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Detection of Anti- β 1-AR Autoantibodies in Heart Failure by a Cell-Based Competition ELISA

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Rationale: Autoantibodies directed against the second extracellular loop of the cardiac β 1-adrenergic receptor (β 1-AR) are thought to contribute to the pathogenesis of dilated cardiomyopathy (DCM) and Chagas heart disease. Various approaches have been used to detect such autoantibodies; however, the reported prevalence varies largely, depending on the detection method used.

Objective: We analyzed sera from 167 DCM patients (ejection fraction <45%) and from 110 age-matched volunteers who did not report any heart disease themselves, with an often used simple peptide-ELISA approach, and compared it with a novel whole cell-based ELISA, using cells expressing the full transgene for the human β 1-AR. Additionally, 35 patients with hypertensive heart disease with preserved ejection fraction were investigated.

Methods and Results: The novel assay was designed according to the currently most reliable anti-TSH receptor antibody-ELISA used to diagnose Graves disease (“third-generation assay”) and also detects the target antibodies by competition with a specific monoclonal anti- β 1-AR antibody (β 1-AR MAb) directed against the functionally relevant β 1-AR epitope. Anti- β 1-AR antibodies were detected in \approx 60% of DCM patients and in \approx 8% of healthy volunteers using the same cutoff values. The prevalence of these antibodies was 17% in patients with hypertensive heart disease. Anti- β 1-AR antibody titers (defined as inhibition of β 1-AR MAb-binding) were no longer detected after depleting sera from IgG antibodies by protein G adsorption. In contrast, a previously used ELISA conducted with a linear 26-meric peptide derived from the second extracellular β 1-AR loop yielded a high number of false-positive results precluding any specific identification of DCM patients.

Conclusions: We established a simple and efficient screening assay detecting disease-relevant β 1-AR autoantibodies in patient sera yielding a high reproducibility also in high throughput screening. The assay was validated according to “good laboratory practice” and can serve as a companion biodiagnostic assay for the development and evaluation of antibody-directed therapies in antibody-positive heart failure. (*Circ Res.* 2012;111:675-684.)

Key Words: heart failure ■ β -adrenergic receptor ■ autoantibodies ■ ELISA ■ adrenergic receptors ■ cardiomyopathy

Evidence has accumulated from both animal- and patient-based studies that autoantibodies targeting the human β 1-adrenergic receptor (β 1-AR) may play an important role in the development and clinical course of progressive cardiac dilatation and failure.¹⁻⁶

β 1-ARs are G protein-coupled receptors that trigger signaling via adenylate cyclase, cAMP, and PKA. This signaling pathway regulates the sarcoplasmic calcium concentration and increases cardiomyocyte contractility.

During recent years, it has been demonstrated independently by various groups that a relevant subset of these autoantibodies bind to the second extracellular loop of the β 1-AR and recognize a native receptor conformation.^{3,4,7,8}

Such conformational anti- β 1-AR-(ECII) antibodies have been shown to be functionally active and may be capable of stimulating intracellular cAMP production.^{3,5} Moreover, only those anti- β 1-AR autoantibodies that target the second extracellular loop (β 1-AR-ECII) appear to be functionally active. In contrast, antibodies directed against the amino- or carboxy terminus of the receptor protein exert no biological effects.^{1,8,9} The presence of such functionally active, receptor-stimulating anti- β 1-AR-ECII is associated with a markedly worse prognosis in dilated cardiomyopathy (DCM).¹⁰

Autoantibodies directed against the β 1-AR-ECII are found in 30% to 75% of patients with DCM, depending on the respective study or screening method used.^{3,6,10-11} In contrast,

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Non-standard Abbreviations and Acronyms	
β1-AR	β 1 adrenergic receptor
β1-AR-ECII	second extracellular loop of the beta1 adrenergic receptor
anti-β1-AR antibodies	antibodies that bind to the second extracellular loop of β 1-AR
ELISA	enzyme-linked immunosorbent assay
EC₅₀	concentration that induces half-maximal response
HHD	hypertensive heart disease
K_D	affinity constant
LVEF	left ventricular ejection fraction
mAb 23-6-7	mouse monoclonal antibody directed against the second extracellular domain of the β 1-adrenergic receptor
MOI	multiplicity of infection
NC	negative control sample
OD	optical density
PBS	phosphate-buffered saline, pH 7.4
PBS-T	phosphate-buffered saline, pH 7.4, +0.1% Tween 20
PC	positive control
PFA	para-formaldehyde
POD	peroxidase
RT	room temperature
SF9	<i>Spodoptera frugiperda</i> 9
TMB	tetramethylbenzidine
VS	validation sample

a low prevalence of these functionally active anti- β 1-AR-ECII has been observed in healthy individuals, or in patients who had heart failure due to valvular or hypertensive heart disease.⁵ Recently, direct evidence has been obtained in a human-homologous rat model of heart failure, that anti- β 1-AR-ECII may cause a cardiac phenotype resembling human DCM.⁶

The functionally relevant (conformational) epitope within the β 1-AR-ECII has been identified experimentally, characterized, and epitope-specific monoclonal antibodies have been generated. One of the monoclonal antibody clones, termed 23-6-7, exerted a strong stimulatory effect on β 1-AR, thus mimicking the characteristics of assumingly disease-relevant anti- β 1-AR autoantibodies in DCM patients.

Using a competition approach against β 1MAb 23-6-7, we established a novel whole-cell ELISA that specifically detects clinically relevant anti- β 1-AR autoantibodies in patient sera. On comparison of the results obtained with sera from DCM patients versus those obtained with sera from a healthy control population, the specificity of this “third-generation” competition ELISA is markedly superior to the often used simple peptide-based ELISA approach.

Methods

Cell-Based ELISA

Sf9 cells were grown in adhesion culture according to standard cell culture protocols. Cells were detached from culture flasks after 3 to

4 days of growth, when they had reached about 70% to 100% confluence. Afterward, they were centrifuged (400 g, 5 minutes) and resuspended in cell culture medium. Suspended cells were infected with baculovirus (MOI 6), carrying the gene for the human β 1-AR. A transgene-free baculovirus served as control. Cell suspension was directly seeded on poly-L-lysine coated 96-well cell culture plates (Biocoat, No. 356516) at a density of 30 000 cells per well. After 72 hours of incubation, half of the cell culture supernatant (200 μ L/well) was removed and 100 μ L 2 \times para-formaldehyde (PFA) fixation solution (2% PFA in the final solution) was added. Cells were incubated for 15 minutes at room temperature (RT) at constant shaking. Supernatants were removed subsequently, and fixed cells were washed 3 times with PBS (Dulbecco, Cat No. L1820, Biochrom AG)+0.1% Tween 20 (PBS-T). Optionally, the microtiter plates were frozen at -80°C for up to 6 months.

The PFA-fixed cells were blocked with 200 μ L PBS-T+3% milk powder for 1 hour at RT. Afterward, the plates were washed 3 times with PBS-T. Mouse monoclonal anti- β 1-AR antibody (23-6-7 Biogenes) was added, then 23-6-7 binding was competed by addition of human sera from healthy volunteers or from DCM patients, respectively. Positive control samples were provided by defined concentrations of monoclonal rat anti- β 1-AR antibodies, which were also used for competition. After incubation for 2 hours at RT with constant shaking, the cells were washed 3 times with PBS-T and secondary antibody solution (1:5000 in PBS-T+3% milk powder) was added. Plates were incubated for 1 hour at RT. After a further washing step, 3 \times with PBS-T, peroxidase bound in the complex was visualized by tetramethylbenzidine (TMB) substrate solution. After stopping the enzymatic reaction with sulfuric acid, the intensity of the resulting color was determined at 450 nm and at a reference wave length of 595 nm.

The competitive efficacy of human samples, negative control sample (NC, serum from healthy volunteers) and positive control (PC, serum from healthy volunteers spiked with anti- β 1-AR rat 13F6 antibody), respectively, was calculated as percentage inhibition of the mouse antibody (23-6-7) binding. To this end, each OD value was divided by the mouse antibody (23-6-7) value, multiplied by 100, and the resulting values were subtracted from 100.

No reduction in OD value of the (23-6-7) mouse antibody resulted in 0% inhibition, whereas complete OD value reduction corresponded to 100% inhibition.

The assay validation was conducted for the determination of the factor (K) and assay cutoff value. In 3 independent experiments based on the analysis of sera from 20 healthy volunteers, the factor (K)=0.143 was obtained by using equations (1), (2), and (3).

$$(1) \quad \text{Inhibition}\%_{\text{screening cutoff}}$$

$$= \text{mean Inhibition}\%_{\text{row data}} (\text{control samples}) + 2 \times \text{SD}$$

$$(2) \quad K_i = (\text{Inhibition}\%_{\text{screening cutoff } i} - \text{mean Inhibition}\%_{\text{NC } i})$$

$$/ \text{mean Inhibition}\%_{\text{PC } i}$$

$$(3) \quad K = (K_1 + K_2 + K_3) / 3$$

K_i ($i=1-3$) was determined on 3 plates with 20 blank individual samples.

For all further plates “i” the following cutoff formula (4) was applied:

$$(4) \quad \text{Inhibition}\%_{\text{cutoff } i} = \text{mean Inhibition}\%_{\text{NC } i} + K (0.143)$$

$$\times \text{mean Inhibition}\%_{\text{PC } i}$$

This way of Inhibition%_{cutoff} calculation avoided the necessity to analyze a high number of individual blank samples on each plate.

To adjust the Inhibition%_{row data} (sample) from different plates, the respective Inhibition%_{cutoff} must be considered.

$$(5) \quad \text{Inhibition}\% = \text{mean Inhibition}\%_{\text{row data}} (\text{sample})$$

$$- \text{Inhibition}\%_{\text{cutoff}}$$

Radioligand Binding

Baculovirus-infected cells (multiplicity of infection, MOI of 0.02 to 6 active viral particles per cell) were grown in large-size bottles and harvested by scraping off with a cell wiper. Membranes were prepared by centrifugation and resuspended in TRIS-HCl (50 mmol/L, pH 7.4). Radioligand binding was performed by adding iodo-cyano-pindolol (I-CYP) at concentrations of 5 pmol/L to 3760 pmol/L for 90 minutes at 32°C. Nonspecific binding was determined in the presence of bisoprolol (1 μ mol/L each). Incubation samples were filtered by GF/F-Filters (Millipore APFF No. 02500). Filters were washed 4 times with 50 mL Tris-HCl, pH 7.4, and counted.

ELISA Based on a 26-Meric Peptide

Nunc microtiter maxisorp plates were coated with 0.5 μ g/mL peptide, 26-meric peptide HWRAESDEARRCYNDRPKCCD-FVTNR, in 0.1 mol/L Na_2CO_3 or buffer alone for 16 hours at 4°C. After saturation of the wells with PBS supplemented with 3% milk powder and 0.1% Tween 20, human serum from healthy volunteers or from patients with DCM, respectively, were diluted 1:20 in PBS-T+3% BSA+10% FCS and added to the wells. After incubation for 2 hours at RT, the bound antibodies were detected by a secondary antihuman IgG antibody labeled with peroxidase, diluted 1:20 000 in PBS-T+3% milk. Between each step, plates were washed 3 \times with PBS-T. Afterward, 100 μ L of TMB substrate solution was dispensed to all wells. The plate was covered and incubated for 10 to 30 minutes at RT. The enzyme reaction was stopped by addition of 100 μ L stop solution to all wells. The absorbance was read at 450 nm (reference filter, 650 nm). The reduction of color intensity was directly related to the amount of human anti- β 1 receptor antibodies in the sample. Strong positivity was defined as 1.5 \times the background density.

Patients

Patients (n=167) with DCM were included who had been investigated by echocardiography, and their left ventricular ejection fraction had been determined to be <45%. Sera from strictly age-matched subjects (n=110) from a local blood donor bank served as controls; all these subjects had not reported cardiovascular disease on blood sampling. In addition, another group of patients with hypertensive heart disease was included, who had also been investigated by echocardiography, and their left ventricular ejection fraction had been determined to be >60%.

The mean patient age was 60.9 \pm 13.0 years in the DCM group and 59.3 \pm 11.2 years in the volunteer group, which did not differ significantly (2-tailed *t* test). The mean age of the patients with hypertensive heart disease was 72 \pm 7.8 years.

Antibodies

Large-scale production of the mouse monoclonal antibody 23-6-7, batch kDa-250 208 to 001, was performed commercially (Biogenes GmbH, Berlin, Germany). To this end, 8-week-old BALB/c female mice were immunized subcutaneously over a period of 39 days with GST fusion protein linked to a 25-meric peptide, corresponding to the β 1-AR-ECII. The antibodies produced from this hybridoma cell clone were purified by Protein A affinity chromatography and dissolved in PBS. The rat monoclonal antibody clone 13F6 was produced by InVivo Biotech Services GmbH, and the hybridoma cells were generated in our own hands, using GST fusion protein identical to the one used for mouse monoclonal antibodies. Rat antibodies were purified by Protein G affinity chromatography and dissolved in PBS. Goat polyclonal antibodies were generated by Biogenes GmbH, Berlin. The immunization of the goats was carried out by 6 boosts over a period of 133 days by using the same GST fusion protein linked to the 25-meric β 1-AR-ECII peptide. At day 161, the antibody-containing serum was obtained and purified by affinity chromatography using a 25-meric β 1-AR-ECII peptide coupled to CNBr-activated Sepharose 4B (GE Healthcare, cat. 17-0430-01).

Deposit of Mouse Monoclonal Antibody 23-6-7 and Rat Monoclonal Antibody 13F6

Hybridoma cells expressing antibodies 23-6-7 and 13F6 were submitted to DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig). Depositing was carried out according to the rules of the Budapest Treaty (accession numbers DSM ACC3121 and ACC3174, respectively).

Immunoprecipitation

Immunoprecipitation was performed with sera from 20 β 1-AR antibody-positive DCM patients; 200 μ L of sera was incubated with 100 μ L Protein G Sepharose (GE Healthcare 17-0618-05) under shaking for 16 hours at 20°C. After centrifugation of the suspension in an empty column (Pierce Micro-Spin No. 89879) with 500g for 10 minutes at 20°C, the flow-throughs were collected.

Data Analysis

IC₅₀ values and sample concentration were calculated by using standard curve analysis ("4-parameter logistic") from Sigma plot software, version 11. All other calculations were performed with EXCEL software, version 2003/2007.

Results

Novel Cell-Based ELISA Using the Fully Recombinant Human β 1-AR

To provide the native and functionally active β 1-AR as binding target for autoantibodies, SF9 cells were infected with a baculovirus carrying the transgene for the human β 1-AR and coated on 96-well plates. Direct measurement of patients' anti- β -AR autoantibody titers by a simple ELISA approach using infected SF9 cells was not feasible due to a strong background signal. This nonspecific background was probably due to recognition of other cell surface epitopes by the highly diversified human antibody pool. To circumvent this problem, a competition assay was established, in which a high affinity antibody directed against the β 1-AR-ECII was used to generate a specific binding signal at the β 1-AR-expressing SF9 cells, which was then specifically competed by the anti- β 1-AR autoantibodies directed against the same epitope present in patient sera (Figure 1).

Determination of the Density and Affinity of Recombinant Human β 1-ARs

Infected SF9 cells were harvested, and the density and affinity of recombinant receptors were determined by radioligand binding. Whereas only a small nonspecific signal was determined in noninfected or control virus-infected cells, the density was 219 406 \pm 34 456 receptors per cell after infection at an MOI of 0.02 active virus particles per cell, and amounted to 246 221 \pm 95 436 receptors per cell after infection with 0.2 active virus particles per cell, reaching a plateau with higher MOI values (350 376 \pm 30 048 receptors per cell after infection with 2 active virus particles per cell and 330 990 \pm 41 170 receptors per cell after infection with 6 active virus particles per cell). The respective binding affinities of the radioligand (K_D values) were 36, 37, 24, and 32 pmol/L, respectively, and did not vary significantly between the groups.

Also, the affinity of the allosteric binding site for stimulatory anti- β 1-AR antibodies was tested in SF9 cells that had been infected with MOI ranging from 0.2 to 6 active virus

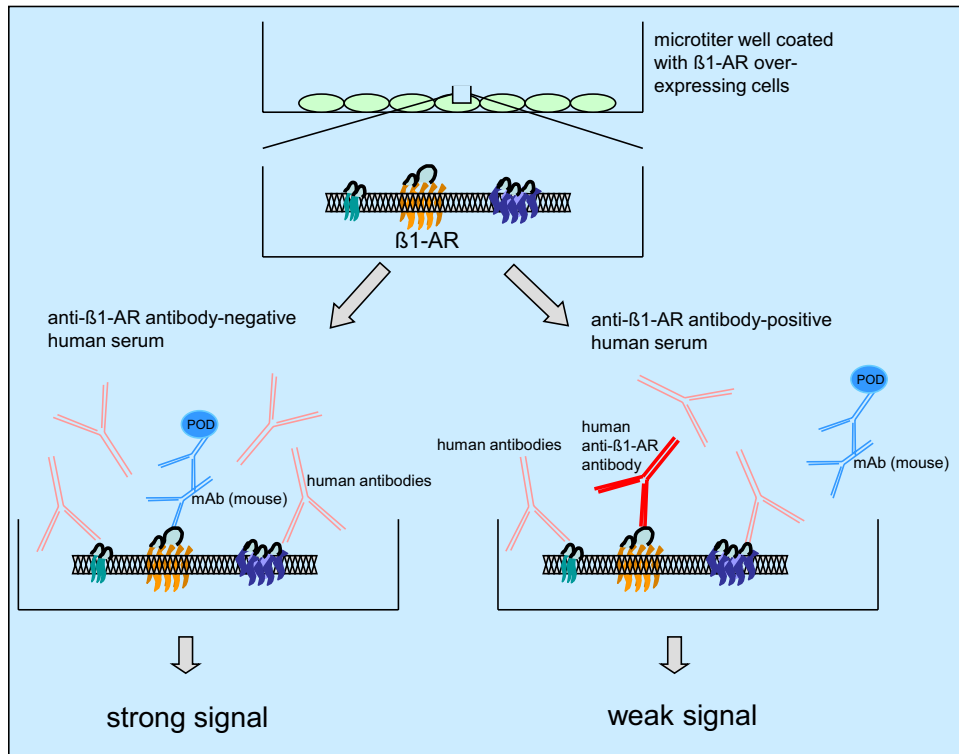


Figure 1. Principle of the ELISA measurement of human anti-β1-AR antibodies via competition of the anti-β1-AR monoclonal antibody 23-6-7. The ELISA mimics the in vivo autoantibody binding characteristics to β1-ARs using a microstate plate format. To avoid cross-binding of other human antibodies to various cellular membrane proteins, a competitive approach was developed: specific human anti-β1-AR antibodies compete with the monoclonal antibody 23-6-7 for the binding to cellular β1-ARs.

particles per cell. In these cells, EC₅₀ values of ELISA binding assays were analyzed using the antibody 23-6-7 (as depicted in Figure 2), and K_D values were calculated. The K_D values ranged between 0.22 and 0.43 nmol/L and did not differ significantly between the groups.

The K_D values did not differ significantly when various passages (23–51 passages) of SF9 cells were used and compared in these experiments.

Identification and Characterization of the Monoclonal Anti-β1-AR Antibody Clone 23-6-7

A prerequisite for such a competition approach was the generation of an antibody with high specificity and affinity to

β1-AR. Various monoclonal mouse antibodies to β1-AR ECII were produced by using a hybridoma cell line-based approach, and clone 23-6-7 was identified as the most promising monoclonal antibody.

To prove the binding specificity, we incubated various concentrations of 23-6-7 on recombinant β1-AR-over-expressing SF9 cells and initially measured its binding characteristics in the absence of any competitor. The results are shown in Figure 2, which illustrates an IC₅₀ value of 0.43 nmol/L. To determine the functionality of the 23-6-7 anti-β1-AR antibody clone, we investigated its ability to activate receptor-mediated intracellular cAMP accumulation through sequential activation of G_s proteins and adenylyl cyclase. One

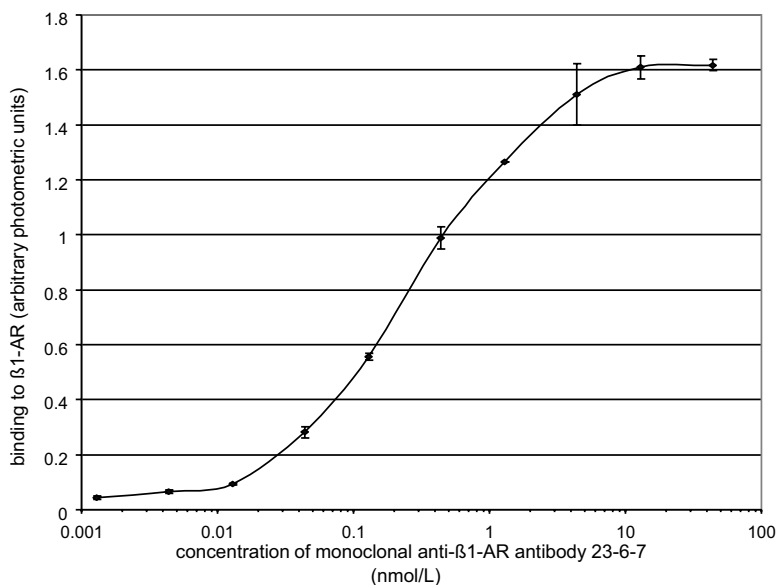


Figure 2. Binding affinity of the monoclonal antibody 23-6-7 to fully recombinant human β1-AR, overexpressed on SF9 cells. Means with SEM of at least 4 independent measurements are plotted.

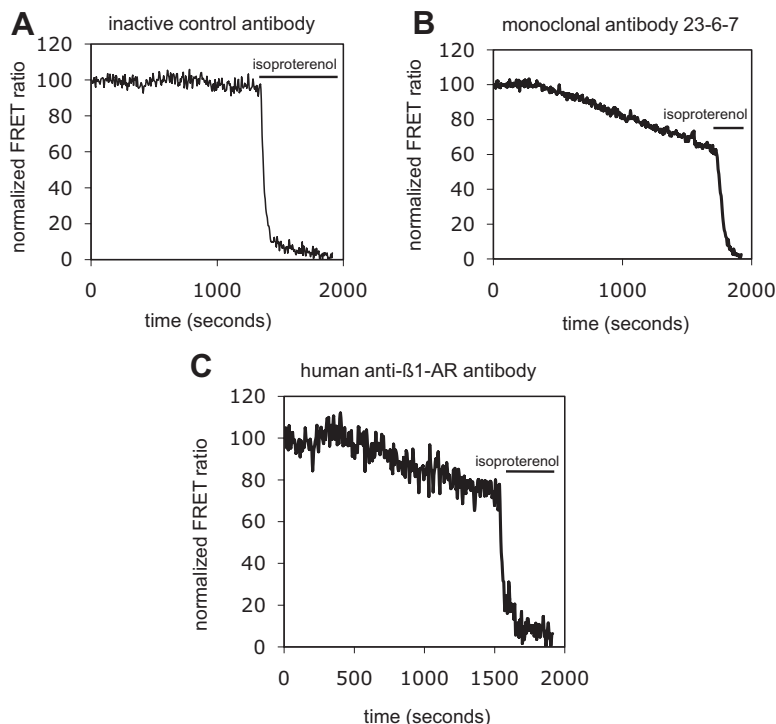


Figure 3. Measurement of cAMP levels by Epac-FRET in human embryonic kidney HEK293 cells stably expressing human β 1-ARs. Representative FRET ratiotracings of independent experiments are presented (% corresponds to the relative change in YFP/CFP intensity ratio). The decrease in FRET reflects an increase in intracellular cAMP. **A**, None of the inactive control antibodies induced a significant cAMP response in living cells. The viability of the cell is proven by additional stimulation by isoproterenol (Iso) at a concentration of 2.5 mol/L at the end of the experiment, which elicits a full cAMP response. **B**, In contrast, addition of antibody 23-6-7 elicited a relevant signal, which corresponds to 38.2% of the maximum possible signal, as was induced by additional administration of Iso at the end of this experiment. **C**, The signal intensity and kinetics were comparable to those from DCM patient sera previously judged anti- β 1-AR antibody-positive.

method to detect this increase in intracellular cAMP is to use fluorescence resonance energy transfer (FRET) between cyan fluorescence proteins (CFP) and yellow fluorescent proteins (YFP) fused to the cAMP-binding domain of Epac1.¹⁰ Addition of clone 23-6-7 clearly activated HEK 293 cells stably expressing human β 1-AR, as determined by using this FRET assay (Figure 3B), with slow kinetics as are typically exerted by anti- β 1-AR autoantibodies from DCM patients (Figure 3C). In contrast, a negative control antibody was ineffective (Figure 3A).

Cell-Based β 1-AR Competition ELISA

Our hypothesis was that the binding of the monoclonal anti- β 1-AR antibody 23-6-7 to β 1-AR-overexpressing SF9 cells should be modified by coincubation with human DCM patient serum (schematic overview in Figure 1). A competitive reduction of the 23-6-7 antibody binding should occur, depending on the presence of anti- β 1-AR antibodies in the respective sample.

As a first step to establish such an assay, polyclonal goat anti- β 1-AR antibodies were spiked to a serum pool derived from healthy volunteers to mimic the presence of human anti- β 1-AR antibodies. To clarify the impact of adding this human serum pool to the assay, the polyclonal goat anti- β 1-AR antibodies were also added in control buffer in an identical assay approach. Figure 4 shows high similarity of the competition curve for both conditions, regarding both dose-dependency and maximum signal. To validate the assay, a negative control, consisting of a serum pool from healthy volunteers, was deemed to be necessary. Assay sensitivity was determined at 10 nmol/L when using the polyclonal goat anti- β 1-AR antibody for competition.

Validation of the β 1-AR ELISA

To warrant interassay comparability, an NC, consisting of pooled human serum samples from healthy volunteers, and a PC, consisting of a human serum pool spiked with rat anti- β 1-AR antibodies (13F6), were measured on each microtiter plate. We used a monoclonal rat (13F6) antibody instead of the polyclonal goat anti- β 1-AR antibody because of its more reproducible availability.

To classify the inhibition (%) of the human serum samples, the plate specific Inhibition%_{cutoff} was considered. Responses varied between individual assays; therefore, cutoff values were modified accordingly. The use of the negative control plus a predetermined factor (K) to assess the cutoff value in

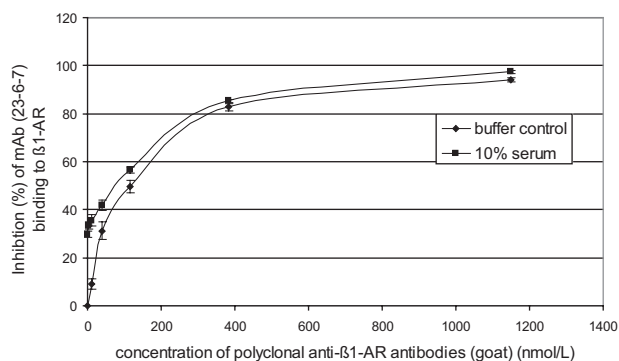


Figure 4. Competition of the binding of the mAb 23-6-7 by polyclonal goat anti- β 1-AR antibodies. Various concentrations of polyclonal goat antibodies were coincubated with the mAb 23-6-7 at a final concentration of 0.26 nmol/L. Addition of 10% serum pool derived from healthy volunteers was compared with buffer control and resulted in a similar dose-dependent effect. Inhibition was exerted by at least 10 nmol/L goat antibodies. Means with SEM of at least 4 independent measurements are plotted.

each assay allowed to correct for changes of the nonspecific binding (NSB) over time. The additional use of the positive control in the cutoff formula allowed for an even better normalization, because only the OD value of the positive control allows an assessment of assay sensitivity.

Assay Cutoff Point Value

The cutoff value was determined statistically based on the level of nonspecific background of the assay and the response of those matrix samples, above which a positive response was detected. In 3 independent experiments, serum samples from 20 healthy volunteers were examined. The mean $+2.0 \times SD$ was calculated to determine the cutoff. To account for some smaller variation between individual assays, an adjusted cutoff value was calculated by multiplying with a specific normalization factor, determined from the prestudy validation data.

Sensitivity

Assay sensitivity was determined as the concentration at which the antibody preparation produced an assay readout equal to the cutoff value. Because it was so far not possible to purify human anti- $\beta 1$ -AR antibodies sufficiently from patient sera, the assay sensitivity was determined by using the polyclonal goat anti- $\beta 1$ -AR antibody, as described before. The cutoff value was determined at approximately 10 nmol/L.

Recovery

To determine recovery, 20 plasma samples from healthy volunteers were spiked with rat 13F6 anti $\beta 1$ -AR antibodies (assay concentration: 760 nmol/L). All 20 samples showed inhibition values above the cutoff point value, with mean coefficients of variation (CV) of 2.54%, and therefore completely fulfilled the criteria for recovery.

Precision

Intra-assay (repeatability) and interassay (intermediate precision) variability was evaluated by using a validation sample (VS) and a PC, both spiked with rat 13F6 antibodies at an assay concentration of 253 nmol/L and 760 nmol/L, respectively. Four replicates were used on each plate, which were carried out on 3 different days. We found a mean intra-assay CV of 4.8% and an interassay CV of 16.2% for the VS and a mean intra-assay CV of 3.6% and an interassay CV of 15.4% for the PC, respectively.

Measurement of the 167 human DCM serum samples and 110 age-matched volunteers in 3 independent measurements resulted in a mean interassay CV of 14.4% for the patient group and of 16.9% for the control group.

Stability

Storage conditions and blood serum sample stability was investigated for the VS. Storage at either 22°C for 3 hours or at 4°C for 16 hours had no negative impact on the measurement of rat 13F6 anti- $\beta 1$ -AR antibodies and resulted in 95.1% and 92.3% recovery compared with the unstressed VS. Also, 3 \times repeated freeze/thaw cycles had no influence on the results of the VS.

Additionally, the stability of anti- $\beta 1$ -AR antibody determination was analyzed in whole blood samples. Ten DCM samples, which were tested positive for anti- $\beta 1$ -AR antibodies, were stored at 20°C for 20 hours and analyzed again. A

recovery of 94.7% (SD \pm 10.4) was determined, thus showing a high antibody stability in whole blood comparable to the stability in serum.

Screening Results of DCM Versus Volunteers and Hypertensive Heart Disease

We evaluated the presence of anti- $\beta 1$ -AR antibodies in 167 DCM patients presenting with a left ventricular ejection fraction (LVEF) $<45\%$ and of 110 age-matched volunteers who reported no known heart disease on blood sampling. In the DCM group, we identified 62.2% of these samples to be positive for relevant anti- $\beta 1$ -AR antibodies and only 8.2% in the age-matched control group (Figure 5A). In a second consecutive study, we also investigated 35 patients with hypertensive heart disease (HHD) and preserved LVEF. In contrast, in the somewhat older HHD patient population, we identified 17% of these samples to be positive (Figure 5C).

To demonstrate that the inhibition values which were determined in the cellular ELISA were actually due to antibodies, 20 anti- $\beta 1$ -AR antibody-positive DCM sera were depleted via Protein G Sepharose to eliminate IgG immunoglobulins. The flow-through from each serum sample was collected and analyzed in comparison to the load (untreated serum) by cellular ELISA. We observed that ELISA-determined anti- $\beta 1$ -AR titers disappeared completely in all investigated antibody-depleted samples (nominal mean Inhibition % was reduced from 13.1% to -31.1% , Figure 6).

Peptide-Based ELISA

A widely used method for determination of anti- $\beta 1$ receptor antibodies in human serum is a peptide-based ELISA, in which a 26-meric peptide (HWWRAESDEARRCYNDPKCCDFVTNR), corresponding to the amino acid sequence (residues 197–222) of the second extracellular loop of the human $\beta 1$ receptor, is immobilized on plastic surfaces to capture specific human anti- $\beta 1$ receptor antibodies. We conducted the same method to clarify the potential as a diagnostic tool for this ELISA assay.

Identical serum samples from DCM and age-matched volunteers were analyzed as used in the cellular SF9 $\beta 1$ -AR ELISA assays.

We observed 29.9% anti- $\beta 1$ -AR antibody positive DCM patients versus 35.5% positive findings in the control group (Figure 5B).

Discussion

Autoantibodies directed against $\beta 1$ -adrenergic receptors seem to play a important role in the pathogenesis of heart failure, particularly in the pathogenesis of DCM and Chagas heart disease.^{1,3,6,8,9} Putative mechanisms by which anti- $\beta 1$ -AR autoantibodies exert their adverse effects on cardiomyocytes are complex and have been intensively investigated over the past 2 decades.

In the present study, we report the establishment of a novel cell-based competitive ELISA for the detection of functionally active human anti- $\beta 1$ -AR autoantibodies. The assay uses the fully native $\beta 1$ -AR protein as target antigen to provide a correct folding of the extracellular domains, which is a basic

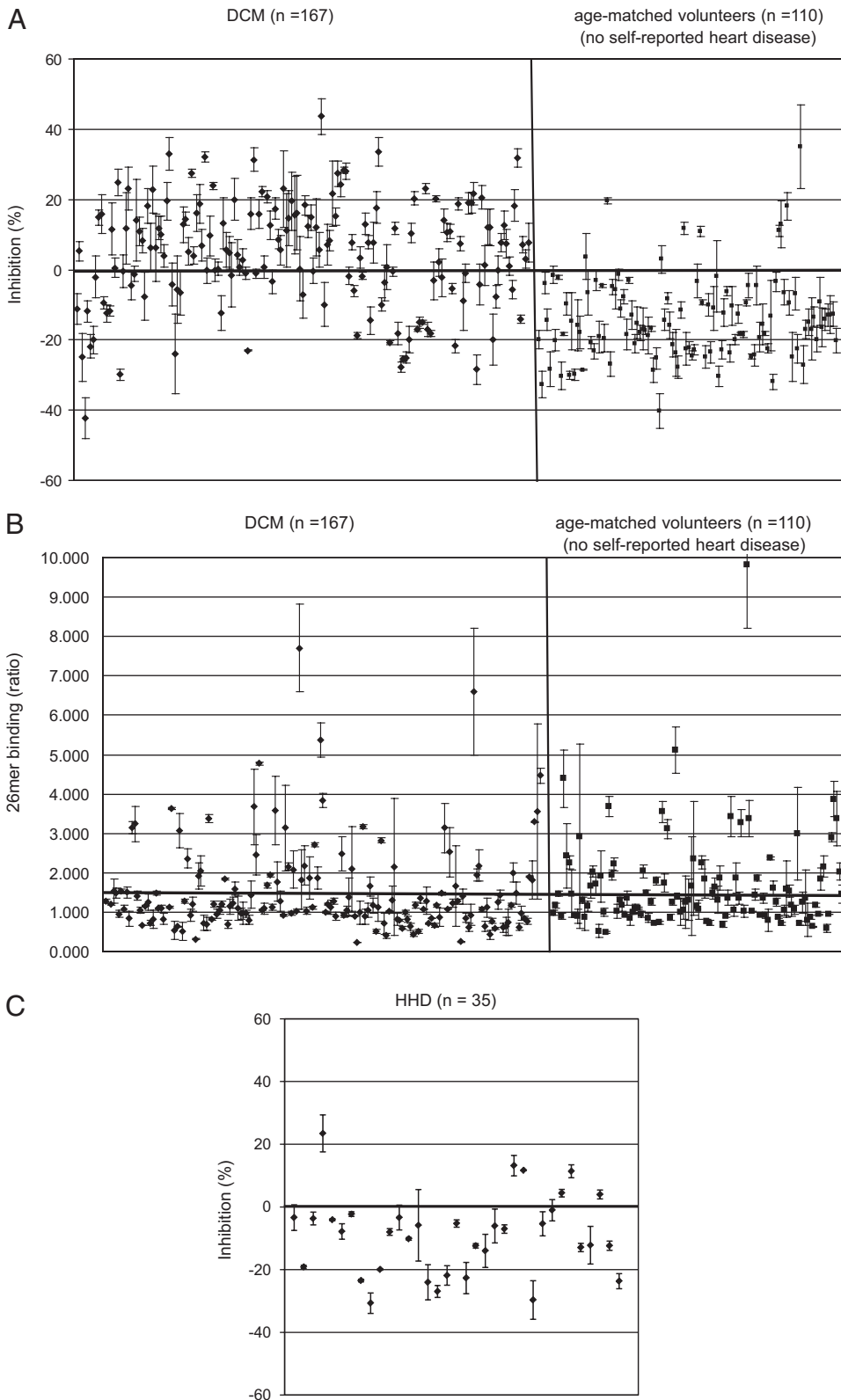


Figure 5. A, Overview of the β 1-AR binding activity of DCM patients (n=167) and control subjects who did not report any known heart disease (n=110) using an ELISA with SF9 cells overexpressing β 1-AR versus control SF9 cells (negative for β 1-AR). The binding activity was determined by measuring the competition with the monoclonal anti- β 1-AR antibody 23-6-7. Means with SEM of 3 independent measurements are plotted. **B, Identical serum samples were analyzed against the 26-mer peptide.** Binding activity was calculated as a ratio (sample OD to 26 mer per sample OD to control well). An anti- β 1-AR antibody-positive score was defined as a ratio of >1.5. Means with SEM of 2 independent measurements are plotted. **C, Overview of the β 1-AR binding activity of HHD patients (n=35), using an ELISA with SF9 cells overexpressing β 1-AR versus control SF9 cells (negative for β 1-AR).**

requirement to identify epitope-specific autoantibodies. To optimize the specificity of the assay, a competitive approach was developed using the monoclonal anti- β 1-AR antibody 23-6-7. This antibody binds to the second extracellular loop of the human β 1-AR and is able to stimulate receptor activity. Functionally relevant human anti- β 1-AR autoantibodies

from patient sera are characterized by their capacity to bind to the same or overlapping epitopes and displace this monoclonal antibody and therefore reduce the ELISA signal. An epitope search by alanine permutation scanning has yielded hints that within the ECII loop of the β 1-AR, the amino acid sequence NDPK should be part of the relevant epitope.

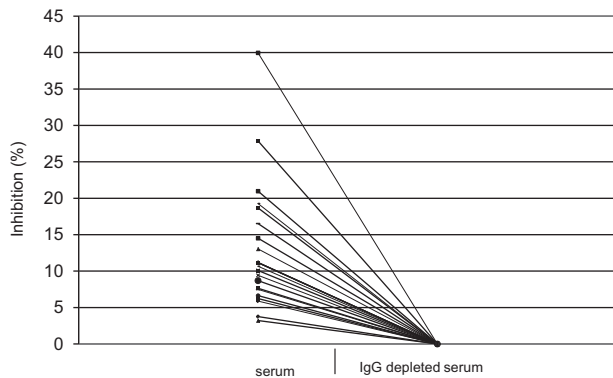


Figure 6. Comparison of the inhibitory effect of unaltered sera and the respective antibody-depleted serum fractions. The 20 original serum samples were tested positive, with a mean inhibition value of 13.1%. In contrast, all protein G-treated samples were tested negative, with a mean inhibition value below cutoff value.

As opposed to classic β_1 -AR agonists such as catecholamines, which bind to the typical receptor binding pocket, the agonistic anti- β_1 -AR autoantibodies bind to the second extracellular loop of the receptor molecule. This atypical binding site is characterized by allosteric binding kinetics, in which no high or low affinity components can be distinguished. This finding was reconfirmed in our experiments which documented a single-phase binding plot of the agonistic monoclonal antibody 23-6-7 (see Figure 2). The absolute receptor densities were counterchecked by radioligand binding, which identified an MOI of 2 active viral particles as sufficient to generate a maximum receptor density on infected sf9 cells. The assay was best reproducible at an MOI of 6 active virus particles, which was therefore used for establishing the assay.

Previous studies used several other screening methods for detecting anti- β_1 -AR autoantibodies. Autoantibodies against the second extracellular loop of the β_1 -AR were found in 30% to 75% of DCM patients in these previous studies.^{1,3,6} The inconsistency among studies may be due to the lack of standardized measurement methods. The definition of antibody positivity depended on divergent screening methods. These assay approaches can be divided into 2 classes: (1) assays that investigate the functional capacity of the antibodies to activate β_1 -AR and (2) assays that analyze the binding characteristics of the autoantibodies to the ECII loop of the β_1 -AR.

Functional assays, which measured, for example, contractility effects on neonatal rat cardiomyocytes or chick embryo hearts or β_1 -AR-mediated cAMP accumulation, were established and adapted to detect functional anti- β_1 -AR antibodies.^{5,6,13–15} All these functional assays are characterized by procedures that are time- and cost-consuming or difficult to standardize and cannot be reasonably used to screen larger patient populations ($n > 1000$) rapidly.

Binding of human anti- β_1 -AR autoantibodies was mostly investigated by using peptide-based ELISAs. To this end, a linear 26-meric peptide, which corresponds to the 2nd extracellular loop (amino acid position 197–222) of the human β_1 -AR, was immobilized onto microtiter plates.^{16,17} This kind of assay is fully high-throughput screening adapted, but its

use as a screening assay with diagnostic relevance had not yet been simultaneously investigated in a larger population of patients and age-matched control subjects who did not report any known heart disease.

The present study represents a new approach to systematically compare these populations on a strictly age-matched basis. Another previous publication had investigated healthy subjects using the same 26-meric β_1 -AR-ECII peptide-based ELISA¹⁸ and found 10% false-positives, but the mean age of that control group was markedly lower (36.6 years) than that of the investigated DCM patient group (55.7 years). These authors¹⁸ as well as many other researchers¹⁹ have found that the number of false-positive biomarker results markedly increases with increasing age of the investigated control population, and this also corresponds to our own experience with several anti- β_1 -AR antibody ELISA assays, so that we would think that our study has the advantage to strictly control for this important parameter. In contrast, in another, even older patient population with HHD, but preserved LVEF, we found a somewhat increased prevalence compared with the control group. This low prevalence for patients who did not present with clinically overt heart failure compares with the results of previous studies.⁵

Another interesting question is the prevalence of autoantibodies in patients with heart failure due to severe coronary artery disease (“ischemic cardiomyopathy,” ICM). This question should be investigated in future studies.

The novel whole cell-based competition ELISA yielded a markedly lower rate of false-positives compared with peptide-based assay. We tested the same DCM and age-matched control population in a simple 26-meric binding assay. We found that 29.9% of DCM patients and 35.5% of age-matched volunteers were tested positive. The result for the DCM group is within the range of data from other studies which investigated anti- β_1 -AR antibodies by using peptide-based ELISAs.^{16,20,21} We suppose that some of the positive findings obtained in the control group are due to cases of existing but yet undiagnosed heart failure, since the prevalence of undiagnosed heart failure with impaired cardiac LVEF (EF $< 35\%$) is about 7.7%; 4% of randomly selected asymptomatic men over 40 years even had an EF $< 30\%$.²² The health status in the volunteers of the present study was checked by a self-reporting questionnaire; an echocardiographic recording of LV function was not feasible for the whole population. Additionally, a number of the results in this group are very probably due to false-positive sera: this percentage seems to be in the lower single-digit range for the novel assay. In contrast, it amounted to about 26% (31% minus about 5% assumed cases of yet undiagnosed heart failure) in the peptide-based assay. Thus, the percentage of false-positives obtained with the novel cell-based competition ELISA compares quite well to what is found with even the most advanced assays for anti-TSH antibodies in Graves disease,²³ whereas it seems to be rather too high for the peptide-based assay.

To verify the assay specification, we conducted a validation procedure according to “good laboratory practice.” Besides the determination of the cutoff values, the precision and reproducibility were verified in detail. In summary, the

assay shows high accuracy (intra-assay comparison as well as in the interassay assessment) with CV values <20% for positive control and validation samples.

The novel approach to use a monoclonal antibody that competes with human anti- β 1-AR antibodies for immobilized native β 1-ARs was conceived very much in parallel to a widely distributed ELISA that measures functionally active, stimulating autoantibodies against the thyroid stimulating hormone (TSH) receptor in Graves disease. This so-called 3rd-generation ELISA has shown the highest sensitivity and robustness and is now commonly used as a gold standard diagnostic assay.^{24,25} The ELISA uses the specific activating monoclonal anti-TSHR antibody M22 and the fully human TSH receptor protein expressed on recombinant cells and has largely replaced the previously used radioimmunoassay (RIA), which was based on a competition for the natural ligand TSH.²⁶ A prerequisite for both RIA and ELISA was the use of the native fully expressed receptor, which provided a conformationally active epitope.

In contrast, several attempts to establish large scale ELISAs based on peptides from the TSH receptor failed. Also, approaches that assessed the direct binding of human serum samples to the recombinant TSH receptor protein were unreliable.²⁷ These findings are very much in line to our results on anti- β 1-AR antibodies.

Similarly, autoantibodies against the acetylcholine receptor that occur in myasthenia gravis are most reliably determined by an assay which uses the full α -bungarotoxin-labeled receptor protein as an antigen.²⁸

The novel assay holds promise to identify especially well those DCM patients who should benefit most from removal of anti- β 1-AR autoantibodies. To this end, several therapeutic approaches have been developed: Several clinical trials with DCM patients showed that elimination of anti- β 1-AR antibodies by immunabsorption (IA) led to an increase in several cardiac functional parameters, followed by an improvement of the New York Heart Association functional class.²⁹ Most notably, these studies found a significant improvement in LVEF for both nonselective antibody removal and selective anti- β 1-AR antibody removal using β 1-AR-peptide-coated columns,^{29–31} inferring that anti- β 1-AR antibodies are the disease-relevant antibodies.

Another approach to lower cardiac autoantibodies is the use of a peptide to induce antigen-specific tolerance and to reduce the response of an overactive immune system, which is currently in clinical development.³²

Both treatment strategies share the need of a reliable diagnostic assay to screen for anti- β 1-AR autoantibodies and thus to reliably identify antibody-positive heart failure patients. It was the scope of the present study to define the experimental basis for future clinical studies, which will have to include larger patient numbers to finally assess the relevance of this parameter as a prognostic marker in heart failure.

Conclusion

We have established a simple and efficient screening assay that identifies relevant autoantibodies directed against the β 1-AR in human sera. These autoantibodies can be detected

and quantified with high reproducibility in a high throughput screening approach. The assay was validated according to “good laboratory practice” and now serves as a companion biodiagnostic assay to develop and control individualized therapies in antibody-positive patients. It can be easily and reproducibly established in any laboratory in the world. Future modifications could include the use of permanently β 1-AR-overexpressing cell lines or of membrane preparations of β 1-AR-overexpressing cells.

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Disclosures

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Novelty and Significance

What Is Known?

- Autoantibodies against β_1 -adrenergic receptors (β_1 -ARs) may contribute to heart failure, such as dilated cardiomyopathy (DCM) and Chagas heart disease.
- Several diagnostic ELISA assays have been used, mostly based on coated peptides.

What New Information Does This Article Contribute?

- A new assay was designed in analogy to the anti-TSH receptor antibody ELISA (Graves disease).
- The full-length β_1 -AR protein is used as the binding target.
- β_1 -AR autoantibodies are detected by competition with a specific monoclonal antibody directed against the functionally relevant epitope.
- The assay had high reproducibility and CV values comparable to other standard assays.
- The ELISA can serve as a companion biodiagnostic assay for the development of novel therapeutic approaches.

Autoantibodies directed against the second extracellular loop of the β_1 -AR are thought to contribute to the pathogenesis of DCM and Chagas heart disease. We analyzed sera from DCM patients and from age-matched volunteers with an often used a peptide-ELISA approach and compared it with a novel whole cell-based ELISA, using cells expressing the full transgene for the human β_1 -AR. This assay was designed according to the most reliable anti-TSH receptor antibody ELISA used to diagnose Graves disease (“third-generation assay”). It detects the target antibodies by competition with a specific monoclonal antibody directed against the functionally relevant β_1 -AR epitope.

Anti- β_1 -AR antibodies were detected in $\approx 60\%$ of DCM patients and in $\approx 8\%$ of healthy volunteers. The novel assay yielded a high reproducibility in high throughput screening and a low number of false-positives. These features render it superior to the existing assays. The assay was validated according to “good laboratory practice” and can serve as a companion biodiagnostic assay for the development and evaluation of antibody-directed therapies in antibody-positive heart failure.