Quantikine® ELISA

Human CNTF Immunoassay

Catalog Number DNT00

For the quantitative determination of recombinant human Ciliary Neurotrophic Factor (CNTF) concentrations in cell culture supernates, serum, and plasma.

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

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INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a polypeptide initially purified from 15-day chick embryo ocular tissue (1). Originally identified as a trophic factor for 8-day chick embryo parasympathetic neurons, CNTF has since been found to be a member of a limited family of neuropoietic cytokines that also includes Leukemia Inhibitory Factor (LIF) and Oncostatin M (OSM) (2, 3). Structurally, these cytokines are characterized by four distinctive α-helical folds commonly associated with growth hormone. CNTF, LIF, and OSM all have similar exon organizations and a distinctive sequence in the D or C-terminal helix (2, 4, 5). For reviews on CNTF, see references 2, 4, 6-9. Although CNTF has no classic signal sequence, cellular secretion may employ non-classical secretion mechanisms such as ABC-transporters or novel signal recognition particle (SRP) interactions (10, 11). Whatever its route of secretion, CNTF has been hypothesized to be a target-derived differentiation factor for developing neurons and glial cells (9, 12, 13). When CNTF is retained intracellularly, the factor has been suggested to be a lesion-associated molecule, providing damaged neurons with trophic or survival support following injury (14-16).

Human CNTF is a 200 amino acid (aa) residue, single chain polypeptide of 22.7 kDa. Based on its predicted aa sequence, CNTF possesses no potential sites for N-linked glycosylation and there is no evidence for any O-linked carbohydrate (17, 18). CNTF contains one cysteine that is not considered essential for activity (17). The molecule is highly conserved across species, as aa sequence comparisons of human with rat and rabbit CNTF show 83% and 87% identity, respectively (17, 19, 20). Accordingly, CNTF shows some degree of species cross-reactivity (18, 21). To date, CNTF has been localized to Schwann cells and type 1 astrocytes (13, 14, 22). Recombinant CNTF has been successfully expressed in *E. coli* and found to retain full biological activity (21, 23-25).

Functionally, CNTF has demonstrated activity as a survival and differentiation factor for cells of the nervous system. It was originally identified by its ability to support the *in vitro* growth of dissociated 8-day chick embryo ciliary (parasympathetic) ganglia neurons (1). Subsequent studies *in vitro* have demonstrated survival-promoting activity on sensory (dorsal root) ganglia neurons, non-autonomic motor neurons and sympathetic motor neurons (7, 26-30). The effects on sympathetic neurons are variable however, as high concentrations of CNTF are reported to induce apoptosis (31). *In vivo* studies have also shown enhanced survival of embryonic non-autonomic motor neurons but not sensory or autonomic (sympathetic and parasympathetic) neurons, suggesting that local *in situ* factors may be important in determining neuronal survival (8, 32). CNTF has also been suggested to be a rescue factor for damaged or axotomized neurons. In particular, motor neuron death that follows axotomy of the rat facial nerve was reversed by applying rat CNTF to the proximal axonal segment (33). Additionally, recombinant human CNTF injected into rat lateral ventricle prevents the death of axotomized medial septal neurons projecting to the hippocampus. Within this tract are axons of cholinergic and GABAergic neurons, and both are protected from degeneration (24, 34). In contrast, application of CNTF to sectioned adult rat hypoglossal nerve did not statistically protect hypoglossal motor neurons from degeneration. These results suggest that some subpopulations of motor neurons may be more responsive to CNTF than others (35).

CNTF has also been identified as a neuronal differentiation factor. It is known that target organs can influence the phenotype of their innervating neurons. Target organs are believed to dictate, through cytokines or other factors, the actual type of neurotransmitter used, neuropeptide produced, and the type of synapses made by other neurons with the target organ-associated neuron (9, 36). CNTF has been demonstrated *in vitro* to induce cholinergic properties in otherwise adrenergic sympathetic motor neurons. This includes the expression of acetylcholine (ACh) as neurotransmitter, and Substance P (SP) and Vasoactive Intestinal Peptide (VIP) as ACh-associated neuropeptides (37-41). The effects of CNTF on non-autonomic sensory neurons is less certain. Dorsal root ganglia cells *in vivo* have been found to increase their expression of SP, while SP and VIP have not been found to be increased in response to CNTF *in vitro* (21, 41).

Although CNTF is generally associated with cholinergic phenotype expression, in conjunction with norepinepherine, CNTF has been shown to promote the adrenergic phenotype in cultures of rat fetal locus coeruleus cells (42). Finally, CNTF is also suggested to play a role in glial differentiation. In cultures of O-2A (oligodendroglia-type 2 astrocytes) progenitors, CNTF promotes the development of either oligodendroglia or type 2 astrocytes, if applied in the presence of extracellular matrix (43). Given that type 1 astrocytes are known to synthesize CNTF, this indicates a CNS role for CNTF in glial cell formation (13, 44). Other activities attributed to CNTF include the maintenance of pluripotentiality in embryonic stem cells (45), the induction of fibrinogen and acute phase plasma proteins in rat hepatocytes (46, 47), the promotion of survival and differentiation of adrenal chromaffin cells, (48) and, like IL-6, the induction of fever after intravenous injection (49).

The α- or low affinity (K_d=1-10 nM) subunit for the human CNTF receptor has been isolated and cloned from neuroblastoma cells (6). This α-component of the CNTF receptor complex is 372 aa residues in length with a predicted molecular weight of 41 kDa. The protein has four potential N-linked glycosylation sites, most of which are believed to be occupied by carbohydrate (50, 51). Unlike other cytokine receptors, the CNTF receptor possesses no cytoplasmic domain and cellular attachment is achieved through a membrane glycosyl-phosphatidylinositol linkage (52). Consistent with its lack of a cytoplasmic domain, the CNTF Rα requires interaction with other membrane-bound components to produce a biological response to CNTF binding. The fully functional high affinity (Kd=0.01-0.1 nM) (6, 23, 53) receptor complex for CNTF is now known to consist of the CNTF Rα in association with the LIF receptor beta (LIF Rβ) and with gp130, a signal transducing subunit common to a number of cytokine receptor complexes, including IL-6, LIF, and IL-11 (53, 54). Soluble forms of the CNTF Rα exist, probably due to phospholipase activity on the cell membrane (7, 52). Cells recognized to normally express CNTF receptors include skeletal muscle cells and neurons. Within neurons generally, CNTF Rα has been found on autonomic and dorsal root (sensory) ganglia, anterior thalamus, layer V of the neocortex, the pars reticulata of the substantia nigra, the dentate gyrus plus subiculum of the hippocampal region, and most notably, on both upper and lower motor neurons (51, 55). CNTF Rα is also expressed during embryological development by mitotically active neuronal precursors in both PNS neural crest-derived progenitors and CNS neuroepithelium (55).

CNTF has become of interest because of its documented survival activity for neurons. Notably, CNTF has been reported to greatly reduce many of the morphological and functional changes in mice afflicted with progressive motor neuropathy (pmn) (56). This newly identified autosomal recessive disease has the potential to be a satisfactory small animal model for human amyotrophic lateral sclerosis (57).

The Quantikine® Human CNTF Immunoassay is a 3.5-4.5 hour solid phase ELISA designed to measure recombinant human CNTF in cell culture supernates, serum, and plasma. It contains recombinant human CNTF and has been shown to accurately quantitate the recombinant factor.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human CNTF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CNTF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human CNTF 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 CNTF 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.
- It is important that the calibrator diluent selected for the standard curve be consistent with the samples being assayed.
- 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 CNTF Microplate	890266	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human CNTF.	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 CNTF Standard	890269	2 vials of recombinant human CNTF in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard fo each assay.
Human CNTF Conjugate	890268	21 mL of a polyclonal antibody specific for human CNTF conjugated to horseradish peroxidase with preservatives.	May be stored for up to1 month at 2-8 °C.*
Assay Diluent RD1-9	895167	11 mL of a buffered protein solution with preservatives. *May contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.*	
Calibrator Diluent RD5P Concentrate	895151	21 mL of a buffered protein base with preservatives. *For cell culture supernate samples. Use diluted 1:5 in this assay.*	
Calibrator Diluent RD6-3	895165	21 mL of animal serum with preservatives. *For serum/plasma samples.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. *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.
- 100 mL and 500 mL graduated cylinders.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- **Polypropylene** test tubes for dilution of standards.
- Human CNTF Controls (optional; R&D Systems®, Catalog # QC20).

PRECAUTIONS

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 - To prevent loss of CNTF in cell culture supernates prior to assay, it is recommended that media be supplemented with at least 2% fetal bovine serum. 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.

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

REAGENT PREPARATION

Bring all reagents to room temperature before use.

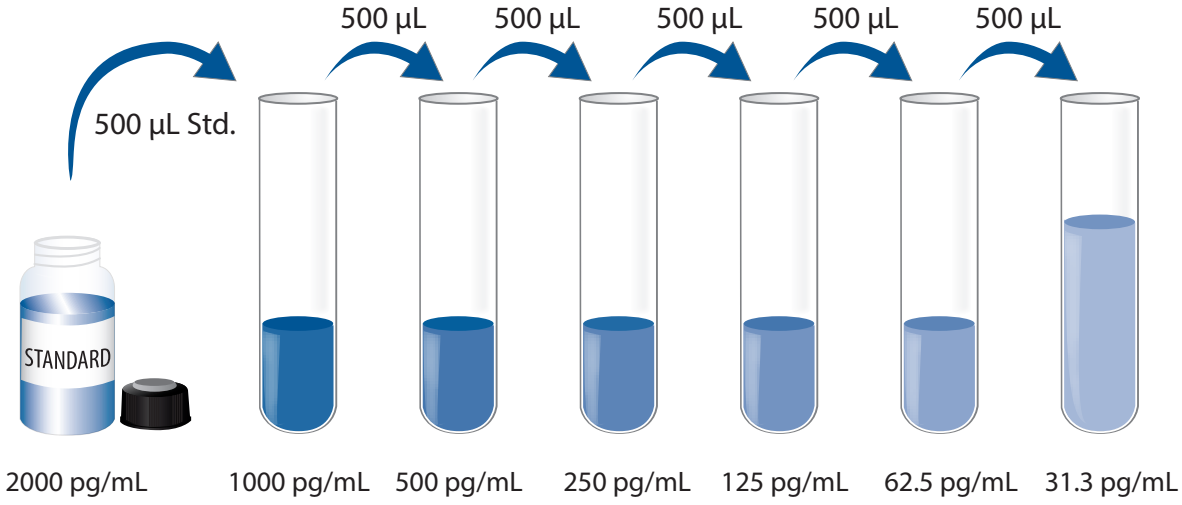
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human CNTF Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human CNTF Standard with Calibrator Diluent RD5P (diluted 1:5) (*for cell culture supernate samples*) or Calibrator Diluent RD6-3 (*for serum/plasma samples*). This reconstitution produces a stock solution of 2000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of Calibrator Diluent RD5P (diluted 1:5) (*for cell culture supernate samples*) or Calibrator Diluent RD6-3 (*for serum/plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human CNTF Standard (2000 pg/mL) 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 samples, standards, and controls be assayed in duplicate.

1. Prepare all reagents and working standards 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 RD1-9 to each well. Warm the Assay Diluent to room temperature and mix well if precipitate is present.
4. Add 200 µL of standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 aspiration or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human CNTF Conjugate to each well. Cover with a new adhesive strip.
   For Cell Culture Supernate Samples: Incubate for 1 hour at room temperature.
   For Serum/Plasma Samples: Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. 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.).

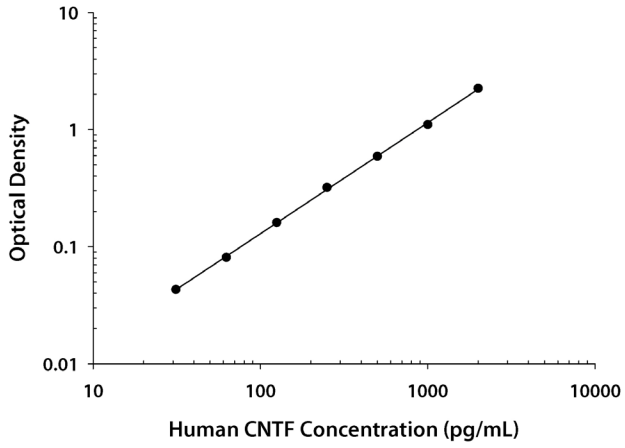
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points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

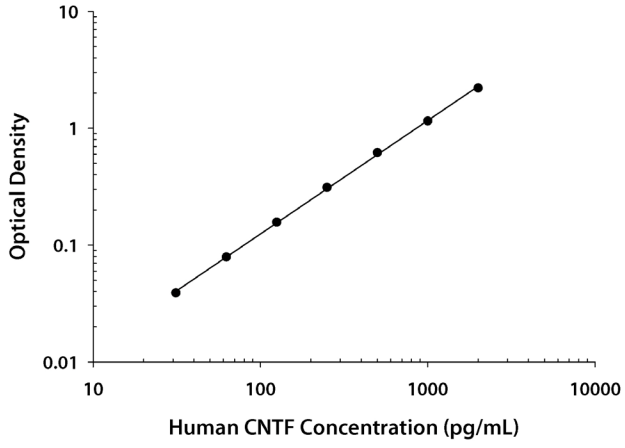
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.015 0.015	0.015	—
31.3	0.061 0.054	0.058	0.043
62.5	0.098 0.093	0.096	0.081
125	0.179 0.173	0.176	0.161
250	0.343 0.327	0.335	0.320
500	0.622 0.591	0.606	0.591
1000	1.132 1.108	1.120	1.105
2000	2.306 2.220	2.263	2.248

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.018 0.020	0.019	—
31.3	0.058 0.059	0.058	0.039
62.5	0.099 0.098	0.098	0.079
125	0.179 0.173	0.176	0.157
250	0.330 0.331	0.330	0.311
500	0.629 0.647	0.638	0.619
1000	1.173 1.173	1.173	1.154
2000	2.224 2.228	2.226	2.207

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)	110	461	1295	128	399	821
Standard deviation	5.0	10.2	31.4	10.1	26.8	52.6
CV (%)	4.5	2.2	2.4	7.9	6.7	6.4

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)	103	406	1201	206	671	1388
Standard deviation	4.4	12.0	28.2	19.6	47.8	93.9
CV (%)	4.3	3.0	2.3	9.5	7.1	6.8

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	99	92-115%
Serum (n=5)	95	85-101%
EDTA plasma (n=5)	90	78- 97%
Heparin plasma (n=5)	92	82- 98%
Citrate plasma (n=5)	87	71-125%

SENSITIVITY

The minimum detectable dose (MDD) of human CNTF is typically less than 8 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.

LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of human CNTF and diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=5)	Serum (n=5)	EDTA plasma (n=5)	Heparin plasma (n=5)	Citrate plasma (n=5)
1:2	Average % of Expected	98	95	99	100	100
	Range (%)	93-103	89-98	96-102	95-104	97-104
1:4	Average % of Expected	97	96	101	100	102
	Range (%)	93-100	85-102	95-104	94-105	96-106
1:8	Average % of Expected	98	101	102	101	102
	Range (%)	94-103	87-107	97-106	94-107	95-107
1:16	Average % of Expected	93	95	99	99	101
	Range (%)	86-99	84-99	91-102	90-104	95-105

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human CNTF produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Thirty-four samples from apparently healthy volunteers were evaluated for the presence of human CNTF in this assay. No endogenous levels were detected. No medical histories were available for the donors used in this study.

SPECIFICITY

This assay recognizes recombinant human CNTF.

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

Recombinant human:

CLC/CNTF Rα Chimera
CNTF Rα
gp130
IL-6
IL-6 R
IL-11
LIF
LIF Rα
OSM

Recombinant mouse:

IL-6
LIF

Recombinant rat:

CNTF
CNTF Rα

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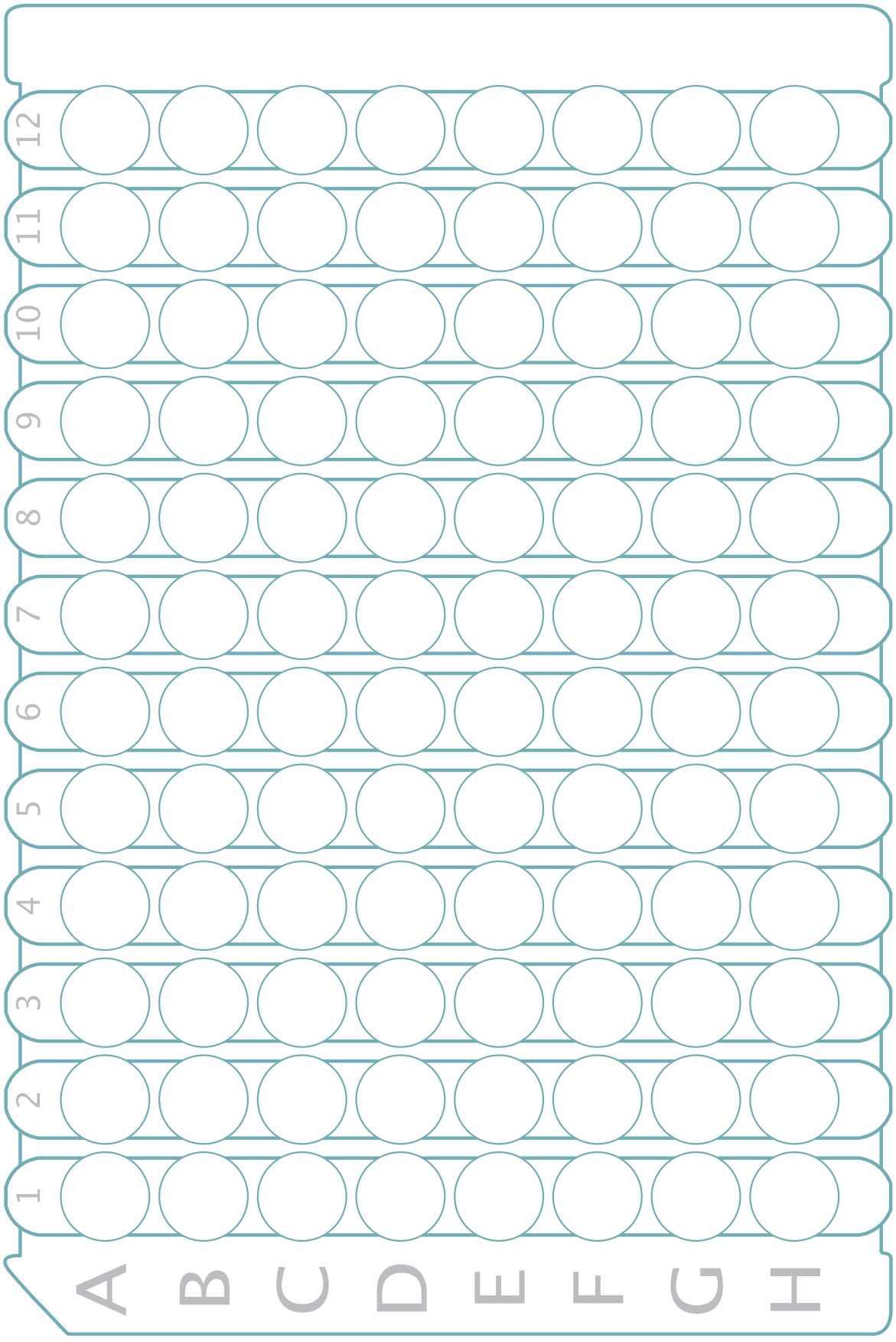
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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