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Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 945–959

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Validation of a rat pheochromocytoma (PC12)-based cell survival assay for determining biological potency of recombinant human nerve growth factor

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Received 8 March 1999; received in revised form 25 June 1999; accepted 5 July 1999

Abstract

A method has been validated, according to the Guidelines of the International Conference on Harmonization (ICH), for precise quantitation of the biological activity of recombinant human nerve growth factor (rhNGF) for lot release testing. The assay is based on the survival of a subclone of rat pheochromocytoma PC12 cells (PC12-CF) in response to rhNGF. Cell survival is measured by monitoring the reduction, by living cells, of the alamarBlue™ dye into a red form which is highly fluorescent. The assay is simple, has high throughput (performed in 96-well microtiter plates) and shows reproducible dose-response curves in the concentration range of 0.2–50 ng/ml. The method was validated for its linearity, accuracy, precision, robustness, and to meet current regulatory requirements. The assay demonstrated good linearity, yielding a coefficient of determination of 0.9902. Sample recovery studies demonstrated an accuracy ranging from 96 to 98%. The repeatability of the assay and intermediate precision had coefficients of variation (CV) of $<9\%$. The assay was stability-indicating since it was able to detect changes in rhNGF samples degraded by protease treatment and in a number of isolated rhNGF variants. Robustness was demonstrated by the relative insensitivity of the assay to small deliberate changes in key method parameters. The validation data, provided in this manuscript, indicate that the newly described bioassay for rhNGF is robust, accurate, precise, and suitable for lot release potency testing of rhNGF.

Keywords: rhNGF; Potency assay; PC12; alamarBlue™; Method validation

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0731-7085/99/$ - see front matter
PII: S0731-7085(99)00225-3

1. Introduction

Nerve Growth Factor (NGF) is a neurotrophic factor that mediates activities such as survival,differentiation, and proliferation of neurons in both the central and peripheral nervous system [1–3]. Signal transduction initiated by NGF is mediated through specific cell surface receptors [4]. NGF binds to two distinct cell surface receptors: the high affinity trkA receptor, which is a tyrosine kinase signal-transducing receptor [5] and the low affinity p75NGF receptor whose role in NGF-induced signal transduction remains unclear [6]. The three-dimensional crystal structure of NGF has been elucidated and many of the sites responsible for binding to its specific receptors, trkA and p75, have been identified [7–13].

The majority of the in vivo experiments have been carried out with NGF purified from mouse submaxillary glands because of the scarcity of human NGF [14]. With the advent of recombinant DNA technology, it is now possible to produce large quantities of human NGF to conduct such studies. To aid in the purification of recombinant NGF and to assess its potency before preclinical and clinical studies, a convenient bioassay is essential.

Common bioassays for NGF involve quantitating the neurite outgrowth response of dorsal root ganglia from 9-day-old chick embryos [2] and the in vitro survival of dissociated neurons [15,16]. These methods are labor intensive, subjective, and require a constant supply and maintenance of embryos. An alternative to these primary neuronal cultures is the use of the cell line PC12, a clone of rat pheochromocytoma. Prolonged incubation of these cells with NGF causes a dose-dependent differentiation into cells exhibiting many properties of sympathetic neurons, including neurite outgrowth [17]. In spite of the convenience of using this cell line, this assay suffers from low precision and low throughput due to the tendency of PC12 cells to adhere to each other and to the subjectivity associated with manual counting. Additional methods were subsequently described in which NGF, in a dose-dependent manner, prevented the death of PC12 cells cultured under serum-free conditions [18,19]. These methods involved either counting cells using a hemacytometer, which is somewhat subjective and of low throughput [18] or using crystal violet staining as cell viability indicator, which requires fixed cells [19].

In this paper, we describe an improved bioassay that addresses many of these problems and which is suitable for the characterization and quantification of NGF bioactivity in a quality control environment. The assay is performed in 96-well microtiter plates, uses a subclone of PC12 cells selected for its lack of tightly packed cell clumps, and an alamarBlue™ fluorescence method for determining NGF-stimulated cell survival under serum-free conditions. alamarBlue™, which is blue and non-fluorescent in its oxidized state, is reduced by the intracellular environment into a red form that is highly fluorescent [20]. The changes in color and fluorescence are proportional to the number of live cells.

The intent of this paper is to describe an approach, developed in our laboratory, for the validation of this new bioassay for NGF activity. The method was validated for accuracy, precision, linearity, specificity, stability-indicating properties, and robustness. Validation was performed in accordance with the Guidelines of the International Conference on Harmonization (ICH) [21,22] and under current good manufacturing practices (cGMPs) as required in a quality control laboratory [23].

2. Experimental

2.1. NGF and variants of NGF

Recombinant human nerve growth factor (rhNGF) was expressed in Chinese hamster ovary (CHO) cells at Genentech [24]. Although the gene for NGF encodes a protein of 120 amino acids, rhNGF used in these studies has, primarily, 118 amino acids, due to enzymatic processing at the C-terminus during production. Small amounts of species with 120 and 117 amino acids copurified with the 118-amino acid homodimer. Each of these species, designated as: (1–118) rhNGF; (1–120) rhNGF and (1–117) rhNGF; was isolated as

described previously [24]. Mono-oxidized NGF (Met^{37}O-rhNGF) and an isoaspartate derivative (β-Asp^{93}-rhNGF) were purified as described previously [25]. rhNGF exists in solution as a dimer, and the N-terminal residues of each rhNGF monomer are susceptible to conversion from serine to glycine, leading to three dimer forms: the homodimer Ser^1-rhNGF/Ser^1-rhNGF, the heterodimer Ser^1-rhNGF/Gly^1-rhNGF and the homodimer Gly^1rhNGF/Gly^1-rhNGF [26].

2.2. Materials

Gentamicin sulfate, glutamine, horse serum, RPMI 1640 medium, and HEPES (*N*-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) were purchased from GIBCO-BRL (Grand Island, NY). Bovine serum albumin (BSA), *tert*-butyl hydroperoxide (THBP), methionine, and Trypan blue were purchased from Sigma Chemical (St. Louis, MO), fetal bovine serum from Hyclone Laboratories (Logan, UT), alamarBlue™ reagent from Accumed International (Westlake, OH); and lysyl endopeptidase from Wako (Richmond, VA).

2.3. Maintenance of cell cultures

The rat pheochromocytoma cell line, PC12, was obtained from American Type Tissue Culture, Rockville, MD. Although this cell line performed well, a subclone, designated PC12-CF, was isolated in our laboratory by employing limited dilution and selecting for better adherence and a lack of tightly packed cell clumps. Cells were banked at passage 14 and used between passages 16 and 47 for these studies. This subclone forms loose cell clumps that can be dissociated easily by gentle shaking. PC12-CF stock cultures were maintained in tissue culture flasks in RPMI 1640 medium with 5% heat-inactivated (56°C for 30 min) fetal bovine serum, 10% heat-inactivated horse serum, 2 mM glutamine and 10 mM HEPES (pH 7.2). PC12-CF cells were cultured at 37°C in a humidified 5% CO_2 incubator and routinely subcultured once a week. Cells were generally 97–100% viable as measured by Trypan blue exclusion method.

2.4. Cell survival assay

The assay was performed under serum-free conditions in assay medium (AM) consisting of RPMI 1640 supplemented with 0.1% BSA, 2 mM glutamine, 20 mM HEPES (pH 7.2), and 100 μg/ml gentamicin sulfate. PC12-CF cells were dislodged from stock flasks by gentle shaking, centrifuged, washed twice in AM, and resuspended at a density of 4×10^5 cells/ml. In a typical assay, 50 μl of AM, 50 μl of cell suspension (20 000 cells/well), and 50 μl of various concentrations of rhNGF (0.2–50 ng/ml) were added to triplicate wells (unless otherwise indicated) of flat bottomed 96-well tissue culture plates and incubated for 2 days at 37°C and 5% CO_2. Thirty microliters of alamarBlue™ (undiluted) were then added and the incubation continued for 5 h. The plates were allowed to cool to room temperature for 10 min on a shaker and the fluorescence was read using a 96-well fluorometer (Cambridge Technology Model 7620), with excitation at 530 nm and emission at 590 nm. Results, expressed in relative fluorescence units (RFU), were plotted against rhNGF concentrations using a 4-parameter curve-fitting program (KaleidaGraph), and the sample concentrations were computed from the standard curve. All rhNGF concentrations shown throughout this study refer to the concentration in the 50 μl added to the well, before the addition of alamarBlue™. rhNGF assay controls targeting 1.5 ng/ml were included on each plate when appropriate.

2.5. Statistical analysis of data

Precision profiles, plots of the estimated within plate coefficient of variation of calibrated concentration, were constructed for each assay plate [27], based on the standards. Variance components (day-to-day within analyst, plate-to-plate within day, analyst-to-analyst, and well-to-well within plate variability) were estimated separately at each of the three concentration levels, using the restricted maximum likelihood method with analyst, day, and plate as factors and calculated concentration as the response [28]. Relative potency of degraded samples and variants was estimated at a

single concentration (single point method) and by standard parallel line bioassay techniques [29]

2.6. Preparation of degraded samples of rhNGF

Samples of rhNGF were prepared using various stress conditions to stimulate degradation. These conditions included elevated temperature, exposure to light, mechanical stress, extremes of pH, oxidative conditions and protease treatment. rhNGF was subjected to thermal degradation by incubation at 45 ± 2°C for 30 days. Light degradation was performed by subjecting a sample of rhNGF to intense light in a light box for 7 days. Mechanical stress was achieved by shaking rhNGF samples at ambient temperature (25 ± 2°C) for 7 days. rhNGF samples were also subjected to acidic pH (3.0) or basic pH (8.0) for 15 days at 5 ± 3°C. For chemical oxidation, a sample of rhNGF was exposed to a solution of 70% aqueous *tert*-butyl hydroperoxide (TBHP) for 3 h at ambient temperature. Protease treatment was performed using lysyl endopeptidase, a proteolytic enzyme that cleaves proteins specifically on the C-terminal side of lysine residues. Briefly, rhNGF samples (1 mg/ml) were diluted 1:1 with a 2 × enzyme digestion buffer (200 mM Tris, pH 8.2), followed by the addition of lysyl endopeptidase solution to a give a final value of 0.04 enzyme AU/mg of rhNGF. The digests were then incubated at 37 ± 2°C. Aliquots were removed at 60- and 120-min time points, and the digestion was stopped by acidifying the digest solutions with 10% TFA (trifluoroacetic acid) for a final concentration of 0.5% TFA. These conditions resulted in partial digestion of rhNGF. All degraded samples were stored at −60°C following treatment and analyzed analytically for extent of modification (data not shown).

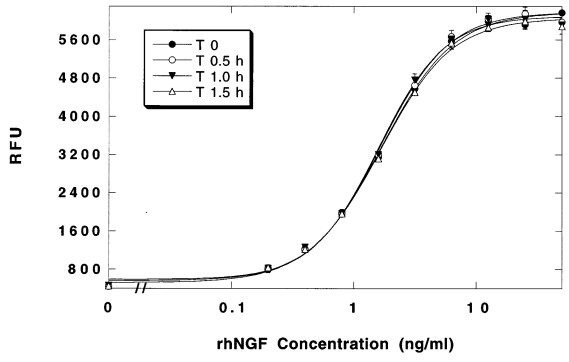

Fig. 1. Effect of cell suspension storage time. Cell seeding suspension was prepared and either used immediately (0 h) or stored at ambient temperature for 0.5, 1 and 1.5 h prior to seeding plates. Cells were then cultured in serumfree medium with varying concentrations of rhNGF as described in Section 2. rhNGF standard curves for each condition were compared. Data represent the mean RFU value of triplicate wells at each rhNGF concentration (± SD).

3. Results

3.1. Robustness

The robustness of the assay was evaluated to measure its ability to remain unaffected by small but deliberate variations in method parameters. The following parameters were studied: cell suspension storage time, serum and tissue culture plate vendors, cell stock density, cell age in flask, cell passage number, cell seeding density, assay incubation time and alamarBlue™ incubation time. For each parameter, the performance of the assay was evaluated by quantitating a control sample of rhNGF (0.085 mg/ml) relative to the rhNGF reference material and determining its percent recovery. If the method is robust, the performance of the assay should remain unchanged.

Because the performance of a cell-based assay depends strongly on the consistency of the cellular responses, various parameters relating to the PC12CF cells were evaluated. First, we investigated the effect of storing the cell seeding suspensions at ambient temperature for up to 1.5 h, i.e. whether there was deterioration of the cell suspension and/or if there was a specific time to seed cells in order to obtain consistent assay performance. PC12-CF cell seeding suspension was prepared and either used immediately (0 h) or stored at ambient temperature for 0.5, 1 and 1.5 h prior to seeding plates. The resultant curves, shown in Fig. 1, indicated that rhNGF caused a dose-dependent increase in viable cell number as measured by a 15-fold increase in relative fluorescence units

Table 1
Effect of cell suspension storage on quantitation of rhNGF[a]

	Cell suspension storage at ambient temperature			
	0 h	0.5 h	1 h	1.5 h
Mean (mg/ml)	0.085[b]	0.086	0.083	0.086
SD	0.003	0.005	0.006	0.007
CV (%)	4	6	7	8
Activity (%)[c]	100	101	97	101

[a] Four independent dilutions of a control sample of rhNGF (0.085 mg/ml) were prepared and tested on three separate days. Each dilution was tested at two concentrations targeting the range of the assay and applied to triplicate wells. Each day, the mean of these six determinations was used for the quantitation of the rhNGF control sample.

[b] The data represent the twelve determinations over the three assay days.

[c] Percent activity = (rhNGF bioassay concentration ÷ 0.085) × 100%.

Table 2
Effect of cell passage level on quantitation of rhNGF[a]

	Passages from working cell bank	
	Early (2–4)	Late (31–33)
Mean (mg/ml)	0.080[b]	0.079
SD	0.004	0.002
CV (%)	5	3
Activity (%)[c]	94	93

[a] Three independent dilutions of a control sample of rhNGF (0.085 mg/ml) were prepared and tested on three separate days. Each dilution was tested at three concentrations targeting the range of the assay and applied to triplicate wells. Each day, the mean of these nine determinations was used for the quantitation of the rhNGF control sample.

[b] The data represent the nine determinations over the three assay days.

[c] Percent activity = (rhNGF bioassay concentration ÷ 0.085) × 100%.

(RFU) over the media blank. The curves, for each condition, were superimposable and the quantitation of the control sample of rhNGF wasclose to the expected value of 0.085 mg/ml, ranging from 0.083 to 0.086 mg/ml (97–101% activity) with coefficients of variation (CV) of 4–8% (Table 1). Although we recommend that cell seeding suspensions be used as soon as possible after preparation, there was no indication that the cells stored at ambient temperature for as long as 1.5 h, had deteriorated in any way. Second, different serum vendors and/or lots can cause variability in the maintenance of cells in culture. It is common practice to order a large quantity of a single serum lot once it passes sterility and cell growth promotion testing. The effects of different sera were evaluated by testing different lots of serum obtained from different vendors (Gibco, Hyclone, and Sigma). The results indicated that no differences in cell viability and assay performance were observed among the different sources (data not shown).

Third, cell stock density and tissue culture plate vendors were evaluated. No changes in assay performance were observed when cell stock density, at the time of the harvest, varied from 0.3 to 2.6×10^5 cells/cm^2, when cell stocks were maintained for 3–8 days in culture before harvest, or when cells were seeded in 96-well microtiter plates obtained from three different vendors (Costar, Falcon, Corning) (data not shown).

Fourth, it is well known that cell lines can change their responsiveness to growth factors if they are cultured for long periods. Therefore, experiments were conducted to compare assay performance when cells from early and later passages were used. The PC12-CF cells used in these studies were at passages 2, 3, 4, 31, 32, and 33, beyond the working bank. The assay results from the later passages were compared to those of earlier passages by comparing quantitation of a control sample of rhNGF. As shown in Table 2, the values averaged 0.080 and 0.079 mg/ml (94 and 93% activity) using cells from passages 2–4 and 31–33, respectively, with the CV of $< 5\%$. The magnitude of the response (i.e. fold-increase in RFU) was very similar for cells from early or later passages (data not shown). These data suggested that the PC12-CF cells were stable in culture for at least 33 passages over an 8-month period.

Finally, to test whether the number of cells seeded per well had an influence on the assay performance, replicate microtiter plates of PC12-

Table 3
Effect of cell seeding density on quantitation of rhNGF[a]

	Cell seeding density (10^4 cells/well)				
	1.2	1.6	2.0	2.4	2.8
Mean (mg/ml)	0.082[b]	0.083	0.080	0.077	0.078
SD	0.008	0.007	0.004	0.003	0.002
CV (%)	9	9	5	4	3
Activity (%)[c]	96	98	94	91	92

[a] Three or four independent dilutions of a control sample of rhNGF (0.085 mg/ml) were prepared and tested on three separate days. Each dilution was tested at two or three concentrations targeting the range of the assay and applied to triplicate wells. Each day, the mean of these nine or twelve determinations was used for the quantitation of the rhNGF control sample.

[b] The data represent the ten determinations over the three assay days.

[c] Percent activity = (rhNGF bioassay concentration ÷ 0.085) × 100%.

CF cells at various seeding concentrations ranging from 12 000 to 28 000 cells/well were compared. The standard curves obtained for each cell seeding concentration are shown in Fig. 2. The high and low plateaus increased from about 3800 to 7200 and 300 to 400 RFU, respectively, as the cell seeding density was increased from 12 000 to 28 000 cells/well. The curves were also shifted as the cell density was increased, as indicated by an increase in ED_{50} values, ranging from 1.4 to 2.3 ng/ml as the cell seeding densities increased from 12 000 to 28 000 cells/well, respectively. This was expected since more cells would require more rhNGF for survival. Despite the marked shift in the placement of the standard curves on the *x*-axis, there was almost no difference in the quantitation of the control sample of rhNGF run on these plates. The control sample averaged 0.077–0.083 mg/ml (91–98% activity) with the CV ranging from 3 to 9% (Table 3). However, because too few cells produced a shallow response and too many cells resulted in higher backgrounds, the optimal cell seeding concentration for subsequent experiments was chosen to be 20 000 cells/well. In conclusion, the cellular component of this assay is very robust.

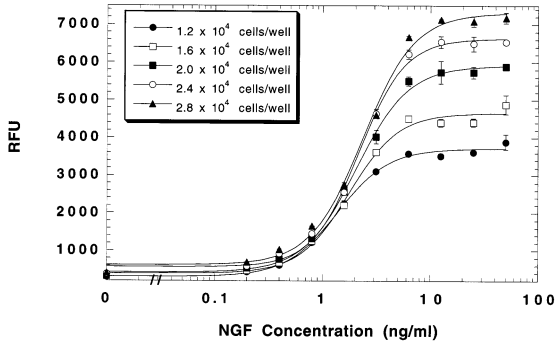

Fig. 2. Effect of cell seeding concentration. Cells were seeded at various density of 1.2, 1.6, 2.0, 2.4 and 2.8×10^4 cells/well, and then were cultured in serum-free medium with varying concentrations of rhNGF as described in Section 2. rhNGF standard curves for each condition were compared. Data represent the mean RFU value of triplicate wells at each rhNGF concentration ($\pm$ SD).

Table 4
Effect of assay incubation time on quantitation of rhNGF[a]

	Incubation time (h)		
	44 h	48 h	52 h
Mean (mg/ml)	0.090[b]	0.091	0.093
SD	0 004	0 .004	0.004
CV (%)	4	4	4
Activity (%)[c]	105	107	109

[a] Three or four independent dilutions of a control sample of rhNGF (0.085 mg/ml) were prepared and tested on three separate days. Each dilution was tested at two or three concentrations targeting the range of the assay and applied to triplicate wells. Each day, the mean of these nine or twelve determinations was used for the quantitation of the rhNGF control sample.

[b] The data represent the ten determinations over the three assay days.

[c] Percent activity = (rhNGF Bioassay concentration ÷ 0.085) × 100%.

In order to optimize the duration of incubation with rhNGF, PC12-CF cells were tested for survival after 44, 48, or 52 h of incubation. Quantitation of the control sample of rhNGF is summarized in Table 4 and ranged from 0.090 to 0.093 mg/ml (105–109% activity) with a CV of 4%. Routinely, assays were incubated for 48 h post rhNGF inoculation but an indication of rhNGF effect was evident as early as 24 h (data not shown).

To optimize the duration of alamarBlue™ incubation, PC12-CF cells were exposed to various doses of rhNGF (0.2 ng to 50 ng/ml) or media alone for 48 h, followed by an alamarBlue™ incubation of 4–6 h. The resultant curves are shown in Fig. 3. As expected, the high plateaus of the curves increased with the time of alamarBlue™ incubation, resulting in a 12- to 20-fold magnitude of stimulation. However, quantitation of the control sample of rhNGF, in each condition, was very similar and close to the expected value of 0.085 mg/ml, ranging from 0.081 to 0.085 mg/ml with a CV of 4–7% (data not shown). While a 4–6 h-alamarBlue™ incubation was most suitable and selected for quantitation in routine assays, an increase in fluorescence could be read as early as 2 h after the addition of the dye (data not shown).

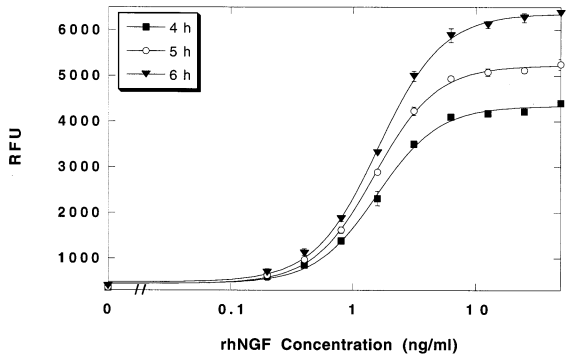

Fig. 3. Effect of alamarBlue™ incubation time. Cells were cultured in serum-free medium with varying concentrations of rhNGF for 2 days at 37°C as described in Section 2. alamarblue™ was then incubated for 4, 5, and 6 h. rhNGF standard curves for each condition were compared. Data represent the mean RFU value of triplicate wells at each rhNGF concentration (± SD).

In summary, all the parameters studied demonstrated that this method was robust. An assay format utilizing 20 000 cells/well, an incubation period of 44–52 h, and an alamarBlue™ incubation of 4–6 h, was used in the validation studies. The validation studies included determination of accuracy, precision, linearity, specificity, and stabilityindicating properties of the assay.

3.2. Accuracy

The accuracy of the method was determined by preparing spiked placebo samples containing three different concentrations of rhNGF which targeted 80, 100, and 120% of the intended concentration of the product (0.1 mg/ml). Each sample was tested in four separate assays by two analysts in two different labs (total of 16 assays). On any given day, samples were diluted to three concentrations in the linear portion of the assay range. Each dilution was added to triplicate wells on two different plates. Freshly prepared rhNGF standard curves were included on each plate and used for sample quantitation. To assess the accuracy of the assay, the measured rhNGF concentrations were divided by the expected concentrations and expressed as percentage recoveries. Results are shown in Table 5. Mean activities of 0.077, 0.099, and 0.115 mg/ml were obtained for the samples compared to expected activities of 0.080, 0.100, and 0.119 mg/ml, respectively. This represents percentage recovery ranging from 96 to 98%.

3.3. Precision

Precision was evaluated from the 16 experiments performed in two laboratories by four analysts, using different cell stocks, pipets, plates, equipment, and medium. The following contributions to the variability were then determined: repeatability (intra-assay variability) and intermediate precision (interassay variability).

For repeatability, well-to-well within plate, plate-to-plate within day and day-to-day within analyst variability was assessed, resulting in a CV ranging from 7 to 9%, 2–4%, and 5–8%, respectively (Table 6). For intermediate precision, an evaluation of the individual results obtained be-

Table 5
rhNGF recovery

Analyst[a]	Day		Expected potencies (mg/ml)					
			0.080		0.100		0.119	
			Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
1	1		0.090[b]	0.083	0.117	0.108	0.127	0.123
	2		0.071	0.072	0.106	0.102	0.130	0.129
	3		0.072	0.078	0.098	0.105	0.109	0.111
	4		0.081	0.082	0.113	0.108	0.133	0.118
		Mean[c]		0.079		0.107		0.123
2	1		0.075	0.078	0.096	0.097	0.109	0.114
	2		0.080	0.077	0.101	0.097	0.122	0.111
	3		0.077	0.068	0.097	0.089	0.114	0.107
	4		0.070	0.076	0.087	0.091	0.102	0.105
		Mean		0.075		0.094		0.111
3	1		0.077	0.076	0.098	0.094	0.108	0.109
	2		0.081	0.083	0.106	0.102	0.118	0.124
	3		0.081	0.085	0.106	0.109	0.128	0.125
	4		0.086	0.075	0.106	0.096	0.135	0.127
		Mean		0.081		0.102		0.122
4	1		0.074	0.074	0.111	0.107	0.093	0.092
	2		0.069	0.070	0.084	0.088	0.104	0.100
	3		0.068	0.061	0.084	0.083	0.098	0.101
	4		0.075	0.075	0.086	0.082	0.119	0.122
		Mean		0.071		0.091		0.104
		Overall mean[d]		0.077		0.099		0.115
		SD		0.004		0.007		0.009
		Recovery (%)		96		98		96

[a] Analysts 1 and 2 were in Laboratory 1; analysts 3 and 4 were in Laboratory 2.
[b] Each value is the mean of one assay, nine wells per plate per day.
[c] Each value is the mean of the results from both plates for the specified number of days shown.
[d] Each value is the overall mean of both plates from all four analysts over all the days tested.

tween the analysts in each laboratory demonstrated that consistent potency measurements were achieved over the range studied, with the CV ranging from 5 to 7%.

Based on these results, a sample assayed on each of 3 days, one plate per day, should have an overall CV of $< 10\%$.

Because there were only two laboratories, it was not possible to estimate inter-laboratory precision (reproducibility). However, quantitation was very similar among the four analysts and across the laboratories (see Table 5).

Table 6
Repeatability and intermediate precision[a]

Experiment	Expected potencies (mg/ml)		
	0.080	0.100	0.119
Repeatability			
Well-to-well within plate (%)	7.1	8.5	9.3
Plate-to-plate within day (%)	3.9	2.3	2.0
Day-to-day within analyst (%)	5.0	7.0	7.6
Intermediate precision			
Analyst-to-analyst (%)	4.9	6.6	6.9

[a] The statistical analysis of the rhNGF recovery study presented in Table 5 was performed on data expressed in mg/ml.

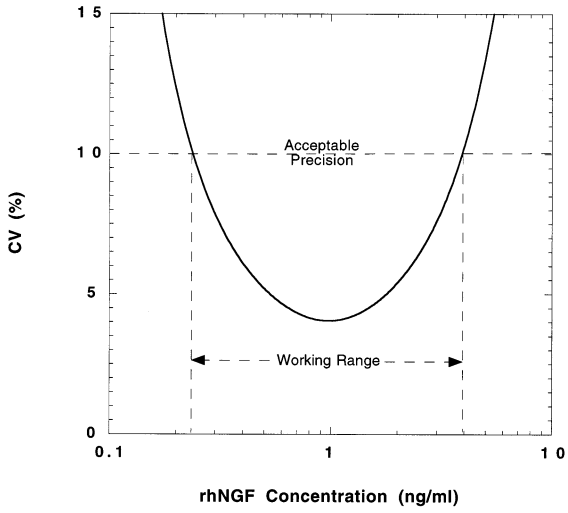

Fig. 4. Example of a precision profile. Coefficients of variation (%CV) are plotted against rhNGF concentrations. The 'working range' of the assay is defined as that range of rhNGF concentrations within which the CV of triplicate measurements is $< 10\%$.

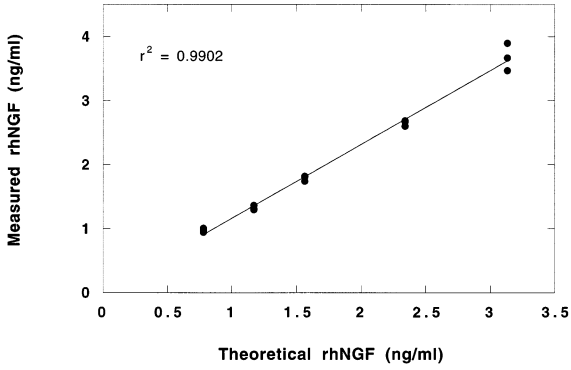

Fig. 5. Linearity of the rhNGF bioassay. Five dilutions of a sample of rhNGF targeting the assay range were quantitated from the standard curve. Each dilution was tested in triplicate. The measured rhNGF concentrations were plotted against the theoretical rhNGF concentrations.

3.4. Range

Precision profiling [28] showed that an intra-plate CV of 10% or lower may be achieved across the range of about 0.3–4.0 ng/ml for samples runin triplicates. Reducing the number of replicates from three to two had little effect on intra-plate precision. Fig. 4 shows a typical precision profile for a plate. These concentrations generally correspond to approximately ED_{10}–ED_{80}. The best estimated within plate CV should be at a concentration near 1.1–1.2 ng/ml.

3.5. Linearity

To evaluate the linearity of the method, three assays were performed in which five dilutions of a sample of rhNGF targeting concentrations ranging from 0.78 to 3.13 ng/ml were quantitated. Each dilution was applied to three wells on two plates each day. The measured concentrations were plotted against the theoretical concentrations of each dilution, as shown in Fig. 5 ($r^2 = 0.9902$). Measured concentrations of all dilutions were within 6% of theoretical.

3.6. Specificity

A number of cytokines, growth factors and potential contaminants were tested for their effects on the assay either alone or in combination with rhNGF. Our primary concern was to test Genentech marketed and clinical products for interference or activity on the PC12-CF cells. With the exception of recombinant human insulin-like growth factor, which is known to be mitogenic for the PC12 cells [30], none of the agents tested alone had any effect on the survival of the PC12-CF cells, nor did they alter the response to 1.5 ng/ml rhNGF (data not shown). Therefore, this assay is suitable to quantitate rhNGF biological activity in relatively pure rhNGF preparations and in those that are not likely to contain significant contamination by other factors. If it is necessary to assay impure samples, we would recommend assaying samples in the presence or absence of NGF neutralizing antibodies.

3.7. Stability-indicating properties

Two different sample sets (stressed samples and production variants) were used to test this method as a stability-indicating assay. In order for a sample quantitation to be valid, it is important

that the response curve of the sample be parallel to the response curve of the standard, as outlined in both the European Pharmacopeia (EP) [31] and the United State Pharmacopoeia (USP) [32]. For this reason, samples were tested using full standard curves ranging from 0.2 to 50 ng/ml; relative potency was estimated by the single point method(where the sample dilution targeted a single mid-range concentration) and compared to the parallel line bioassay method (three dilutions each in the linear portion of the curve for the standard and the sample).

The first set of samples consisted of rhNGF samples subjected to several modes of degradation, including elevated temperature, exposure to light, mechanical stress, extremes of pH, oxidative degradation, and enzymatic treatment. Both methods gave similar results and are summarized in Table 7. Exposure to high temperature (45°C for 30 days) or to fluorescent light for 7 days at ambient temperature had no effect on bioactivity, with recoveries ranging from 91 to 101%. Samples that were subjected to oxidation by *tert*-butyl hydroperoxide, mechanical agitation, or hydrolytic degradation by acid or base also retained full activity in the assay, with recoveries ranging from 104 to 120%. Enzymatic digestion with lysyl endopeptidase caused a 26–41% loss of activity as the digestion incubation time increased from 60 to 120 min, which was a significant decrease.

The second sample set consisted of variants of rhNGF occurring during production. The (1–120) rhNGF variant exhibited lowered activity (48%) relative to the reference material (Fig. 6A; Table 7). The β-Asp^{93}-rhNGF variant also showed a decrease in potency to 59–64% of reference

Table 7
Stability-indicating properties

rhNGF samples and conditions		Cell survival activity (%)	
		Single point[a]	Parallel line[b]
rhNGF reference material		100[c]	100
Degraded samples			
Control	<-60°C	98	103
Thermal	45°C, 30 days	101	91
Light exposed	Intense light	93	94
Mechanical	23–27°C, 7 days	104	104
Oxidative	TBHP	120	118
pH variation	Acidic (pH 3)	116	120
	Basic (pH 8)	108	112
Lysyl endopeptidase	37°C, 60 min	74	73
	37°C, 120 min	59	60
Isolated variants			
(1–118) rhNGF		92	99
(1–120) rhNGF		48	NA[d]
Met^{37}O-rhNGF		91	97
β-Asp^{93}-rhNGF		59	64
(1–117) rhNGF		111	118
Ser^{1}-rhNGF/Ser^{1}-rhNGF		94	87
Ser^{1}-rhNGF/Gly^{1}-rhNGF		72	76
Gly^{1}-rhNGF/Gly^{1}-rhNGF		62	NA[d]

[a] The mid-range dilution (duplicate or triplicate wells) was quantitated from the standard curve.
[b] Parallel line bioassay method was performed as described previously [29].
[c] Each data point represents the mean of three experiments performed on three separate days. The activity of rhNGF reference material was set to 100%.
[d] NA: not applicable. Activity could not be determined since a significant ($P<0.025$) degree of nonparallelism was observed between test and reference standard curves.

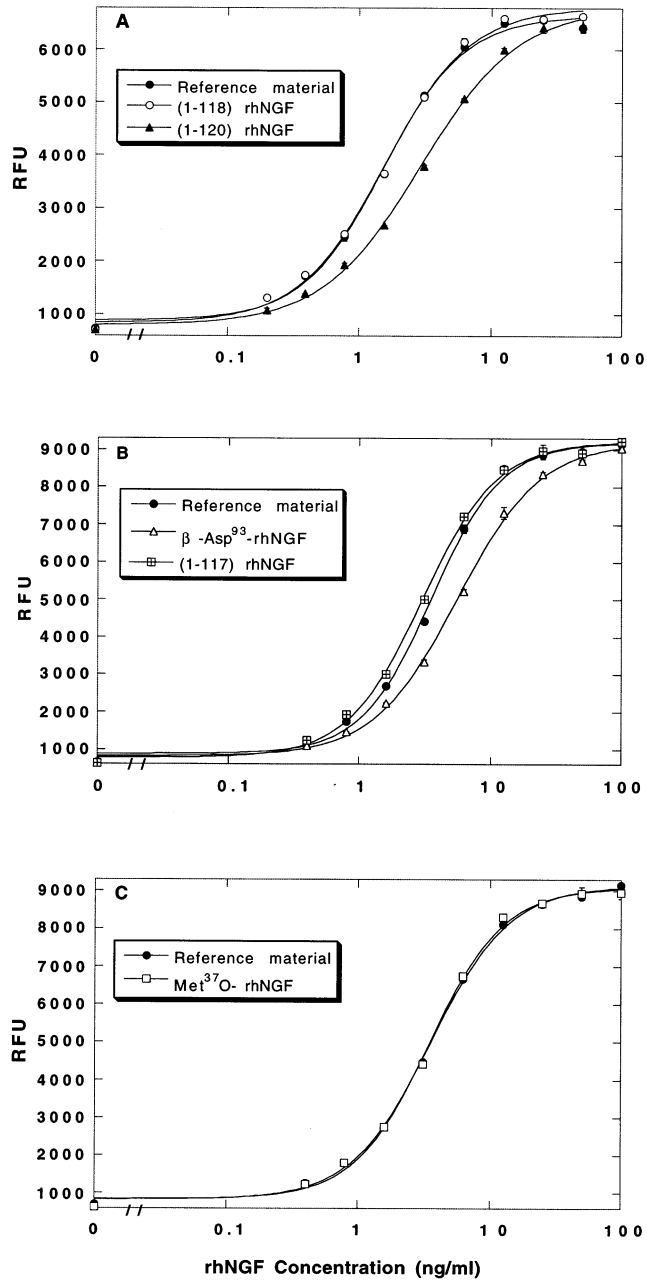

Fig. 6. Cell survival activities of rhNGF variants. Each variant was tested on the same microtiter plate as the reference material and analyzed on three separate days. The figures show representative curves for each sample. Each data point represents the mean of two determinations. The error bars represent the range.

(Table 7; Fig. 6B). Asp^{93} is adjacent to the variable loop region 94–98 in the 3-dimensional structure of NGF. This loop has been shown to be important for binding to the trkA receptor [9,10] and hence, the isomerization from Asp to β-Asp may affect the cell survival activity of this

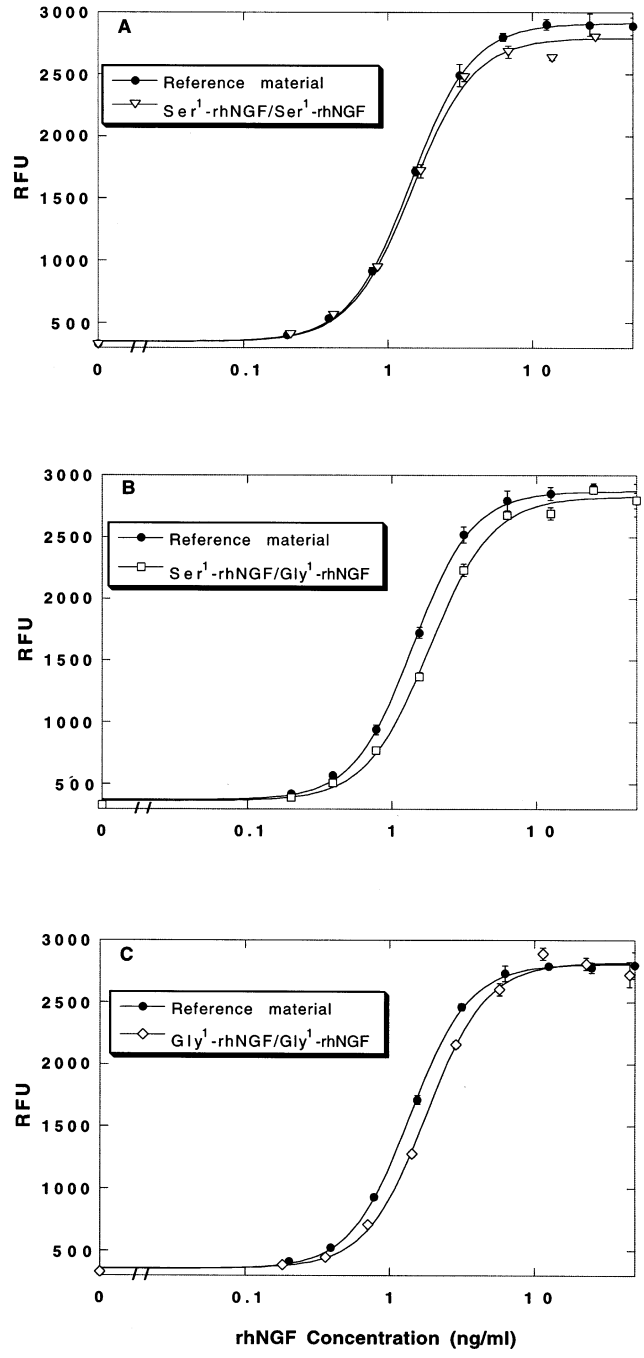

Fig. 7. Cell survival activities of rhNGF variants. Each variant was tested on the same microtiter plate as the reference material and analyzed on three separate days. The figures show representative curves for each sample. Each data point represents the mean of three determinations $\pm$ SD.

variant. Met^{37}O-rhNGF (1–118) rhNGF and (1–117) rhNGF were equipotent to the reference material with activities ranging from 91 to 118% (Table 7; Fig. 6B and C). The activity of the homodimer Ser^1-rhNGF/Ser^1-rhNGF (87–94%) was also similar to that of the reference material (Table 7; Fig. 7A). The heterodimer Ser^1-rhNGF/Gly^1-rhNGF and the homodimer Gly^{1}rhNGF/Gly^1-rhNGF, in which the N-terminal serine is converted to glycine, were less potent at 72–76% and 62% of the reference material, respectively (Table 7; Fig. 7B and C). The data suggested that the conversion of the N-terminal residue had a significant effect on the biological activity in the purified variant. This is consistent with the influence of the N-terminal nine amino acids for biological activity [12,13]. In conclusion, the PC12 cell survival assay was able to detect changes in activity when using purified rhNGF variants and proteolyzed NGF samples. Most of the stressed samples retained full activity, even though they exhibited analytical changes (data not shown); thus, the assay must be used in conjunction with other analytical tools for lot release of rhNGF products.

Except for the (1–120) rhNGF and Gly^1-rhNGF/Gly^1-rhNGF variants, all samples shown in Table 7 were parallel to the reference material in the linear portion of the curve and had comparable activity whether they were quantitated by single point or parallel line methods. Visually, the (1–120) rhNGF curve appeared nonparallel to that of the rhNGF reference material (Fig. 6A). However, the Gly^1-rhNGF/Gly^1-rhNGF curve did appear to be parallel to that of the reference (Fig. 7C). This may be because the lowest concentration used in the parallel line analysis for the test material was somewhat outside the linear portion of the curve (three concentrations were always used for each sample).

4. Discussion

Most of the methods described in earlier studies for assaying NGF have been developed in a research environment with little investigation devoted to method optimization or validation. Development of a convenient, robust, accurate and precise procedure is important for the determination of NGF biological activity in quality control laboratories.

Bioassays described in the literature, which use PC12 cells, have measured neurite outgrowth as the cellular response to NGF. Most recently, however, an in vitro bioassay for NGF based on 24 h survival of PC12 cells was reported [19]. Our proposed method presents several advantages over that assay: it is more sensitive ($EC_{50} \sim 0.5$ vs. 6 ng/ml), yields a broader dose-response range (12- to 20-fold vs. 5-fold increase in signal) and uses a less cumbersome method (alamarBlue™ method versus crystal violet staining) to measure cell survival. Selection of an endpoint of cell survival is often the major source of variation in potency results. The advantages of the alamarBlue™ method, which has been successfully used in a number of assays to assess cell viability [33,34], are that it is non-toxic, and does not require washing steps or medium changes, which often can negatively affect the overall assay precision. In addition, alamarBlue™ can be used with suspended or adherent cells. Thus, there is no requirement for the microtiter plates to be precoated in order to obtain adherent cells. Furthermore, our method involves fewer steps due to use of the PC12-CF subclone. Some of the characteristics of this clone are: (i) absence of cell clumps; (ii) ease of maintenance (no trypsinization step, thus minimizing the possibility of proteolysis of cell surface receptors) and (iii) stability over a long period of time (assay performance was unchanged after passaging cells in culture up to 33 times over an 8-month period). In contrast, the crystal violet-based assay described by Robinson et al. [19] requires multiple steps such as repeated centrifugations in order to remove large clumps present in the cell preparation (often resulting in cell loss), trypsinization of the cells, and pre-coating of the culture plates to enable the cells to adhere strongly to the plastic surface. In conclusion, our proposed method is much simpler and is more suitable for high throughput measurement.

The parallel line analysis is essential for unknown or poorly characterized samples. However, recombinant proteins are manufactured by well-

controlled, validated processes that have been shown to yield a consistent product, and we selected the single point method for our rhNGF products. Numerous analytical methods are employed as part of lot release and stability testing which can be more sensitive to detecting variants or degraded forms of the molecule, such as aggregates, fragments, N- or C-terminal variants and charge variants. The rhNGF potency assay, described in this report, is used in conjunction with these analytical methods for release of rhNGF product lots in a quality control environment. A parallel line approach may be necessary for evaluation of the product shelf life.

Based on results of the validation study, the following assay format was selected for lot release of recombinantly produced, consistently pure rhNGF: each rhNGF lot will be tested on 3 days, one vial per day, one plate per day, with one sample dilution applied to four replicates per plate. A single dilution targeting mid-range will be used to estimate relative potency. Appropriate assay controls will be included on each plate.

These validation results demonstrated that the assay is robust, easily transferable, acceptably precise, and maintains low assay variation. The accuracy of the assay is excellent, ranging from 96 to 98%. The assay has stability-indicating properties as evidenced by the ability to detect changes in rhNGF samples degraded by protease treatment and in a number of rhNGF variants. The high quality of this assay was demonstrated by its ability to distinguish between activities of two N-terminal variants which differed by a single amino acid, the Ser^1-rhNGF/Ser^1-rhNGF versus the Ser^1-rhNGF/Gly^1-rhNGF variant (Table 7).

Acknowledgements

We wish to thank Marian Eng and Victor Ling for purifying and characterizing the rhNGF variants, Alex Gee, Connie Diaz and Wassim Nashabeh for providing stressed samples, Ida Louie and Carina Rickel for their contributions in performing assays.

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