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Authors

De La Rosa, Rosemarie
Vazquez, Sergio
Tachachartvanich, Phum
et al.

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Cell-Based Bioassay to Screen Environmental Chemicals and Human Serum for Total Glucocorticogenic Activity

Rosemarie de la Rosa[1,*], **Sergio Vazquez**[1], **Phum Tachachartvanich**[1], **Sarah I. Daniels**[1], **Fenna Sillé**[2], **Martyn T. Smith**[1]

[1]Division of Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA

[2]Department of Environmental Health and Engineering, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD

Abstract

Glucocorticoids (GCs) are steroid hormones that have systemic effects that are mediated by the glucocorticoid receptor (GR). Environmental chemicals that disrupt GR signaling and/or GC homeostasis could adversely affect the health of human and non-human vertebrates. A major challenge in identifying environmental chemicals that alter GR signaling and/or GC homeostasis is a lack of adequate screening methods. Here, we developed a cell-based bioassay to measure total glucocorticogenic activity (TGA) of environmental chemicals and human serum. Human MDA-MB-231 breast cancer cells were stably transfected with a luciferase reporter gene driven by three tandem glucocorticoid-response elements. Dose-response curves for 6 GCs and 4 non-GC steroid hormones were generated to evaluate specificity of the bioassay. Cells were also optimized to measure TGA of 176 structurally diverse environmental chemicals and human serum samples in a high-throughput format. Reporter activity was GC specific and induced 400-fold by 1 μM dexamethasone. Furthermore, three of the screened chemicals (3,4,4'-trichlorocarbanilide, isopropyl-N-phenylcarbamate, and benzothiazole derivative 2-(4-chlorophenyl)-benzothiazole) potentiated cortisol-induced GR activity. Serum TGA estimates from the bioassay were highly correlated with a cortisol enzyme-linked immunosorbent assay. This work establishes an *in vitro* method to rapidly screen environmental chemicals and human serum for altered glucocorticogenic activity. Future studies can utilize this tool to quantify the joint effect of endogenous GCs and environmental chemicals.

Keywords

biomarkers; endocrine disruptors; in vitro toxicology; stress response; environmental toxicology

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[*]Address correspondence to rmd1025@berkeley.edu.

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INTRODUCTION

Glucocorticoids are steroid hormones that affect the cardiovascular, metabolic immune, reproductive, and central nervous systems (Sapolsky et al. 2000). GC secretion is regulated by the hypothalamus-pituitary-adrenal (HPA) axis in a circadian and stress-associated manner (Biddie et al. 2012). Cortisol is the predominant GC produced by humans and its excess production is associated with multiple chronic diseases, such as atherosclerosis, diabetes, and depression (McEwen 1998). The physiological effects of GCs are mediated by the glucocorticoid receptor (GR), which is expressed in nearly every cell of the body (Pujols et al. 2002). The ubiquitous expression of GR in almost all human tissues highlights the importance of this biological pathway for human health. Upon ligand-binding, GR undergoes a conformational change that allows it to dissociate from the heat-shock protein complex and translocate to the nucleus where it activates transcription of genes containing glucocorticoid response elements (GREs) (Evans 1988). GR influences transcription of 10-20 percent of the human genome (Oakley and Cidlowski 2013). Additionally, GR is highly conserved across vertebrates (Stolte et al. 2006). Consequently, altered GR signaling can have drastic effects on gene transcription profiles and cellular function in humans and non-human vertebrates.

Various xenobiotic compounds modify human GR signaling (Odermatt and Gumy 2008; Gulliver 2017). For example, synthetic GCs are often prescribed to treat many inflammatory and autoimmune diseases (Coutinho and Chapman 2011). Synthetic GCs have also been detected in waste and surface water samples collected globally, suggesting broad environmental exposure to these compounds (Schriks et al. 2010; Kolkman et al. 2013; Macikova et al. 2014; Suzuki et al. 2015; Jia et al. 2016). Macikova et al. estimated a 0.9–83 ng/mL increase in glucocorticoid plasma levels among fish exposed to river water in Switzerland and the Czech Republic (Macikova et al. 2014). In addition to synthetic GCs, *in silico* and *in vitro* methods have also demonstrated that environmental compounds can bind and affect GR transcriptional activity. Some examples include organochlorine and pyrethroid pesticides, parabens, phthalates, bisphenols, and organotins (Gumy et al. 2008; Kolšek et al. 2014; Zhang et al. 2016, 2017; Kojima et al. 2019). Furthermore, the Tox21 program tested over 8000 chemicals and identified 569 potential agonists and 472 antagonists of GR (US EPA 2017). Environmental exposures can also influence human GR signaling in target cells by modifying circulating cortisol levels. There is some evidence that environmental exposures, such as lead and organophosphate pesticides, can alter basal cortisol levels in humans (Fortin Marie C. et al. 2012; Cecchi et al. 2012; Braun et al. 2014; Tamayo y Ortiz et al. 2016). However, the number of epidemiological studies examining this relationship remains limited.

Cell-based bioassays are one approach to identify environmental chemicals that impact human GR signaling. This method relies on cells that contain a reporter gene driven by a GRE, which produces a measurable response proportional to the degree of GR activation. A luciferase reporter model is frequently used since the assay is rapid, simple, relatively inexpensive, sensitive, and has a broad linear range (Smale 2010). Not only has this technology helped to identify chemicals that interfere with receptor-mediated effects, but studies have also demonstrated that GR bioassays can quantify total glucocorticogenic

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activity (TGA) in human serum and plasma (Raivio et al. 2002; Vermeer et al. 2003; Kajantie et al. 2004; Turner et al. 2010; Perogamvros et al. 2011; Fejerman et al. 2016). While these studies highlight that cell-based bioassays can be used to detect differences in serum TGA, they are not without limitations. For instance, most bioassays were generated with mammalian cell lines that lack endogenous GR expression (e.g. COS-7, U2OS, HEK293, and CV-1) (Sedlák et al. 2011; Campana et al. 2015). Cellular context is important since the absence of cell-specific co-regulators could impact GR transcriptional activity (Weikum et al. 2017). Furthermore, the high level of homology between the glucocorticoid, mineralocorticoid, androgen, and progesterone receptors makes it particularly difficult to design specific bioassays (Kino 2017). For example, the MDA-Kb2 cell line is a commercially available bioassay derived from human breast cancer cells that responds to both androgens and GCs (Wilson et al. 2002). These bioassays could be improved by selecting a cell line devoid of homologous nuclear receptors and that endogenously expresses GR to preserve cellular context and attain specificity.

This paper describes the development of a novel GR bioassay to measure TGA of environmental chemicals and human serum. We stably transfected MDA-MB-231, a triple negative breast cancer cell line, with a luciferase reporter gene driven by three tandem GREs. These cells endogenously express high levels of GR and lack the androgen and progesterone receptors (Horwitz et al. 1978). We first characterized specificity of the bioassay by testing the dose-response of 6 GCs, a GR antagonist, and 4 non-GC steroid hormones. The bioassay was then optimized to measure TGA of 176 structurally diverse environmental chemicals using a high-throughput screening approach. Lastly, we developed a protocol to measure TGA of human serum samples. These results were also compared to estimates from a cortisol enzyme-linked immunosorbent assay (ELISA) and the MDA-Kb2 bioassay. Our work establishes the first specific, breast cancer-derived GR bioassay and demonstrates two applications of this tool.

METHODS

Chemicals

Dexamethasone (cat. #D4902, CAS: 50-02-2), hydrocortisone (cortisol; cat. #H0888, CAS: 50-23-7), betamethasone (cat. #34166, CAS: 378-44-9), prednisolone (cat. #P6004, CAS: 50-24-8), triamcinolone (cat. #T6376, CAS: 124-94-7), corticosterone (cat. #27840, CAS: 50-22-6), mifepristone (cat. #M8046, CAS: 84371-65-3), aldosterone (cat. #A9477, CAS: 52-39-1), progesterone (cat. #P8783, CAS: 57-83-0), and hydroxyflutamide (cat. #H4166, CAS: 52806-53-8) were all purchased from Sigma-Aldrich. A dihydrotestosterone mimic (Cl-4AS-1; cat. #3812, CAS: 188589-66-4) and 17 β-estradiol (cat. # 2824, CAS: 50-28-2) were also obtained from Tocris Biosciences. Compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The chemical library was provided by Dr. Bruce Hammock (University of California, Davis) and is described in detail by (Morisseau et al. 2009). Briefly, the library contained 176 compounds obtained from Chem Service Inc. and Sigma Chemical Co at 10mM in DMSO. Chemicals included in the library plates are listed in Supplemental Material Table 1. All tested compounds had purity>95%.

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Cell culture

The human triple negative breast cancer cell line, MDA-MB-231, was obtained from the Cell Culture Facility at the University of California, Berkeley and authenticated using short tandem repeat profiling. MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) at 37°C in an incubator with 5% CO_2.

MDA-Kb2 (ATCC CRL-2713), a human triple negative breast cancer cell line, was obtained from the American Tissue Culture Collection (ATCC). MDA-Kb2 cells were cultured in Leibovitz's L-15 (L-15) (Gibco) supplemented with 10% FBS at 37 °C in an incubator without CO_2. One week prior to luciferase experiments, cells were maintained in phenol red-free L-15 supplemented with 10% charcoal-dextran FBS.

Stable transfection

The pGRE-Luc2P plasmid was provided by Dr. Zdenek Dvorak (Palacky University) and contained a luciferase reporter gene driven by three tandem GREs (Novotna et al. 2012). MDA-MB-231 cells were seeded at a density of 2.5×10^4 in a 60 mm culture dish and transfected the following day with 5ug of pGRE-Luc2P using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were placed on selection media containing 0.5mg/ml of hygromycin B (Calbiochem) 24 hours post-transfection. Selection media was changed every 2-3 days for two weeks. Surviving cells were cloned by limited dilution in 96-well plates and maintained on selection media for two more weeks. Clones were transferred to 24 well plates and kept under selection for an additional three weeks prior to assessing dexamethasone-induced luciferase activity. The clone with the largest fold-change and stable luciferase expression for 24 passages in the absence of antibiotic selection was renamed 231GRE. These cells were routinely cultured in DMEM supplemented with 10% FBS at 37°C in an incubator with 5% CO_2.

Screening total glucocorticogenic activity (TGA) of chemicals

One week prior to luciferase experiments, 231GRE cells were maintained in phenol red-free DMEM (Hyclone) supplemented with 10% charcoal-dextran FBS (Atlanta Biologicals) to reduce interference from hormones present in media. 231GRE cells were seeded at a density of 2.5×104 cells/well in white 96-well plates (Thermo Scientific Nunc). The next day, cells were incubated with chemical treatments for 18 hours at 37°C. Chemical treatments included: vehicle (DMSO, 0.1%), dexamethasone (10 pM-10 μM), cortisol (100 pM-100 μM), betamethasone (10 pM-10 μM), prednisolone (10 pM-10 μM), triamcinolone (10 pM-10 μM), corticosterone (100 pM-200 μM), aldosterone (10 pM-100 μM), estradiol (10 pM-10 μM), dihydrotestosterone (10 pM-10 μM), or progesterone (10 pM-10 μM). Cells were also treated with RU486 (10 pM-10 μM), a competitive antagonist, in the presence of 100nM dexamethasone. Following the incubation period, cells were rinsed with PBS and lysed (1x cell lysis buffer, Promega). Luciferase activity was measured using a Berthold Centro XS3 LB 960 microplate luminometer with automatic injection of Luciferase Assay Reagent (Promega). All chemical treatments were conducted in triplicate and repeated as three independent experiments. The chemical library screen was performed in part with the High-Throughput Screening Facility at UC Berkeley, which provided the Agilent V11 Bravo

Automated Liquid Handler and the Thermo Scientific Multidrop Combi Reagent Dispenser. The 10mM chemical library plate was diluted in DMSO to concentrations of 1 mM, 100 μM, 10 μM, and 1 μM with the Agilent V11 Bravo liquid handler. For the agonist screen, 2 μL of the chemical libraries were transferred to 998μL of phenol-red free DMEM using a liquid handler. This same instrument was used to transfer 100μL of diluted compounds to the assay plates, bring the final volume in each well to 200μL. All agonist assay plates included media only, negative control (0.1% v/v DMSO only), and positive control (100nM cortisol) wells. Cortisol was selected as a positive control since it is the endogenous GR ligand.

The library compounds were also screened in the presence of 100nM cortisol to test for antagonism. This concentration was selected based on the half-maximal response concentration (EC_{50}) of cortisol. For this antagonist screen, chemical libraries were diluted 1:250 in phenol-red free DMEM and 50μL of diluted compounds were transferred to assay plates. An additional 50μL of cortisol-containing media was dispensed to all wells at a final volume and concentration of 200μL and 100nM, respectively. Each antagonist assay plate included media only, DMSO only, 100nM cortisol, and 100nM RU486 control wells.

231GRE cells were dispensed into 96-well plates with a Multidrop Combi Reagent Dispenser (Thermo Scientific) and treated the following day. There were three replicate plates for each treatment. The final concentration of chemicals for both assays ranged from 1nM-10μM. All assay plates were covered with a rayon film (VWR) and incubated at 37°C for 18 hours prior to measuring luciferase activity with a BioTek Cytation 5 microplate reader.

Luminescence measured in negative control wells was averaged and subtracted from all values on the plate. Background corrected relative light units (RLUs) were then normalized by dividing by luminescence measured in the positive control well. Cytotoxic concentrations of compounds, assessed by PrestoBlue assay, were not included in the statistical analysis.

Cell viability assay

PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific) was used to evaluate cytotoxicity of all tested compounds. 231GRE cells were plated in black clear bottom 96-well plates (Thermo Fisher Scientific) and allowed to attach overnight. Cells were treated and incubated at 37°C for 18 hours. Afterwards, the media was removed and replaced with 100μL media containing PrestoBlue diluted 1:10 and incubated for two hours at 37°C before measuring fluorescence (excitation/emission: 560/590 nM) with a BioTek Cytation 5 microplate reader.

Measuring serum TGA

Serum samples were collected from healthy individuals four times approximately 2-4 months apart over a one-year period. A sample size of N=12 healthy subjects were included in the study (exclusion criteria for volunteer subjects were chronic illness or pregnancy at the time of blood draws). A single blood sample was obtained from fasted participants between the hours of 8-10AM. Serum was collected with glass BD Vacutainer® tubes and stored at −80°C until analysis. The Internal Review Board within the University of

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California Berkeley's Human Research Protection Program approved data collection for this study and informed consent was obtained from all participants.

231GRE cells were seeded at 2.7×10^4 cells/well in white 96-well plates and incubated at 37°C for 24 hours. Media was then removed and 100 μL of hormone-depleted media containing diluted human serum was added to wells in quadruplicate. Plates were incubated at 37°C overnight prior to measuring luciferase activity. Cortisol standards were included on each plate at the following concentrations: 0 (0.1% v/v DMSO only), 3.13, 6.25, 12.5, 25, 50nM. Readings for quadruplicate samples were averaged and converted to cortisol concentrations based on standard curves fit with a four parameter logistic regression. These concentrations were then multiplied by the dilution factor of 10 to obtain cortisol equivalent values (nM).

A similar protocol was used to screen human serum with MDA-Kb2 cells for comparison with 231GRE results. Details of this assay are described in Fejerman et al. 2016. Since the androgen receptor is highly expressed in MDA-Kb2 cells, treatments were performed in the presence of the androgen receptor antagonist, hydroxyflutamide (OHF), to distinguish between androgenic and glucocorticogenic activity. Briefly, cells were cultured in Leibovitz's-15 (L-15) medium with 10% FBS one week prior to plasma addition. External sources of androgens and glucocorticoids were then removed by treating cells for 1 week with L-15 medium containing 10% charcoal–dextran serum. Cells were seeded at 2.7×10^4 cells/well in white 96-well plates and incubated at 37°C for 24 hours. Media was then removed and 100 μL of hormone-depleted media containing diluted human serum was added to wells in quadruplicate. Serum from female and male subjects was diluted 10 and 40-fold, respectively, to limit androgen receptor activation. Cortisol standards were also included on each plate. Plates were incubated at 37°C overnight prior to measuring luciferase activity. Cortisol equivalent values were estimated for each sample using the cortisol standard curve and multiplying by the sample dilution factor.

Serum TGA from both GR cell-based assays were compared to cortisol concentrations measured by competitive enzyme-linked immunoassay (ELISA) (Cayman Chemical, Ann Arbor, MI). The assay was run according to standard manufacturer's instructions using human serum samples diluted 1:250 in ELISA buffer provided with the kit. Samples were run in duplicate and serum cortisol concentrations were calculated using a standard curve fit with a logistic four-parameter model. The intra-assay and inter-assay variability was 4.8% and 0.1%, respectively.

Statistics

Dose–response curves were fit with a four-parameter Hill function using the R drc package to obtain half-maximal activity concentration (AC_{50}) and maximum response values (Ritz et al. 2015). All positive hits in the chemical screen had a statistically significant AC_{50} <10μM. In the agonist screen, positive hits were defined as chemicals that induced a statistically significant increase in the maximum response parameter (p-value <0.05). Antagonists were defined as chemicals that inhibited activation of the reporter by cortisol. Chemicals that enhanced cortisol-induced reporter activity were also considered hits.

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Comparisons between human serum treatments and media controls were made using one-way ANOVA with Dunnet's post-hoc test. Differences with p-value <0.05 were considered statistically significant. A coefficient of variation (CV=100×standard deviation/mean) was computed for each serum sample to assess technical variability. Concordance correlation coefficients (and 95% confidence intervals) were calculated to compare cortisol equivalent values obtained by the bioassays to concentrations measured by ELISA. This estimate evaluates how far the observed data deviates from the line of perfect concordance. Values closer to 1 indicated very good agreement between the bioassay and ELISA results.

A random effects model was used to identify sources of variability in measured serum TGA. The interindividual, intraindividual, and within sample variability components were defined by the following equation:

$$Y_{hij} = \log(X_{hij}) = \mu_y + \alpha_h + \beta_i + \gamma_{hi} + \varepsilon_{hij}$$

for h=1,…,12 individuals; i= 1, 2, 3, 4 time points; and j= 1, 2, 3, 4 replicate samples, where X_{hij}= the serum TGA levels for the hth individual at the ith time point for the jth replicate and Y_{hij} represents the natural log transformation of X_{hij}. In this model, μ_y represents the true logged mean of serum TGA and α_h represents the random effect of the hth individual. β_i represents the time-specific random effect and γ_{hi} is the random effect of the hth individual at the ith timepoint. Lastly, ε_{hij} is the random-error effect of the jth replicate sample from the hth individual at the ith timepoint. It is assumed that α_h, β_i, γ_{hi}, and ε_{hij} are mutually independent and normally distributed with means of zero. The respective variance components of α_h, β_i, γ_{hi}, and ε_{hij} are σ_h^2 (intra-individual variability) σ_i^2 (variability over time), σ_{hi}^2 (intra-individual variability), and σ_e^2 (within sample + residual variability).

RESULTS

Characterization of 231GRE cell line

MDA-MB-231 cells were stably transfected with the pGRE-Luc2P reporter plasmid. Five monoclonal cell lines were generated and evaluated for dexamethasone-induced luciferase activity. Clone #7 exhibited the highest reporter activity in response to dexamethasone (10μM) with a fold change of 438 (Supplemental Material Figure 1). Luciferase activity was also detected in this clone for up to 24 passages, demonstrating stable integration of the reporter plasmid (Supplemental Material Figures 2). Therefore, clone #7 was renamed 231GRE based on its wide dynamic range and stability.

Specificity of the 231GRE cell line was then evaluated by testing multiple GCs and other non-GC steroid hormones (Figure 1). Dose-response curves were generated for six GCs: dexamethasone (DEX), cortisol, betamethasone (BMZ), prednisolone (PRED), triamcinolone (TAC), and corticosterone. All GCs induced luciferase activity and produced EC_{50} values comparable to previously published bioassays (Table 1). Dexamethasone-induced reporter activity was also suppressed by RU486, a GR antagonist. Aldosterone (ALDO), a mineralocorticoid hormone, elicited a response since it is a partial GR agonist. As expected, estradiol (E2), progesterone (PROG), and dihydrotestosterone (DHT) had no

effect on reporter activity. Collectively, these results demonstrate specificity of the 231GRE bioassay for GR ligands.

High-throughput screen of environmental chemicals

One potential application of 231GRE cells is high-throughput screening of environmental chemical libraries. 231GRE cells were treated with 10-fold serial dilutions of a library containing 176 structurally diverse environmental chemicals at concentrations ranging from 1nM-10μM. Cortisol (100nM) was the positive control and consistently induced GR activation. However, none of the environmental compounds produced a statistically significant increase in reporter activity (Supplemental Table 2). 231GRE cells were also treated with library compounds in the presence of 100nM cortisol to test for antagonism. Except for the positive control (RU486), none of the screened compounds inhibited cortisol-induced GR activity (Supplemental Table 3). Interestingly, three compounds (isopropyl-N-phenylcarbamate, 3,4,4'-trichlorocarbanilide, and 2-(4-chlorophenyl)-benzothiazole) potentiated the effect of 100nM cortisol (Figure 2, Supplemental Table 3). Although the chemical screen did not identify any GR agonists or antagonists, this approach indicated chemicals that enhanced cortisol-induced GR activity.

Measuring Serum TGA

The 231GRE bioassay was also optimized to measure TGA of human serum. Cells were treated with human serum obtained from a healthy individual that was diluted 10, 20, 30, 40 and 50-fold in cell culture media. All tested dilutions induced a statistically significant increase in reporter activity above the media only control (Figure 3). Relative luciferase units were then converted to cortisol equivalent values and directly compared to the concentration measured by ELISA (Table 2). The cortisol concentration was best estimated by serum diluted 10 and 20-fold. Variability in cortisol equivalent values was also lowest in the 1:10 serum dilution (CV=2.7%). Therefore, serum was diluted 10-fold for all other experiments.

Serum TGA was then measured in samples collected from twelve healthy individuals at four different time points over a one-year period with the 231GRE bioassay. Cortisol equivalent values obtained with 231GRE were also compared to estimates from the MDA-Kb2 bioassay and concentrations determined by ELISA (Figure 4). The concordance correlation coefficients (r_c) between the 231GRE and ELISA concentrations were all statistically significant and ranged from 0.63 to 0.92. However, concordance between MDA-Kb2 and ELISA concentrations was much lower (r_c range: 0.08-0.19) and yielded 95% confidence intervals that contained zero, indicating a lack of statistical significance. These comparisons demonstrate that serum cortisol concentrations were more closely approximated by the 231GRE bioassay than by MDA-Kb2.

The 231GRE bioassay was also used to identify sources of variability in serum TGA (Figure 5). Variability between replicates was minimal with intra-assay CVs ranging from 0.5-11.2% (median: 4.4%). A random effects model estimated that the inter-individual variability, time-specific variability, intra-individual variability, and within-sample variability were 63.36%, <0.01%, 34.67%, and 1.97%, respectively. These results demonstrate that serum TGA varied

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more between individuals than within the same individual over a one-year period and that technical variability of the 231GRE bioassay was remarkably low.

DISCUSSION

This paper describes the development of a specific and stable breast cancer-derived GR bioassay called 231GRE. We demonstrated that 231GRE cells were highly specific for GR ligands and did not respond to compounds from other steroid classes. Furthermore, all GR ligands had EC_{50} values comparable with those reported for other stable reporter cell lines. We also conducted a high-throughput screen of 176 environmental chemicals with 231GRE. None of the tested compounds were agonists or antagonists of GR. However, we identified three chemicals that potentiated cortisol-induced GR activity. Our work is also the first to use a GR-specific bioassay to measure TGA in human serum. ELISA cortisol concentrations were in high concordance with 231GRE estimates. Collectively, these results highlight two applications of the 231GRE cell line and how it could be used to measure the total effect of endogenous GCs and environmental chemicals present in human serum on GR signaling.

To our knowledge, this is the first specific GR bioassay generated in a breast cancer cell line. The only other available stable breast cancer-derived GR bioassay is the MDA-Kb2 cell line, which contains a reporter driven by the mouse mammary tumor virus (MMTV) promoter that responds to both androgens and GCs (Wilson et al. 2002). To overcome this limitation, we performed the stable transfection with a luciferase reporter gene driven by three tandem GREs, which provides greater specificity than the MMTV promoter. Additionally, MDA-MB-231 cells were selected for the bioassay since it endogenously expresses highly levels of GR and lacks homologous nuclear receptors, such as the progesterone and androgen receptors (Horwitz et al. 1978). MDA-MD-231 cells are frequently utilized in breast cancer research and were recently used to show how GR activation promotes breast cancer metastasis and reduced survival in mice (Obradovi et al. 2019). Therefore, our GR bioassay provides a valuable tool to evaluate the contribution of environmental exposures in the development and progression of breast cancer.

Our work identifies three environmental chemicals that potentiate the effect of cortisol on GR activity. For example, we observed that 3,4,4'-trichlorocarbanilide (triclocarban) increased the amount of reporter activity induced by 100nM cortisol. Another study conducted with MDA-Kb2 cells also found that 2μM triclocarban enhanced cortisol-induced GR activity . Triclocarban is an antimicrobial agent that was recently banned by the US Food and Drug Administration in 2016 from consumer antiseptic washes due to its endocrine disrupting effects. However, this compound is still allowed in household plastics, industrial cleaning and hospital supplies, and other personal care products not covered by the regulation (Halden et al. 2017). Based on our findings, future regulation of triclocarban and other industrial chemicals should consider the additive effect of these compounds on endogenous hormone signaling. Enhanced cortisol-induced GR activity was also observed with the carbamate herbicide isopropyl-N-phenylcarbamate (Propham) and the benzothiazole derivative 2-(4-chlorophenyl)-benzothiazole. Christodoulou et al. 2018 identified benzothiazole derivatives that influenced GR activity through allosteric binding. Moving forward, molecular docking is a promising approach to evaluate whether these

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compounds bind GR and modulate receptor-ligand interactions. Additionally, larger screens should be conducted to identify other environmental chemicals that enhance cortisol-induced GR signaling.

Although 231GRE cells did not identify any agonists or antagonists of GR in this small chemical library, cortisol and RU486 control wells produced consistent results on reporter activity across all plates demonstrating that the assay can be used to identify GR agonists and antagonists in a high-throughput setting. It is intriguing that chemicals previously shown to modulate GR activity had no effect when tested in 231GRE cells. For example, 142 chemicals included in the library that were also tested in Tox21. Of these, 17 and 21 compounds were active in Tox21 GR-BLA ratio agonist and antagonist assays, respectively. According to the CompTox Chemicals Dashboard, all agonist and most antagonists (except chlorodane and toxaphene) had AC50 values greater than cytotoxic concentrations and did not display activity below cytotoxic levels (Supplemental Material Table 4). We observed a 55% and 45% reduction in cortisol-induced activity with 10 μM of chlorodane and toxaphene but AC50 values were not statistically significant, possibly because this was the only concentration that reduced TGA. Additionally, pyrethroids (bifenthrin, λ-cyhalothrin, cypermethrin, resmethrin) and organochlorine pesticides (o,p′-DDT, p,p′-DDT, methoxychlor) antagonized GR transcriptional activation in a Chinese hamster ovarian cell line that contained a MMTV-luciferase reporter (Zhang et al. 2016). One possible explanation for this discrepancy is that we used AC50 values obtained from a dose response curve to define hits, whereas this study tested chemicals at a single 10 μM dose. A titration-based high-throughput screening approach has been shown to be more efficient for large chemical libraries since single-concentration screens often produce false positives and false negatives (Inglese et al. 2006). Supplemental Material Table 4 shows that resmethrin, methoxychlor, and p,p’-DDT were also active antagonists in Tox21 but had AC50 values above cytotoxic level, suggesting that these compound only inhibit GR activity at concentrations 10μM.” Zhang et al. also showed that various pesticides had differential effects on downstream GR target genes, even within the same cell line. Therefore, the effect of chemicals on GR transcription may vary by gene promoter. It is also possible that the 231GRE bioassay lacks certain cofactors that influence the effect of chemicals on GR transcriptional activity. However, additional studies are needed to evaluate the influence of environmental chemicals on GR signaling across multiple cell types.

Our work provides the field with a tool to specifically measure TGA of human serum. This technique is a rapid, sensitive, and cost-effective method to quantify the total effect of endogenous GCs present in serum (e.g. cortisol and corticosterone) on GR activity. Serum cortisol concentrations are routinely measured using antibody-based methods, such as enzyme-linked immunosorbent assay (ELISA), or by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). While both of these techniques quantify the amount of a specific compound present in a biospecimen, they often require sample preparation, are expensive, and do not measure the biological effect of exogenous and endogenous compounds present in serum (Xu et al. 2014). Alternatively, several studies thus far have used cell-based bioassays to detect differences in serum TGA (Raivio et al. 2002; Vermeer et al. 2003; Kajantie et al. 2004; Turner et al. 2010; Perogamvros et al. 2011; Fejerman et al. 2016). For example, elevated serum TGA were detected after synthetic GC

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administration (Raivio et al. 2002). Therefore, cell-based bioassays such as ours can be used to quantify the overall net effect of both endogenous and exogenous molecules present in human serum. This method can also be coupled with other analytical approaches (e.g. LC-MS/MS) to identify environmental GR agonists and antagonists present in human serum (Smith et al. 2015; Smith et al. 2019).

Comparisons with a cortisol ELISA demonstrated that plasma concentrations were more closely approximated by 231GRE than MDA-Kb2. It should be noted that although concordance between MDA-Kb2 and ELISA was relatively low (r_c=0.14), these two assays were still highly correlated (r=0.6). This means that both bioassays can be used to infer relative differences in serum TGA, but absolute differences are more accurately estimated by 231GRE. Our results also suggest that 231GRE may be more sensitive to GR activation than MDA-Kb2. However, the reason for this is unclear. There is some evidence that the androgen receptor (AR) and GR influence each other's transcriptional activity (Chen et al. 1997). Therefore, androgens present in serum and/or hydroxyflutamide might interfere with GR activation in MDA-Kb2, since these cells also express AR.

Characterizing sources of variation in biomarkers is important when designing epidemiological studies. Therefore, we measured sources of variability in serum TGA levels using samples collected from healthy adults. Intra-assay CVs were below 15% for all tested serum samples indicating low technical variability of the 231GRE assay. Reproducibility of the bioassay was further supported by the fact that within-sample variance only accounted for <2% of variability in serum TGA. Interestingly, serum TGA varied less within the same individual (intra-individual) than between individuals (inter-individual). This result corresponds with previous research that reported a high level of individual stability in morning cortisol concentrations (Huizenga et al. 1998). Evidence from twin studies suggests that genetic factors may play a role in regulating morning cortisol levels (Maxwell et al. 1969; Meikle et al. 1988). Consequently, the high amount of inter-individual variability in serum TGA may reflect genetic differences in cortisol secretion and regulation between individuals. Collectively, these results suggest that our bioassay is reproducible and can be used to measure serum TGA in epidemiological studies.

In summary, we developed a novel method that can be used to identify environmental chemicals that modulate GR transcriptional activity and to screen human serum samples for differences in TGA. This low-cost and rapid method can be used to monitor glucocorticoid levels in humans and the broader environment (potentially fish plasma and water samples). Future studies can also use this assay to predict mixture effects of GR ligands since TGA is an effect-based measure of all active compounds present in a matrix. Given the significance of the GR pathway in human health and in development of disease, greater emphasis should be placed on identifying environmental chemicals that perturb GR signaling and GC homeostasis.

Supplementary Material

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Data availability statement

Data, associated metadata, and calculation tools are available from the corresponding author (rmd1025@berkeley.edu).

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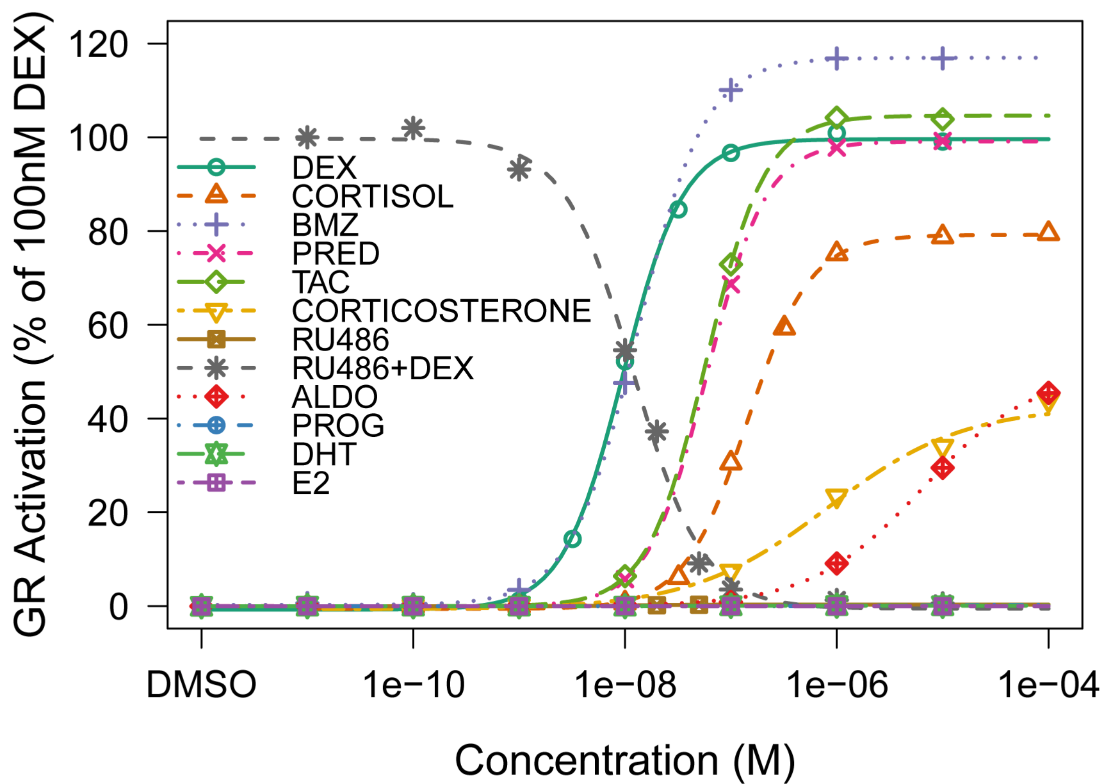

Figure 1.

Dose-response curves of glucocorticoid receptor ligands and other steroid hormones. Plots represent mean data from three independent experiments (N=3) that were fit with a 4-parameter hill function.

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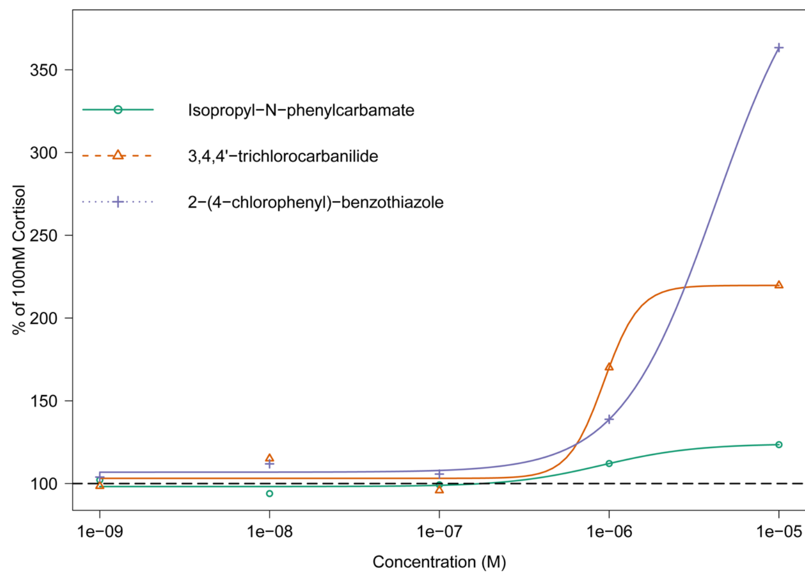

Figure 2.
Dose-response curves for chemicals that enhanced cortisol-induced GC activity. Data from triplicate wells for each concentration were used to plot 4-parameter Hill functions. The dashed line represents baseline activity of cortisol alone (100%).

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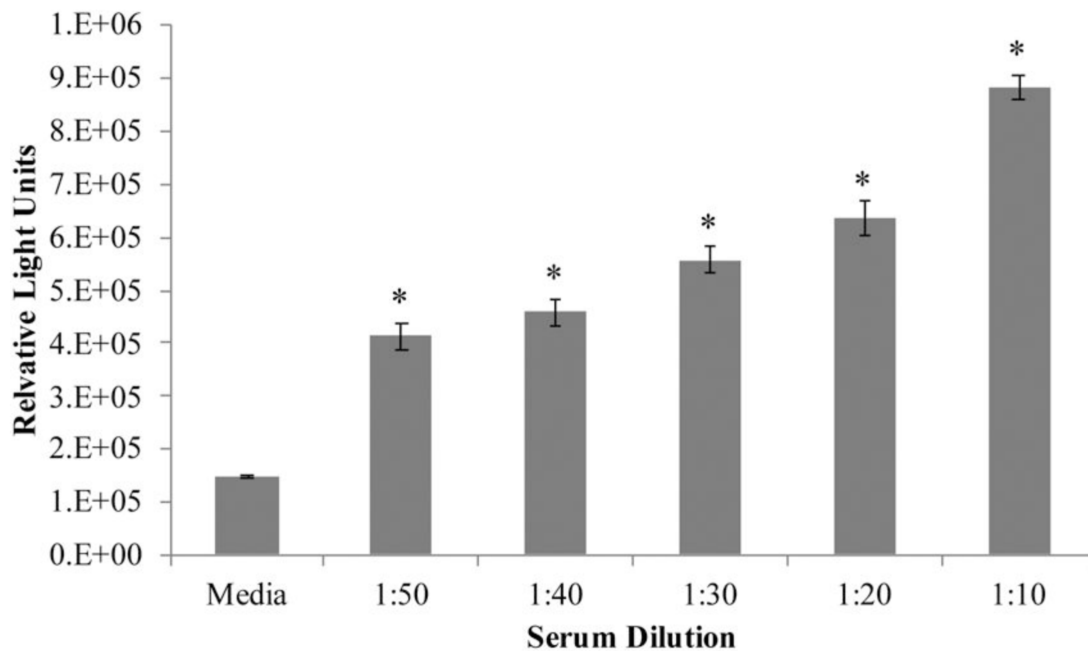

Figure 3.

Response of 231GRE cells treated with diluted human serum. The bars represent the average relative luciferase units measured for each dilution factor. Error bars represent the standard error of the mean (SEM) of quadruplicate wells. * Indicates a statistically significant difference between serum dilution and media only control (P-value <0.05).

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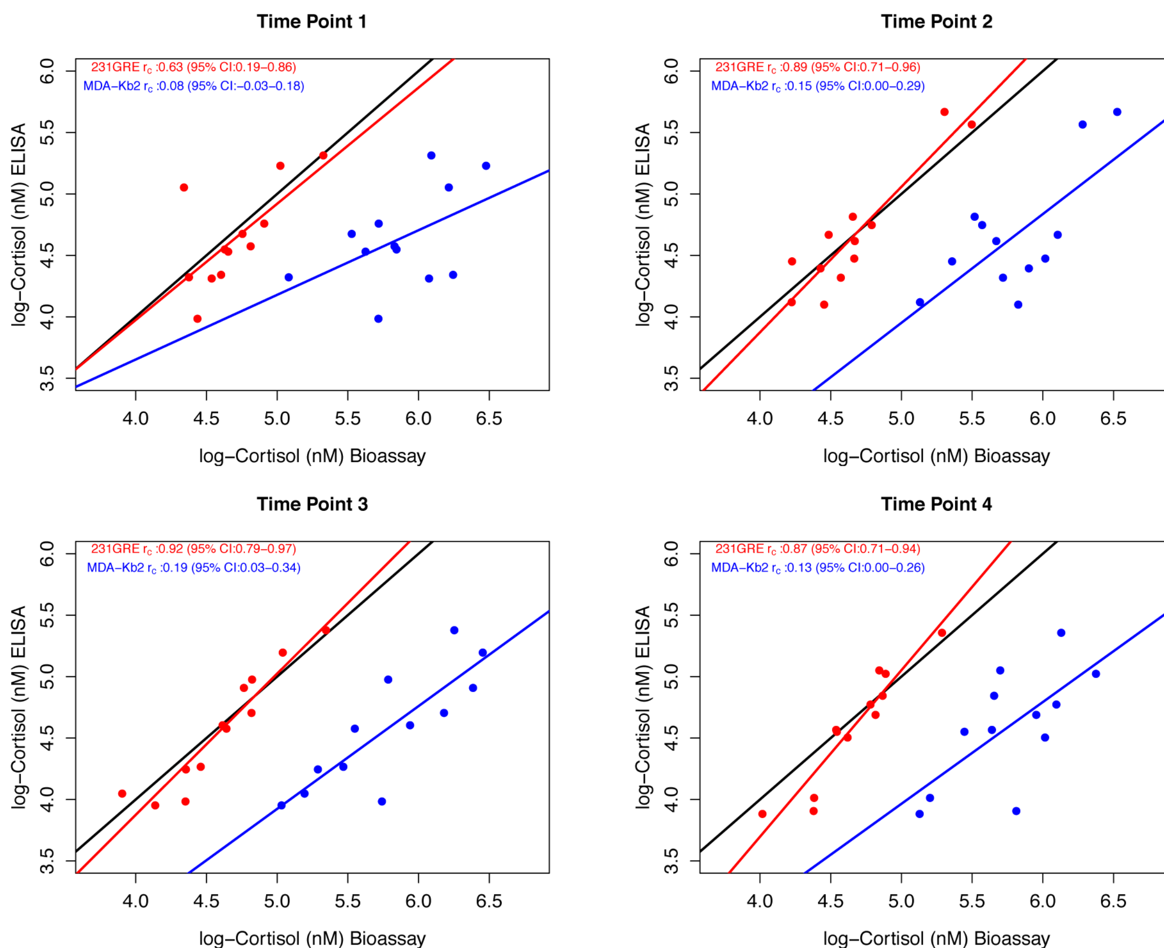

Figure 4.

Correlation between serum GC activity and cortisol ELISA concentrations for each time point. Cortisol equivalent values from the bioassays were plotted on the x-axis and ELISA concentrations on the y-axis. All concentrations are on the logarithmic scale. Fit lines for 231GRE (red) and MDA-Kb2 (blue) were also plotted and compared to the black identity line (y=x). Concordance correlation coefficients for each bioassay are included in the plot legends.

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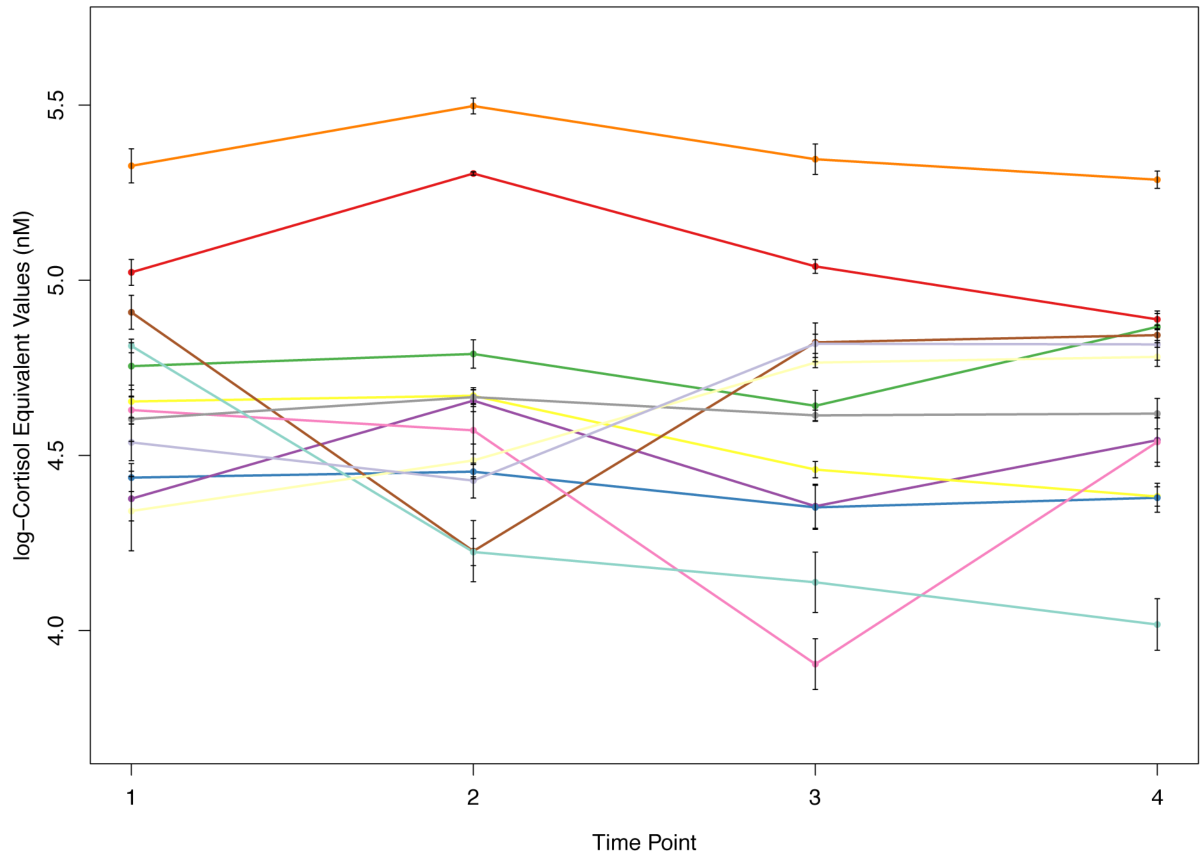

Figure 5.

Variability in serum GC activity. Cortisol equivalent values were plotted by time point. Each line represents a different individual. Error bars represent the standard deviation of quadruplicate wells.

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Table 1.

Comparison of EC_{50} (M) values for GR ligands between 231GRE and other published stable GR bioassays

Chemical	231GRE	AZ-GR (Novotna et al. 2012)	GR-BLA (TOX21)	GR-CALUX (Bovee et al. 2011)
Dexamethasone (DEX)	9.5E-9	9.5E-9	2.8E-9	2.2E-9
Cortisol	1.4E-7	6.6E-8	3.8E-8	3.7E-8
Betamethasone (BMZ)	1.3E-8	1.5E-8	4.9E-9	1.1E-8
Prednisolone (PRED)	6.0E-8	4.5E-8	2.3E-8	1.2E-8
Triamcinolone (TAC)	5.9E-8	5.4E-8	3.0E-8	1.9E-8
Corticosterone	8.2E-7	2.4E-7	8.0E-8	8.0E-8
Aldosterone (ALDO)	6.4E-6	1.1E-6	-	5.0E-7

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Table 2.

Comparison of ELISA concentration to cortisol equivalent (CortEq) values for human serum dilutions

Dilution	CortEq (nM)	SD	CV (%)
1:10	93	3.5	3.7
1:20	144	9.6	6.6
1:30	196	11.4	5.8
1:40	225	14.7	6.5
1:50	260	19.6	7.5
ELISA	118		