

Quantikine[®] ELISA

Mouse/Rat RGM-C/Hemojuvelin Immunoassay

Catalog Number MRGMC0

For the quantitative determination of mouse and rat Repulsive Guidance Molecule C (RGM-C) concentrations in cell culture supernates, serum, and plasma.

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

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY.....	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS.....	2
MATERIALS PROVIDED & STORAGE CONDITIONS	3
OTHER SUPPLIES REQUIRED	4
PRECAUTIONS.....	4
SAMPLE COLLECTION & STORAGE	4
SAMPLE PREPARATION.....	4
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
CALCULATION OF RESULTS.....	7
TYPICAL DATA.....	7
PRECISION	8
RECOVERY.....	8
LINEARITY.....	8
SENSITIVITY	9
CALIBRATION	9
SAMPLE VALUES.....	9
SPECIFICITY.....	10
REFERENCES.....	10

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.

614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.

19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.

24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Repulsive Guidance Molecule C (RGM-C), also known as Hemojuvelin (HJV), is a GPI-anchored membrane glycoprotein that plays a critical role in iron homeostasis (1, 2). Polymorphisms of Hemojuvelin cause type 2A juvenile hemochromatosis, a hereditary disorder characterized by excessive iron accumulation (3). Hemojuvelin is predominantly expressed in hepatocytes and striated muscle (4, 5). It is initially presented on the cell surface with partial glycosylation, but then is subsequently internalized, modified in glycosylation, and cleaved. The mature molecule is an approximately 50 kDa disulfide-linked heterodimer of a 15-20 kDa N-terminal fragment and a 35 kDa C-terminal fragment (5-8). Soluble forms of Hemojuvelin can be produced by additional cleavage at distinct sites within the C-terminal fragment. Cleavage by Matriptase 2 (following Arg288 in mouse) generates a 32 kDa molecule, and proprotein convertases (e.g. Furin) cleave at a compound RxxR motif (aa 319-328 in mouse) to generate a 40 kDa molecule (9). Shedding of both forms from either liver or muscle releases heterodimeric Hemojuvelin into the circulation, a process that requires its interaction with the transmembrane protein Neogenin (5, 7, 10, 11). Circulating levels of soluble Hemojuvelin are elevated in chronic kidney disease patients undergoing hemodialysis (12).

In the liver, Hemojuvelin is required for the expression of Hepcidin, a peptide hormone that limits circulating iron levels and promotes tissue iron accumulation (1, 4, 13). Hepcidin inhibits cellular efflux of iron by inducing the degradation of the transporter Ferroportin (13). This action inhibits the release of iron by macrophages which phagocytose senescent erythrocytes, by hepatocytes which store iron, and by intestinal epithelial cells which transfer dietary iron to the blood. Circulating Hemojuvelin opposes the effect of membrane bound Hemojuvelin; it suppresses the expression of Hepcidin and promotes a rise in blood iron levels (7). High blood iron levels, in turn, inhibit the production of soluble Hemojuvelin (7). Like its proteolytic shedding, the effect of Hemojuvelin on iron accumulation is dependent on Neogenin (6). In contrast to liver-expressed Hemojuvelin, skeletal muscle Hemojuvelin does not regulate Hepcidin expression or iron homeostasis (14).

Hemojuvelin also functions as a coreceptor for bone morphogenetic proteins (BMPs). It binds to BMP-2, -4, -6 and enhances signaling through the receptors ALK2, ALK3, ActRIIA, and BMPRII (15, 16). BMPs induce Hepcidin upregulation in the liver, an effect which is enhanced by the presence of Hemojuvelin and its interaction with Neogenin (15, 17). The 40 kDa soluble form of Hemojuvelin can bind BMP-2 and BMP-6 directly and block BMP-induced Hepcidin expression, while the 36 kDa Matriptase 2-cleaved form binds more weakly and does not block Hepcidin expression (9). Disease-related point mutations in Hemojuvelin can prevent its internal cleavage, its ability to interact with Neogenin, and its ability to modulate BMP signaling (5, 6, 15). Mature mouse Hemojuvelin shares 89% and 97% amino acid sequence identity with human and rat Hemojuvelin, respectively (18, 19). An alternatively spliced isoform of mouse Hemojuvelin lacks the N-terminal 219 amino acids.

The Quantikine Mouse/Rat RGM-C/Hemojuvelin Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse and rat RGM-C in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse RGM-C and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural RGM-C showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural RGM-C.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse/rat RGM-A/C has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any RGM-C present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat RGM-C is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of RGM-C bound in the initial step. The sample values are then read off the standard curve.

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.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard 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.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse/Rat RGM-A/C Microplate	893968	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse or rat RGM-A/C.	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.*
Mouse/Rat RGM-C Conjugate	894582	12 mL of a polyclonal antibody specific for mouse/rat RGM-C conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse/Rat RGM-C Standard	894583	20 ng of recombinant mouse RGM-C in a buffered protein base with preservatives; lyophilized.	
Mouse/Rat RGM-C Control	894584	Recombinant mouse RGM-C in a buffered protein base with preservatives; lyophilized. The concentration range of mouse RGM-C after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1-55	895066	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-18	895335	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

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

Some components in this kit contain ProClin[®] 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. Please refer to the MSDS 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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.
Icteric samples are not suitable for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 50 μ L of sample + 150 μ L of Calibrator Diluent RD5-18.

All trademarks and registered trademarks are the property of their respective owners.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

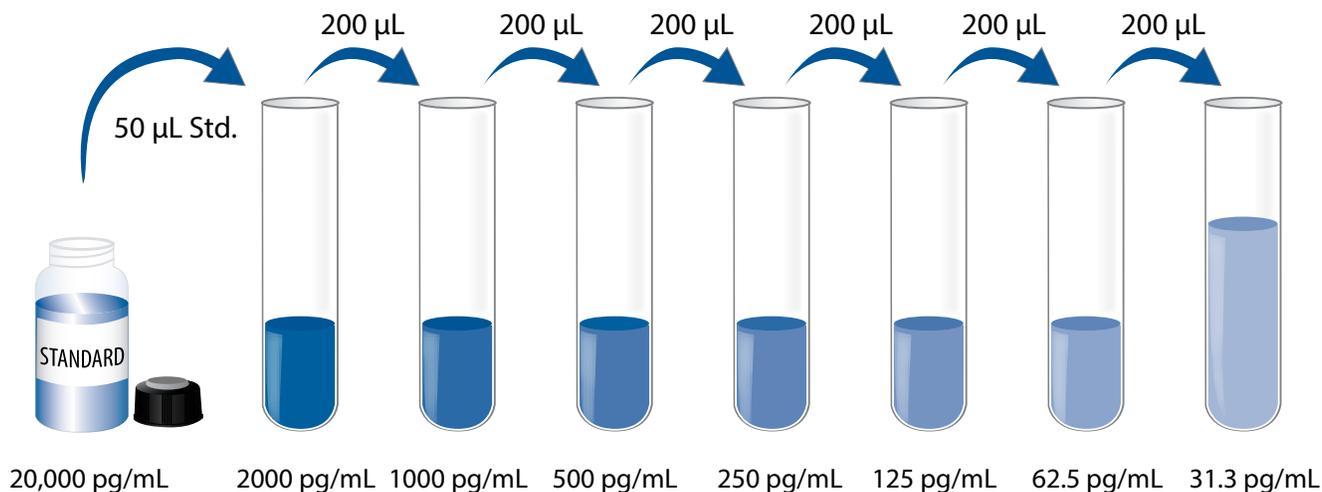
Mouse/Rat RGM-C Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

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 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. 100 μ L of the resultant mixture is required per well.

Mouse/Rat RGM-C Standard - Reconstitute the Mouse/Rat RGM-C Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 450 μ L of Calibrator Diluent RD5-18 into the 2000 pg/mL tube. Pipette 200 μ L into the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-18 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, Control, and samples be assayed in duplicate.

1. Prepare all reagents, standards, Control, and samples as directed by 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 RD1-55 to each well.
4. Add 50 μL of Standard, Control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. 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 towels.
6. Add 100 μL of Mouse/Rat RGM-C Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 μL of Stop Solution to each well. 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.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

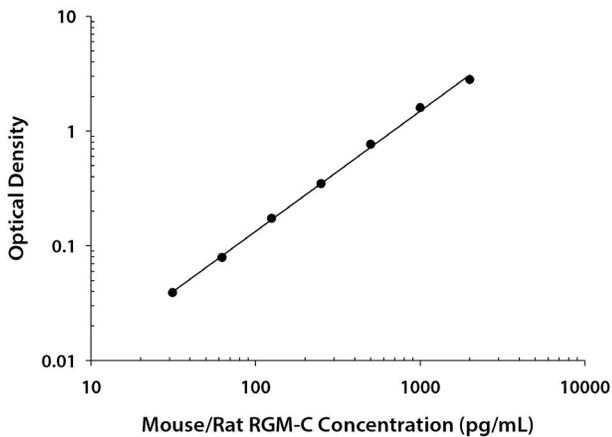
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse/rat RGM-C concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.013 0.014	0.014	—
31.3	0.050 0.055	0.053	0.039
62.5	0.092 0.093	0.093	0.079
125	0.184 0.189	0.187	0.173
250	0.351 0.372	0.362	0.348
500	0.769 0.791	0.780	0.766
1000	1.601 1.612	1.607	1.593
2000	2.813 2.821	2.817	2.803

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 kit components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	75	244	751	73	228	726
Standard deviation	3.60	6.10	23.0	5.35	16.6	47.8
CV (%)	4.8	2.5	3.1	7.3	7.3	6.6

RECOVERY

The recovery of RGM-C spiked to levels throughout the range of the assay in various matrices was evaluated.

Mouse Samples	Average % Recovery	Range
Cell culture samples (n=4)	98	85-110%
Serum* (n=4)	105	88-120%
EDTA plasma* (n=4)	100	80-120%
Heparin plasma* (n=4)	100	83-116%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of RGM-C were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

Mouse Samples		Cell culture supernates (n=2)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	97	103	105	107
	Range (%)	91-102	97-110	96-112	94-112
1:4	Average % of Expected	89	102	107	104
	Range (%)	87-90	91-111	90-117	85-117
1:8	Average % of Expected	94	106	103	101
	Range (%)	93-94	90-119	83-114	81-114
1:16	Average % of Expected	92	108	106	105
	Range (%)	89-94	93-120	85-119	88-119

*Samples were diluted prior to assay as directed in the Sample Preparation section.

Note: Rat samples were evaluated and no significant difference in linearity or recovery was observed from the data above.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of RGM-C ranged from 1.18-4.46 pg/mL. The mean MDD was 2.25 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse RGM-C produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of RGM-C in this assay.

Mouse Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	2143	1064-3088	658
EDTA plasma (n=5)	3219	1700-4748	1212
Heparin plasma (n=5)	2134	1844-2508	267

Rat Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	3587	2532-5656	955
EDTA plasma (n=5)	3832	2952-4616	686
Heparin plasma (n=5)	3074	2764-3328	218

Cell Culture Supernates:

Tissues from individual mice or rats were removed and rinsed in PBS and kept on ice. The tissue was homogenized using a tissue homogenizer and seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of RGM-C.

Tissue Type	(pg/mL)
Mouse liver	92.8
Rat Liver	174

C2C12 mouse myoblast cells were cultured in DMEM supplemented with 10% fetal bovine serum (undifferentiated) or DMEM supplemented with 2% equine serum (muscle cell differentiated) for 6 days. Aliquots of the cell culture supernates were removed and assayed for RGM-C.

Cell Type	(pg/mL)
Undifferentiated	450
Muscle Cell Differentiated	727

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat RGM-C.

The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 200 ng/mL in a mid-range RGM-C control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Activin RIB
BMP-4
BMP-6
BMPR-IA
BMPR-IB
Neogenin
RGM-B

Recombinant rat:

UNC5H2

Recombinant human:

Activin RIB
BMP-2

Recombinant human RGM-C cross-reacts approximately 13% in this assay.

Recombinant mouse RGM-A interferes at concentrations > 50 ng/mL in this assay.

REFERENCES

1. Zhang, A.S. (2010) *Adv. Nutr.* **1**:38.
2. Corradini, E. *et al.* (2009) *Cytokine Growth Factor Rev.* **20**:389.
3. Santos, P.C. *et al.* (2012) *Int. J. Mol. Sci.* **13**:1497.
4. Niederkofler, V. *et al.* (2005) *J. Clin. Invest.* **115**:2180.
5. Kuninger, D. *et al.* (2006) *J. Cell Sci.* **119**:3273.
6. Zhang, A.S. *et al.* (2005) *J. Biol. Chem.* **280**:33885.
7. Lin, L. *et al.* (2005) *Blood* **106**:2884.
8. Maxson, J.E. *et al.* (2009) *Blood* **113**:1786.
9. Maxson, J.E. *et al.* (2010) *J. Biol. Chem.* **285**:39021.
10. Zhang, A.S. *et al.* (2007) *J. Biol. Chem.* **282**:12547.
11. Enns, C.A. *et al.* (2012) *J. Biol. Chem.* **287**:35104.
12. Rumjon, A. *et al.* (2012) *Am. J. Nephrol.* **35**:295.
13. Huang, F.W. *et al.* (2005) *J. Clin. Invest.* **115**:2187.
14. Chen, W. *et al.* (2011) *Blood* **117**:6319.
15. Babitt, J.L. *et al.* (2006) *Nat. Genet.* **38**:531.
16. Xia, Y. *et al.* (2008) *Blood* **111**:5195.
17. Zhang, A.S. *et al.* (2009) *J. Biol. Chem.* **284**:22580.
18. Niederkofler, V. *et al.* (2004) *J. Neurosci.* **24**:808.
19. Schmidtmer, J. and D. Engelkamp (2004) *Gene Exp. Patterns* **4**:105.