Quantikine® ELISA

Human Thrombopoietin Immunoassay

Catalog Number DTP00B

For the quantitative determination of human Thrombopoietin (Tpo) 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

Human thrombopoietin (Tpo), also known as megakaryocyte growth and development factor (MGDF), Mpl-ligand, and megakaryocyte colony stimulating factor (MK-CSF), is a 60-70 kDa, 332 amino acid (aa) residue glycosylated polypeptide that is believed to play a key role in the development of megakaryocytes (1-4). Secreted principally by hepatocytes (5) and bone marrow stromal cells (6), the Tpo molecule can be structurally and functionally divided into N- and C-terminal halves. The N-terminal half of Tpo is structurally analogous to the erythropoietin (Epo) molecule, showing approximately 25% aa residue identity and conservation of a number of cysteines. Functionally, this area is suggested to be responsible for receptor binding (1, 2, 7). The C-terminal region, on the other hand, is structurally characterized by a high percentage of serine and proline residues and contains a number of potential N-linked glycosylation sites. The C-terminus is believed to contribute to overall molecule stability and circulating half-life (1, 2, 8). Across species, there is marked conservation of aa residues, with human Tpo demonstrating 75%, 73% and 76% aa identity with rat (9), mouse (2) and dog (2) Tpo, respectively. The conservation of aa structure accounts for the cross-species bioactivity noted on human and mouse cells.

An 84 kDa receptor for Tpo, Mpl, has been identified in both humans and mice (12-14). In humans, two forms of the receptor have been noted, the longest of which is 607 aa residues in length, with a 463 aa extracellular region, a 22 aa transmembrane segment, and a 122 aa cytoplasmic domain rich in proline and serine (12). The shorter receptor form differs from the longer form only in that the cytoplasmic domain is only 66 amino acids in length with a high proline content (12). The extracellular region can be divided into two subdomains. The N-terminal subdomain contains the Tpo binding site and four conserved cysteines, characteristic of the hematopoietin receptor superfamily. The juxtamembrane subdomain shows a WS-X-WS box (specifically, WS-S-WS), also characteristic of members of the hematopoietin receptor superfamily (12). Relative to the human, the mouse receptor is almost identical in length (608 aa residues) and shows 81% overall aa identity, 78% in the extracellular region (13). Both the human and the mouse *c-mpl* gene show a possibility for an alternative splice event that could produce a soluble form of the receptor (13, 14). Cells known to express Mpl include platelets, megakaryocytes and $CD34^+CD61^+CD41a^+$ megakaryocyte progenitor cells (15), BFU-E cells (16), plus the HEL erythroid (12), MO-7E (15), and CMK (17) cell lines.

Functionally, Tpo seems to increase the absolute number of megakaryoctes (18), to increase megakaryocyte viability (19), and to shorten the time necessary for cell cycling (18). Tpo does not seem to contribute to pro-platelet formation (19, 21). Circulating Tpo has been measured in both normal and pathophysiological conditions, including idiopathic thrombocytopenia purpura (ITP) (22-24), essential thrombocythemia (ET) (25), liver cirrhosis (26), amegakaryocytic thrombocytopenia (AMT) (27), and aplastic anemia (AA) (22-25). While Tpo levels may be regulated by megakaryocyte counts, and platelets may play a role in regulating plasma Tpo levels, there does not seem to be a direct correlation between platelet count and blood Tpo levels (22, 24, 26-28).

The Quantikine® Human Thrombopoietin Immunoassay is a 4.5 hour solid phase ELISA designed to measure human Tpo in cell culture supernates, serum, and platelet-poor plasma. It contains NS0-expressed recombinant human Tpo and antibodies raised against the recombinant factor. Results obtained using natural human Tpo showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural human Tpo.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Tpo has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Tpo present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Tpo 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 Tpo 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, 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Tpo Microplate	892470	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Tpo.	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 Tpo Conjugate	892471	21 mL of a polyclonal antibody specific for human Tpo conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Tpo Standard	892472	Recombinant human Tpo in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-1	895143	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-11	895489	21 mL of a buffered protein base with preservatives. *Use undiluted for serum/ plasma samples. Use diluted 1:5 for cell culture supernate samples.*	
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	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.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards.
- Human Tpo Controls (optional; R&D Systems®, Catalog # QC176).

PRECAUTIONS

Calibrator Diluent RD6-11 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 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 at 2-8 °C 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 ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

The Tpo receptor is biologically active and present on platelets. Therefore, to measure circulating levels of Tpo, 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.

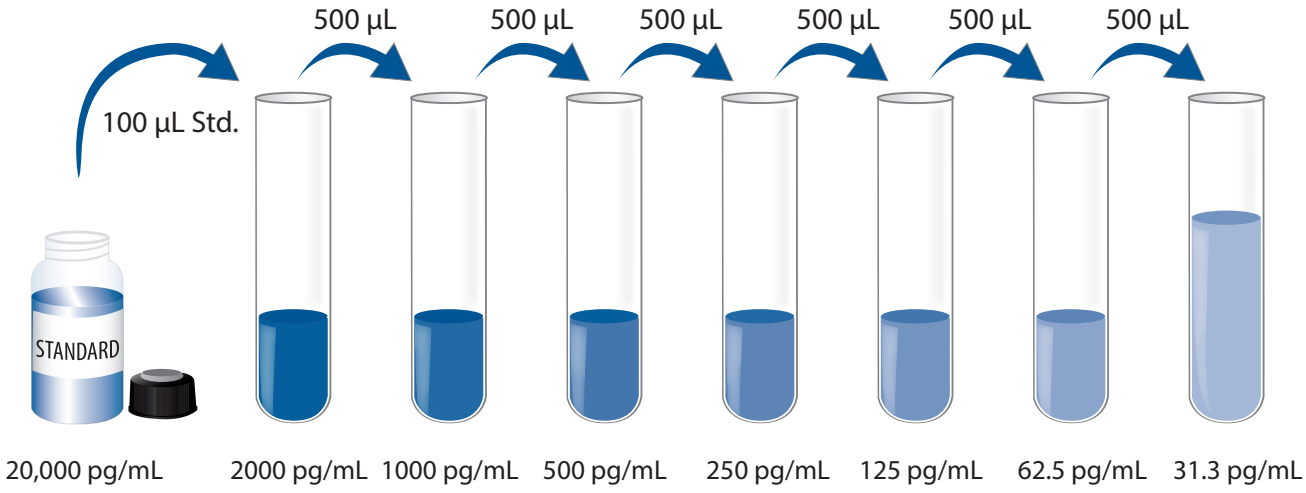
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD6-11 (diluted 1:5) - **For cell culture supernate samples only.** Add 5.0 mL of Calibrator Diluent RD6-11 to 20 mL of deionized or distilled water to prepare 25 mL of Calibrator Diluent RD6-11 (diluted 1:5).

Human Tpo Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Tpo Standard with 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 RD6-11 (diluted 1:5) (*for cell culture supernate samples*) or Calibrator Diluent RD6-11 (*for serum/plasma samples*) into the 2000 pg/mL tube. Pipette 500 µL of the appropriate calibrator diluent 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. 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, samples, and controls be assayed in duplicate.

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 50 µL of Assay Diluent RD1-1 to each well.
4. Add 200 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature. 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 Human Tpo Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
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. **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.

CALCULATION OF RESULTS

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

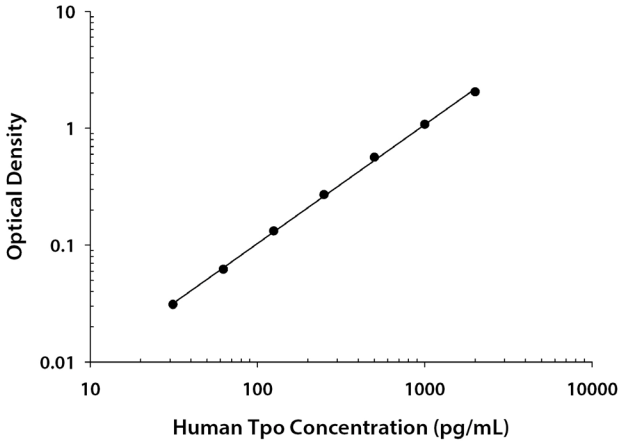
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Tpo 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

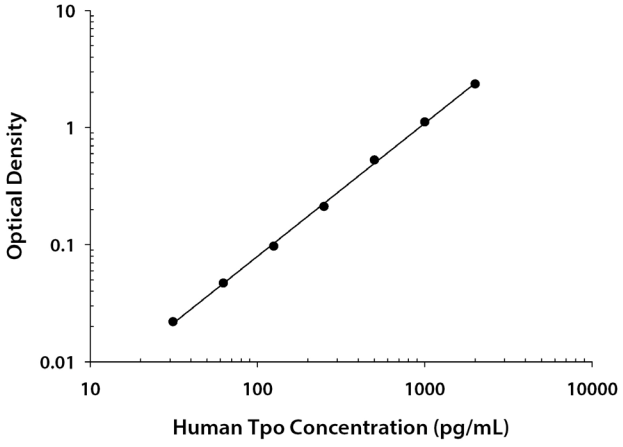
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.061 0.061	0.061	—
31.3	0.091 0.093	0.092	0.031
62.5	0.119 0.126	0.123	0.062
125	0.189 0.196	0.193	0.132
250	0.329 0.333	0.331	0.270
500	0.622 0.626	0.624	0.563
1000	1.118 1.159	1.139	1.078
2000	2.038 2.179	2.109	2.048

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.061 0.061	0.061	—
31.3	0.083 0.083	0.083	0.022
62.5	0.106 0.110	0.108	0.047
125	0.156 0.160	0.158	0.097
250	0.272 0.273	0.273	0.212
500	0.567 0.613	0.590	0.529
1000	1.159 1.196	1.178	1.117
2000	2.387 2.443	2.415	2.354

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 forty 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	40	40	40
Mean (pg/mL)	204	579	1140	203	555	1070
Standard deviation	13.7	15.1	52.6	19.3	48.7	92.8
CV (%)	6.7	2.6	4.6	9.5	8.8	8.7

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	194	512	983	187	517	970
Standard deviation	17.5	16.5	26.8	16.3	39.2	63.9
CV (%)	9.0	3.2	2.7	8.7	7.6	6.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	99	89-109%
Serum (n=4)	102	90-111%
Platelet-poor EDTA plasma (n=4)	95	85-102%
Platelet-poor Heparin plasma (n=4)	93	86-106%

LINEARITY

To assess linearity of the assay, samples spiked with high concentrations of human Tpo were diluted with the appropriate 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)	Heparin plasma (n=4)
1:2	Average % of Expected	110	101	108	104
	Range (%)	104-115	98-104	103-111	98-115
1:4	Average % of Expected	110	102	107	103
	Range (%)	106-115	97-111	101-115	94-115
1:8	Average % of Expected	103	92	98	99
	Range (%)	96-108	90-96	93-107	92-109
1:16	Average % of Expected	102	89	98	100
	Range (%)	94-112	83-97	88-104	88-104

SENSITIVITY

Eighty-four assays were evaluated and the minimum detectable dose (MDD) of human Tpo ranged from 2.78-18.5 pg/mL. The mean MDD was 7.45 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.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human Thrombopoietin produced at R&D Systems®.

SAMPLE VALUES

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

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=38)	74.2	74	ND-228
Platelet-poor EDTA plasma (n=34)	91.8	21	ND-196
Platelet-poor Heparin plasma (n=37)	82.8	19	ND-168

ND=Non-detectable

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 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 culture supernate were removed on days 1 and 5 and assayed for levels of human Tpo. No detectable levels were observed.

HepG2 human hepatocellular carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. An aliquot of the culture supernate was removed, assayed for human Tpo, and measured 81.9 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Tpo.

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

Recombinant human:
Epo
Epo R
Tpo R

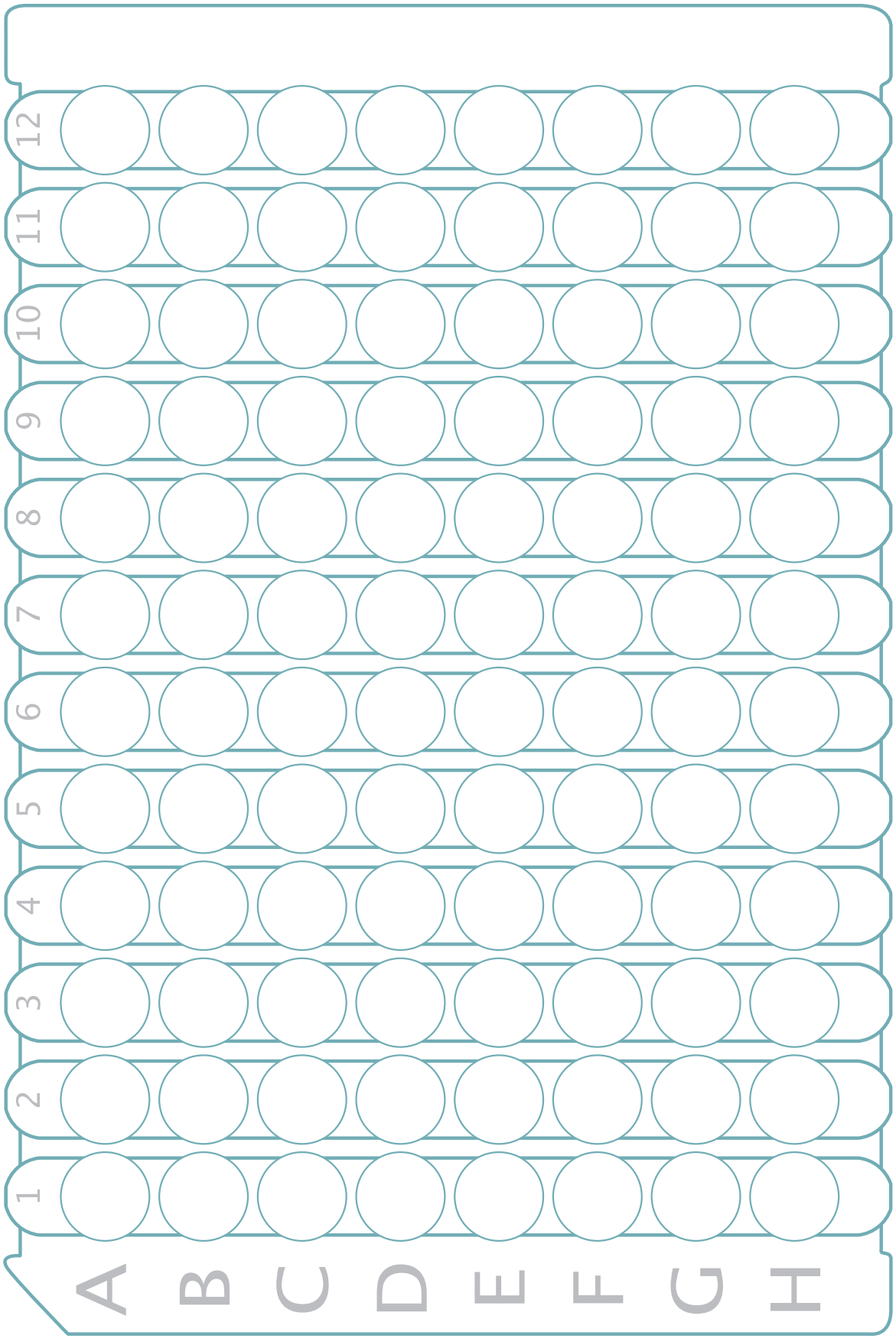
Recombinant mouse:
Epo
Tpo

REFERENCES

1. de Sauvage, F.J. *et al.* (1994) Nature **369**:533.
2. Bartley, T.D. *et al.* (1994) Cell **77**:1117.
3. Kaushansky, K. *et al.* (1994) Nature **369**:568.
4. Wendling, F. *et al.* (1994) Nature **369**:571.
5. Shimada, Y. *et al.* (1995) Exp. Hematol. **23**:1388.
6. Nagahisa, H. *et al.* (1996) Blood **87**:1309.
7. Sohma, Y. *et al.* (1994) FEBS Lett. **353**:57.
8. Lok, S. *et al.* (1994) Nature **369**:565.
9. Ogami, K. *et al.* (1995) Gene **158**:309.
10. Kato, T. *et al.* (1995) J. Biochem. **118**:229.
11. Miyazaki, H. (1996) Cancer Chemother. Pharmacol. **38**(Suppl):S74.
12. Vigon, I. *et al.* (1992) Proc. Natl. Acad. Sci. USA **89**:5640.
13. Vigon, I. *et al.* (1993) Oncogene **8**:2607.
14. Mignotte, V. *et al.* (1994) Genomics **20**:5.
15. Debli, N. *et al.* (1995) Blood **85**:391.
16. Kieran, M.W. *et al.* (1996) Proc. Natl. Acad. Sci. USA **93**:9126.
17. Shinohara, A. *et al.* (1996) Oncology **53**:426.
18. Angchaisuksiri, P. *et al.* (1996) Br. J. Haematol. **93**:13.
19. Ito, T. *et al.* (1996) Br. J. Haematol. **94**:387.
20. Itoh, R. *et al.* (1996) Br. J. Haematol. **94**:228.
21. Choi, E.S. *et al.* (1996) Br. J. Haematol. **95**:227.
22. Kosugi, S. *et al.* (1996) Br. J. Haematol. **93**:704.
23. Kunishima, S. *et al.* (1996) Eur. J. Hematol. **57**:68.
24. Ichikawa, N. *et al.* (1996) Thromb. Haemost. **76**:156.
25. Tahara, T. *et al.* (1996) Br. J. Haematol. **93**:783.
26. Shimodaira, S. *et al.* (1996) Thromb. Haemost. **76**:545.
27. Mukai, H.Y. *et al.* (1996) Thromb. Haemost. **76**:675.
28. Fielder, P.J. *et al.* (1996) Blood **87**:2154.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES