Quantikine® ELISA

Human Erythropoietin Immunoassay

Catalog Number DEPRUO

For the quantitative determination of Erythropoietin (Epo) concentrations in human serum and EDTA 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

Erythropoietin (Epo), a glycoprotein (~30,400 Daltons) produced primarily by the kidney, is the principal factor regulating red blood cell production (erythropoiesis) in mammals. Renal production of Epo is regulated by changes in oxygen availability. Under conditions of hypoxia, the level of Epo in the circulation increases and this leads to increased production of red blood cells.

The over-expression of Epo may be associated with certain pathophysiological conditions (1, 2). Polycythemia exists when there is an overproduction of red blood cells (RBCs). Primary polycythemias, such as polycythemia vera, are caused by Epo-independent growth of erythrocytic progenitors from abnormal stem cells and low to normal levels of Epo are found in the serum of affected patients. On the other hand, various types of secondary polycythemias are associated with the production of higher than normal levels of Epo. The overproduction of Epo may be an adaptive response associated with conditions that produce tissue hypoxia, such as living at high altitude, chronic obstructive pulmonary disease, cyanotic heart disease, sleep apnea, high-affinity hemoglobinopathy, smoking, or localized renal hypoxia (1, 2). In other instances, excessive Epo levels are the result of production by neoplastic cells. Cases of increased Epo production and erythrocytosis have been reported for patients with renal carcinomas (3), benign renal tumors (4), Wilms' tumors, hepatomas (5), liver carcinomas (6), cerebellar hemangioblastomas (3, 7, 8), adrenal gland tumors (9), smooth muscle tumors (3, 9), and leiomyomas (10).

Deficient Epo production is found in conjunction with certain forms of anemias. These include anemia of renal failure and end-stage renal disease (1, 2, 11), anemias of chronic disorders such as chronic infections (1), autoimmune diseases (1), rheumatoid arthritis (12), AIDS (13), malignancies (14), anemia of prematurity (2), anemia of hypothyroidism (2), and anemia of malnutrition (2). Many of these conditions are associated with the generation of IL-1 and TNF-α, factors that have been shown to be inhibitors of Epo activity (1, 15). Other forms of anemias, on the other hand, are due to Epo-independent causes and affected individuals show elevated levels of Epo (2). These forms include aplastic anemias, iron deficiency anemias, thalassemias, megaloblastic anemias, pure red cell aplasias, and myelodysplastic syndromes.

The Quantikine® Human Epo ELISA uses a monoclonal antibody and a polyclonal antibody conjugate in a sandwich ELISA format. The assay is designed to measure Epo levels in serum or EDTA plasma in less than 4.5 hours, or less than 2.5 hours using the shaker protocol.

PRINCIPLE OF THE ASSAY

The Quantikine® Human Epo ELISA is based on the double-antibody sandwich method. Microplate wells, precoated with a mouse monoclonal antibody specific for Epo are incubated with specimen or standard. Erythropoietin binds to the immobilized antibody on the plate. After removing excess specimen or standard, wells are incubated with a rabbit anti-Epo polyclonal antibody conjugated to horseradish peroxidase. During the second incubation, the antibody-enzyme conjugate binds to the immobilized Epo. Excess conjugate is removed by washing. A chromogen is added to the wells and is oxidized by the enzyme reaction to form a blue colored complex. The reaction is stopped by the addition of acid, which turns the blue to yellow. The amount of color generated is directly proportional to the amount of conjugate bound to the Epo antibody complex, which, in turn, is directly proportional to the amount of Epo in the specimen or standard. The absorbance of this complex is measured and a standard curve is generated by plotting absorbance versus the concentration of the Epo standards. The Epo concentration of the unknown specimen is determined by comparing the optical density of the specimen to the standard curve. The standards used in this assay are recombinant human Epo calibrated against the Second International Reference Preparation (67/343), a urine-derived form of human erythropoietin.

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.
- In order to minimize within assay variation, it is recommended that the assay be pipetted within 15 minutes.
- If samples generate values higher than the highest standard, dilute the samples with Specimen Diluent and repeat the assay.
- Any variation in 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 Epo Microplate	893440	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Epo.	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 Epo Conjugate	893441	21 mL of a polyclonal antibody specific for human Epo conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Epo Standard 0 mIU/mL	893442	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 2.5 mIU/mL	893443	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 5.0 mIU/mL	893444	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 20.0 mIU/mL	893445	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 50.0 mIU/mL	893446	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 100.0 mIU/mL	893447	2.1 mL of a buffered protein base with preservatives.	
Human Epo Standard 200.0 mIU/mL	893448	2.1 mL of a buffered protein base with preservatives.	
Human Epo Assay Diluent	895166	11 mL of a buffered protein base with preservatives.	
Human Epo Specimen Diluent	895149	26 mL of a protein stabilized buffer with preservatives.	
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	895926	11 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

- Pipettes and pipette tips.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm (required for the Shaker Protocol).
- 500 mL graduated cylinder.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Absorbent pad or paper towels for blotting the wells.
- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 600 nm.
- Deionized or distilled water.
- A computer capable of 4 parameter logistic curve fitting for data reduction.
- **Polypropylene** test tubes for dilution of samples.

PRECAUTIONS

Some components of this kit contain 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.

SPECIMEN COLLECTION & STORAGE

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

Serum - Use a serum separator or clot tube and allow specimens to coagulate at room temperature. Centrifuge at 760 x g* for 15 minutes at room temperature within 30 minutes of collection to avoid hemolysis.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge specimens at 760 x g* for 15 minutes at room temperature within 30 minutes of collection.

It is recommended that each laboratory standardize the assay using either serum or EDTA plasma specimens.

Lipemic, grossly hemolyzed, or contaminated specimens may yield inaccurate results and should not be tested with this procedure. Further, no drugs have been investigated for assay interference.

Refer to CLSI Guideline: *Procedures for the Handling and Processing of Blood Specimens* (CLSI Document H18-A; current volume)

* $g = (1.118 \times 10^{-5})$ (radius in cm) $(rpm)^2$

SPECIMEN PREPARATION

Use polypropylene tubes.

If a serum or plasma specimen is above 200 mIU/mL, dilute with the Specimen Diluent. For example:

- For specimens with Epo concentrations between 200 mIU/mL and 2000 mIU/mL, a 10-fold dilution of the specimen is necessary. Dilute 25 µL of specimen with 225 µL of the Specimen Diluent.
- For specimens with Epo concentrations in excess of 2000 mIU/mL, a higher dilution will be necessary to bring them within the range of the standard curve (*i.e.* 20-fold, 40-fold, *etc.* dilution).

To determine the Epo concentration of the serum or plasma specimen, multiply the result obtained by the dilution factor.

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. Discard any unused, prepared Substrate Solution.

ASSAY PROCEDURE

Bring all reagents and specimens to room temperature before use. It is recommended that all specimens, standards, and controls be assayed in duplicate. Benchtop and shaker protocols are provided. The same protocol must be used throughout the assay.

1. Prepare all reagents as directed in the previous section.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Pipette 100 µL of Human Epo Assay Diluent into each well.
4. Add 100 µL of standard, control, or specimen per well. Gently tap the plate frame for approximately 1 minute to mix the well contents. Cover the plate with the adhesive strip provided. A plate layout containing a sample diagram of standards, controls, and specimens is shown on page 12.
   For Benchtop Protocol: Incubate for 2 hours ± 5 minutes at room temperature.
   For Shaker Protocol: Incubate for 1 hour ± 5 minutes at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Thoroughly aspirate or decant the contents from each well. Blot dry on clean paper toweling. **Do not wash.**
6. Add 200 µL of Human Epo Conjugate to each well. Cover the plate with a new adhesive strip.
   For Benchtop Protocol: Incubate for 2 hours ± 5 minutes at room temperature.
   For Shaker Protocol: Incubate for 1 hour ± 5 minutes at room temperature on a horizontal orbital microplate shaker.
7. Aspirate each well and wash, repeating the process three times for a total of 4 washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential for 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.
8. Add 200 µL of Substrate Solution to each well (**Note:** *Substrate Solution* ***must*** *be used within 15 minutes of preparation*). Incubate for 20-25 minutes at room temperature **on the benchtop.**
9. Add 100 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density (O.D.) of each well within 15 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 600 nm. If wavelength correction is not available, subtract readings at 600 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

Read the absorbance of each well on a microplate reader using 450 nm as the primary wavelength and 600 nm as the reference wavelength (540, 570, or 650 nm is acceptable).

Average the duplicate readings for each standard, control, and specimen and subtract the average 0 mIU/mL standard optical density (O.D.).

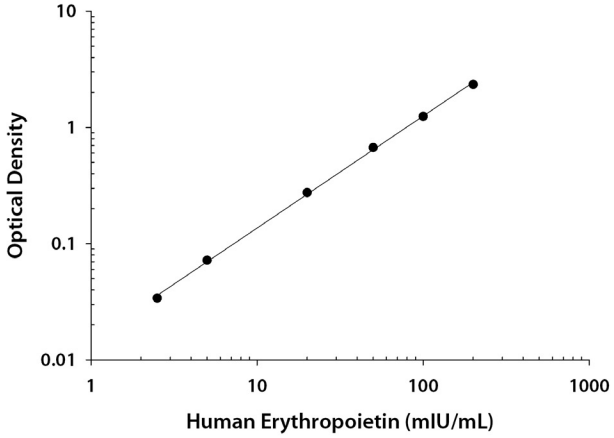
Create a standard curve by reducing the data using software capable of generating a four parameter logistic (4PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y axis against the concentration on the x axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the Epo concentrations versus the log of the O.D., and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Report values for each unknown that reads within the range (2.5-200 mIU/mL) of the assay. For unknown values above the range, see Specimen Preparation section. For values below the range, report as undetectable or < 2.5 mIU/mL.

TYPICAL DATA

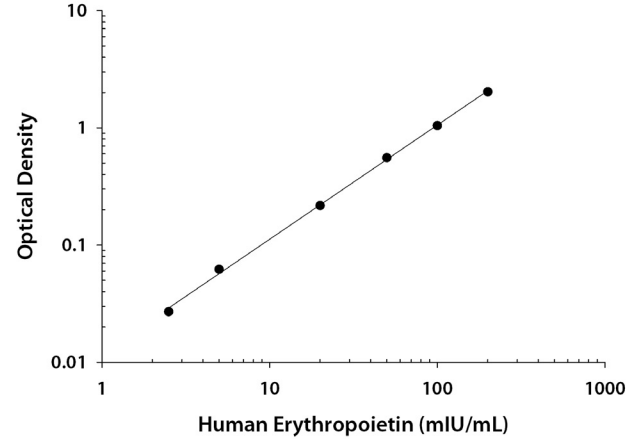
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

BENCHTOP PROTOCOL



(mIU/mL)	O.D.	Average	Corrected
0	0.072		
0	0.074	0.073	—
2.5	0.106		
2.5	0.108	0.107	0.034
5.0	0.144		
5.0	0.146	0.145	0.072
20	0.342		
20	0.353	0.348	0.275
50	0.743		
50	0.746	0.744	0.671
100	1.298		
100	1.340	1.319	1.246
200	2.366		
200	2.463	2.414	2.341

SHAKER PROTOCOL



(mIU/mL)	O.D.	Average	Corrected
0	0.045		
0	0.047	0.046	—
2.5	0.070		
2.5	0.076	0.073	0.027
5.0	0.107		
5.0	0.108	0.108	0.062
20	0.263		
20	0.263	0.263	0.217
50	0.597		
50	0.608	0.602	0.556
100	1.081		
100	1.098	1.090	1.044
200	2.008		
200	2.136	2.072	2.026

PRECISION

Intra-assay Precision (Precision within an assay)
Four samples of known concentration were tested thirty times in each protocol to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Four samples of known concentration were tested in thirty separate assays in each protocol to assess inter-assay precision. Assays were performed by at least three technicians using three lots of components.

	Benchtop Protocol				Shaker Protocol			
Sample	1	2	3	4	1	2	3	4
Mean (mIU/mL)	5.82	16.0	25.2	79.0	6.45	16.2	26.4	84.2
Intra-assay Precision (%CV)	4.95	2.84	5.22	3.06	7.82	3.02	2.18	2.42
Inter-assay Precision CV (%)	8.25	4.30	5.90	4.24	10.30	3.74	4.20	3.71

RECOVERY

Recovery was estimated by addition of recombinant human Epo into ten plasma and ten serum specimens. The percent recovery of the added Epo was calculated from the equation:

$$\% \text{ recovery} = \frac{\text{measured value after addition - measured value before addition}}{\text{measured value of the added material}}$$

Mean recoveries are shown in the following table. The overall mean for the two specimen types and two assay protocols was 100 ± 12%.

Specimen Type	Protocol	n	Amount Added	Mean Recovery
Serum	Benchtop	10	52.2 mIU/mL	100%
	Shaker	10	57.5 mIU/mL	93%
Plasma (EDTA)	Benchtop	10	51.8 mIU/mL	102%
	Shaker	10	55.9 mIU/mL	105%

LINEARITY

Five matched serum and plasma specimens containing elevated Epo concentrations were diluted with Specimen Diluent and assayed using the Quantikine® Human Epo ELISA. Diluted specimens demonstrated very good linearity when compared to neat concentrations of Epo.

		Benchtop Protocol		Shaker Protocol	
		EDTA Plasma	Serum	EDTA Plasma	Serum
1:2	Average % of Expected	102	101	101	103
	Range (%)	95-115	95-109	96-110	98-110
1:4	Average % of Expected	103	104	101	102
	Range (%)	97-123	100-117	92-111	92-111
1:8	Average % of Expected	105	104	102	101
	Range (%)	96-132	94-126	91-114	91-109
1:16	Average % of Expected	103	99	97	95
	Range (%)	93-129	87-126	88-106	81-110

SENSITIVITY

The sensitivity (minimum detectable dose) of the Quantikine® Human Erythropoietin ELISA is typically less than 0.6 mIU/mL. This was determined by adding two standard deviations to the mean optical density of twenty replicates of the zero standard and calculating the corresponding concentration from the standard curve.

SAMPLE VALUES

Samples from apparently healthy volunteers were evaluated for the presence of human Epo in this assay. No medical histories were available for the donors used in this study.

Serum (n=123)	EDTA plasma (n=123)
3.3-16.6 mIU/mL	3.1-14.9 mIU/mL

SPECIFICITY

The complete sequence of the Epo protein was compared with sequences in the Protein Identification Resource and the Swiss-Protein data bases. Recombinant and natural human Epo sequences are identical; no significant homology with other human proteins was found. When assayed in the Quantikine® Human Epo ELISA, the WHO standard 88/574 (recombinant human Epo) showed similar reactivity relative to WHO standard 67/343 (natural human Epo).

Each of the following analytes was spiked to 1 µg/mL in Specimen Diluent and run as an unknown in the assay. No cross-reactivity was observed.

Recombinant human:

ANG
β-ECGF
FGF basic
GROα
IFN-γ
IGF-I
IGF-II
IL-1β
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-6 R
IL-8
IL-9
IL-10
IL-11
LIF
MCP-1
M-CSF
MIP-1α
MIP-1β
OSM
PDGF-AA
PDGF-AB
PDGF-BB
RANTES
SLPI
TGF-β3
TNF-α
TNF RI

Recombinant mouse:

EGF
IL-1β
IL-3
IL-4
IL-5
IL-9
MIP-1α
MIP-1β
SCF
TNF-α

Recombinant canine:

TGF-β3

Recombinant amphibian:

TGF-β5

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine TGF-β1
porcine TGF-β1.2
porcine TGF-β2

Interference was tested by spiking serum, plasma or Specimen Diluent with selected amounts of the following substances and analyzing for the presence of Epo in the Quantikine® Human Epo ELISA using both the benchtop and shaker protocols. Endogenous and additional levels of these substances are detailed in the following table. To determine if the added substance interfered with assay performance, the recovery of Epo levels in each specimen was calculated. Recoveries averaged 102.5 ± 4.7% and ranged from 88.6-117.7%.

Protein	Normal Range*	Amount Added
α-1-acid glycoprotein	50-140 mg/dL	30 and 80 mg/dL
α-1-antitrypsin	150-400 mg/dL	200 mg/dL
α-2-macroglobulin	70-430 mg/dL	36 mg/dL
Albumin	3.5-5.0 g/dL	3 g/dL
Bilirubin (unconjugated)	0-0.8 mg/dL (adult)	20 mg/dL
Gamma Globulin	0.7-1.5 g/dL	3 and 5 g/dL
Hemoglobin	< 2.5 mg/dL	15, 30, and 45 mg/dL
Triglycerides	35-160 mg/dL	~2920 and ~14600 mg/dL
Transferrin	200-400 mg/dL	200 mg/dL

* Tietz, Fundamentals of Clinical Chemistry, 2nd Edition, Copyright 1976

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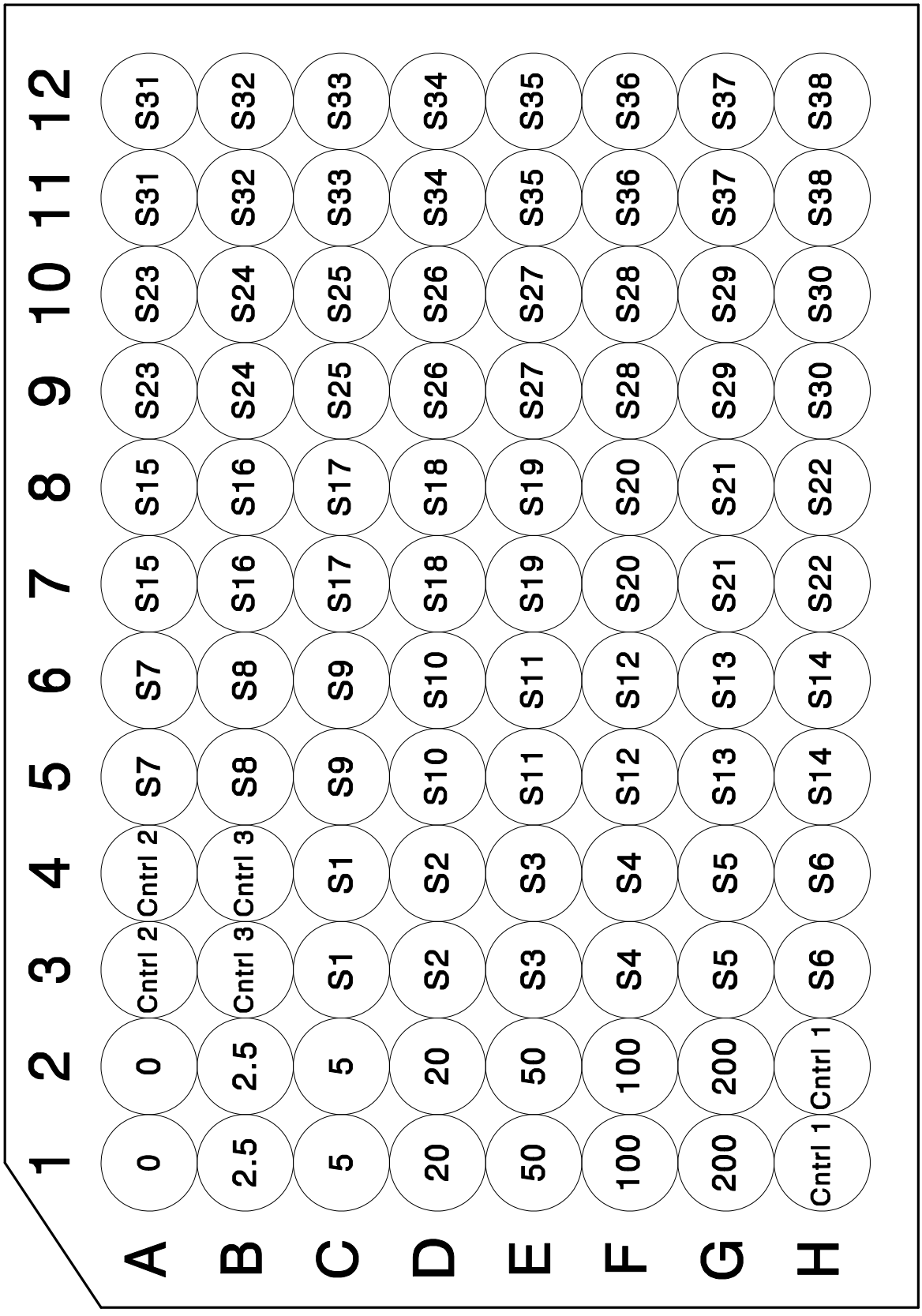
PLATE LAYOUT

A sample diagram for standards, controls, and specimens is shown below.



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