



Promega

Technical Bulletin

BDNF E_{max}[®] ImmunoAssay System

INSTRUCTIONS FOR USE OF PRODUCTS G7610 AND G7611.



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BDNF E_{max}[®] ImmunoAssay System

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1. Description

The BDNF E_{max}[®] ImmunoAssay System is designed for the sensitive and specific detection of BDNF in an antibody sandwich format (1; Figure 1). In this format, flat-bottom 96-well plates are coated with Anti-BDNF Monoclonal Antibody (mAb) to bind soluble BDNF. The captured BDNF binds the second, specific, BDNF polyclonal antibody (pAb). After washing, the amount of specifically bound pAb is detected using a species-specific anti-IgY antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. Unbound conjugate is removed by washing, and following an incubation with a chromogenic substrate, the color change is measured. The amount of BDNF in the test solution is proportional to the color generated in the oxidation-reduction reaction. This system can be used to quantitate BDNF in tissue culture supernatants, plasma, serum, urine and tissue extracts.

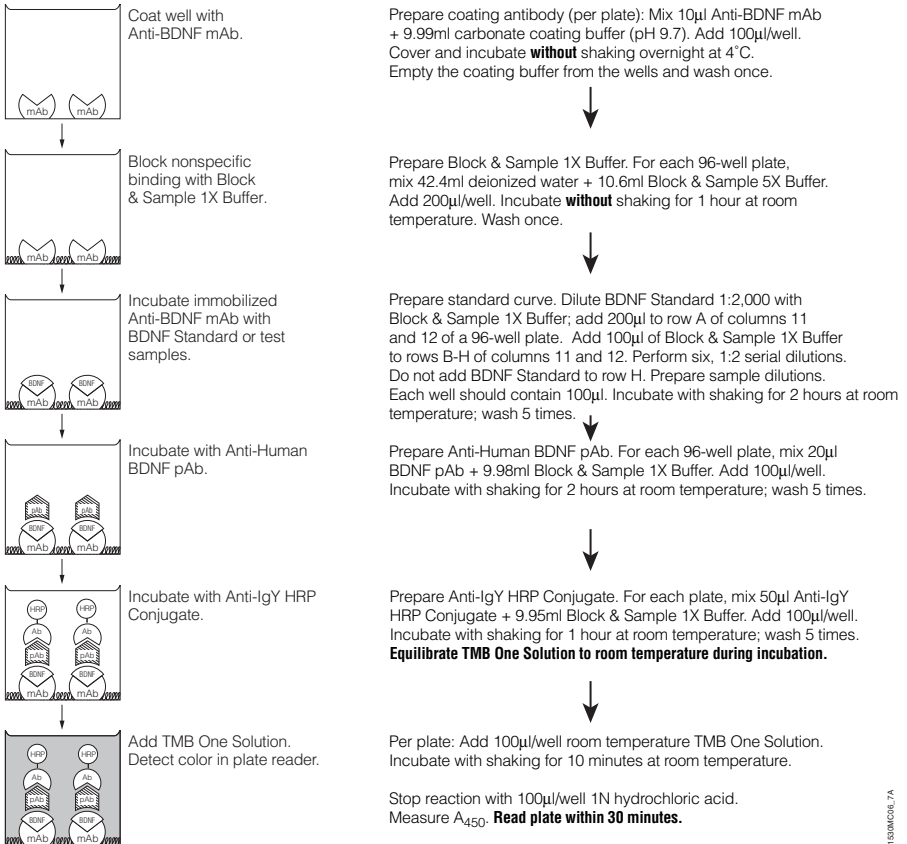


Figure 1. Schematic representation of the BDNF E_{max}® ImmunoAssay System.

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

The BDNF E_{max}® ImmunoAssay System offers several benefits:

- **Specificity:** Specific detection of BDNF; typically less than 3% cross-reactivity with other related neurotrophic factors (NGF, NT-3 and NT-4) at 100ng/ml.
- **Sensitivity:** Detects a minimum of 15.6pg/ml of BDNF.
- **Flexibility:** Available in sizes for five or two 96-well ELISA plates; can configure plates as desired.
- **High Value:** Optimized reagents and protocol.

Biological Roles of BDNF

The neurotrophin BDNF, a 27kDa protein originally derived from human brain that shares high sequence homology with NGF, NT-3 and NT-4/5 (2), influences many neuron types in the CNS (3,4). BDNF was first shown to promote the outgrowth of spinal sensory neurons (5) but has since been shown to support the survival and outgrowth of sensory, ganglion, dopaminergic, cholinergic, GABAergic and motor neurons (3). BDNF can signal the differentiation of pluripotent neural crest cells into sensory neurons (6). It shows no supportive effects on NGF-sensitive sympathetic neurons. In fact, trigeminal neurons, which are responsive to BDNF and NT-3 early in development, switch their dependency to that of NGF alone (7). Whereas BDNF or NGF promote the survival of chicken embryonic dorsal root ganglion neurons, these neurons eventually diverge into two distinct subpopulations that depend on one or the other of these same neurotrophins (8,9).

BDNF is produced primarily in the brain and spinal cord by glial cells (10) but is also produced by Schwann cells associated with peripheral motor neurons (11). BDNF is found at much lower levels in heart, lung and platelets (12,13). BDNF, as all neurotrophins, binds to p75^{NTR} (the low-affinity neurotrophin receptor) and may initiate programmed cell death by acting through this receptor (14). However, it activates signal transduction by dimerization and autophosphorylation of the TrkB receptor (15). More recently, it has been shown that BDNF (and NT-3) induces message for neuritin, a glycosyl-phosphatidylinositol-anchored protein that is expressed in post-mitotic differentiating neurons (16). For a review on BDNF and other neurotrophins, see reference 14.

2. Product Components and Storage Conditions

The BDNF E_{max}[®] ImmunoAssay System is offered in two sizes, designed to accommodate two or five 96-well plates. Both systems contain the same reagents, except Cat.# G7611 contains more of each component.

Product	Size	Cat.#
BDNF E _{max} [®] ImmunoAssay System	2 × 96 wells	G7610

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

- 20µl Anti-BDNF mAb
- 22ml Block & Sample 5X Buffer
- 50µl BDNF Standard
- 20µg Anti-Human BDNF pAb
- 100µl Anti-IgY HRP Conjugate
- 25ml TMB One Solution


2. Product Components and Storage Conditions (continued)

Product	Size	Cat.#
BDNF E _{max} [®] ImmunoAssay System	5 × 96 wells	G7611

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

- 50µl Anti-BDNF mAb
- 54ml Block & Sample 5X Buffer
- 50µl BDNF Standard
- 50µg Anti-Human BDNF pAb
- 250µl Anti-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. Return each component to 4°C immediately after use. Avoid refreezing reagents. After dilution, use reagents the same day.

 **Do not** add any preservatives (e.g., sodium azide) to these solutions before or after dilution, as they may interfere with the assay.

3. General Considerations

The BDNF E_{max}[®] ImmunoAssay System uses the following protocols. Plate coating requires an overnight incubation. The remainder of the assay incubations are performed the following day and require 7-8 hours to complete. The room-temperature steps in this assay were optimized at 18-23°C. When transferring the BDNF Standard and experimental samples to the plate, take care not to disturb or scratch the surface of the wells, as this may dislodge the coating mAb and result in significant loss of signal. If unfamiliar with the technique, practice the pipetting procedures on a trial plate. For consistent results, dilute samples using Block & Sample 1X Buffer.

Binding and recovery of BDNF from mouse brain homogenates has not been fully characterized. Using the BDNF ELISA system with mouse brain homogenates may not be appropriate. See reference 18 for information on using this system with mouse brain homogenates.

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.

4. Sample Preparation

The BDNF E_{max}[®] ImmunoAssay System may be used to quantitate BDNF in tissue culture supernatants, plasma, serum, urine and 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 Section 8.B. Further processing of samples by acidification and subsequent neutralization with base may increase the amount of detectable BDNF in extracts from a variety of samples, specifically brain and serum samples (17). For BDNF, the mechanism by which this occurs is not known. Acid treatment is a species- and tissue-specific phenomenon and also can lead to a decrease in detection of BDNF levels. 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 total free BDNF. **To measure the amount of free mature BDNF in your samples, proceed directly to the ELISA protocol in Section 5.A without acid treatment.** To assay total BDNF, acid-treat, then neutralize the samples as described below before proceeding with the ELISA protocol. **Do not attempt to acid-treat the BDNF Standard.**

Acid Treatment Procedure (optional)

 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.B) to approximately pH 2.6, then neutralizes them to approximately pH 7.6. Depending on the amount of protein in the samples, additional dilutions may or may not require the use of Block & Sample 1X Buffer, which contains a proprietary carrier protein, to minimize loss of BDNF.

For low-protein matrices, we recommend direct acid treatment at 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 on the species. We suggest adding 110–125µl 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°C or -70°C.

4. Sample Preparation (continued)

Materials to Be Supplied by the User

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

- 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 μ l of 1N HCl for each 50 μ 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 μ l of 1N NaOH per 50 μ l of sample. Check the pH to ensure that it is approximately 7.6.

5. Protocol for BDNF Quantitation

Materials to Be Supplied by the User

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

- 96-well (flat-bottom) ELISA plate (see Note in Section 5.A, Step 1)
- carbonate coating buffer (pH 9.7)
- plate sealer
- TBST wash buffer
- 1N hydrochloric acid
- microplate reader capable of monitoring absorbance at 450nm
- pipettors capable of accurately delivering volumes of 1 μ l-1ml
- multichannel pipettor
- wash bottle or automated plate washer (DYNEX Technologies UltraWash Plus or equivalent)
- plate shaker (DYNEX Technologies Micro-Shaker® II or equivalent)
- 50ml (for better mixing) or 15ml polypropylene tubes for dilutions

5.A. Plate Coating

1. In a 15ml or 50ml polypropylene tube, add exactly 10 μ l of Anti-BDNF mAb to 9.99ml of carbonate coating buffer (pH 9.7) to prepare enough reagent for each full 96-well plate. **Mix thoroughly**, but avoid creating excess bubbles. Use a multichannel pipettor to add 100 μ l to each well of a polystyrene ELISA plate.

Hint: Keep the undiluted Anti-BDNF mAb on ice once removed from 4°C storage.

Note: For best well-to-well accuracy, we recommend a high-quality name brand polystyrene ELISA plate, such as Thermo Labsystems Microtiter® Immunoassay Microplates (Immulon® 4; Cat.# 3855), Nunc-Immuno™ MaxiSorp™ (Cat.# 439454) and Corning Costar® (Cat.# 3590) plates. There are no observable differences in the performance of plates from these manufacturers.

2. Seal the wells with a plate sealer, and incubate **without shaking** overnight at 4°C.

Note: This assay is optimized for the carbonate coating buffer prepared as described in Section 8.B; other buffers may give poor results.

5.B. Preparing Block & Sample 1X Buffer

Each 96-well plate requires approximately 53ml of Block & Sample 1X Buffer to be used on the second day. Prepare Block & Sample 1X Buffer by mixing 42.4ml of deionized water with 10.6ml of Block & Sample 5X Buffer. Use aseptic transfer techniques when using the Block & Sample 5X Buffer stock solution. Mix gently and completely by inversion prior to use.

5.C. Blocking the Plate

 **Do not allow wells to dry out completely between steps.**

1. Remove the coated plate from the refrigerator. Flick out contents of the wells, and slap the plate upside down three times on a paper towel to help clear the wells. Vigorously wash all wells with TBST wash buffer using an automated plate washer, wash bottle or multichannel pipettor. For manual washing, fill each well with TBST wash buffer, flick out contents over a sink, and slap the plate three times on a paper towel. Add 200 μ l of Block & Sample 1X Buffer to each well using a multichannel pipettor. Do not touch or scratch the surface of the wells where antibody is bound to the plate.

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

2. Incubate at room temperature for one hour **without shaking**.

5.D. Preparing the BDNF Standard Curve



A standard curve must be performed on each plate.

The BDNF Standard provided with this system will generate a linear standard curve from 7.8–500pg/ml. **Use only values that are within the linear range to determine the BDNF concentration of the test samples.**

The BDNF Standard is supplied at a concentration of 1µg/ml. Accurately dilute the supplied BDNF Standard 1:2,000 in Block & Sample 1X Buffer to achieve a concentration of 500pg/ml. For example, transfer 10µl of undiluted standard into 390µl of Block & Sample 1X Buffer (1:40 dilution), then transfer 10µl of this solution into 490µl of Block & Sample 1X Buffer (1:50 dilution). This dilution series protocol results in a 1:2,000 dilution of the standard.

Hint: Keep the undiluted BDNF Standard on ice once removed from 4°C storage.

1. Following plate blocking, flick out contents of the wells over a sink. Slap the plate three times upside down on a paper towel to remove residual liquid, and wash once with TBST wash buffer as described in Section 5.C, Step 1. Designate two columns of wells (16 wells) for the standard curve. To prepare the BDNF standard curve within the assay plate, add 100µl/well of Block & Sample 1X Buffer to wells in rows B through H in the two columns designated for the standard curve (Figure 2).
2. Add 200µl of the diluted BDNF Standard (500pg/ml) to the first well in each column designated for the standard curve (row A).
3. Immediately perform serial 1:2 dilutions (100µl/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 BDNF. The final concentrations (in duplicate) within the plate will be 0–500pg/ml (Figure 2).

	Test Samples										BDNF Standard Curve		
	1	2	3	4	5	6	7	8	9	10	11	12	pg/ml
A	○	○	○	○	○	○	○	○	○	○	○	○	500
B	○	○	○	○	○	○	○	○	○	○	○	○	250
C	○	○	○	○	○	○	○	○	○	○	○	○	125
D	○	○	○	○	○	○	○	○	○	○	○	○	62.5
E	○	○	○	○	○	○	○	○	○	○	○	○	31.3
F	○	○	○	○	○	○	○	○	○	○	○	○	15.6
G	○	○	○	○	○	○	○	○	○	○	○	○	7.8
H	○	○	○	○	○	○	○	○	○	○	○	○	0

0826MOD6.7A

Figure 2. Recommended ELISA plate format for standard curve and unknown samples.

5.E. Addition of Sample

We recommend starting with a 1:4 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 (perhaps in triplicate) and subsequently reassay all positive samples not on the linear portion of the curve to determine the exact BDNF concentration.

Where the sample carrier solution may contribute nonspecific sources of BDNF (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 BDNF standard curve, add 100µl of samples (acid-treated or untreated, whichever is appropriate for your system. See Section 4 for the acid treatment of samples) to each remaining well.

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

2. Seal the wells with a plate sealer, and incubate the plate for 2 hours at room temperature with shaking (400 ± 100rpm).

Note: Best results are obtained using a plate shaker. Although plates may be incubated without shaking, a slight loss in sensitivity of the assay may occur.

3. Wash the plate five times with TBST wash buffer as described in Section 5.C, Step 1.

5.F. Addition of Anti-Human BDNF pAb

1. In a 15ml or 50ml polypropylene tube, add 20µl of Anti-Human BDNF pAb to 9.98ml 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 100µl of diluted Anti-Human BDNF pAb to each well, being careful not to touch or scratch the surfaces of the wells.

Hint: Keep the undiluted Anti-Human BDNF pAb on ice once removed from 4°C storage.

2. Seal the wells with a plate sealer, and incubate for 2 hours at room temperature with shaking.

Note: Best results are obtained using a plate shaker. Although plates may be incubated without shaking, a slight loss in sensitivity of the assay may occur.

3. Wash the plate five times with TBST wash buffer as described in Section 5.C, Step 1.

5.G. Addition of Anti-IgY HRP Conjugate

1. In a 15ml or 50ml polypropylene tube, accurately add 50µl of Anti-IgY HRP Conjugate to 9.95ml of Block & Sample 1X Buffer (1:200 dilution) to prepare enough reagent for a full 96-well plate. **Mix thoroughly**, and avoid creating excess bubbles. Using a multichannel pipettor, add 100µl of diluted Anti-IgY HRP Conjugate to each well, being careful not to disturb the bottom or sides of the wells.

Hint: Keep the undiluted Anti-IgY HRP on ice once removed from 4°C storage.

2. Incubate for 1 hour at room temperature with shaking (400 ± 100rpm).
Note: During this incubation, equilibrate the TMB One Solution to room temperature.
3. Wash the plate five times with TBST wash buffer as described in Section 5.C, Step 1.

5.H. Color Development

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

1. Add 100µl of room-temperature TMB One Solution to each well using a multichannel pipettor.
2. Incubate at room temperature with shaking for 10 minutes. A blue color will form in the wells.
3. Stop the reactions by adding 100µl of 1N hydrochloric acid to wells in the same order in which substrate was added in Step 2. The blue color 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 reactions. See Figure 3 for a representative BDNF standard curve.

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

5.I. Representative Standard Curve

⚠ A standard curve must be performed on each plate.

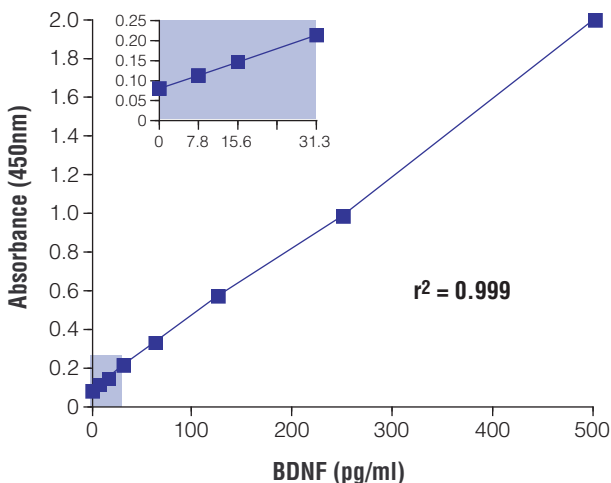


Figure 3. Representative BDNF standard curve obtained using the BDNF E_{max}[®] ImmunoAssay System. The inset is an enlargement of the 0-31.2pg/ml portion of the graph.

6. Troubleshooting

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

Symptoms	Causes and Comments
Sample absorbance above range of standard curve	Sample was too concentrated. Further dilute the sample. Assay multiple dilutions of each sample to ensure that at least one point will fall in the useful range of the standard curve.
Sample absorbance below range of standard curve	Sample was too dilute. Reassay at a higher sample concentration.
High absorbance in all samples	BDNF was present in buffer or medium. Perform negative control reactions containing carrier solution but no sample whenever BDNF may be present in buffer or medium.
Low absorbance in all samples	Incorrectly diluted component. Recheck the dilutions of each assay component.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Variability in replicate samples	<p>Technique problems in performing assay. Add stop solution to wells in same order as enzyme substrate.</p> <hr/> <p>Change pipette tips before adding each reagent.</p> <hr/> <p>Perform additional replicates.</p> <hr/> <p>Check calibration of the pipettor.</p>
Low activity in BDNF Standard	<p>Improper storage. The standard is stable, if stored undiluted, for six months at -20°C and three months at 4°C.</p>

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8. Appendix

8.A. Performance Characteristics of the BDNF E_{max}[®] ImmunoAssay System

Cross-Reactivity of the BDNF E_{max}[®] ImmunoAssay System

The BDNF E_{max}[®] ImmunoAssay System demonstrates very low cross-reactivity with related neurotrophic factors, NGF, NT-3 and NT-4/5, at concentrations as high as 100ng/ml, as shown below.

Neurotrophic Factor*	Actual Concentration	Cross-Reactivity
NGF	100ng/ml	<3%
NT-3	100ng/ml	<3%
NT-4/5	100ng/ml	<3%

*To evaluate the specificity of this assay system, 100ng/ml of NGF, NT-3 and NT-4/5 were tested for binding using the protocols as described in Sections 3-5. Results are expressed as the mean of triplicate determinations.

Intra-Assay Comparison

Three concentrations of BDNF were diluted in Block & Sample 1X Buffer and assayed by one operator for a total of eight determinations each.

	BDNF		
	Low	Medium	High
N	8	8	8
Mean (pg/ml)	28.6	53.3	286.1
SD (pg/ml)	2.53	1.53	6.4
CV (%)	8.8	2.9	2.2

N = sample size, SD = standard deviation,
CV = coefficient of variance

8.B. Composition of Buffers and Solutions

carbonate coating buffer

0.025M sodium bicarbonate
0.025M sodium carbonate

Adjust pH to 9.7 using 1N HCl or 1N NaOH.

DPBS (per liter)

0.2g KCl
8.0g NaCl
0.2g KH_2PO_4
1.15g Na_2HPO_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 KCl, NaCl, KH_2PO_4 and Na_2HPO_4 to a final volume of 1 liter. Adjust pH to 7.35 using 1N HCl or 1N NaOH. Add $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and mix thoroughly. Add $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and mix thoroughly.

1N hydrochloric acid

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

TBST wash buffer

20mM Tris-HCl (pH 7.6)
150mM NaCl
0.05% (v/v) Tween® 20

lysis buffer

137mM NaCl
20mM Tris-HCl (pH 8.0)
1% NP40
10% glycerol
1mM PMSF
10µg/ml aprotinin
1µg/ml leupeptin
0.5mM sodium vanadate

8.C. Related Products

E_{max} ® ImmunoAssay Systems and Buffers

Product	Size	Cat.#
NGF E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7630
	5 × 96 wells	G7631
NT-3 E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7640
	5 × 96 wells	G7641
GDNF E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7620
	5 × 96 wells	G7621
TGFβ ₁ E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7590
	5 × 96 wells	G7591
TGFβ ₂ E_{max} ® 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.#
Block & Sample 5X Buffer*	54ml	G3311
TMB One Solution*	100ml	G7431
rhBDNF	5 μ g	G1491
Anti-Human BDNF pAb	200 μ g	G1641
Anti-Human NT-3 pAb	200 μ g	G1651
rhNT-3	5 μ g	G1501
mNGF, 2.5S	100 μ g	G5141
	10 μ g	G5142
Anti-NGF mAb	100 μ g	G1131
	20 μ g	G1132
Anti-Pan Trk pAb	200 μ g	G1581
Anti-Human p75 pAb	200 μ g	G3231
rhGDNF	5 μ g	G2781
Anti-Human GDNF pAb	200 μ g	G2791
Anti-TGF β ₁ pAb	100 μ g	G1221
Anti-VACHT pAb	100 μ g	G4481
Anti- β III Tubulin mAb	100 μ g	G7121
Anti-GFAP pAb	100 μ g	G5601

*For Laboratory Use.

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