prior to performing the ELISA assay can lead to a decrease or complete loss of detection signals (reviewed in Ref. 2). Here we demonstrate that bovine serum albumin (BSA) present in the AOB buffer is the primary element that impedes the detection signal in the commercial AT_1R ELISA kit.
- Sensitivity and specificity of ELISA as a reliable platform are confirmed through the Adsorption with Crossmatch cells and Elution (AXE) experiment. This experiment defines only the antibodies bound to the specific antigens on the cell surface. By transferring the eluted antibodies to the AT_1R-Ab detection ELISA tray, we successfully detected those antibodies.

References:

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2. Xu, Q., Johnson, K. P., Hardiman, M., Helmick, D., & Zeevi, A. (2023). Loss of anti-AT1R reactivity in ELISA post-adsorption - False reactivity or interference in the assay? Human Immunology, 84(4), 286–289.
3. ThermoFisher Sci, (n.d.). Deciphering the Loss of Anti-Angiotensin Type 1 Receptor Reactivity in Enzyme-Linked Immunosorbent Assay Post-Adsorption. P519 Human Immunology, Volume 84, Supplement, September 2023, p142-143.

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