Parameter™

Total Nitric Oxide and Nitrate/Nitrite Assay

Catalog Number KGE001
SKGE001
PKGE001

For the quantitative determination of Nitric Oxide (NO) concentrations in cell culture supernates, serum, plasma, and urine.

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 biological activities of Nitric Oxide (NO) were first widely appreciated when it was identified as the endothelial-derived relaxing factor (EDRF) responsible for the potent vasodilating properties of stimulated endothelia (1-3). Since then, NO has been recognized as a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. It is a gaseous free radical with a short half-life *in vivo* of a few seconds or less. Therefore, the levels of the more stable NO metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), have been used in the indirect measurement of NO in biological fluids (4-7). Altered levels of NO have been shown to be associated with sepsis, reproduction, infection, hypertension, exercise, type 2 diabetes, hypoxia, and cancer (8-16).

Catalyzed by enzymes of the Nitric Oxide Synthase (NOS) family, NO is produced via the reaction of L-Arginine, NADPH, and O_2 to NO and Citrulline (17-20). Members of the NOS family include neuronal (nNOS/NOS1), endothelial (eNOS/NOS3), and inducible (iNOS/NOS2) (21). As the name implies, nNOS is highly expressed in neurons of the central and peripheral nervous systems, and has also been described in other cell types including skeletal muscle myocytes, lung epithelial cells, and skin mast cells (22-29). eNOS is highly expressed by endothelial cells and may also be found in neurons, dermal fibroblasts, epidermal keratinocytes, thyroid follicular cells, hepatocytes, and smooth muscle cells (28, 30-34). iNOS is expressed in a wide range of cell types including chondrocytes, epithelial cells, hepatocytes, glial cells, and several cell types of the immune system (22, 35-37). In general, eNOS and nNOS are constitutively expressed and regulated by Ca^{2+}/Calmodulin, while iNOS is induced by endotoxin and inflammatory cytokines, and exhibits a relative insensitivity to Ca^{2+} (21, 38).

Because it is lipid soluble, NO is not stored but is synthesized *de novo* and freely diffuses across lipid membranes. NO has the potential to mediate its effects on target cells via several different mechanisms. For instance, NO-mediated activation of the enzyme Guanylyl Cyclase (GC) catalyzes the formation of the second messenger Guanosine 3′,5′-cyclic Monophosphate (cGMP). cGMP is implicated with a range of biological functions such as regulating smooth muscle contractility, cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, angiogenesis, and the activity of cyclic nucleotide-gated channels (39-47). NO also functions as an anti-tumor and anti-microbial agent via mechanisms that include its conversion to peroxynitrite ($ONOO^-$), the formation of S-nitrosothiols, and the depletion of arginine (35). Another putative role for NO includes the suppression of mitochondrial respiration through the inhibition of Cytochrome Oxidase (48). NO may also modify protein activity through post-translational nitrosylation via the attachment of an NO moiety to the thiol side chain of cysteine residues (49, 50).

R&D Systems®' Total Nitric Oxide kit has two assay options. Endogenous nitrite is measured in the first option. In the second option, nitrate is converted to nitrite using nitrate reductase and total nitrite is measured. To obtain the nitrate concentration, endogenous nitrite is subtracted from the total nitrite value.

PRINCIPLE OF THE ASSAY

This assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm (51).

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 Reaction Diluent (diluted 1:10) and repeat the assay.
- Any variation in diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.
- Variations in sample collection, processing, and storage may cause sample value differences.

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.
- Pre-rinse the pipette tip when pipetting.
- Precautions should be taken to avoid the contamination of samples or buffers with outside sources of nitrites or nitrates. Possible sources of contamination include skin, saliva, food, drink, water, or laboratory supplies such as pipette tips, gloves, etc.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary to avoid contamination.
- Microfiltration devices used for the deproteinization of samples can contain trace amounts of chemicals that may interfere in this assay. Rinse these devices by filtering 0.5 mL of high purity water through the membrane just prior to sample filtration.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	CATALOG # KGE001	CATALOG # SKGE001	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Uncoated Microplate	892880	2 plates	12 plates	96 well polystyrene microplates (12 strips of 8 wells).	Return unused wells to the storage bag. May be stored at 2-8 °C or at room temperature.
Nitrate Reductase	892873	1 vial	6 vials	Lyophilized Nitrate Reductase.	Aliquot and store for up to 1 month at ≤ -20 °C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
NADH	892875	2 vials	12 vials	Lyophilized reduced β-Nicotinamide adenine dinucleotide. **Must be stored in the dark.**	
Nitrate Reductase Storage Diluent	892874	1 vial	6 vials	1.2 mL/vial of a buffer containing glycerol.	May be stored for up to 1 month at 2-8 °C.*
Nitrite Standard	892886	1 vial	6 vials	0.5 mL/vial of a Sodium Nitrite solution (2000 µmol/L) in buffer.	
Nitrate Standard	892876	1 vial	6 vials	0.5 mL/vial of a Sodium Nitrate solution (2000 µmol/L) in buffer.	
Reaction Diluent Concentrate	892877	1 vial	6 vials	30 mL/vial of a 10-fold concentrated buffer containing detergent. *Use diluted 1:10 in this assay.*	
Griess Reagent I	892878	1 vial	6 vials	12 mL/vial of Sulfanilamide in 2 N hydrochloric acid.	
Griess Reagent II	892879	1 vial	6 vials	12 mL/vial of *N*-(1-Naphthyl) ethylenediamine in 2 N hydrochloric acid.	
Plate Sealers	N/A	4 strips	24 strips	4 adhesive strips.	

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

KGE001 contains sufficient materials to run one Nitrite and one Nitrate assay.
SKGE001 (SixPak) contains sufficient materials to run six Nitrite and six Nitrate assays.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PKGE001). PharmPaks contain sufficient materials to run 50 Nitrite and 50 Nitrate assays. Specific vial counts of each component may vary. Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 nitrite and 50 nitrate 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.

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Uncoated Microplate	892880	100 plates
Nitrate Reductase	892873	50 vials
NADH	892875	25 vials
Nitrate Reductase Storage Diluent	892874	50 vials
Nitrite Standard	892886	25 vials
Nitrate Standard	892876	25 vials
Reaction Diluent Concentrate	892877	50 vials
Griess Reagent I	892878	50 vials
Griess Reagent II	892879	50 vials
Plate Sealers	N/A	100 sheets
Package Inserts	751331	2 booklets

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 540 nm, with the correction wavelength set at 690 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- 37 °C incubator.
- Vortex mixer.
- 500 mL graduated cylinder.
- 10,000 molecular weight cut-off filters (Millipore, Catalog # UFC501096) and 1.5 mL microcentrifuge tubes (non-sterile) for use in sample preparation.
- Test tubes for dilution of standards and samples.
- Controls (optional; R&D Systems®, Catalog # QC134 or Catalog # QC135).

PRECAUTION

Griess Reagents I and II supplied with this kit are acidic and contain organic chemicals. Refer to the Safety Data Sheets for proper handling information. Wear protective gloves, clothing, eye, and face protection when using these reagents.

INTERFERENCE (see references 51-55)

The Griess reaction involves an oxidation and a nucleophilic reaction. Any reagents or substances that impact the Griess reaction will also likely alter color development or absorption. Examples of interfering substances are anticoagulants, azide, ascorbic acid, and sulfhydryl-containing compounds such as cysteine and glutathione. If concentrations of these compounds are expected to exceed 10 µM in the sample, nitrate/NO_3^- and nitrite/NO_2^- recovery should be determined using the nitrate and nitrite standards provided in the kit. Nitrate and nitrite concentrations similar to those used for the standard curve should be added to both the buffer containing the suspected interfering compound(s), and to an equivalent buffer that does not contain the suspected interfering compound. If significant differences are found in the buffer containing the interfering substance(s), the magnitude of the effect should be noted and suitable corrections made to the unknown samples.

In vivo, nitrite is converted to stable nitrate by hemoglobin. Thus nitrite levels are typically very low. With enzymatic conversion by nitrate reductase, all nitrite and converted nitrate is assayed, providing a comprehensive overview of total NO production. Problems with the enzyme assay include the presence of enzyme inhibitors and a failure to convert all of the nitrate to nitrite. Thus, strict controls should accompany all steps in the assay.

The nitrate salt concentration must be considered when choosing a tissue culture media. Some media may contain unacceptably high levels of nitrate that may contribute to an over-estimation of NO production. A parallel situation may occur *in vivo*. Absorbed dietary nitrates may inflate NO estimates. Absorbance interference is also a potential problem, however tissue culture media that uses phenol red as a pH indicator does not interfere with the Griess Reaction.

Proteins are also known to interfere with the Griess reaction. It is recommended that proteins be removed before analysis. Common deproteinizing protocols involve the use of $ZnSO_4$ or 35% sulfosalicylic acid or ultrafiltration using 10,000 molecular weight (MW) cut-off filters. R&D Systems® has tested and found 10,000 MW cut-off filtration of protein-containing samples is the preferred method of deproteinizing samples for use in this assay.

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 - Use a serum separator tube (SST). Centrifuge 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.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter. Assay immediately or aliquot and store at ≤ -80 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Samples should be 10,000 MW filtered* and diluted prior to assay.

Serum and plasma samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of filtered sample + 100 µL of Reaction Diluent (diluted 1:10)**.

Cell culture supernate and urine samples require at least a 5-fold dilution. A suggested 5-fold dilution is 50 µL of filtered sample + 200 µL of Reaction Diluent (diluted 1:10)**.

Note: Some cell culture supernate and urine samples may contain high concentrations of nitrate and may require up to a 20-fold dilution.

Note: Samples containing 0.5-1.0 mM NADPH may require at least a 10-fold dilution in Reaction Diluent (diluted 1:10)**. In samples containing > 1.0 mM NADPH, the NADPH should be oxidized prior to the Griess reaction. See reference 56 for an example of an appropriate oxidation procedure.

*See the Interference section.
**See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use. Use deionized or distilled water when reconstituting or diluting reagents to avoid nitrite/nitrate contamination.

Reaction Diluent (diluted 1:10) - Add 30 mL of Reaction Diluent Concentrate to 270 mL of deionized or distilled water to prepare 300 mL of Reaction Diluent (diluted 1:10).

Nitrate Reductase -

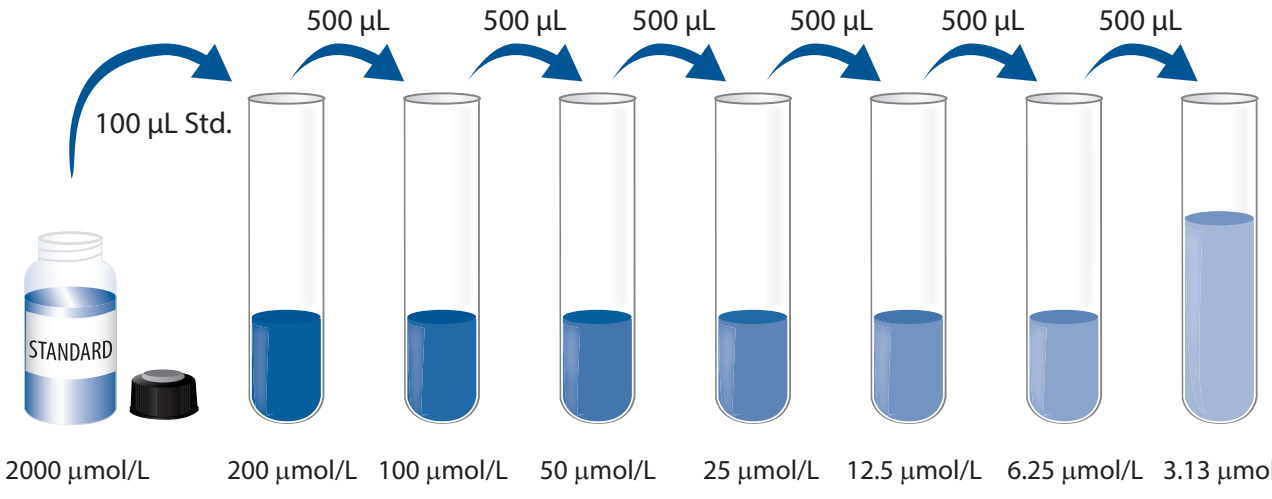
Reconstitution - Reconstitute the Nitrate Reductase with 1.0 mL Nitrate Reductase Storage Diluent. Vortex vigorously and allow to sit for 15 minutes at room temperature. Vortex again and allow to sit for an additional 15 minutes at room temperature. Vortex again and use immediately.

Dilution - Immediately before use, dilute the Nitrate Reductase using the following equation. Determine the number of standard and sample wells to be used (do not include blank wells). All samples and standards should be assayed in duplicate.

A. Nitrate Reductase (µL) = (# of wells + 2) x 5.0 µL.
B. Reaction Diluent (diluted 1:10) (µL) = Volume from step A x 4.
C. Add volumes from steps A and B to a clean test tube and vortex.
D. Place on ice and use within 15 minutes of dilution.

NADH Reagent - Reconstitute the NADH with 5.0 mL of deionized or distilled water. Allow the NADH to sit for 3 minutes with gentle agitation prior to use. **Use within 15 minutes or place on ice.**

Nitrite/Nitrate Standard - Pipette 900 µL of Reaction Diluent (diluted 1:10) into the 200 µmol/L tube. Pipette 500 µL into the remaining tubes. Use the appropriate 2000 µmol/L standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 200 µmol/L standard serves as the high standard. Reaction Diluent (diluted 1:10) serves as the blank (0 µmol/L).



NITRITE ASSAY PROCEDURE

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

This assay procedure measures the concentration of endogenous nitrite present in the sample.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, and return them to the storage bag.
3. Add 50 µL of Reaction Diluent (diluted 1:10) to the Blank wells.
4. Add 50 µL of Nitrite Standard or sample* to the remaining wells.
5. Add 50 µL of Reaction Diluent (diluted 1:10) to all wells.
6. Add 50 µL of Griess Reagent I to all wells.
7. Add 50 µL of Griess Reagent II to all wells. Mix well by gently tapping the side of the plate.
8. Incubate for 10 minutes at room temperature.
9. Determine the optical density (O.D.) of each well using a microplate reader set at 540 nm (wavelength correction at 690 nm).

NITRATE REDUCTION ASSAY PROCEDURE

This assay procedure measures total nitrite by converting nitrate to nitrite. To determine the nitrate concentration in the sample, the endogenous nitrite concentration measured from the Nitrite Assay Procedure must be subtracted from the converted nitrite concentration measured in this procedure.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, and return them to the storage bag.
3. Add 50 µL of Reaction Diluent (diluted 1:10) to the Blank wells.
4. Add 50 µL of Nitrate Standard or sample* to the remaining wells.
5. Add 25 µL of NADH to all wells.
6. Add 25 µL of diluted Nitrate Reductase** to all wells. Mix well and cover with the adhesive strip provided.
7. Incubate for 30 minutes at 37 °C.
8. Add 50 µL of Griess Reagent I to all wells.
9. Add 50 µL of Griess Reagent II to all wells. Mix well by gently tapping the side of the plate.
10. Incubate for 10 minutes at room temperature.
11. Determine the optical density (O.D.) of each well using a microplate reader set at 540 nm (wavelength correction at 690 nm).

*Samples require dilution and filtration. See the Sample Preparation section.
**Nitrate Reductase must be diluted. See the Reagent Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average blank O.D.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) 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 concentration 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.

Calculate the concentration of nitrite corresponding to the mean absorbance from the Nitrite standard curve.

To determine the concentration of nitrate in the sample:

a. Measure the endogenous nitrite concentration (X µmol/L) using the Nitrite Assay Procedure.

b. Measure the total nitrite concentration (Y µmol/L) after the conversion of nitrate to nitrite using the Nitrate Reduction Assay Procedure.

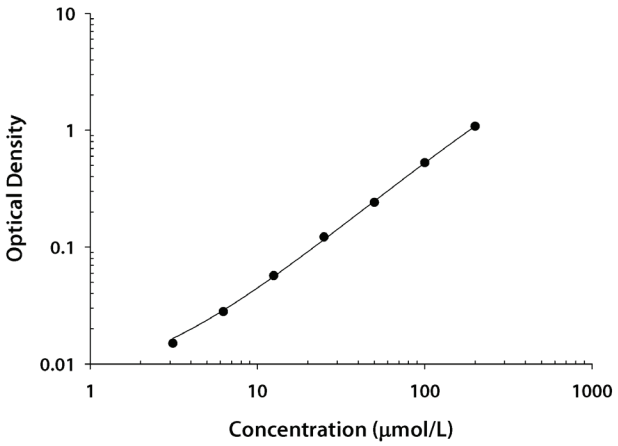
c. Determine the nitrate concentration in the sample by subtracting the endogenous nitrite concentration from the total nitrite concentration.

Nitrate concentration = (Y-X) µmol/L

Since 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.



(µmol/L)	O.D.	Average	Corrected
0	0.014 0.016	0.015	—
3.13	0.030 0.030	0.030	0.015
6.25	0.041 0.044	0.043	0.028
12.5	0.070 0.073	0.072	0.057
25	0.133 0.140	0.137	0.122
50	0.254 0.257	0.256	0.241
100	0.528 0.557	0.543	0.528
200	1.071 1.118	1.095	1.080

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (µmol/L)	30.0	77.2	138.0	31.5	79.9	138.7
Standard deviation	0.76	1.10	2.20	1.46	2.91	4.78
CV (%)	2.5	1.4	1.6	4.6	3.6	3.4

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum (n=10)	95	89-101%
Plasma (n=25)	98	87-110%

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

Sample Type	Average % Recovery	Range
Cell culture supernates (n=10)	107	91-115%
Serum (n=10)	109	98-118%
Plasma (n=20)	104	92-117%
Urine (n=10)	101	87-112%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of the nitrate standard were serially diluted with the Reaction Diluent (diluted 1:10) to produce samples with values within the dynamic range of the assay. Samples were filtered and diluted prior to assay. See the Sample Preparation section.

		Cell culture supernates (n=10)	Serum (n=10)	EDTA plasma (n=10)	Heparin plasma (n=10)	Citrate plasma (n=5)	Urine (n=10)
1:2	Average % of Expected	98	101	99	99	99	100
	Range (%)	96-102	96-105	96-103	92-105	95-101	96-103
1:4	Average % of Expected	99	101	98	98	99	102
	Range (%)	94-104	98-103	95-102	89-104	96-101	97-105
1:8	Average % of Expected	101	105	102	102	101	107
	Range (%)	93-111	101-109	99-108	92-109	95-105	99-119
1:16	Average % of Expected	106	112	111	108	106	108
	Range (%)	102-121	104-118	100-119	92-120	96-111	——

SENSITIVITY

Fifty-two assays were evaluated and the minimum detectable dose (MDD) ranged from 0.09-0.78 µmol/L. The mean MDD was 0.25 µmol/L.

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.

SAMPLE VALUES

Samples were filtered and diluted and then evaluated for the presence of NO_2^- in the nitrite assay. All samples measured below the lowest standard, 3.13 µmol/L. Samples were also evaluated for the presence of total nitric oxide (NO_2^-/NO_3^-) in the nitrate reduction assay. Samples were filtered and diluted prior to this assay. See the Sample Preparation section. Results are shown in the table below:

Sample Type	Mean (µmol/L)	Range (µmol/L)	Standard Deviation (µmol/L)
Cell culture supernates (n=10)	502	11-850	414
Serum (n=25)	37	13-97	22
EDTA plasma (n=25)	37	10-92	21
Heparin plasma (n=25)	36	10-90	20
Citrate plasma (n=8)	30	11-81	23
Urine (n=10)	1396	369-2684	877

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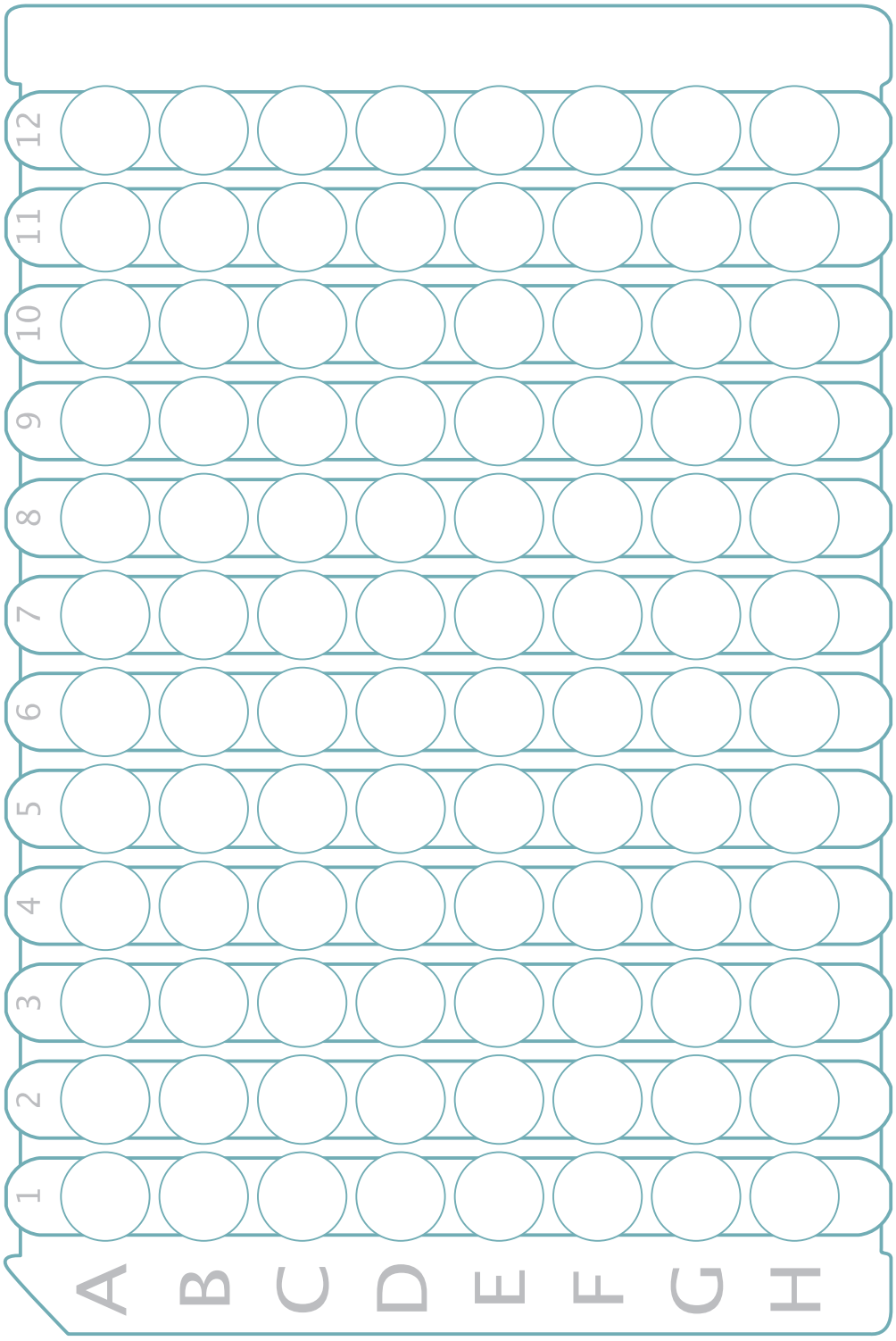
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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