



Promega

Technical Bulletin

NT-3 E_{max}[®] ImmunoAssay System

INSTRUCTIONS FOR USE OF PRODUCTS G7640 AND G7641.



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NT-3 E_{max}[®] ImmunoAssay System

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 of this system. E-mail techserv@promega.com.

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1. Description

The NT-3 E_{max}[®] ImmunoAssay System is designed for the sensitive and specific detection of Neurotrophic Factor-3 (NT-3) in an antibody sandwich format (1; Figure 1). Briefly, flat-bottom 96-well plates are coated with Anti-Human NT-3 pAb that binds soluble NT-3. A quick wash removes any unbound NT-3. Captured NT-3 is bound by a second specific antibody, Anti-NT-3 mAb. After washing, a tertiary antibody conjugated to horseradish peroxidase, Anti-Mouse IgG, HRP Conjugate, is added and binds the sandwich complex. Finally, the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) is added, and the amount of specifically bound NT-3 is detected indirectly by HRP-catalyzed color development using TMB. The amount of NT-3 is proportional to the color generated in the coupled oxidation-reduction reaction and is quantitated using a standard curve generated with known amounts of NT-3. Using this system, NT-3 can be quantitated.

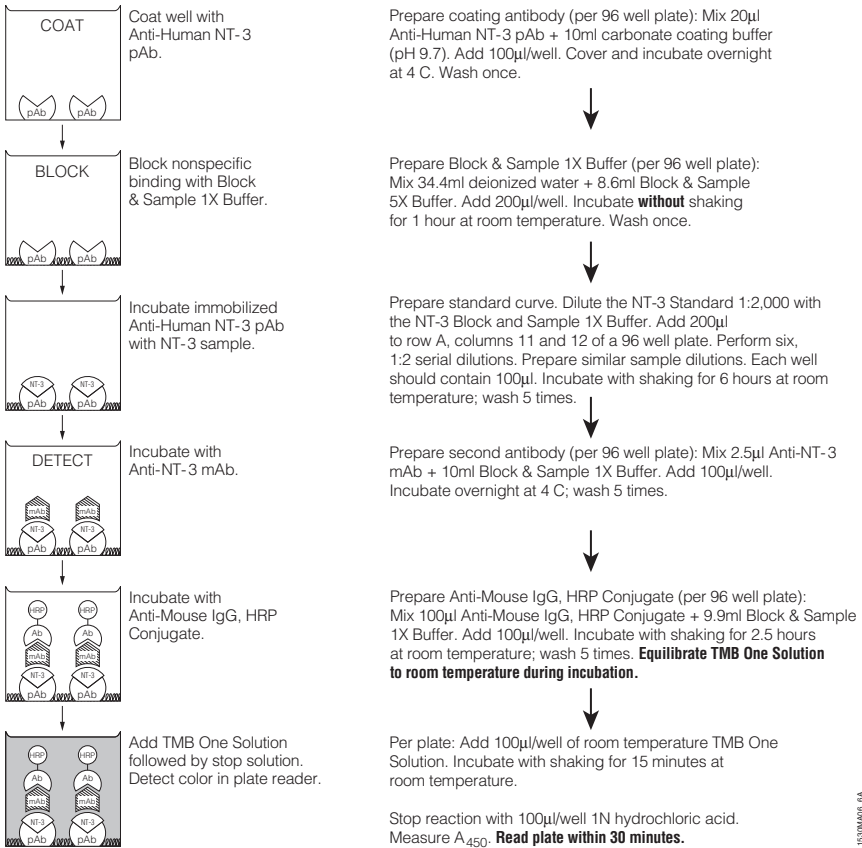


Figure 1. Schematic representation of the NT-3 E_{max}[®] ImmunoAssay System.

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

The NT-3 E_{max}[®] ImmunoAssay System offers several benefits:

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

2. Product Components

The NT-3 E_{max}[®] ImmunoAssay System is offered in two sizes – designed to accommodate five or two 96-well plates. Both systems contain the same reagents, except Cat.# G7641 contains more of each component.

Note: The NT-3 Standard supplied with these systems is recombinant human NT-3.

Product	Size	Cat.#
NT-3 E _{max} [®] ImmunoAssay System	2 × 96 wells	G7640

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

- 20µg Anti-Human NT-3 pAb
- 22ml Block & Sample 5X Buffer
- 20µl NT-3 Standard
- 10µl Anti-NT-3 mAb
- 200µl Anti-Mouse IgG, HRP Conjugate
- 25ml TMB One Solution

Product	Size	Cat.#
NT-3 E _{max} [®] ImmunoAssay System	5 × 96 wells	G7641

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

- 50µg Anti-Human NT-3 pAb
- 54ml Block & Sample 5X Buffer
- 50µl NT-3 Standard
- 15µl Anti-NT-3 mAb
- 500µl Anti-Mouse IgG, 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.

3. General Considerations

The NT-3 E_{max}[®] ImmunoAssay System has been tested using the following protocols. Plate coating requires an overnight incubation. Plates are blocked for 1 hour, and samples are incubated for 6 hours the next day. Incubation with Anti-Human NT-3 mAb is performed overnight. When transferring the NT-3 Standard and test samples to the plate, take care not to disturb or scratch the surface of the wells, as this may dislodge the coating antibodies and result in significant loss of signal. If unfamiliar with the technique, practice the pipetting procedures 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 NT-3 E_{max}[®] ImmunoAssay System may be used to quantitate NT-3 in tissue culture supernatants or tissue extracts. Since this assay uses an anti-mouse IgG conjugate, avoid using tissues containing high levels of IgG such as mouse serum, plasma and spleen. Store test samples frozen at -20°C before use. Avoid multiple freeze-thaw cycles. Remove any particulate matter from the samples by centrifugation prior to the assay.

Tissue extracts may be prepared using the lysis buffer described in Section 8.B. Acidification and subsequent neutralization with base may increase the amount of detectable NT-3 in extracts from a variety of tissues (2). For NT-3, the mechanism of this effect is not known at this time, but it may be due to the release of NT-3 from its receptors. Increased NT-3 detection following acid treatment is a species- and tissue-specific phenomenon and can, in some instances, lead to a decrease in detection of NT-3 levels. Therefore, it is important to **test the acid treatment procedure for any given species and tissue to determine the benefit, if any, of pretreatment.**

Note: This assay is designed to measure free NT-3. **To measure the amount of free mature NT-3 in your samples, proceed directly to the ELISA protocol in Section 5.A without acid treatment.** To assay total NT-3, acid-treat, then neutralize the samples as described in the procedure below before proceeding with the ELISA protocol. **Do not attempt to acid-treat the NT-3 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) to approximately pH 2.6, 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 proprietary carrier protein, to minimize loss of NT-3.

For low-protein matrices, we recommend direct acid treatment to a pH of 2.0-3.0 for 15-20 minutes. Following neutralization with NaOH, subsequent dilutions, if necessary, 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 μ 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°C or -70°C.

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. Verify that the pH is 3.0 or lower.
3. Mix and incubate at room temperature for 15 minutes.
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 NT-3 Quantitation

Materials to Be Supplied by the User

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

- 96-well (flat-bottom) ELISA plates
- carbonate coating buffer
- plate sealer
- TBST wash buffer
- 1N hydrochloric acid
- microplate reader capable of monitoring absorbance at 450nm
- multichannel pipettor
- pipettors capable of accurately delivering volumes of 2 μ l–1ml
- 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

Note: For best well-to-well accuracy, we recommend a high-quality, name-brand, polystyrene ELISA plate, such as Nunc MaxiSorp™ plates (Cat.# 439454) and Microtiter®-Immunoassay microplates (Thermo Labsystems Cat.# 3855). 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 Preparation

1. Per 96-well plate, prepare the primary coating antibody solution by adding 20 μ l of Anti-Human NT-3 pAb to 10ml of carbonate coating buffer in a 15ml or 50ml polypropylene tube. **Mix thoroughly**, but avoid creating excess bubbles. Use a multichannel pipettor to add 100 μ l of Anti-NT-3 pAb coating solution to each well of a polystyrene ELISA plate.

Hint: Keep the undiluted Anti-NT-3 pAb on ice once removed from 4°C storage.

2. Seal wells with a plate sealer, and incubate 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

The Block & Sample Buffer should be brought to room temperature and mixed well before use. Each 96-well plate requires approximately 43ml of Block & Sample 1X Buffer, to be used on the second day. To prepare Block & Sample 1X Buffer, place 34.4ml of deionized water in a 50ml polypropylene tube. Aspirate 8.6ml of Block & Sample 5X Buffer with a sterile pipettor, being careful not to contaminate the stock solution, and add it to the water. 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 the well contents, and slap the plate upside down 3 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 the contents over a sink and slap the plate 3 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 a plate washer for consistent results.

2. Incubate at room temperature for 1 hour **without** shaking.

5.D. Preparing the NT-3 Standard Curve

! A standard curve must be performed on each plate.

The NT-3 Standard provided with this system will generate a linear standard curve in the range of 4.7–300pg/ml. **Use only values that are within the linear range to determine the NT-3 concentration of test samples.**

The NT-3 Standard is supplied at a concentration of 0.6 μ g/ml. Accurately dilute the supplied NT-3 Standard 1:2,000 in Block & Sample 1X Buffer to achieve a concentration of 300pg/ml. Dilute the standard in a stepwise manner. For example, dilute 10 μ l of the standard in 390 μ l Block & Sample 1X Buffer, then dilute 10 μ l of this in 490 μ l of Block & Sample 1X Buffer for a final dilution of 1:2,000.

Hint: Keep the undiluted NT-3 Standard on ice once removed from 4°C storage.

1. Following plate blocking, flick out the well contents over a sink. Slap the plate 3 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 NT-3 standard curve within the assay plate, add 100 μ l/well of the Block & Sample 1X Buffer to wells in rows B through H in the two columns designated for the standard curve (Figure 2).
2. Add 200 μ l of diluted NT-3 Standard (300pg/ml) to the first well (row A) in each column designated for the standard curve.
3. Immediately perform serial 1:2 dilutions (100 μ l/well) in the two columns designated for the standard curve. Mix by pipetting before transferring to the next well. In the last set of wells for the standard curve, do not add any NT-3. The final concentrations (in duplicate) in the NT-3 control columns will be 0–300pg/ml (Figure 2).

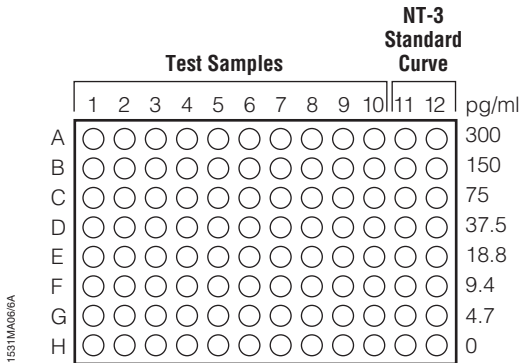


Figure 2. Recommended ELISA plate format for NT-3 standard curve and test samples.

5.E. Addition of Sample

We recommend preparing 1:2 serial dilutions of your test samples. Alternatively, screen samples in triplicate at a single concentration, and re-assay all positive samples to determine the appropriate NT-3 concentration.

Where the sample carrier solution may contribute nonspecific sources of NT-3 (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 NT-3 standard curve, add 100µl of test sample to each of the remaining wells.



Add samples as quickly as possible to minimize evaporation.

2. Seal wells with a plate sealer, and incubate the plate for 6 hours at room temperature with shaking (500 ± 100rpm).

Note: Best results are obtained using a plate shaker.

3. Wash all wells five times with TBST wash buffer as described in Section 5.C, Step 1. Immediately proceed to the next step.

5.F. Addition of Anti-NT-3 mAb

1. In a 15ml or 50ml polypropylene tube, add 2.5µl of Anti-NT-3 mAb to 10ml of Block & Sample 1X Buffer (1:4,000 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-NT-3 mAb to each well, being careful not to touch or scratch the surface of the wells.

Hint: Keep the undiluted Anti-NT-3 mAb on ice one removed from 4°C storage.

2. Seal wells with a plate sealer and incubate overnight at 4°C without shaking.
3. The next day, wash the plate five times with TBST wash buffer as described in Section 5.C, Step 1.

5.G. Addition of Anti-Mouse IgG, HRP Conjugate

1. Prepare a fresh 10ml working solution of Block & Sample 1X Buffer by combining 8ml of deionized water and 2ml of Block & Sample 5X Buffer in a 15ml polypropylene tube. Use care not to contaminate the stock solution. Mix gently and completely by inversion prior to use.
2. In a 15ml or 50ml polypropylene tube, accurately add 100µl of the stock Anti-Mouse IgG, HRP Conjugate to 9.9ml of Block & Sample 1X Buffer (1:100 dilution) to prepare enough reagent for a full 96-well plate. **Mix thoroughly**, but avoid creating excess bubbles. Add 100µl of diluted Anti-Mouse IgG, HRP Conjugate to each well with a multichannel pipettor, being careful not to disturb the surface of the wells.
Hint: Keep the undiluted Anti-Mouse IgG, HRP Conjugate on ice once removed from 4°C storage.

3. Incubate at room temperature for 2.5 hours with shaking (500 ± 100rpm).
Note: Best results are obtained using a plate shaker. Also, during this incubation, equilibrate the TMB One Solution to room temperature.
4. Wash the plate five times with TBST wash buffer as described in Section 5.C, Step 1.

5.H. Color Development

Caution: Avoid direct contact (especially of skin and eyes) with TMB One Solution and 1N hydrochloric acid.

1. Add 100µl of room-temperature TMB One Solution to each well using a multichannel pipettor.
2. Incubate with shaking at room temperature for 15 minutes.
3. Stop the reaction by adding 100µl of 1N hydrochloric acid to each well in the same order that substrate was added. The color should change to yellow as the pH decreases. 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 NT-3 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.I. Representative Standard Curve

⚠ Prepare a standard curve for each plate. Best results are obtained using the average value of replicate samples.

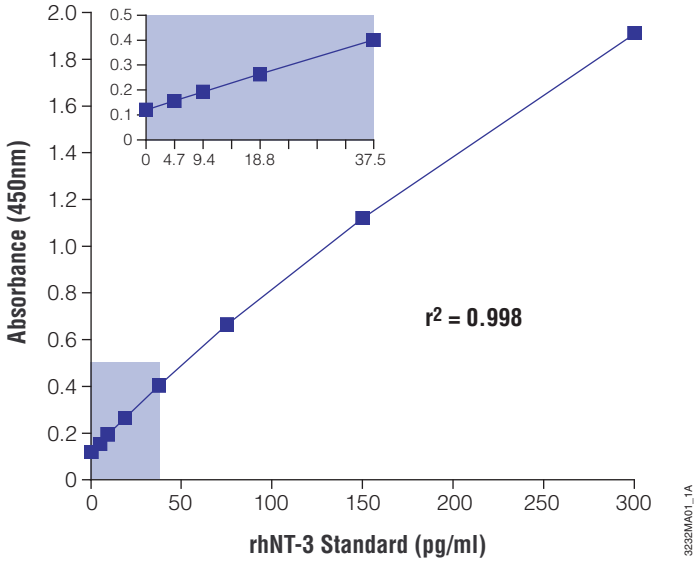


Figure 3. Representative NT-3 standard curve obtained using the NT-3 E_{max}[®] ImmunoAssay System. The inset is an enlargement of the 0–37.5pg/ml portion of the graph.

6. Troubleshooting

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

Symptoms	Causes and Comments
Sample absorbance was above the 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 within the useful range of the standard curve.
Sample absorbance was below the range of standard curve	Sample was too dilute. Re-assay at a higher sample concentration.
High absorbance in all samples	Color reaction was too fast. If absorbance exceeds the dynamic range of plate reader, repeat assay with a shorter color development time or greater dilution of the Anti-Mouse IgG, HRP Conjugate, or use a plate reader with a greater dynamic range.
Low absorbance in all samples	Color reaction was too slow or color reaction was stopped too soon. Increase duration of the color development reaction, or use a less dilute solution of Anti-Mouse IgG, HRP Conjugate, as described in Section 5.G.
Variability in replicate samples	Technique problems in performing assay. Add 1N hydrochloric acid to wells in same order as TMB substrate. Change pipette tips before adding each reagent. Perform additional replicates. Ensure that all wells are washed completely. Verify calibration of pipettor.
Low activity in NT-3 Standard	Improper storage. The standard is stable if stored undiluted for six months at -20°C and three months at 4°C.

7. References

- Hornbeck, P. (1994) In: *Current Protocols in Immunology* **1**, Coico, R., ed., John Wiley & Sons, Inc., Unit 2.1.
- Okragly, A.J. and Haak-Frendscho, M. (1997) An acid-treatment method for the enhanced detection of GDNF in biological samples. *Exp. Neurol.* **145**, 592-6.

8. Appendix

8.A. Performance Characteristics of the NT-3 E_{max}[®] ImmunoAssay System

Cross-Reactivity of the NT-3 E_{max}[®] ImmunoAssay System

The NT-3 E_{max}[®] ImmunoAssay System demonstrates very low cross-reactivity with the structurally related growth factors 2.5S Nerve Growth Factor (NGF), Recombinant, Human Brain Derived Neurotrophic Factor (BDNF) and Recombinant, Human Neurotrophin-4 (NT-4) at concentrations as high as 10ng/ml, as shown in the following table.

Neurotrophic Factor*	Actual Concentration	Cross-Reactivity
NT-4	10ng/ml	0.11%
NGF	10ng/ml	0.11%
BDNF	10ng/ml	0.11%

*To evaluate the specificity of this assay system, 10ng of Murine 2.5S NGF (Cat.# G5141), rhBDNF (Cat.# G1491) and rhNT-4 (Cat.# G1511) were tested for binding using the protocols as described in Section 5. Results are expressed as the mean of triplicate determinations.

Intra-Assay Comparison

Three concentrations of rhNT-3 were diluted in Block & Sample 1X Buffer and assayed by a single operator for a total of 8 determinations each. The apparent concentrations are described in the following table.

	rhNT-3 (pg/ml)		
	30	70	225
N	8.0	8.0	8.0
Mean (pg/ml)	30.0	71.0	227.0
SD (pg/ml)	2.0	2.0	4.0
CV (%)	5.2	2.2	1.5

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 the pH to 9.7 (\pm 0.1) 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 the 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 the $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; mix thoroughly, then 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 of deionized water.

lysis buffer

137mM NaCl

20mM Tris-HCl (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[®]-20

8.C. Related Products

E_{max} ® ImmunoAssay Systems

Product	Size	Cat.#
NGF E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7630
	5 × 96 wells	G7631
GDNF E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7620
	5 × 96 wells	G7621
BDNF E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7610
	5 × 96 wells	G7611
TGF β ₁ E_{max} ® ImmunoAssay Systems	2 × 96 wells	G7590
	5 × 96 wells	G7591
TGF β 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

8.C. Related Products (continued)

Items Available Separately

Product	Size	Cat.#
Block & Sample 5X Buffer*	54ml	G3311
Anti-Human NT-3 pAb	200µg	G1651
rhNT-3	5µg	G1501
mNGF, 2.5S	100µg	G5141
	10µg	G5142
Anti-NGF mAb	100µg	G1131
	20µg	G1132
rhBDNF	5µg	G1491
Anti-Human BDNF pAb	200µg	G1641
Anti-Rat CNTF pAb	200µg	G1631
Anti-Pan Trk pAb	200µg	G1581
Anti-TrkB In pAb	100µg	G1561
Anti-Human p75 pAb	200µg	G3231
rhGDNF	5µg	G2781
Anti-Human GDNF pAb	200µg	G2791
Anti-TGFβ ₁ pAb	100µg	G1221
Anti-βIII Tubulin mAb	100µg	G7121
Anti-GFAP pAb	100µg	G5601
Anti-VACHT pAb	100µg	G4481

*For Laboratory Use.

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