Quantikine® ELISA

Human HTRA2/Omi Immunoassay

Catalog Number DHTR20

For the quantitative determination of human HtrA serine peptidase 2 (HTRA-2) 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.

TABLE OF CONTENTS

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

HTRA2, also known as Omi, is a widely expressed 50 kDa serine protease with both pro-apoptotic and neuroprotective functions (1, 2). HTRA2 is synthesized with a 31 amino acid (aa) mitochondrial targeting peptide, a 102 aa propeptide with a hydrophobic membrane-associating segment, a protease domain, and a C-terminal PDZ domain (3). Alternative splicing of human HTRA2 generates additional isoforms with deletions in the protease and/or PDZ domains, or carry a 64 aa substitution of the PDZ domain and a portion of the protease domain (4). The association of HTRA2 into noncovalent homotrimers is required for its proteolytic activity (5). HTRA2 is largely found in the mitochondria but also on the cytoplasmic face of the endoplasmic reticulum (ER) (6-8). In the mitochondria, it fills a protective role by cleaving pro-apoptotic proteins and misfolded proteins which abnormally translocate to the mitochondrial intermembrane space (9-11). HTRA2 deficiency compromises ATP production by disrupting the function of the mitochondrial ATP synthase (12).

Following apoptotic stimulation, HTRA2 undergoes autocatalytic cleavage to remove its propeptide, and the remaining 35 kDa portion translocates into the cytosol (6, 7, 13, 14). This processing generates a new N-terminus with an AVPS motif resembling that of other pro-apoptotic proteins (*e.g.* Grim, Reaper, and Smac/Diablo) (6, 7, 13, 14). Processed human HTRA2 shares 95% aa sequence identity with mouse and rat HTRA2. Processed HTRA2 binds several members of the Inhibitor of Apoptosis Protein family in the cytosol. This exposes the HTRA2 active site and enhances its proteolytic activity (15). HTRA2 cleaves the IAPs and interferes with their normal ability to inhibit caspase activity (6, 7, 13, 14, 16, 17). The relief of caspase inhibition enables rapid progression of the apoptotic cascade (17, 18). HTRA2 additionally cleaves and inactivates Parkin and the anti-apoptotic WT1 (19, 20). HTRA2 activity is regulated by several protein interactions. While still in the mitochondrion, full length HTRA2 is activated by and then cleaves the anti-apoptotic Hax1 (9, 21). HTRA2 activity is increased by its binding to Presenilin-1 (8, 22) or its phosphorylation by select p38 isoforms, ERK1, Cdc2, or Cdk5 (3, 23, 24). In contrast, its proteolytic and proapoptotic activities are decreased by Akt1,2 mediated phosphorylation (25).

In contrast to its pro-apoptotic function, HTRA2 plays a neuroprotective role in the brain. It is upregulated during neurogenesis and brain development and in the brains of patients with Parkinson's disease and other neurodegenerative disorders (26, 27). Mnd2 mice, which carry an inactivating HTRA2 mutation, exhibit extensive neurodegeneration which can be experimentally corrected by neuronal overexpression of HTRA2 (28, 29). HTRA2 cleavage of MEK serves a tissue protective role by suppressing the inflammatory activation of microglia (30). HTRA2 is involved in multiple steps of amyloid metabolism and protection against the development of Alzheimer's disease (31). It cleaves the amyloid precursor protein (APP) as well as the APP751 and APP695 isoforms and the related APLP1 and APLP2 (8, 11). In addition, HTRA2 binds to Presenilin-1 and -2 of the gamma-Secretase complex and inhibits gamma-Secretase mediated production of the Amyloid β-peptide 1-42 (Aβ) from APP (31, 32). This reduces mitochondrial accumulation of amyloidogenic Aβ. HTRA2 preferentially binds, cleaves, and prevents the further aggregation of oligomeric forms of Aβ in the mitochondria (26, 33). In turn, Aβ oligomers reduce the trimerization, proteolytic, and pro-apoptotic activity of HTRA2 (26).

The Quantikine Human HTRA2/Omi Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human HTRA2 in cell culture supernates, serum, and platelet-poor plasma. It contains *E. coli*-expressed recombinant human HTRA2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human HTRA2 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 naturally occurring HTRA2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human HTRA2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any HTRA2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human HTRA2 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 HTRA2 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.
- 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.
- 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
HTRA2 Microplate	894544	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human HTRA2.	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.*
HTRA2 Standard	894546	2 vials (20 ng/vial) of recombinant human HTRA2 in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a new standard for each assay.
HTRA2 Conjugate	894545	21 mL of monoclonal antibody specific for human HTRA2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1S	895137	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P Concentrate	895151	21 mL of a concentrated buffered protein base with preservatives. *Used undiluted in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric 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.
- Test tubes for dilution of standards and samples.
- Human HTRA2 Controls (optional; available from R&D Systems).

PRECAUTIONS

HTRA2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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 or heparin as an anticoagulant. Centrifuge for 15 minutes 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 ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Do not use hemolyzed samples.

HTRA2 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of HTRA2, 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.

SAMPLE PREPARATION

Serum and platelet-poor plasma samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5P Concentrate.

REAGENT PREPARATION

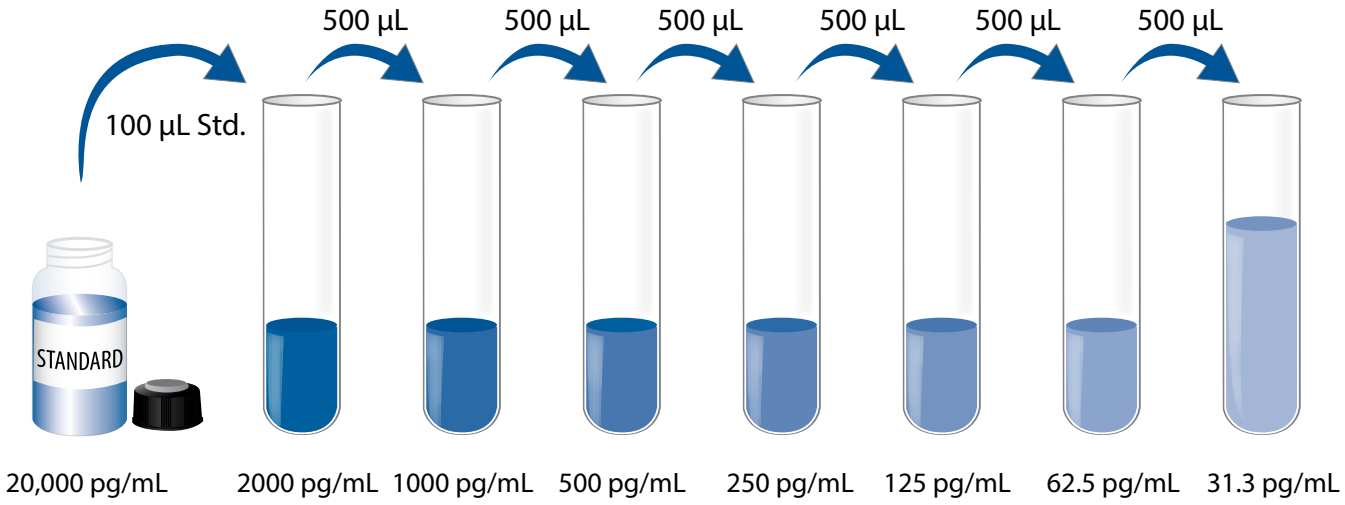
Bring all reagents to room temperature before use.

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 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.

HTRA2 Standard - Reconstitute the HTRA2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5P Concentrate into the 2000 pg/mL tube. Pipette 500 µL of Calibrator Diluent RD5P Concentrate into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P Concentrate 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, samples, and controls be assayed in duplicate.

Note: *HTRA2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.*

1. Prepare all reagents, working standards, and samples 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 100 µL of Assay Diluent RD1S 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. A plate layout is provided to record standards and samples assayed.
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 200 µL of HTRA2 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 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

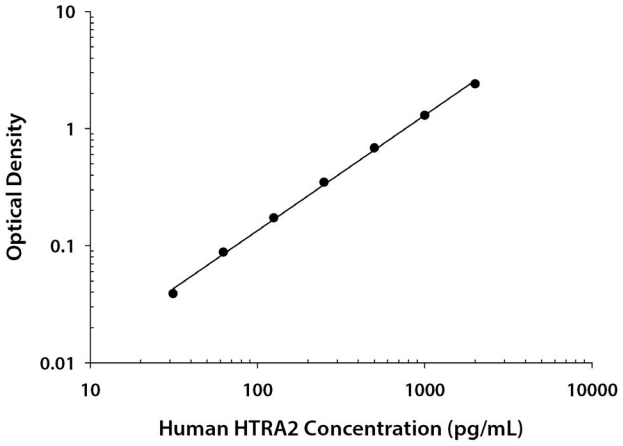
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human HTRA2 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

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	0.025 0.035	0.030	—
31.3	0.067 0.070	0.069	0.039
62.5	0.117 0.118	0.118	0.088
125	0.201 0.204	0.203	0.173
250	0.372 0.384	0.378	0.348
500	0.712 0.714	0.713	0.683
1000	1.305 1.347	1.326	1.296
2000	2.393 2.480	2.437	2.407

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	284	582	1108	280	567	1089
Standard deviation	15.7	15.9	28.6	25.1	41.3	65.3
CV (%)	5.5	2.7	2.6	9.0	7.3	6.0

RECOVERY

The recovery of human HTRA2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	92	86-101%
Serum* (n=4)	91	80-111%
EDTA plasma* (n=4)	90	79-100%
Heparin plasma* (n=4)	89	81-95%

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

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human HTRA2 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	Platelet-poor EDTA plasma* (n=4)	Platelet-poor heparin plasma* (n=4)
1:2	Average % of Expected	101	104	101	102
	Range (%)	96-106	100-106	95-106	97-106
1:4	Average % of Expected	96	105	99	101
	Range (%)	88-103	101-108	89-106	92-105
1:8	Average % of Expected	94	102	98	99
	Range (%)	85-101	93-108	87-103	89-104
1:16	Average % of Expected	86	96	93	93
	Range (%)	81-93	89-103	80-102	85-99

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

SENSITIVITY

Forty-nine assays were evaluated and the minimum detectable dose (MDD) of human HTRA2 ranged from 1.07-13.2 pg/mL. The mean MDD was 4.79 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 *E. coli*-expressed recombinant human HTRA2 produced at R&D Systems.

SAMPLE VALUES

Serum/Platelet-Poor Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human HTRA2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=35)	412	189-2003	306
Platelet-poor EDTA plasma (n=35)	479	233-1870	282
Platelet-poor heparin plasma (n=35)	405	187-1870	289

Cell Culture Supernates - Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% bovine calf serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for natural human HTRA2.

	Day 1 (pg/mL)	Day 6 (pg/mL)
Unstimulated	197	187
Stimluated	101	489

SW480 human colorectal adenocarcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, and 5% CO_2. An aliquot of the cell culture supernate was removed, assayed for natural human HTRA2, and measured 129 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human HTRA2.

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 HTRA2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Cystatin E/M
Cystatin F
Cystatin S
Cystatin SA
Cystatin SN
HES-4
HTRA1
RTN1-A
SMAC
XIAP
XIAP (BIR2)
XIAP (BIR3)
cIAP-1 (HIAP-2)
cIAP-2 (HIAP-1)

Recombinant mouse:

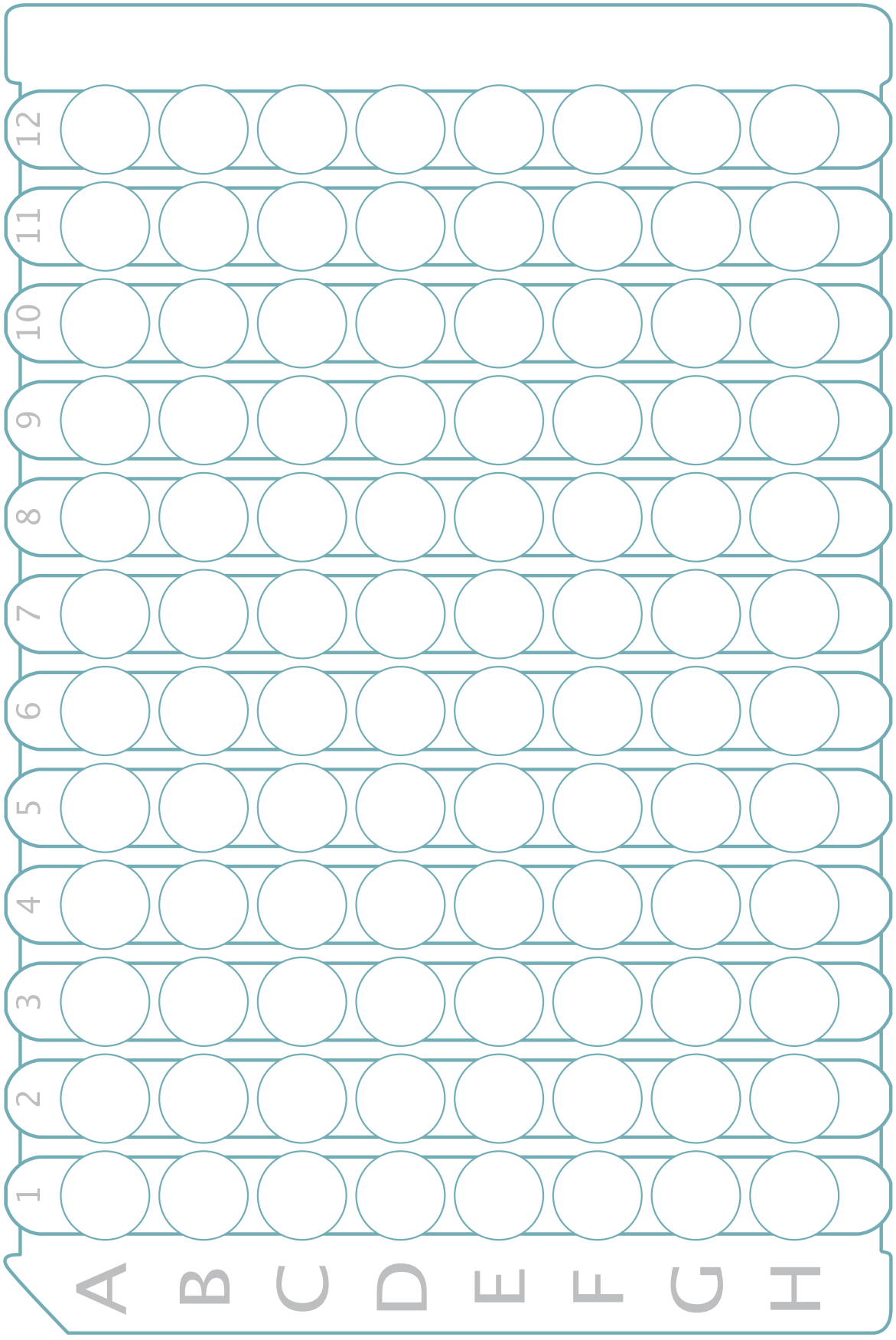
Cystatin A
Cystatin B
Cystatin C
Cystatin E/M

REFERENCES

1. Kilbride, S.M. and J.H.M. Prehn (2012) Oncogene **30**:2703.
2. Desideri, E. and L.M. Martins (2012) Int. J. Cell Biol. **2012**:607929.
3. Faccio, L. *et al.* (2000) J. Biol. Chem. **275**:2581.
4. Faccio, L. *et al.* (2000) Genomics **68**:343.
5. Nam, M. *et al.* (2006) Exp. Mol. Med. **38**:36.
6. Hegde, R. *et al.* (2001) J. Biol. Chem. **277**:432.
7. Verhagen, A. *et al.* (2001) J. Biol. Chem. **277**:445.
8. Huttunen, H.J. *et al.* (2007) J. Biol. Chem. **282**:28285.
9. Chao, J. *et al.* (2008) Nature **452**:98.
10. Radke, S. *et al.* (2008) J. Biol. Chem. **283**:12681.
11. Park, H. *et al.* (2006) J. Biol. Chem. **281**:34277.
12. Plun-Favreau, H. *et al.* (2012) Cell Death Dis. **3**:e335.
13. Seong, Y.M. *et al.* (2004) J. Biol. Chem. **279**:37588.
14. Martins, L. *et al.* (2002) J. Biol. Chem. **277**:439.
15. Martins, L.M. *et al.* (2003) J. Biol. Chem. **278**:49417.
16. Yang, Q. *et al.* (2003) Genes Dev. **17**:1487.
17. Srinivasula, S. *et al.* (2003) J. Biol. Chem. **278**:31469.
18. MacKenzie, S.H. and A.C. Clark (2012) Adv. Exp. Med. Biol. **47**:55.
19. Park, H. *et al.* (2009) Biochem. Biophys. Res. Commun. **387**:537.
20. Hartkamp, J. *et al.* (2010) Mol. Cell **37**:159.
21. Cilenti, L. *et al.* (2004) J. Biol. Chem. **279**:50295.
22. Gupta, S. *et al.* (2004) J. Biol. Chem. **279**:45844.
23. Fitzgerald, J.C. *et al.* (2012) Cell Death Differen. **19**:257.
24. Plun-Favreau, H. *et al.* (2007) Nat. Cell Biol. **9**:1243.
25. Yang, L. *et al.* (2007) J. Biol. Chem. **282**:10981.
26. Liu, M. *et al.* (2009) J. Cell Sci. **122**:1917.
27. Kawamoto, Y. *et al.* (2008) J. Neuropathol. Exp. Neurol. **67**:984.
28. Jones, J.M. *et al.* (2003) Nature **425**:721.
29. Kang, S. *et al.* (2012) Cell Death Differen. **20**:259.
30. Hu, Q. *et al.* (2012) Sci. Signal. **5**:ra61.
31. Zhou, Z. *et al.* (2011) Cell Adh. Migr. **5**:280.
32. Behbahani, H. *et al.* (2010) Neurochem. Int. **57**:668.
33. Kooistra, J. *et al.* (2009) J. Alzheimers Dis. **17**:281.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES