



**Promega**

## Technical Bulletin

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# GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System

INSTRUCTIONS FOR USE OF PRODUCTS G7620 AND G7621.



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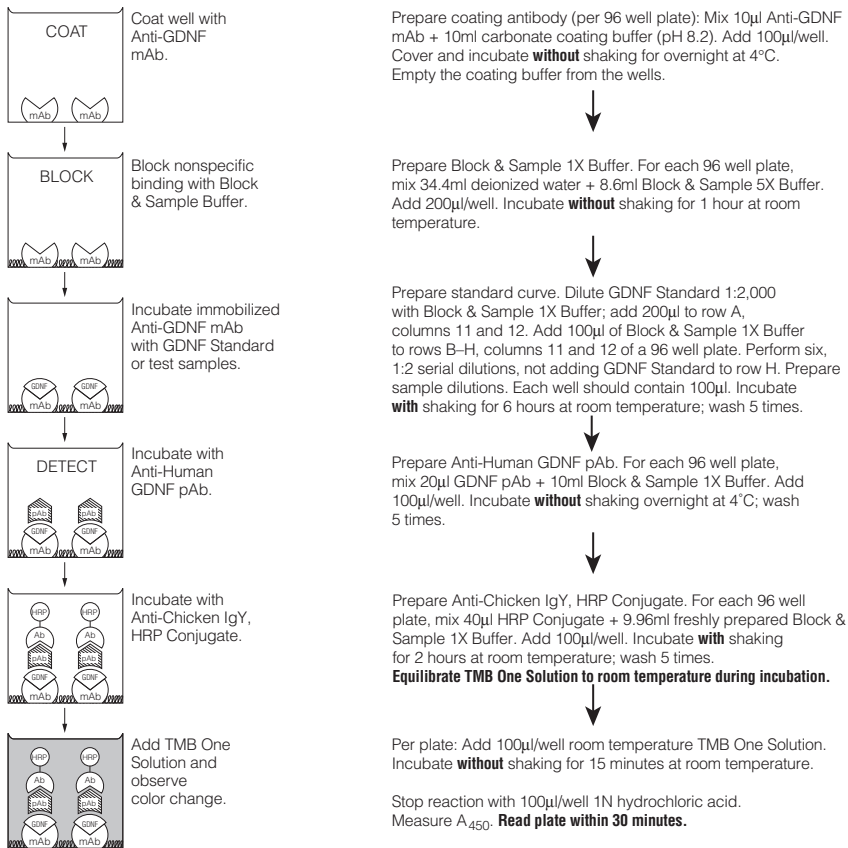
# GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System

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## 1. Description

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System is designed for the sensitive and specific detection of GDNF in an antibody sandwich format (1; Figure 1). In this format, 96-well plates are coated with Anti-GDNF Monoclonal Antibody (mAb), which binds soluble GDNF. The captured GDNF is bound by a second, specific polyclonal antibody (pAb). After washing, the amount of specifically bound pAb is then detected using a species-specific antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. The unbound conjugate is removed by washing, and following an incubation with a chromogenic substrate, the color change is measured. The amount of GDNF in the test solutions is proportional to the color generated in the oxidation-reduction reaction. Using this system, GDNF in tissue culture supernatants or tissue homogenates can be quantitated.



1977MA11\_7A

**Figure 1. Schematic representation of the GDNF E<sub>max</sub>® ImmunoAssay System.**

For a detailed protocol, or when using this system for the first time, please read Sections 3 through 6 carefully.

The GDNF E<sub>max</sub>® ImmunoAssay System offers several benefits:

- **Specificity:** Specific detection of GDNF; typically less than 3% cross-reactivity with other neurotrophic factors at 10ng/ml (Figure 4).
- **Sensitivity:** Detects a minimum of 31.2pg/ml of GDNF.
- **Flexibility:** Available in sizes for two or five 96-well ELISA plates; can configure plates as desired.
- **High Value:** Optimized reagents and protocol.

## Biological Roles of GDNF

GDNF is processed from a 211-amino acid precursor protein to form a mature homodimer of two 134-amino acid subunits. GDNF possesses seven cysteine residues and forms three disulfide bonds resulting in a 'cysteine knot' quarternary structure. Discovered by Lin and colleagues in 1993 and shown to be a potent survival factor for dopaminergic neurons (2), GDNF has since been shown to be a potent survival factor for spinal motoneurons (3-5), locus ceruleus noradrenergic neurons (6) and distinct subpopulations of peripheral sensory, sympathetic and parasympathetic neurons (7,8). A surprising finding from studies in GDNF-null mice was that the animals failed to develop kidneys or form an enteric nervous system (9-11).

GDNF interacts with the receptor tyrosine kinase, Ret, formerly an orphan receptor (12-17). GDNF only binds Ret through a membrane-tethered accessory protein, identified as GDNFR- $\alpha$  (12,13). Since the members of this multi-component receptor family are growing, new terminology has been suggested. The GFR $\alpha$  nomenclature committee has proposed that GDNFR- $\alpha$  be referred to as GFR $\alpha$ -1, for TGF $\beta$ -related neurotrophic factor receptor 1, and other such accessory factors be named accordingly (18). A review of GDNF can be found in reference 19, and a review of the GDNF/neurturin receptor complex can be found in reference 20.

### Selected Citation Using the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System

- Peden, C.S., Burger, C., Muzyczka, N. and Mandel, R.J. (2004) Circulating anti-wild-type Adeno-associated Virus Type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. *J. Virol.* **78**, 6344-59.

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System was used to measure GDNF levels in adenovirus-infected rats. Data are displayed graphically as picograms of GDNF per milligram of brain tissue.

For additional peer-reviewed articles that cite use of the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System, visit: [www.promega.com/citations/](http://www.promega.com/citations/)

## 2. Product Components and Storage Conditions

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System is offered in two sizes, which differ only in the quantity of reagents, sufficient for either 5 plates or 2 plates. Catalog numbers and quantity of reagents are listed below.

**Note:** The GDNF Standard supplied with this system is recombinant human GDNF.

Product	Size	Cat.#
GDNF E <sub>max</sub> <sup>®</sup> ImmunoAssay System	2 × 96 wells	G7620

Each system contains sufficient reagents for 160 determinations plus standards (plates not included). Includes:

- 20µl Anti-GDNF mAb
- 22ml Block & Sample 5X Buffer
- 20µl GDNF Standard, 2µg/ml
- 20µg Anti-Human GDNF pAb
- 80µl Anti-Chicken IgY, HRP Conjugate
- 25ml TMB One Solution

Product	Size	Cat.#
GDNF E <sub>max</sub> <sup>®</sup> ImmunoAssay System	5 × 96 wells	G7621

Each system contains sufficient reagents for 400 determinations plus standards (plates not included). Includes:

- 50µl Anti-GDNF mAb
- 54ml Block & Sample 5X Buffer
- 45µl GDNF Standard, 2µg/ml
- 50µg Anti-Human GDNF pAb
- 200µl Anti-Chicken IgY, HRP Conjugate
- 2 × 25ml TMB One Solution

**Storage Conditions:** When stored at -20°C in its original package, the product expires on the date listed on the product label. The product must not be used beyond this date. Once thawed and stored at 4°C, the product is stable for three months (except the GDNF Standard). The GDNF Standard should always be stored at -20°C. Return each component to its correct storage temperature immediately after use. Avoid refreezing reagents. After dilution, use reagents the same day.

**Note:** The GDNF Standard should be stored at -20°C.

### 3. General Considerations

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System has been tested using the following three-day protocol. Coating the plate with the GDNF mAb requires an overnight incubation; the Anti-Human GDNF pAb incubation also is performed overnight.

When serially diluting the GDNF Standard and experimental samples within the plate, take care not to disturb or scratch the bottoms or sides of the wells with the pipette tips. If unfamiliar with the technique, practice the pipetting procedure on a trial run.

#### Limitations of the Assay

- For research use only. Not for use in diagnostic procedures.
- Absorbance values beyond the range of the standard curve are not valid.
- For consistent results, dilute samples using the Block & Sample 1X Buffer.

### 4. Sample Preparation

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System may be used to quantitate GDNF in tissue culture supernatants, plasma, serum or tissue extracts. Store experimental samples frozen at -20°C before use. Avoid multiple freeze-thaw cycles. Remove particulates from samples by centrifugation before use in the assay.

Tissue extracts may be prepared using the lysis buffer described in the Appendix (Section 8.A). Further processing of the samples by acidification and subsequent neutralization may increase the amount of detectable GDNF in extracts from a variety of samples (21). For GDNF, the mechanism of this procedure is likely to be disruption of the ligand:receptor interaction (21). Acid treatment is a species- and tissue-specific phenomenon and may lead to a decrease in detection of GDNF levels in some samples. Therefore, it is important to **test the acid treatment procedure for any given species and sample type to determine the benefit of pretreatment.**

**Note:** This assay is designed to measure both free and total GDNF. **To measure the amount of free GDNF in your samples, proceed directly to the ELISA protocol in Section 5.A without acid treatment.** To assay for total GDNF, acid-treat and then neutralize the samples as described in the procedure below before proceeding with the ELISA protocol. **Do not attempt to acid treat the GDNF Standard.**

### Acid Treatment Procedure



Test the acid treatment procedure for any given species and sample type.

This procedure acidifies samples diluted 1:5 in Dulbecco's PBS (DPBS, Section 8.A) to approximately pH 2.6 and then neutralizes them to approximately pH 7.6. Depending on the amount of carrier protein in the samples, additional dilutions may or may not require the use of Block & Sample 1X Buffer (which contains a carrier protein) to minimize loss of GDNF.

For low protein matrices such as urine or serum-free media, we recommend direct acid treatment to a pH of 2.0–3.0 for 15–20 minutes. Following neutralization with NaOH, subsequent dilutions should be done with Block & Sample 1X Buffer before adding samples to the assay plate.

For all matrices, verify that the pH is 3.0 or lower using pH paper. In animal sera, the amount of 1N HCl required to lower the pH will vary depending upon the species. We suggest adding 110–125 $\mu$ l of 1N HCl per milliliter of **undiluted** serum or plasma and checking the pH before adding additional amounts of acid. Samples can be acid-treated in advance and stored at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ .

### Materials to Be Supplied by the User

(Solution compositions are provided in Section 8.A.)

- DPBS
- 1N HCl, reagent grade
- 1N NaOH, reagent grade

**Caution:** HCl and NaOH are caustic. Avoid contact with skin or eyes.

1. Dilute the sample by adding 4 volumes of DPBS.
2. Add 1 $\mu$ l of 1N HCl for each 50 $\mu$ l of diluted sample. Check the pH to ensure that it is less than 3.0.
3. Mix and incubate for 15 minutes at room temperature.
4. Neutralize by adding 1 $\mu$ l of 1N NaOH per 50 $\mu$ l of sample. Check the pH to ensure that it is approximately 7.6.

## 5. Protocol for GDNF Quantitation

### Materials to Be Supplied by the User

(Solution compositions are provided in Section 8.A.)

- 96-well (flat-bottom) ELISA plate (MaxiSorp™ or Microtiter®-immunoassay plates)
- carbonate coating buffer
- plate sealer
- TBST wash buffer
- 1N hydrochloric acid
- microplate reader capable of monitoring absorbance at 450nm
- pipettors capable of accurately delivering volumes of 1µl-1ml
- multichannel pipettor
- wash bottle or automated plate washer (DYNEX UltraWash Plus or equivalent)
- plate shaker (DYNEX MICRO-SHAKER® II or equivalent)
- 50ml (for better mixing) or 15ml polypropylene tubes for dilutions

**Note:** For best well-to-well accuracy, we recommend a high-quality, name brand polystyrene ELISA plate, such as Nunc-Immuno™ MaxiSorp™ (Nunc Cat.# 439454) and Microtiter®-Immunoassay (Immulon® 4; Thermo LabSystems Cat.# 3855) plates. There are no observable differences in the performances of these plates. We do not recommend the use of Corning Costar® or Thermo Electron Immulon®-2 plates.

### 5.A. Plate Coating

1. In a 15ml or 50ml polypropylene tube, add exactly 10µl of the Anti-GDNF mAb (coat antibody) to 10ml of carbonate coating buffer to prepare enough reagent for each full 96-well plate. Mix thoroughly, but avoid creating excess bubbles. Use a multichannel pipettor to add 100µl to each well of a polystyrene ELISA plate.

**Hint:** Keep the undiluted Anti-GDNF mAb on ice when removed from 4°C storage.

2. Seal the wells with a plate sealer and incubate overnight at 4°C.

**Note:** This assay is optimized using the carbonate coating buffer prepared with a pH of 8.2 (Section 8.A); other buffers may give poor results.

### 5.B. Preparing Block & Sample 1X Buffer

Each 96-well plate requires a total of 43ml of Block & Sample 1X Buffer to be used the second day. This volume includes 3ml to determine the analyte (GDNF) standard curve.

1. For each 96-well plate, add 34.4ml of deionized water to a clean 50ml polypropylene tube. Remove 8.6ml of the Block & Sample 5X Buffer with a sterile pipette, being careful not to contaminate the stock solution, and add it to the 50ml tube. Mix gently and completely, by inversion, prior to use.

### 5.C. Blocking the Plate



Do not allow wells to dry out completely between steps.

- Over a sink, flick out the contents of the wells and slap the plate upside down 3 times on a paper towel to help clear the wells. Add 200 $\mu$ l of Block & Sample 1X Buffer to each well using a multichannel pipette. Do not touch or scratch the surface of the wells where antibody has bound to the plate.
- Incubate at room temperature for 1 hour **without** shaking.

### 5.D. Preparing the GDNF Standard Curve

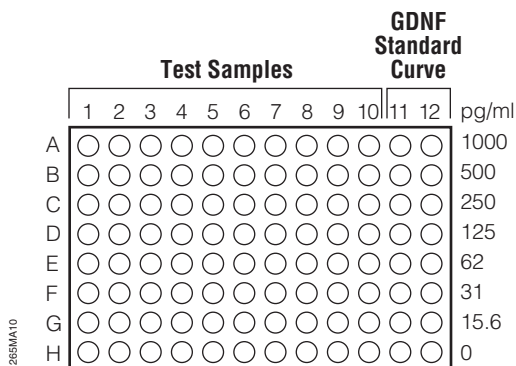


A standard curve must be performed on each plate.

The GDNF standard curve is linear between 15.6 and 1,000pg/ml of the GDNF Standard. The GDNF Standard is supplied at a concentration of 2 $\mu$ g/ml. Accurately dilute the supplied GDNF Standard 1:2,000 in Block & Sample 1X Buffer to achieve a concentration of 1,000pg/ml. For example, mix the standard well after thawing, and pipet 10 $\mu$ l of GDNF Standard into 390 $\mu$ l of Block & Sample 1X Buffer (1:40 dilution); then pipet 10 $\mu$ l of this solution into 490 $\mu$ l of Block & Sample 1X Buffer for a final dilution of 1:2,000.

**Hint:** Keep the undiluted GDNF Standard on ice after thawing.

- Following plate blocking, flick out the contents of the wells over a sink. Slap the plate 3 times upside down on a paper towel to remove residual liquid. Designate two columns of wells (16 wells) for the standard curve. To prepare the GDNF standard curve within the assay plate, add 100 $\mu$ l/well of the Block & Sample 1X Buffer to wells B through H in the two columns designated for the standard curve (Figure 3).
- Add 200 $\mu$ l of the diluted GDNF Standard (1,000pg/ml) to the first well in each column designated for the standard curve.



**Figure 2. Recommended ELISA plate format for GDNF standard curve and test samples.**

3. Immediately perform serial 1:2 dilutions (100 $\mu$ l/well) down the plate in the columns designated for the standard curve. In the last set of wells for the standard curve, do not add any GDNF. The final concentrations (in duplicate) within the plate will be 0-1,000pg/ml.

#### 5.E. Addition of Sample

We recommend starting with a 1:2 dilution of each test sample and preparing 1:2 serial dilutions per column in the ELISA plate. Alternatively, you may wish to screen samples at a single concentration and subsequently re-assay all positive samples to determine the exact GDNF concentration.

Where the sample carrier solution may contribute nonspecific sources of GDNF (such as serum in culture media), we also recommend performing a series of negative control reactions containing the carrier solution alone.

1. After preparing the GDNF Standard curve, add 100 $\mu$ l of the test samples to each of the remaining wells.

**Note:** Add samples as quickly as possible to minimize evaporation.

2. Incubate the plate for 6 hours at room temperature with shaking (500rpm).

**Note:** Best results are obtained using a plate shaker. Plates may be incubated without shaking, although a slight loss of assay sensitivity may occur.

#### 5.F. Washing Procedure

1. Using the recommended wash buffer (Section 8.A), wash all wells vigorously using an automated plate washer. Alternatively, plates can be washed by entirely filling each well with wash buffer, flicking out the contents over a sink and slapping the plate 3 times on a paper towel. Repeat this wash procedure an additional 4 times for a total of 5 washes. Immediately proceed to the next step.

**Note:** We strongly recommend the use of an automated plate washer for consistent results.

#### 5.G. Addition of Anti-Human GDNF pAb

1. In a 15ml or 50ml polypropylene tube, add 20 $\mu$ l of the Anti-Human GDNF pAb to 10ml of Block & Sample 1X Buffer (1:500 dilution) to prepare enough reagent for a full 96-well plate. **Mix thoroughly**, but avoid creating excess bubbles. Use a multichannel pipettor to add 100 $\mu$ l of the diluted GDNF pAb to each well, being careful not to touch or scratch the bottom or sides of the wells.

2. Incubate overnight at 4°C without shaking.

3. Wash as described in Section 5.F.

**Hint:** Keep the undiluted Anti-Human GDNF pAb on ice when removed from 4°C storage.

#### 5.H. Addition of Anti-Chicken IgY, HRP Conjugate

1. For each 96-well plate, add 8ml of deionized water and 2ml of the Block & Sample 5X Buffer into a clean 15ml or 50ml polypropylene tube. Mix gently and completely by inversion prior to use.
2. Accurately add 40 $\mu$ l of the stock Anti-Chicken IgY, HRP Conjugate to 9.96ml of Block & Sample 1X Buffer (1:250 dilution) to prepare enough reagent for a full 96-well plate. **Mix thoroughly**, and avoid creating excess bubbles. Using a multichannel pipettor, add 100 $\mu$ l of the diluted Antibody Conjugate to each well, being careful not to disturb the bottom or sides of the wells.

**Hint:** Keep the undiluted Anti-Chicken IgY, HRP Conjugate on ice when removed from 4°C storage.

3. Incubate for 2 hours at room temperature with shaking.

**Note:** During this incubation equilibrate the TMB One Solution to room temperature.

4. Wash as described in Section 5.F.

#### 5.I. Color Development

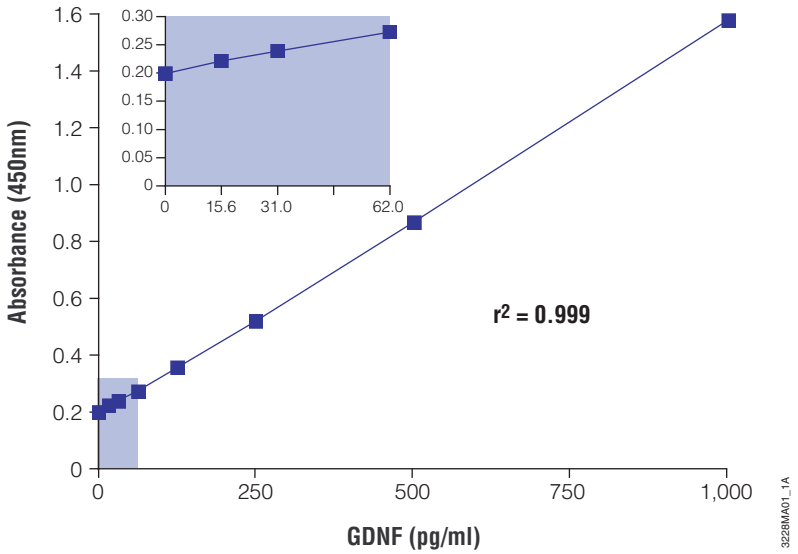
**Caution:** Avoid contact of the TMB One Solution and 1N hydrochloric acid with skin and eyes.

1. Add 100 $\mu$ l of room temperature TMB One Solution to each well using a multichannel pipette.
2. Incubate at room temperature for 15 minutes. Do not shake.
3. Stop the reaction by adding 100 $\mu$ l of 1N hydrochloric acid to the wells in the same order in which substrate was added in Step 2. The blue coloration will change to yellow upon acidification. Take care to avoid creating bubbles.
4. Record the absorbance at 450nm on a plate reader within 30 minutes of stopping the reaction. See Figure 3 for a representative GDNF standard curve.

**Note:** The exterior bottom of the plate must be optically clean for accurate measurement. Wash the **exterior bottom** with 70% ethanol if necessary.

### 5.J. Representative Standard Curve

**!** A standard curve must be prepared for each plate.



**Figure 3. Representative GDNF standard curve obtained using the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System.** The inset is an enlargement of the 0-62.5pg/ml portion of the graph.

## 6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: [www.promega.com](http://www.promega.com). E-mail: [techserv@promega.com](mailto:techserv@promega.com)

Symptoms	Causes and Comments
Sample absorbance is above range of standard curve	<p>Sample is too concentrated. Further dilute the sample.</p> <p>Assay multiple dilutions of each sample to ensure that at least one point will fall in the useful range of the standard curve.</p>
Sample absorbance is below range of standard curve	Sample is too dilute. Re-assay at a higher sample concentration.
Low absorbance in all samples	Incorrectly diluted component. Verify the dilutions for each component of the assay.
Low activity in GDNF Standard	Improper storage. The standard is stable if stored undiluted for nine months at -20°C.
Variability in replicate samples	<p>Technique problems in performing assay. Ensure that all wells are washed completely.</p> <p>Add stop solution to wells in same order as TMB substrate.</p> <p>Change pipette tips before adding each reagent.</p> <p>Perform additional replicates.</p> <p>Check calibration of the pipettor.</p>

## 7. References

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## 8. Appendix

### 8.A. Composition of Buffers and Solutions

#### carbonate coating buffer

0.025M sodium bicarbonate

0.025M sodium carbonate

Adjust the pH to 8.2 using 1N HCl or 1N NaOH.

#### Dulbecco's PBS buffer (per liter)

0.2g KCl

8.0g NaCl

0.2g  $\text{KH}_2\text{PO}_4$

1.15g  $\text{Na}_2\text{HPO}_4$

133mg  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

100mg  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

Add room temperature deionized water to a final volume of 1 liter to the KCl, NaCl,  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ .

Adjust pH to 7.35 using 1N HCl or 1N NaOH, if necessary. Add the  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , mix thoroughly; then add the  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and mix thoroughly.

#### 1N hydrochloric acid

Add 82.7ml concentrated hydrochloric acid to 917.3ml of deionized water.

#### lysis buffer

137mM NaCl

20mM Tris (pH 8.0)

1% NP40

10% glycerol

1mM PMSF

10 $\mu\text{g}/\text{ml}$  aprotinin

1 $\mu\text{g}/\text{ml}$  leupeptin

0.5mM sodium vanadate

#### TBST wash buffer

20mM Tris-HCl (pH 7.6)

150mM NaCl

0.05% (v/v) Tween<sup>®</sup> 20

### 8.B. Performance Characteristics of the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System

#### Intra-Assay Comparison

Three concentrations of rhGDNF (indicated in parentheses) were diluted in Block & Sample 1X Buffer and assayed by one operator for a total of 6 determinations each. The apparent concentrations are described in the table below.

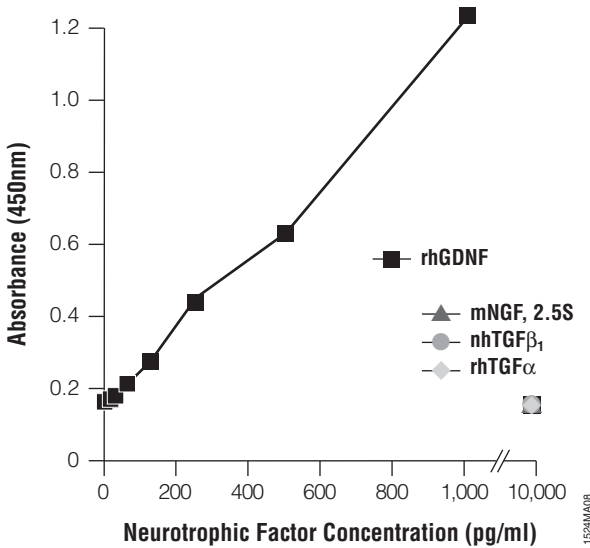
	GDNF		
	Low (80)	Medium (250)	High (500)
N	6	6	6
Mean (pg/ml)	79	255	502
SD (pg/ml)	4	16	18
CV (%)	5.1	6.2	3.6

N = sample size, SD = standard deviation,

CV = coefficient of variance

### Cross-Reactivity of the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System

The GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System demonstrates very low cross-reactivity with the structurally related growth factors, Natural Human Transforming Growth Factor  $\beta$ 1 (TGF $\beta$ 1), Recombinant Human Transforming Growth Factor  $\alpha$  (rhTGF $\alpha$ ) and NGF, at concentrations as high as 10ng/ml, as demonstrated in Figure 4.



**Figure 4. Specificity of the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System.** To evaluate the specificity of this assay system, 10ng each of Murine 2.5S NGF (Cat.# G5141), an unrelated neurotrophic factor, and rhTGF $\alpha$  and natural human TGF $\beta$ 1, structurally related growth factors, were tested for binding following the protocols described in this Technical Bulletin. The results are expressed as the mean of triplicate determinations. These findings illustrate the specificity of the GDNF E<sub>max</sub><sup>®</sup> ImmunoAssay System, which showed no detectable binding of functionally or structurally related proteins at concentrations as high as 10ng/ml.

### 8.C. Related Products

#### E<sub>max</sub><sup>®</sup> ImmunoAssay Systems

Product	Size	Cat.#
BDNF E <sub>max</sub> <sup>®</sup> ImmunoAssay Systems	2 × 96 wells	G7610
	5 × 96 wells	G7611
NGF E <sub>max</sub> <sup>®</sup> ImmunoAssay Systems	2 × 96 wells	G7630
	5 × 96 wells	G7631
NT-3 E <sub>max</sub> <sup>®</sup> ImmunoAssay Systems	2 × 96 wells	G7640
	5 × 96 wells	G7641
TGFβ <sub>1</sub> E <sub>max</sub> <sup>®</sup> ImmunoAssay Systems	2 × 96 wells	G7590
	5 × 96 wells	G7591
TGFβ <sub>2</sub> E <sub>max</sub> <sup>®</sup> ImmunoAssay System	5 × 96 wells	G7600

#### Primer Pairs

Product	Size	Cat.#
β-Actin Primer Pair	20 reactions	G5740
CNTF Primer Pair	20 reactions	G5770
NT-3 Primer Pair	20 reactions	G6801
p75 Primer Pair	20 reactions	G6861

#### Items Available Separately

Product	Size	Cat.#
TMB One Solution*	100ml	G7431
Block & Sample 5X Buffer*	54ml	G3311
rhGDNF	5μg	G2781
Anti-Human GDNF pAb	200μg	G2791
Anti-TGFβ <sub>1</sub> pAb	100μg	G1221
mNGF, 2.5S	100μg	G5141
Anti-NGF mAb	100μg	G1131
	20μg	G1132
Anti-Human NT-3 pAb	200μg	G1651
rhNT-3	5μg	G1501
Anti-Human BDNF pAb	200μg	G1641
rhBDNF	5μg	G1491

\*For Laboratory Use.

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**8.C. Related Products, Items Available Separately (continued)**

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
Anti-Rat CNTF pAb	200µg	G1631
Anti-TrkB In pAb	100µg	G1561
Anti-Pan Trk pAb	200µg	G1581
Anti-Human p75 pAb	200µg	G3231
Anti-VACHT pAb	100µg	G4481
Anti-βIII Tubulin mAb	100µg	G7121
Anti-GFAP pAb	100µg	G5601

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