Technical Bulletin

NGF E_{max}® ImmunoAssay System

INSTRUCTIONS FOR USE OF PRODUCTS G7630 AND G7631.

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NGF E_{max}® ImmunoAssay System

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I. Description

The NGF (Nerve Growth Factor) E_{max}® ImmunoAssay System is designed for the sensitive and specific detection of NGF in an antibody sandwich format (1) (Figure 1). In this format, flat-bottom 96-well plates are coated with Anti-NGF Polyclonal Antibody (pAb), which binds soluble NGF. The captured NGF is bound by a second specific monoclonal antibody (mAb). After washing, the amount of specifically bound mAb is detected using a species-specific antibody conjugated to horseradish peroxidase (HRP) as a tertiary reactant. The unbound conjugate is removed by washing, and following incubation with a chromogenic substrate, the color change is measured. The amount of NGF in the test solutions is proportional to the color generated in the oxidation-reduction reaction. Using this system, NGF in tissue culture supernatants or tissue extracts can be quantitated in the range of 3.9–250pg/ml.

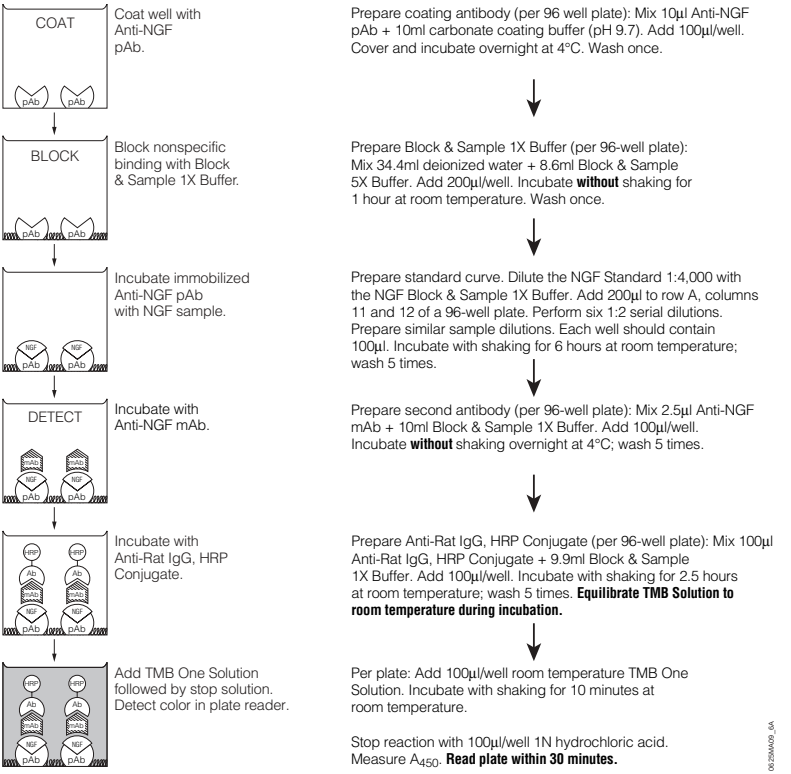

Figure 1. Schematic representation of the NGF E_{max}® ImmunoAssay System. For a detailed protocol, or when using this system for the first time, please read Sections III–VI carefully.

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

- **Specificity:** Specific detection of NGF; typically less than 3% cross-reactivity with other neurotrophic factors at 10ng/ml.
- **Sensitivity:** Detects a minimum of 7.8pg/ml of NGF.
- **Flexibility:** Available in sizes for two or five 96-well ELISA plates; can configure plates as desired.
- **High Value:** Optimized reagents and protocol.

II. Product Components and Storage Conditions

The NGF 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.# G7631 contains more of each component.

Product	Size	Cat.#
NGF E_{max}® ImmunoAssay System	2 × 96 wells	G7630

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

- 20µl Anti-NGF pAb
- 22ml Block and Sample 5X Buffer
- 20µl NGF Standard
- 10µg Anti-NGF mAb
- 200µl Anti-Rat IgG, HRP Conjugate
- 25ml TMB One Solution
- 1 Protocol

Product	Size	Cat.#
NGF E_{max}® ImmunoAssay System	5 × 96 wells	G7631

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

- 50µl Anti-NGF pAb
- 54ml Block and Sample 5X Buffer
- 50µl NGF Standard
- 20µg Anti-NGF mAb
- 500µl Anti-Rat IgG, HRP Conjugate
- 2 × 25ml TMB One Solution
- 1 Protocol

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 to these diluted solutions, as they may interfere with the assay.

III. General Considerations

The NGF E_{max}® ImmunoAssay System has been tested using the following protocols. Plate coating requires an overnight incubation. Plates are blocked for one hour and samples are incubated for six hours the next day. Incubation with Anti-NGF mAb also is performed overnight. When transferring the NGF standard and the experimental 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 plate.

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.

IV. Sample Preparation

The NGF E_{max}® ImmunoAssay System may be used to quantitate NGF in tissue culture supernatants or tissue extracts. The system uses an anti-rat IgG conjugate that may demonstrate cross-reactivity with samples containing mouse IgG or human IgG, resulting in increased absorbance readings. Avoid using samples containing high levels of IgG such as serum, plasma and spleen. 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.

Avoid using samples containing high levels of IgG such as serum, plasma and spleen.

Acidification, and subsequent neutralization with base, has been shown to increase the amount of detectable NGF in extracts from a variety of tissues from several species (2–4). For NGF, the acidification may cause proteolysis of the 7S to the 2.5S form, the release of NGF from soluble receptors or both. Regardless, acid treatment can be performed in vitro, and it can increase the level of detectable factor. Increased NGF detection following acid treatment is a species- and tissue-specific phenomenon and can, in some instances, lead to a decrease in detection of NGF levels. Therefore, it is important to **test the acid treatment procedure for any given species and tissue to determine the benefit of pretreatment.**

Note: This assay is designed to measure free NGF. **To measure the amount of free mature NGF in your samples, proceed directly to the ELISA protocol in Section V.A without acid treatment.** To assay for total NGF, 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 NGF Standard.**

Acid Treatment Procedure

This procedure acidifies samples diluted 1:5 in Dulbecco's PBS (DPBS) 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 to minimize loss of NGF.

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 110–125µl 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.

Materials to Be Supplied by the User
(Solution compositions are provided in Section VIII.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 1µl of 1N HCl for each 50µl of diluted sample. Verify that the pH is 3.0 or lower.
3. Mix and incubate for 15 minutes at room temperature.
4. Neutralize by adding 1µl of 1N NaOH per 50µl of sample. Check the pH to ensure that it is approximately 7.6.

V. Protocol for NGF Quantitation

Materials to Be Supplied by the User
(Solution compositions are provided in Section VIII.B.)

- 96-well (flat bottom) ELISA plate
- 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: This assay has been tested using Nunc MaxiSorp™ plates (Nunc Cat.# 439454) and Microtiter®-Immunoassay Microplates (Immulon® 4; Thermo Labsystems Cat.# 3855). There are no observable differences in the performances of plates from these manufacturers. For best well-to-well accuracy, we recommend a high-quality, name-brand polystyrene ELISA plate.

V.A. Plate Coating

1. In a 15ml or 50ml polypropylene tube, add exactly 10µl of the Anti-NGF pAb 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-NGF pAb 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 has been optimized using the carbonate coating buffer prepared as described in Section VIII.B; other buffers may give poor results.

V.B. Preparing Block & Sample 1X Buffer

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.

V.C. Blocking the Plate

1. Remove the coated plate from the refrigerator. Flick out the 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 the contents over a sink and slap the plate three times on a paper towel. Add 200µl 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.

V.D. Preparing the NGF Standard Curve

The NGF Standard provided with this system will generate a linear standard curve from 3.9—250pg/ml. Use only values that are within the linear range to determine the NGF concentration of the test samples. The NGF Standard is supplied at a concentration of 1µg/ml. Accurately dilute the supplied NGF Standard 1:4,000 in Block & Sample 1X Buffer to achieve a concentration of 250pg/ml. For example, dilute 10µl of the NGF Standard into 790µl of Block & Sample 1X Buffer (1:80 dilution), then dilute 10µl of this into 490µl of the Block & Sample 1X Buffer for a final dilution of 1:4,000.

1. Following plate blocking, flick out the 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 V.C, Step 1. Designate two columns of wells (16 wells) for the standard curve. To prepare the NGF standard curve within the assay plate, add 100µl/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 200µl of the diluted NGF Standard (250pg/ml) to the first well in each column designated for the standard curve.
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 NGF. The final concentrations (in duplicate) within the plate will be 0–250pg/ml (Figure 2).

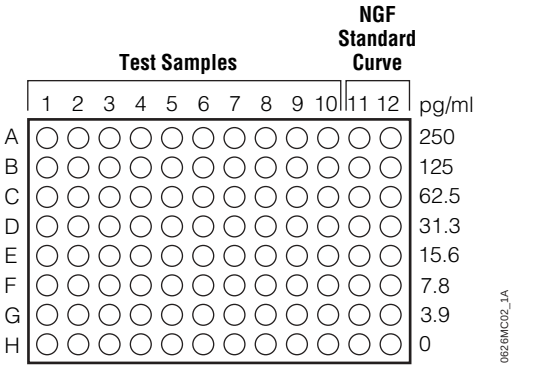

Figure 2. Recommended ELISA plate format for standard curve and test samples.

V.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, screen samples at a single concentration and subsequently reassay all positive samples not on the linear portion of the curve to determine the exact NGF concentration.

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

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

2. Seal the wells with a 96-well plate sealer and incubate the plate for six hours at room temperature with shaking (500 ± 100rpm).

   Note: Best results are obtained using a plate shaker. Alternatively, plates may be incubated at 37°C without shaking, although a slight loss in sensitivity of the assay may occur.
   Do not stack plates when incubating at 37°C.

3. Wash all wells five times with TBST wash buffer as in Section V.C, Step 1.

V.F. Addition of Anti-NGF mAb

1. In a 15ml or 50ml polypropylene tube, add 2.5µl of the Anti-NGF 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 the diluted Anti-NGF mAb to each well, being careful not to touch or scratch the bottom or sides of the wells.

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

3. The next day, wash all wells five times with TBST wash buffer as described in Section V.C, Step 1.

V.G. Addition of Anti-Rat 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. Again, use care not to contaminate the stock solution. Mix gently and completely by inversion prior to use.

   Hint: Keep the Anti-NGF mAb and Anti-Rat IgG, HRP Conjugate on ice when removed from 4°C storage.

2. In a 15ml or 50ml polypropylene tube, accurately add 100µl of the stock Anti-Rat 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** and avoid creating excess bubbles. Using a multichannel pipettor, add 100µl of the diluted Antibody Conjugate to each well, being careful not to disturb the bottom or sides of the wells.

3. Incubate for 2.5 hours at room temperature with shaking (500 ± 100rpm).

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

   Hint: During this incubation, equilibrate the TMB One Solution to room temperature.

4. Wash all wells five times with TBST wash buffer as described in Section V.C, Step 1.

V.H. Color Development

1. Add 100µl of the room temperature TMB One Solution to each well using a multichannel pipet.

2. Incubate at room temperature with shaking for 10 minutes.

3. Stop the reaction by adding 100µl of 1N hydrochloric acid to the wells in the same order in which TMB One Solution was added in Step 1 above. Blue will change to yellow upon acidification. Take care to avoid creating bubbles.

   Caution: Take care to avoid contact of the TMB One Solution and 1N hydrochloric acid with skin and eyes.

4. Record the absorbance at 450nm on a plate reader within 30 minutes of stopping the reaction. See Figure 3 for a representative NGF 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.

V.I. Representative Standard Curve

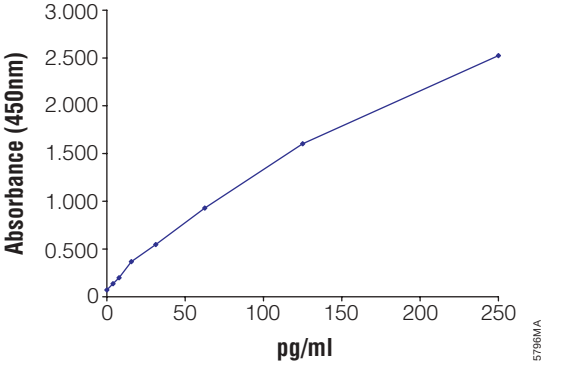

Figure 3. Representative NGF standard curve obtained using the NGF E_{max}® ImmunoAssay System.

VI. 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 is above range of standard curve	Sample 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 is below range of standard curve	Sample too dilute. Re-assay at a higher sample concentration.
High absorbance in all samples	NGF present in buffer or media. Perform negative control reactions containing carrier solution but no sample whenever NGF may be present in buffer or media.
	Color reaction too long. If absorbance exceeds dynamic range of plate reader, repeat assay with a shorter color development time, or use a plate reader with a greater dynamic range.
	Mouse, human or rat samples containing high levels of IgG were used. Avoid using samples containing high levels of IgG such as serum, plasma and spleen. See Section IV for more details.

VI. Troubleshooting (continued)

Symptoms	Causes and Comments
Low absorbance in all samples	Color reaction too slow: • Increase duration of color development reaction. • Recheck the dilutions of each component of the assay.
Variability in replicate samples	Technique problems in performing the assay: • Ensure that all wells are washed completely. • Allow plate to warm to room temperature for 10–15 minutes before starting blocking procedure. • Add stop solution to wells in the same order as TMB One Solution. • Change pipette tips before adding each reagent. • Perform additional replicates. • Check calibration of the pipettor.
Low activity in NGF Standard	Improper storage. The standard is stable, if stored undiluted, for six months at –20°C and three months at 4°C.

VII. References

1. Hornbeck, P. (1994) In: *Current Protocols in Immunology*, Vol. 1, Coico, R. ed., John Wiley & Sons, Inc. Unit 2.1.
2. Zettler, C. *et al*. (1996) Detection of increased tissue concentrations of nerve growth factor with an improved extraction procedure. *J. Neurosci. Res.* **46**, 581–94.
3. *Acid Treatment of NGF Samples* (1996) *Neural Notes* **II**(3), 23.
4. 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.

VIII. Appendix

VIII.A. Performance Characteristics of the NGF E_{max}® ImmunoAssay System

Cross-Reactivity of the NGF E_{max}® ImmunoAssay System

The NGF E_{max}® ImmunoAssay System demonstrates very low cross-reactivity with structurally related growth factors Human Recombinant Brain Derived Neurotrophic Factor (rhBDNF), Neurotrophin-3 (rhNT-3) and Neurotrophin-4 (rhNT-4) at concentrations as high as 10ng/ml, as shown below.

Neurotrophic Factor	Actual Concentration (ng/ml)	% Cross-Reactivity
BDNF	10	<0.078
NT-3	10	<0.078
NT-4	10	<0.078

To evaluate the specificity of this assay system, 10ng/ml of rhBDNF (Cat.# G1491), NT-3 (Cat.# G1501) and rhNT-4 were tested for binding using the protocols as described in Sections III–V. Results are expressed as the mean of triplicate determinations.

Intra-Assay Comparison

Acid-treated samples were diluted in Block & Sample 1X Buffer and assayed by one operator for a total of eight determinations each.

	NGF	
	Sample 1	Sample 2
N	8	8
Mean (pg/ml)	194	404
SD (pg/ml)	8	17
CV (%)	4.1	4.2

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

VIII.B. Composition of Buffers and Solutions

1N hydrochloric acid

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

carbonate coating buffer

0.025M	sodium bicarbonate
0.025M	sodium carbonate

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

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

DPBS (per liter)

0.2g	KCl
8.0g	NaCl
0.2g	KH_2PO_4
1.15g	Na_2HPO_4
133mg	$CaCl_2 \cdot 2H_2O$
100mg	$MgCl_2 \cdot 6H_2O$

Add room temperature deionized water to a final volume of 1 liter to the KCl, NaCl, KH_2PO_4 and Na_2HPO_4. Adjust pH to 7.35 using 1N HCl or 1N NaOH, if necessary. Add the $MgCl_2 \cdot 6H_2O$, mix thoroughly; then add the $CaCl_2 \cdot 2H_2O$, and mix thoroughly.

TBST wash buffer

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

VIII.C. Related Products

E_{max}® ImmunoAssay Systems

Product	Size	Cat.#
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
BDNF E_{max}® ImmunoAssay Systems	2 × 96 wells	G7610
	5 × 96 wells	G7611
$TGF\beta_1$ E_{max}® ImmunoAssay Systems	2 × 96 wells	G7590
	5 × 96 wells	G7591
$TGF\beta_2$ E_{max}® ImmunoAssay System	5 × 96 wells	G7600

VIII.C. Related Products (continued)

Items Available Separately

Product	Size	Cat.#
TMB One Solution*	100ml	G7431
Block & Sample 5X Buffer*	54ml	G3311
mNGF, 2.5S	100µg	G5141
	10µg	G5142
mNGF, 7S	100µg	G5151
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
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-βIII Tubulin mAb	100µg	G7121
Anti-GFAP pAb	100µg	G5601

*For Laboratory Use.

Primer Pairs and Amplification Reagents

Product	Size	Cat.#
β-Actin Primer Pair	20 reactions	G5740
CNTF Primer Pair	20 reactions	G5770
TrkB Primer Pair	20 reactions	G5790
NT-3 Primer Pair	20 reactions	G6801
p75 Primer Pair	20 reactions	G6861
Access RT-PCR System*	20 reactions	A1260
AccessQuick™ RT-PCR System*	20 reactions	A1701

*For Laboratory Use.

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