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Amplification of the Total Coding Sequence of the *NF1* Gene from Peripheral Blood Lymphocyte RNA

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder with a prevalence of ~1 in 3500. It is characterized by multiple neurofibromas, café-au-lait spots, and Lisch nodules of the iris with a high variability in phenotypic expression.^(1,2) The *NF1* gene was isolated by positional cloning.^(3–5) It is >350 kb in size and encodes a transcript of 11–13 kb. The complete coding sequence is 8457 bp long and contains at least 51 exons.⁽⁶⁾ Analysis of the exon–intron structure of the gene has led to the identification of two alternatively spliced transcripts, GRD II with a 63-bp insertion (exon 23a) and 3'ALT with a 54-bp insertion (exon 48a).^(3,6,7) The search for disease-causing mutations in the gene has proved difficult mainly because of the large size and the high mutation rate of the gene.⁽⁸⁾ Here, we describe a reverse transcriptase–polymerase chain reaction (RT–PCR)-based strategy for amplifying the total coding sequence of the *NF1* gene from peripheral lymphocyte RNA. This amplification should facilitate the further search for mutations in the *NF1* gene.

The strategy involves an initial cDNA synthesis step, followed by two consecutive rounds of PCR amplification. Total RNA was prepared from peripheral blood lymphocytes.⁽⁹⁾ The first-strand cDNA was synthesized by reverse transcription in a 60- μ l reaction containing 300 ng of random hexamers (Pharmacia Biotech), 12 μ l of 5 \times first-strand buffer (GIBCO BRL), 6 μ l of 0.1 M DTT, 15 μ l of 5 mM dNTPs (Pharmacia Biotech), 200 units of Moloney murine leukemia virus (Mo-MLV) reverse transcriptase (GIBCO BRL), 40 units of RNase inhibitor (Boehringer Mannheim), and ~300 ng of total lymphocyte RNA. The reaction was incubated at 42°C for 10 min, 37°C for 60 min, heated at 95°C for 6 min, and cooled in ice for 2 min or stored at –20°C.

Two-round PCR amplifications were performed using the primer sets as listed in Table 1. For the first amplification, each 20- μ l reaction was assembled including 2 μ l of 10 \times PCR buffer (500 mM KCl, 100 mM Tris-HCl, and 20 mM MgCl₂), 200 ng of upper primer, 200 ng of lower primer, 2 units of *Taq* DNA polymerase (Amersham), 10% DMSO or 10% glycerol, and 8 μ l of the first-strand cDNA. In a Perkin-Elmer Cetus thermal cycler, 30 cycles of PCR were programmed at 94°C for 1 min, 55°C for 1

min, and 72°C for 2 min following an initial step of 94°C for 3 min and with a final extension of 72°C for 10 min.

The second amplification was accomplished in a total volume of 50 μ l for each reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.0–2.0 mM MgCl₂, 500 ng of nested upper primer, 500 ng of nested lower primer, 0.2 mM dNTPs, 2.5 units of *Taq* DNA polymerase, 6% DMSO or 10% glycerol, and 1 μ l of the corresponding product of the first amplification reactions (F1 for SF1 and SF2; F2 for SF3; sf3, sf4, and sf5; F3 for SF4, sf6, and sf7; F4 for SF5, sf8, and sf9). PCR was performed as in the first amplification but with different annealing temperatures for different sets of primers as described in Table 1. The amplified products were subjected to electrophoresis on 2% agarose gels.

The second amplification allowed the construction of five or nine overlapping fragments that span an 8694-bp cDNA sequence of the gene (Figs. 1 and 2). The sf5 reaction product was seen as two close bands (Fig. 2). This product is expected to contain the 63-bp insertion region of the GRD II. Because the 63-bp sequence has an *AluI* site, we digested the product with this enzyme to show the presence of the amplified GRD II. The digested product generated an expected band of 450 bp specific for the 63-bp sequence (Fig. 3).

Roberts et al.⁽¹⁰⁾ have used a similar two-round PCR-based strategy to amplify the entire coding sequence of the dystrophin gene from peripheral blood lymphocyte total RNA. Our protocol for the initial reverse transcription step differs from that reported by Roberts et al. in three major points: We utilized random hexamers for the first-strand cDNA synthesis in a single reverse transcription; the primer annealing step at 65°C was also omitted; and we used less quantity of reverse transcriptase, which enabled us to achieve efficient reverse transcription without the inhibitive activity of the excess of reverse transcriptase or its contaminants in the subsequent PCR reaction.⁽¹¹⁾

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¹CRC Chromosome Molecular Biology Group, Department of Biochemistry, University of Oxford, Oxford OX1 3QU UK.

TABLE 1 Primers for Amplification of the *NF1* Gene cDNA by RT-PCR

Reaction ^a	Name	Sequence (5' → 3')	Base position ^b	Exon position	Product (bp)	AT (°C) ^c	MgCl ₂ (mM)	
First-round PCR	F1	F1U F1L	CCAGCCGCGCTCTCAATCTCTA ATCCTGCTGCACTATCCATAGA	-396--375 1996-2017	1 (5' Utrl) 12b-13	2413	55	2.0
	F2	F2U F2L	GAGATTAGCTCACAAATG AGTTTTGAACCTCCGTGCAACATA	1714-1731 4759-4782	11-12a 27b-28	3069 (3132)	55	2.0
	F3	F3U F3L	GAATCGAAAGGGGCTTGAAGTTA TGGCTGTAATTTGGTTAGTGCTATTA	4238-4260 6821-6846	24 37	2609	55	2.0
	F4	F4U F4L	CATTGCCTTCCGTTCCAGTTAC CCTCCCCTTTCTTTGTCAGTTGT	6462-6483 8702-8724	34 49 (3' Utrl)	2263 (2317)	55	2.0
	SF1	SF1U SF1L	GGGAGCCTGCACTCCACAGA AATGCCTTTTCCAGGCTATTTATA	-127--108 636-659	1 (5' Utrl) 4-5	786	60	2.0
	SF2	SF2U SF2L	GCCTCTGGGGTTTTATTTTCTCT GGAACTTCTATCTGCCTGCTTATT	412-434 1840-1863	4 12a-12b	1452	60	1.5
	SF3	SF3U SF3L	CATCTGCAAGAAATTAAGTATCATC TTTGGAAAGTCCCAGCTTGGTAGA	1740-1765 4715-4737	12a 27b	2998 (3061)	62	1.5
	SF4	SF4U SF4L	T CAGGAGAAAATTTGGGCAGTATCT T CAGGTCCTTTTAAGCAACTCTC	4479-4502 6763-6785	26 37	2307	60	1.5
SF5	SF5U SF5L	CTCATGGGCAGATAAAGCAGATA AAAACAGGAAGTGCAGCATTACA	6716-6738 8545-8567	36 49 (3' Utrl)	1852 (1906)	60	1.5	
Second-round PCR (1)	sf3	sf3U sf3L	= SF3U CATCATTGTTTCAATGCTAGCTTGC	2952-2976	17	1237	60	1.0
	sf4	sf4U sf4L	GACTGATACCAATACTCAATTTGTAG GCCAAGCTGTTGCCCTCGGAAG	2858-2884 3822-3842	17 22	984	60	1.0
	sf5	sf5U sf5L	AACAAGGCACAGAATTTGACACA = SF3L	3563-3585	21	1175 (1238)	60	1.5
	sf6	sf6U sf6L	= SF4U TTGTGTTTTGGCATCATCATTAT	5822-5844	31	1366	62	1.5
	sf7	sf7U sf7L	CTGCCAACAACACCCTCTTTAT = SF4L	5639-5660	30	1147	64	1.5
	sf8	sf8U sf8L	= SF5U GCCTTTTAGGAGCCTTTGTGTCT	7584-7606	43	891	60	1.5
	sf9	sf9U sf9L	CAACCTTCTCAGGCCAACACTA = SF5L	7519-7540	42	1049 (1103)	60	1.5

^aThe products of reactions F2, SF3, and sf5 contain the alternatively spliced 63-bp insertion, exon 23a; the products of reactions F4, SF5, and sf9 contain the alternatively spliced 54-bp insertion, exon 48a.

^bThe *NF1* cDNA sequence was numbered according to the NF1 Genetic Analysis Consortium.

^c(AT) Annealing temperature.

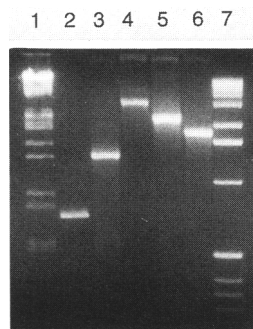


FIGURE 1 Amplification of the *NF1* coding sequence with five overlapping nested primer sets (lane 1) λ DNA (*Hind*III-*Eco*RI) marker; (lanes 2-6) the products of reactions SF1, SF2, SF3, SF4, and SF5; (lane 7) 1-kb ladder.

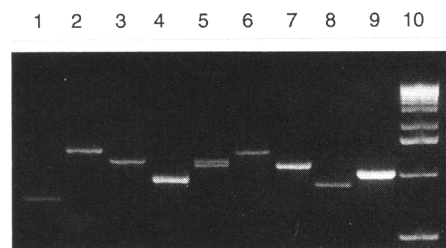


FIGURE 2 Amplification of the *NF1* total coding sequence with nine overlapping nested primer sets. (Lanes 1-9) The products of reactions SF1, SF2, sf3, sf4, sf5, sf6, sf7, sf8, and sf9; (lane 10) 1-kb ladder.

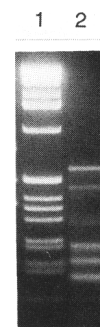


FIGURE 3 Restriction analysis of the alternate transcript GRD II with *Alu*I. (Lane 1) One-kilobase ladder; (lane 2) the digested products of reaction sf5.

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