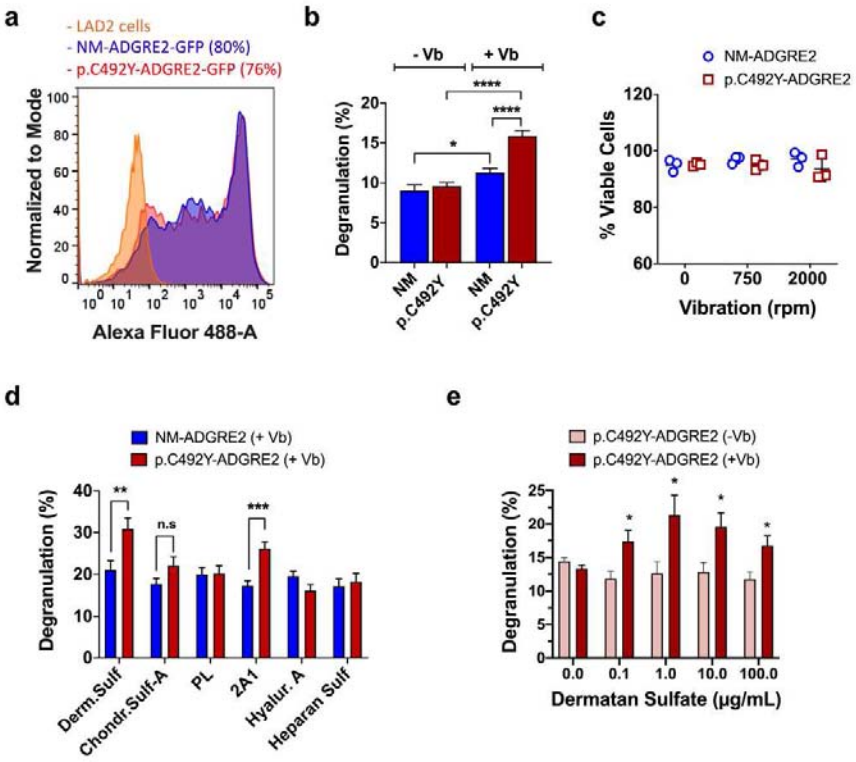

FIGURE S1. Efficiency of transfection for normal and mutated ADGRE2; and characteristics of vibration-induced degranulation. (a) LAD2 cells were transfected with nonmutated (NM)-ADGRE2-GFP or p.C492Y-ADGRE2-GFP and 20 h later efficiency of transfection was determined by FACS. **(b)** β-Hexosaminidase release in response to vibration. Cells were plated in DS-coated dishes and subjected or not to vibration (750 rpm) for 20 min. Data are mean±SEM (n>30). **(c)** Effect of vibration on cell viability. Cells were collected after vibration (20 min) and stained with acridine orange (stains all cells in green) and propidium iodide (stains dead cells in red). Viable and dead cells were counted using a dual fluorescence Cell Counter (Luna-FL). **(d)** Effect of ADGRE2 ligation to stated substrates on vibration-induced degranulation. NM-ADGRE2 or p.C492Y-ADGRE2 cells were plated on wells coated with dermatan sulfate (100 µg/ml), chondroitin sulfate A (100 µg/ml), polylysine (PL; 0.01%), 2A1 monoclonal antibody (50 µg/ml), hyaluronic acid (100 µg/ml) or heparan sulfate (100 µg/ml). **(e)** Degranulation of p.C492Y-ADGRE2 plated on the indicated concentrations of immobilized DS with or without vibration. In b, d and e, cells were vibrated for 20 min at 750 rpm, and degranulation was measured by β-hexosaminidase release. Data are mean±SEM ($n \geq 7$).*p<0.05; **p<0.01;***p<0.001; **** p<0.0001; n.s. not significant.

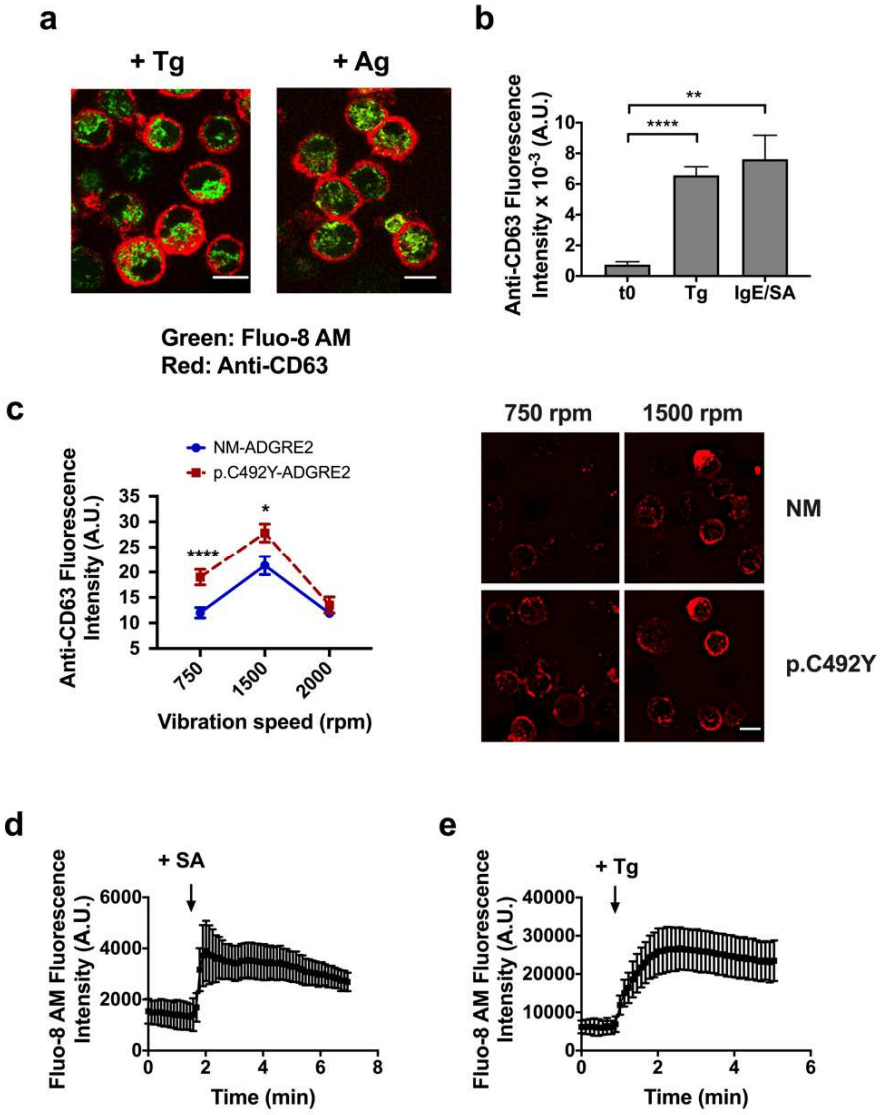

FIGURE S2. Degranulation and calcium changes induced by thapsigargin or by stimulation of the high affinity IgE receptor using confocal microscopy. (a) Degranulation and calcium mobilization induced by 1 µM thapsigargin (Tg) or via stimulation of the high affinity IgE receptor (IgE/SA). Confocal images show the changes in fluorescent intensity of Fluo-8 AM (changes in intracellular calcium) and anti-CD63-APC (degranulation). Scale bar=10 µm. (**b**) Quantification of CD63 exposure after activation with Tg or IgE/SA. For IgE receptor stimulation, LAD2 cells were sensitized with biotinylated-IgE overnight and washed before loading with Fluo-8 AM. Cells were then challenged with 100 ng/mL of streptavidin (SA). Anti-

CD63-APC was added immediately after stimulation and fluorescent intensity was quantified 10 min later. **(c)** Effect of the vibration strength on CD63 cell surface exposure of NM- and p.C492Y-ADGRE2 cells. Shown is anti-CD63-APC fluorescent intensity 10 min after vibration in responding cells (with signals above background fluorescence). Scale bar=10 µm. Data quantification at 2000 rpm was not reliable since cells significantly detached and cell integrity was also partly compromised. This contrasts with the β-hexosaminidase release experiments (done in round wells) where no effects in viability or cell numbers were detected (see Figure S1c), which we believe relates to the different size and geometry of the chambers. **(d, e)** Changes in intracellular calcium induced via stimulation of the high affinity IgE receptor (**d**) or by Tg (**e**) measured by Fluo-8 using confocal microscopy. Data represent mean±SEM, n=5. ** $p<0.005$ and **** $p<0.0001$.

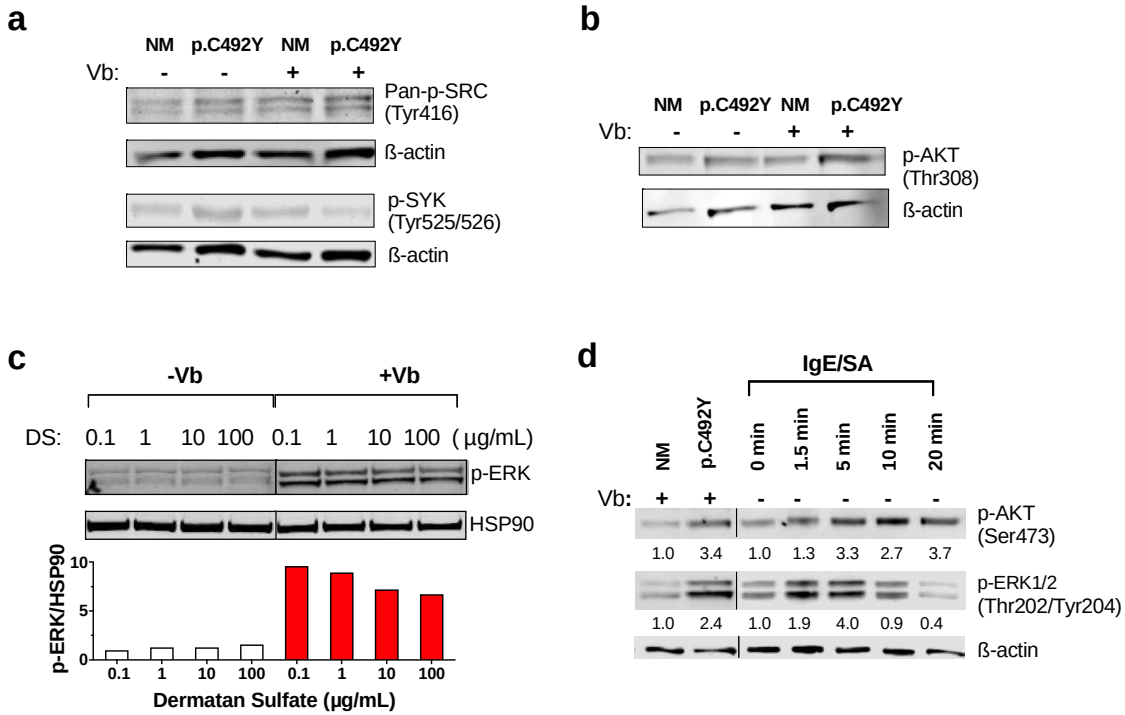

FIGURE S3. Vibration does not activate SRC or SYK but induces similar AKT and ERK1/2 phosphorylation to that mediated by IgE receptor stimulation. DS-bound LAD2 cells expressing nonmutated (NM)- or p.C492Y-ADGRE2 were vibrated (+Vb) for 5 min at 750 rpm. Cell lysates were then obtained and resolved in SDS-PAGE. **(a)** Phosphorylation in SRC family members and SYK. **(b)** AKT phosphorylation in Thr308, a target for phosphoinositide-dependent kinase 1 (PDK1). Blots are from a representative experiment of at least 3 different experiments. **(c)** ERK1/2 phosphorylation after vibration of p.C492Y-ADGRE2 cells on DS coated plates at the indicated concentrations. The histogram shows the quantification of band intensities (normalized by HSP90) and expressed as fold change compared to non-vibrated, NM-ADGRE2- cells plated in 0.1 µg/ml DS. **(d)** Comparison of the extent of AKT and ERK1/2 phosphorylation induced by vibration or activation via the IgE receptor on non-attached cells, done side by side. For IgE receptor stimulation, LAD2 cells were sensitized with biotinylated-IgE overnight, washed and challenged with 100 ng/mL streptavidin (SA).

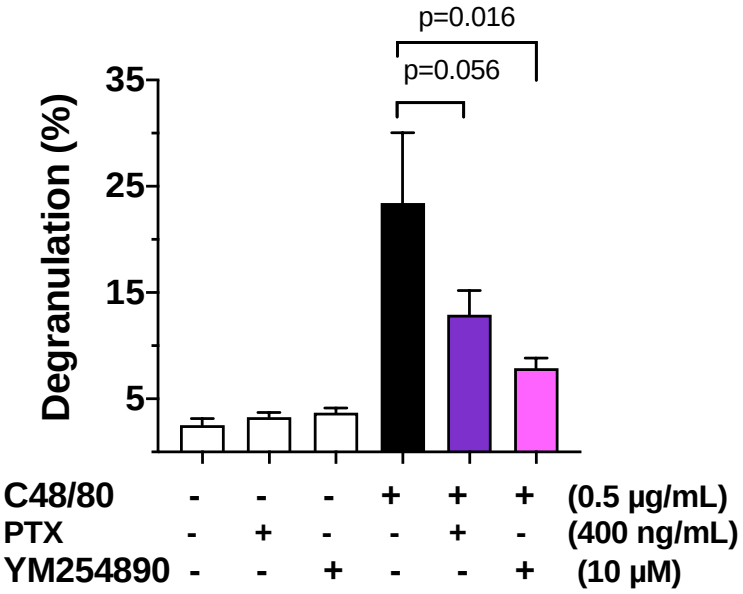

FIGURE S4. Degranulation induced via MRGPRX2 in LAD2 cells is partially inhibited by PTX or YM254890. LAD2 cells were stimulated for 30 min with compound 48/80 (0.5 µg/mL), a ligand for the GPCR involved in pseudoallergic responses, MRGPRX2. Cells were pretreated for 20 min with or without PTX to inhibit $\alpha_{i/o}$-mediated signaling or YM254890 to inhibit $\alpha_{q/11/14}$**.** Degranulation was determined as the percentage of β-hexosaminidase released into the media compared to total cellular content.

SUPPLEMENTARY MATERIALS AND METHODS

Antibodies and inhibitors

Antibodies against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), AKT, phospho-AKT (Ser473), phospho-AKT (Thr308), phospho-SAPK/JNK (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling (Danvers, MA). Anti-β-actin clone AC-15 was from Sigma-Aldrich (St. Louis, MO); IRDye 680RD goat anti-mouse IgG (red) and IRDye 800CW donkey anti-rabbit IgG (green) were from LI-COR Biosciences (Lincoln, NE). Anti-CD63-APC was from ThermoFisher Scientific (Rockford, IL), mouse anti-human CD312 (2A1) was from Bio-Rad (Hercules, CA). Inhibitors for the G protein βγ subunit (gallein), PKC (Go6983), PI3K (LY294002 and wortmannin), MEK/ERK1/2 (U0126), IP3 receptors (2-Aminoethoxydiphenyl borate or 2APB), G protein receptor coupling (Pertussis toxin) and PLC (U73122) and its inactive analog (U73343) were from Tocris (Minneapolis, MN). A selective inhibitor of G protein α_q and α_{11} (YM254890) was from Wako Chemicals (Richmond, VA).

Cell culture

LAD2 cells were cultured in StemPro^{TM}-34 SFM medium containing StemPro^{TM}-34 Nutrient Supplement (Life Technologies, Grand Island, NY), L-glutamine (2 mM), penicillin (100 U/mL)/streptomycin (100 μg/mL) (GIBCO, Grand Island, NY), and 100 ng/mL recombinant human SCF (R&D systems, Minneapolis, MN), as described (Kirshenbaum et al., 2003).

ADGRE2 constructs and LAD2 transfection

Nonmutated-*ADGRE2* and p.C492Y-*ADGRE2* were inserted into the pENTR/D-TOPO entry vector (Invitrogen, Carlsbad, CA) and the constructs were subcloned into the C-terminal Emerald Green Fluorescent Protein (EmGFP)-tagged Vivid Colors pcDNA6.2/C-EmGFP DEST Gateway destination vector (Invitrogen), or into the C-terminal V5-tagged pcDNA3.2/V5 DEST Gateway

destination vector (Invitrogen), using the Gateway LR Clonase II Enzyme Mix (Invitrogen) (Boyden et al., 2016). LAD2 cells (2 x 10^6) were transfected by electroporation using the Amaxa Nucleofector II (Lonza, Walkersville, MD). Cells were washed once with PBS, resuspended in 100 µl of Cell Line Kit V solution (Lonza), mixed with 4 µg of the corresponding *ADGRE2* constructs and electroporated using pre-programed electrical parameters (U-025). After nucleofection, 1 mL of culture media was added to the cuvette and cells were transferred to a 6-well plate containing an additional mL of media. Transfected LAD2 cells were used for experiments within 24 h, as we have observed that electroporation often caused a slow but progressive decline in cell viability 24 hours after transfection, limiting other possible manipulations including knockdown experiments. The transfection efficiency was on the average >70% as determined by FACS analysis of GFP positive cells (see Figure S1a).

Determination of degranulation by β-hexosaminidase release

Culture 96-flat bottom well plates were coated with 100 µg/mL DS (chondroitin sulfate B) (Sigma-Aldrich, St. Louise, MO) in PBS for 6 h at 37 °C or overnight at 4 °C and washed with PBS. In some experiments, wells were coated instead with 100 µg/mL chondroitin sulfate A, 0.01% polylysine, or 50 µg/mL 2A1 monoclonal antibody. Transfected LAD2 cells as described above were immediately plated in culture media (50,000 cells/well) and allowed to attach to the DS-coated dishes overnight. Alternatively, cells were plated on various concentrations of DS, various glycosaminoglycans or polylysine (as indicated in the corresponding figure) for 3 h in pre-warmed HEPES buffer (pH 7.4, 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1.3 mM $MgSO_4 \cdot 7H2O$). Adhered cells were gently washed twice with pre-warmed HEPES buffer. Plates were vibrated in 100 µL of HEPES buffer at 750 rpm for 5-20 min on an orbital shaker (ThermoMixer C, Eppendorf,

Hauppauge, NY) at 37 °C. After vibration, plates were centrifuged at 450 g for 5 min at 4 °C and the supernatant collected to determine the content of released β-hexosaminidase. β-hexosaminidase activity remaining within the cells was also measured and the percentage of released β-hexosaminidase determined as described (Kuehn et al., 2010). The complexity of the experimental design is reflected in a coefficient of variation between degranulation experiments of 16-20%. In some experiments, LAD2 cells were stimulated with the ligand of the Mas-Related G-protein Receptor member X2 (MRGPRX2), compound 48/80 (0.5 μg/mL) (Sigma-Aldrich) in HEPES buffer for 30 min and β-hexosaminidase release determined as above.

Determination of mast cell degranulation by confocal microscopy

LAD2 cells were electroporated with the *ADGRE2-V5* constructs and plated (200,000 cells per well) overnight in DS-coated μ-slide 8-well imaging chambers (Ibidi, Madison, WI). Cell were vibrated at 750 rpm for 5 min on an orbital shaker at 37 °C. Anti-CD63-APC was added (1:20 dilution) after vibration at the time of image recording. Receptor independent degranulation by 1 μM thapsigargin or IgE receptor-activation were used as positive controls. For IgE-mediated stimulation, LAD2 cells were sensitized with 100 ng/ml of biotinylated-IgE overnight, washed and challenged with 100 ng/mL streptavidin (SA). Confocal images were acquired using a Leica TCS SP8 microscope (Leica Microsystems, Exton, PA) equipped with a 63X/1.4NA oil immersion objective, hybrid HyD dectectors, adaptive focus control, and an environmental chamber set to 37 °C with 5 % CO_2. Images were processed using ImageJ (version 1.5a). Individual cells were selected using Region of Interest (ROI) Manager, and APC mean fluorescent intensity per cell was recorded overtime. Background mean fluorescence intensity was subtracted from each individual cell, and the signals from all cells with values above background were averaged overtime.

Calcium flux measurements

Transfected LAD2 cells were plated on black 96-flat bottom well plates (CulturPlate-96 Black, PerkinElmer, Boston, MA) coated with 100 µg/mL DS (50,000 cells/well) and incubated overnight in culture media. Fura-2 AM (5 µM) (Life Technologies, Carlsbad, CA) was added to cells for 30 min. Adhered cells were gently washed twice with HEPES buffer containing 0.04% BSA and 0.3 mM sulfinpyrazone. Plates were vibrated at 750 rpm for 5 min at 37 °C. To determine the minimum and maximal fluorescence signals, 50 µM EGTA or 1 µM thapsigargin, respectively, were added. For IgE receptor stimulation, LAD2 cells were sensitized and challenges as described above. Samples were excited at two wavelengths (340 and 380 nm) to detect Fura-2 bound and unbound to calcium, and fluorescence emission was measured at 510 nm using a Perkin Elmer Wallac 1420 Victor2 microplate reader. The ratio of the fluorescence readings was calculated after subtracting the fluorescence of the cells that were not loaded with Fura-2 AM. Calcium concentration was calculated using the ratio of fluorescence readings (340 and 380 nm) at each time point and the minimal and maximal readings as described (Grynkiewicz et al., 1985).

Alternatively, changes in cytosolic calcium were detected in individual cells by fluorescence of calcium-bound Fluo-8 (Abcam, Cambridge, MA) using a confocal microscope. Following transfection, LAD2 cells were plated overnight on 100 µg/mL DS-coated µ-slide 8-well imaging chambers (250,000 cells per well). Cells were then loaded with 5 µM Fluo-8 AM for 30 min at 37 °C and 5% CO_2. Adhered cells were washed twice with HEPES buffer and baseline fluorescence was then recorded. Chambers were vibrated at 750 rpm for 5 min on an orbital shaker at 37 °C. Prior to imaging, anti-CD63-APC at 1:20 dilution was added to simultaneously track mast cell degranulation. Confocal images were acquired using a Leica TCS SP8

microscope (Leica Microsystems, Exton, PA). Images were processed as described for the degranulation measurements by confocal microscopy.

Phosphorylation of downstream molecules: Immunoblotting

Cells transfected with the *ADGRE2-GFP* constructs were plated in DS (100 µg/mL)-coated 24-well plates (250,000 cells per well) overnight, washed and vibrated in 200 µL HEPES buffer at 750 rpm for 5 min at 37 °C. LAD2 cells were lysed in 2 x Denaturing Sample Buffer (Tkaczyk et al., 2002). Samples were boiled and separated by electrophoresis on 4-12% NuPage Bis-Tris gels (ThermoFisher Scientific). Proteins were transferred to nitrocellulose membranes (0.45 µm pore size, ThermoFisher Scientific) and blots were incubated with the primary antibodies (listed above) overnight at 4 °C on a shaker. Bands were detected using infrared-labeled secondary antibodies and imaging of the bands was performed using an Odyssey CLx Infrared Imaging System (LI-COR Biosciences). Quantification of infrared fluorescence was conducted using Image Studio Lite (version 5.2).

Inhibition experiments

When inhibitors were used to determine their effects on mast cell responses or signaling, LAD2 cells were treated with the corresponding inhibitor or vehicle (0.1% DMSO) 20 min before vibration and maintained in the media during vibration. The concentrations used were as follows: Gö6983 (10 µM), LY294002 (25 µM), Wortmannin (0.1 µM), U0126 (10 µM), U73122 (10 µM), U73343 (10 µM), 2APB (20-50 µM), gallein (10 µM), PTX (400 ng/mL), and YM254890 (10 µM).

Patients and vortex challenge

Patients with VU were enrolled and evaluated at the National Institutes of Health Clinical Center under a protocol approved by the Institutional Review Board of the National Institute of Allergy

and Infectious Diseases (09-I-0126) as described (Boyden et al., 2016). All subjects provided written informed consent. To elicit VU symptoms in a clinical setting, the anterior forearm of a subject was placed horizontally on the 3-inch platform of a laboratory vortex for a 4 min challenge at 2500 rpm. Serial blood draws at baseline and during the 60 min post-challenge period were taken for analysis of PGD_2 by ELISA.

PGD_2 measurements

LAD2 cells were plated (50,000 cells/well) on DS-coated 96- well plates overnight, washed and subjected to vibration in HEPES buffer (750 rpm for 20 min) as described above. Cell-free supernatants or serum sample from patients with VU were analyzed for PGD_2 by competitive enzyme immunoassay (Cayman Chemicals), according to the manufacturer's instructions.