Quantikine® ELISA

Human CXCL1/GROα Immunoassay

Catalog Number DGR00B
SGR00B
PDGR00B

For the quantitative determination of human Growth-Regulated protein alpha (GROα) concentrations in cell culture supernates, serum, and platelet-poor plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The term chemokine refers to a large superfamily of small (8-10 kDa), inducible, secreted, pro-inflammatory cytokines. These cytokines are characterized by four conserved cysteine residues and nearly all of these cytokines have now been shown to chemoattract and activate different leukocyte subsets. The chemokine family of cytokines can be divided into four subfamilies based on the positioning of the cysteine residues. In the chemokine α or "CXC" subfamily, the first two cysteine residues are separated by a variable amino acid (aa). In the chemokine β or "CC" subfamily, the first two cysteine residues are adjacent. Some of the better known chemokine α family members are IL-8, GRO, platelet factor 4, β-thromboglobulin and IP-10. Examples of chemokine β family members include RANTES, MIP-1α, MIP-1β, MCP-1, and I-309. The genes for all of the human chemokine α family members have been mapped to chromosome 4q while those for chemokine β family members are closely linked on human chromosome 17q. Chemokines act primarily on leukocytes, regulating their trafficking. In general, chemokine α family members preferentially chemoattract and activate neutrophils, whereas chemokine β family members are chemotactic for monocytes and T cells (1-3).

GROα, or CXCL1, was initially discovered as a growth-regulated gene that is overexpressed constitutively in tumorigenic cells (4) and transcribed in normal cells only during growth stimulation (5). Independently, a melanoma growth-stimulating activity (MGSA) was cloned from a human melanoma cell line, Hs294T, and found to be identical to human GROα (6). In addition to the initially cloned GROα, two additional human GRO genes, named GROβ or MIP-2α and GROγ or MIP-2β, which share 90% and 86% amino acid (aa) sequence homology, respectively, with GROα have been identified (7, 8). GROα expression is inducible by PDGF and/or by a variety of inflammatory mediators, such as IL-1 and TNF, in monocytes, fibroblasts, melanocytes, mammary epithelial cells, and umbilical vein endothelial cells (4). GROα is also one of several neutrophil-activating factors extracted from human psoriatic scales (9).

The GROα cDNA encodes a 107 aa precursor protein from which the N-terminal 34 aa residues are cleaved to generate the 73 aa residue mature GROα. There are no potential N-linked glycosylation sites in the aa sequence nor does the mature protein appear to undergo any post-translational modifications. GROα exhibits growth stimulatory activity for the human Hs294T melanoma cell line from which it was initially isolated. As a chemokine α member, GROα, like IL-8, is a potent chemoattractant for human neutrophils and stimulates neutrophil degranulation and enzyme release from cytochalasin B-treated human neutrophils (4). It has been demonstrated that GROα can cause a decrease in the expression of interstitial collagens by rheumatoid synovial fibroblasts (10). In *in vivo* experiments, GROα has been shown to induce an acute inflammatory reaction when injected into the foot-pads of endotoxin resistant mice (4).

GROα can bind with high affinity to the type II IL-8 receptor, also called IL-8 RB. However, it will only bind with low affinity to the type I IL-8 receptor (IL-8 RA) (11). Both IL-8 RA and IL-8 RB, like other chemokine receptors, are members of the superfamily of rhodopsin-like, G-protein-coupled receptors containing seven transmembrane domains. A novel GROα-specific receptor that does not bind IL-8 has also been identified in synovial fibroblasts and Hs294T cells (10, 12).

The Quantikine® Human CXCL1/GROα Immunoassay is a 4.25-4.5 hour solid phase ELISA designed to measure human GROα in cell culture supernates, serum, and platelet-poor plasma. It contains *E. coli*-expressed recombinant human GROα and antibodies raised against the recombinant protein. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural human GROα showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human GROα.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human GROα has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any GROα present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human GROα is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of GROα bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the calibrator diluent selected for the standard curve be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DGR00B	CATALOG # SGR00B	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human GROα Microplate	890150	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human GROα.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human GROα Standard	890152	1 vial	6 vials	Recombinant human GROα in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
Human GROα Conjugate	890151	1 vial	6 vials	21 mL/vial of a polyclonal antibody specific for human GROα conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1U	895138	1 vial	6 vials	6 mL/vial of a buffered protein base with preservatives. *May appear cloudy and contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5K	895119	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6-69	896012	1 vial	6 vials	21 mL/vial of diluted animal serum with preservatives. *For serum/plasma samples.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

DGR00B contains sufficient materials to run an ELISA on one 96 well plate.
SGR00B (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDGR00B). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Human GROα Microplate	890150	50 plates
Human GROα Standard	890152	25 vials
Human GROα Conjugate	890151	50 vials
Calibrator Diluent RD5-K	895119	50 vials
or		
Calibrator Diluent RD6-69	896012	50 vials
Assay Diluent RD1U	895138	50 vials
Wash Buffer Concentrate	895126	9 bottles
Color Reagent A	895000	50 vials
Color Reagent B	895001	50 vials
Stop Solution	895032	50 vials
Plate Sealers	N/A	100 sheets
Package Insert	753213	2 booklets

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 2-8 °C centrifuge for plasma preparation
- 2-8 °C incubator for assay procedure
- 500 mL graduated cylinder
- Test tubes for dilution of standards
- Human GROα Controls (optional; R&D Systems®, Catalog # QC101B)

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Caution: *Normal human serum added to cell culture media may contain GROα. For best results, do not use human serum for growth of cell cultures if assaying for GROα production. Because there is no species cross-reactivity of this kit, human GROα levels in culture media containing 10% bovine or fetal bovine serum can be assayed without interference.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using EDTA, heparin, or citrate as an anticoagulant. Centrifuge at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

GROα appears to be present in platelet granules and released upon platelet activation. Therefore, to measure circulating levels of GROα, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood.

REAGENT PREPARATION

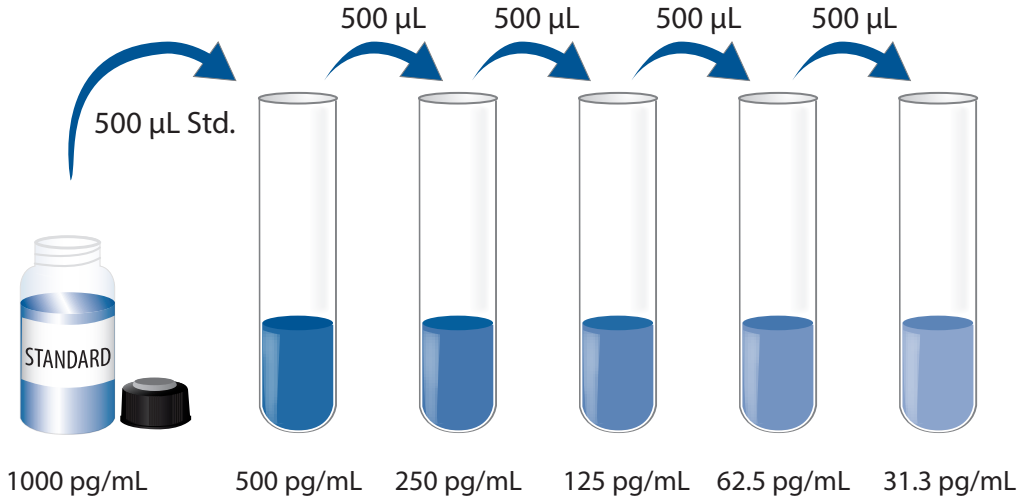
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human GROα Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human GROα Standard with Calibrator Diluent RD5K (*for cell culture supernate samples*) or Calibrator Diluent RD6-69 (*for serum/plasma samples*). This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 µL of Calibrator Diluent RD5K (*for cell culture supernate samples*) or Calibrator Diluent RD6-69 (*for serum/plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human GROα Standard (1000 pg/mL) serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1U to each well. *Contains a precipitate. Mix well before and during use.*
4. Add 200 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. **After 1 minute, empty wells by aspiration or decanting.** Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200 µL of Human GROα Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at **2-8 °C.**
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. **Protect from light.**
   For Cell Culture Supernate Samples: Incubate for 15 minutes at room temperature.
   For Serum/Plasma Samples: Incubate for 30 minutes at room temperature.
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

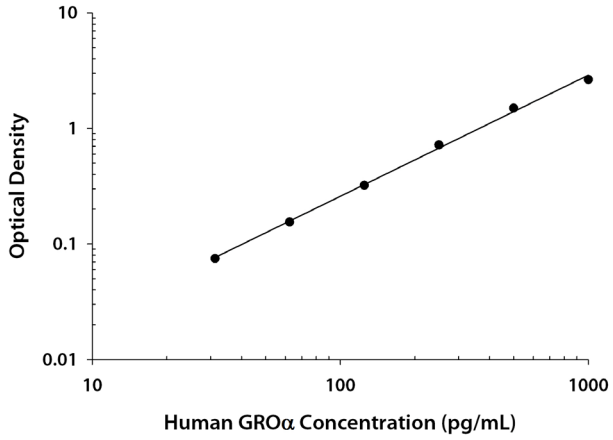
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human GROα concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

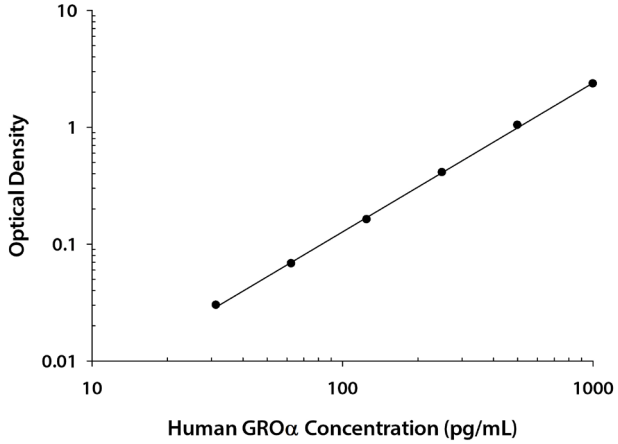
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.006 0.009	0.008	—
31.3	0.074 0.076	0.075	0.067
62.5	0.148 0.162	0.155	0.147
125	0.317 0.326	0.322	0.314
250	0.704 0.735	0.720	0.712
500	1.456 1.542	1.499	1.491
1000	2.642 2.647	2.645	2.637

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.020 0.023	0.022	—
31.3	0.051 0.052	0.052	0.030
62.5	0.090 0.090	0.090	0.068
125	0.184 0.184	0.184	0.162
250	0.428 0.435	0.432	0.410
500	1.055 1.071	1.063	1.041
1000	2.352 2.394	2.373	2.351

PRECISION

Intra-Assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	41.8	140	373	48.7	157	379
Standard deviation	0.91	5.98	15.7	2.90	12.3	29.8
CV (%)	2.2	4.3	4.2	6.0	7.8	7.8

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	61.4	251	736	62.8	252	734
Standard deviation	2.2	6.0	20.0	3.6	11.9	38.9
CV (%)	3.6	2.4	2.7	5.7	4.7	5.3

RECOVERY

The recovery of human GROα spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media	99	92-105%
Serum	98	87-108%
Platelet-poor EDTA plasma	93	86-102%
Platelet-poor Heparin plasma	95	83-109%
Platelet-poor Citrate plasma	90	80-98%

SENSITIVITY

The minimum detectable dose (MDD) of human GROα is typically less than 10 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human GROα were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

				Platele-poor		
		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)
1:2	Average % of Expected	103	103	95	112	99
	Range (%)	100-105	97-107	93-100	110-116	97-103
1:4	Average % of Expected	98	103	92	115	96
	Range (%)	96-101	92-111	87-100	108-122	92-101
1:8	Average % of Expected	95	102	88	115	92
	Range (%)	93-96	90-112	82-96	105-125	83-99
1:16	Average % of Expected	91	104	90	116	95
	Range (%)	90-91	93-119	83-101	105-129	87-101

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human GROα produced at R&D Systems®.

The NIBSC/WHO GROα interim reference preparation 92/722 which was intended as a potency standard, was evaluated in this kit. The interim reference material parallels the Quantikine® standard curve. To convert sample values obtained with the Quantikine® Human CXCL1/GROα kit to approximate NIBSC nominally assigned mass values use the equation below.

NIBSC (92/722) approximate value (pg/mL) = 0.32 x Quantikine® Human GROα value (pg/mL)

SAMPLE VALUES

Serum/Platelet-poor Plasma - Serum and platelet-poor plasma samples from apparently healthy volunteers were evaluated for the presence of human GROα in this assay. No medical histories were available for the donors used in this study. Forty serum samples measured between 32-180 pg/mL with a mean of 93 pg/mL. Ten platelet-poor plasma samples were tested, and all measured less than the lowest Human GROα Standard, 31.3 pg/mL.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and stimulated with 10 µg/mL PHA. Aliquots of the culture supernates were removed on days 1 and 5 and assayed for levels of human GROα.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	76.6	81.4
Stimulated	6856	14,928

It is important to note that sample collection and handling procedures will have a significant impact on measured GROα levels. Refer to the Sample Collection and Storage section for the recommended sample collection procedure.

SPECIFICITY

This assay recognizes natural and recombinant human GROα.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human GROα control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CXCL2/GROβ
CXCL5/ENA-78
CXCL7/NAP-2
CXCL8/IL-8
CCL2/MCP-1
CCL3/MIP-1α
CCL4/MIP-1β
CCL5/RANTES

Recombinant mouse:

CCL3/MIP-1α
CCL4/MIP-1β
CXCL1/KC

Recombinant rat:

CXCL1/CINC-1

Recombinant human GROγ does not interfere but does cross-react approximately 3.5% in this assay.

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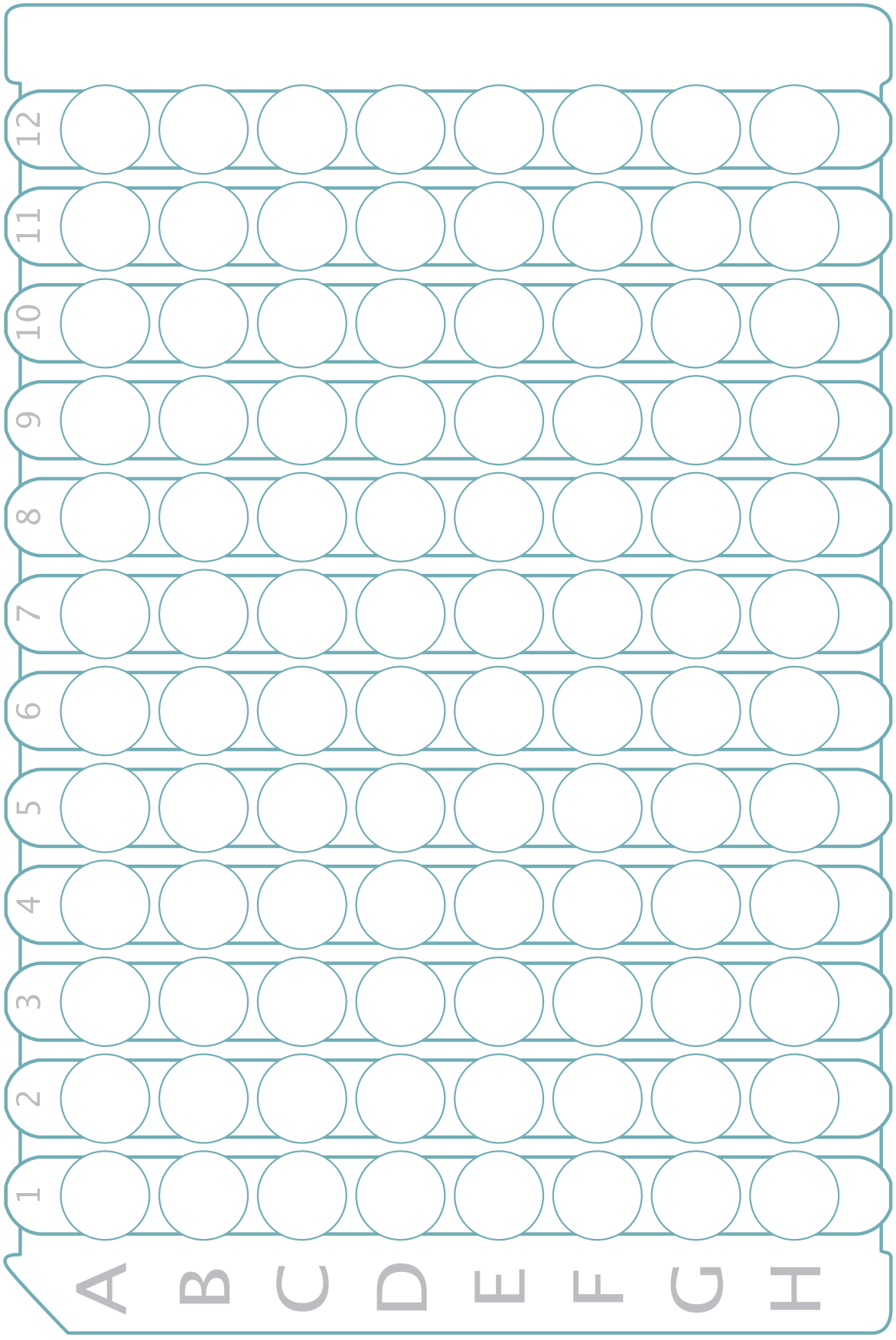
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES