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Development of a practical *NF1* genetic testing method through the pilot analysis of five Japanese families with neurofibromatosis type 1

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Abstract

Objective: Mutation analysis of *NF1*, the responsible gene for neurofibromatosis type 1 (NF1), is still difficult due to its large size, lack of mutational hotspots, the presence of many pseudogenes, and its wide spectrum of mutations. To develop a simple and inexpensive *NF1* genetic testing for clinical use, we analyzed five Japanese families with NF1 as a pilot study. *Methods:* Our original method, CEL endonuclease mediated heteroduplex incision with polyacrylamide gel electrophoresis and silver staining (CHIPS) was optimized for *NF1* mutation screening, and reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the effect of transcription. Also, we employed DNA microarray analysis to evaluate the break points of the large deletion. *Results:* A new nonsense mutation, p.Gln209*, was detected in family 1 and the splicing donor site mutation, c.2850+1G>T, was detected in family 2. In family 3, c.4402A>G was detected in exon 34 and the p.Ser1468Gly missense mutation was predicted. However mRNA analysis revealed that this substitution created an aberrant splicing acceptor site, thereby causing the p.Phe1457* nonsense mutation. In the other two families, type-1 and unique *NF1* microdeletions were detected by DNA microarray analysis. *Conclusions:* Our results show that the combination of CHIPS and RT-PCR effectively screen and characterize *NF1* point mutations, and both DNA and RNA level analysis are required to understand the nature of the *NF1* mutation. Our results also suggest the possibility of a higher incidence and unique profile of *NF1* large deletions in the Japanese population as compared to previous studies performed in Europe.

1.   **Introduction**

Neurofibromatosis type I (NF1 [MIM 162200]) is one of the most common autosomal dominant disorders, occurring with an incidence of 1 in 2,500 to 3,000 individuals, independent of ethnicity, race, and gender [1]. Half of these affected individuals have a *de novo NF1* mutation while the other half carry a mutation that appears as a familial trait [2]. NF1 is characterized by multiple café-au-lait spots, Lisch nodules in the iris, and fibromatous tumors of the skin. Less common but potentially more serious manifestations include plexiform neurofibromas, optical pathway and other central nervous system gliomas, malignant peripheral nerve sheath tumors (MPNST), scoliosis, tibial dysplasia, and vasculopathy [3]. The mean IQ of individuals with NF1 is 88 and learning disabilities are present in at least 50% of those affected [4]. The diagnostic criteria of NF1 developed by a National Institutes of Health Consensus Conference in 1988 are generally accepted for current routine clinical use all over the world [5] and a modified NIH criteria is used in Japan [6].

The disease causative gene, *NF1*, was first identified by Wallace et al. in 1990 [7]. *NF1* is located on chromosome 17q11.2, spans approximately 350 kb of genomic DNA, contains 58 exons, and encodes a 220 to 250 kDa cytoplasmic protein called neurofibromin [2]. The protein function of neurofibromin that is best understood is its role as a negative regulator of the *RAS* proto-oncogene. Neurofibromin acts as a guanosine triphosphatase (GTPase)-activating protein (GAP) and helps maintain the proto-oncogene RAS in the inactive GDP form by accelerating the conversion of GTP-RAS to GDP-RAS through the NF1 GAP-related domain (NF1-GRD). Because RAS is over-activated as a result of the *NF1* loss-of-function mutation, individuals with the disorder have an increased susceptibility to the development of benign and malignant tumors [2,3]. Essentially, the activated RAS protein transmits cell growth signals to

downstream molecules. Recently, the mammalian target of rapamycin (mTOR) has attracted attention as one of these downstream proteins. mTOR is a serine/threonine kinase that acts as a central regulator of many cellular functions, including proliferation, cell size/growth, translation, metabolism, autophagy, angio-genesis, and survival by responding to the availability of nutrients (glucose and amino acids). mTOR was found to be activated in NF1-deficient cells in both humans and mice. The mTOR inhibitor, sirolimus, was shown to dramatically reduce proliferation in human MPNST cell lines as well as completely inhibit the growth of the MPNST developed in a genetic mouse model in vivo [8]. In addition, clinical trials of the mTOR inhibitor on humans have been conducted. It has been reported that sirolimus can alleviate pain in NF1 patients with severe plexiform neurofibromas [9].

Consistent with the "2-hit" model of tumorigenesis, loss of heterozygosity (LOH) has been shown to occur in NF1-associated malignancies, including MPNST, leukemias, pheochromocytomas, and astrocytomas, as well as some benign dermal and plexiform neurofibromas. In neurofibromas, the LOH is seen specifically in involved schwann cells but not in fibroblasts, suggesting that schwann cells are the cells of origin. LOH has also been seen in non-NF1-related malignancies such as breast cancer, colon cancer, and neuroblastomas [2].

Although the causative gene was identified more than 20 years ago, gene analysis of *NF1* remains a challenge for several reasons. First, because *NF1* is a large gene that lacks a mutational hot spot, every coding exon must be analyzed in order for mutation screening to be performed at the DNA level. Additionally, 10 or more *NF1* pseudogenes are present in the human genome, and these highly homologous DNA sequences prevent accurate *NF1* mutation analysis. Furthermore, at the RNA level, mutation analysis is often hampered by nonsense-mediated mRNA decay (NMD) of the mutant allele [10]. Problems with NMD can be

overcome using the protein truncation test (PTT) [11], but the PTT is technically complicated and lacks sufficient sensitivity. Nonetheless, mRNA analysis is essential because a significant number of splicing mutations are caused by deep intronic sequence alternations occurring far from the exon-intone boundary; these mutations are not usually detected in DNA analysis. Finally, mRNA editing, nucleotide substitutions occurring at the mRNA level without associated changes in the DNA sequence, have been reported in the case of NF1 related tumors [12].

In order to gain a better understanding of *NF1*, each researcher has analyzed this mutation using a combination of different methods. To date, several large scale *NF1* mutation studies have been done. However, most of these studied have been performed in Europe and no *NF1* mutational cohort study has yet been conducted in Japan. Fahsold et al. performed the largest ever *NF1* mutation study to the patients of German or Turkish descent [13]. In this study 521 patients with NF1were analyzed, either by temperature-gradient gel electrophoresis (TGGE), PTT, or by direct genomic sequencing of entire coding exons. A total of 278 pathogenic mutations were identified, and mutation-detection efficiencies of these screening methods were found to be similar: 53.7% for TGGE, 47.1% for PTT and 54.9% for entire coding exon sequences. Another example is a recent study performed by Ko et al. in which 60 Korean families with NF1 were studied. Here, entire coding exon sequences were analyzed by direct sequencing at DNA level, also performed reverse transcription polymerase chain reaction (RT-PCR), multiplex ligation-dependent probe amplification (MLPA) and fluorescence in situ hybridization (FISH). In this study, 52 *NF1* mutations were identified, including 30 single base substitutions (12 missense and 18 nonsense), 11 splicing mutations, 7 small insertion or deletions, and 4 gross deletions [14]. As a new trend, next generation sequencing (NGS), is

beginning to be used in *NF1* mutation analysis [15]. Although, this new technology is robust and highly effective in screening large numbers of DNA sequences, the running cost remains expensive even if multiple samples are processed simultaneously. Also, in order to eliminate pseudogenes sequences, it is necessary to close attention to design *NF1* gene specific amplicon primers. Additionally, mutations detected by NGS must be validated by Sanger sequencing because of its low accuracy and, further, current NGS studies of *NF1* provide no mRNA level information. NGS is positioned as one of the expensive DNA screening methods for *NF1* genetic testing, the benefits of which would not outweigh the cost unless it was employed in a massive screening study.

All of these methods are either highly sensitive but expensive, or else inexpensive but lack sufficient sensitivity. Additionally, some of these methods required special equipment and are difficult to perform under standard laboratory conditions. When analyzing large genes, these kinds of problems are common and serve as barriers to the clinical application of genetic testing. To solve this problem, over the past few years we have developed a conventional, highly sensitive and inexpensive mutation screening system for large genes. This new technology, CHIPS (CEL nuclease mediated heteroduplex incision with polyacrylamide gel electrophoresis and silver staining) [16,17], has already been successfully applied to our daily clinical genetic service [18]. The aim of this study is to establish a practicable and inexpensive *NF1* mutation screening system based on CHIPS technology through the pilot analysis of eight patients with NF1 from five independent families, and prepare for a mutational cohort study of Japan and of molecular pathology of NF1 lesions. We adjusted CHIPS in order to maximize the analytical efficiency for the *NF1* gene and included mRNA analysis via RT-PCR. For patients in which we did not find a *NF1* point mutation, we used DNA microarray technology in this study to

evaluate the nature of *NF1* large deletion. We were able to identify the deletion break point on

chromosome 17 in each patient and provided comparative analysis between the patients as well

as to previous reports of *NF1* large deletions in Europe.

2.   Materials and methods

2.1.   Case report

Family 1 (patient 1M, 1D). A 30-year-old woman (patient 1D) has multiple

café-au-lait spots and Lisch nodules but no cutaneous and nodular plexiform neurofibromas. She

speaks and communicates well to others but is unable to read and write. After graduating from

special education school she was employed at a vocational aid center. She suffers from epileptic

seizures on a monthly basis. She also has mild scoliosis and left hemiplegia. Her brain magnetic

resonance image (MRI) is normal. Her mother (patient 1M) also has NF1 but has no intellectual

disability or epilepsy. She works as nurse at the hospital. She has multiple café-au-lait spots and

cutaneous neurofibromas. Brain and spinal MRI results appeared normal. One brother and the

father of patient 1M also have café-au-lait spots and are suspected to have NF1, but details are

not certain.

Family 2 (patient 2). Patient 2 is a 16-year-old girl currently attending high school at a

special support education school. She suffers from severe intellectual disability, speaks few

words, has immense difficulty communicating with others and demonstrates aggressive

behavior. Hundreds of café-au-lait spots indicate NF1, a brain MRI study showed unidentified

bright objects (UBOs) in the bilateral medial part of globus pallidus, and magnetic resonance

angiography (MRA) showed quasi-moyamoya disease caused by the obstruction of left middle cerebral artery. She also has epilepsy which is controlled by anti epileptic drugs, and her aggressive behavior is treated with risperidone. This patient has no family history of NF1.

Family 3 (patient 3M, 3S). Patient 3S is a 12-year-old boy. He also has severe intellectual disability with autistic spectrum disorder. He cannot speak, is unable to use gestures and does not understand simple orders. He takes medications to treat his epilepsy and aggressive behavior. Although the results of his brain MRI were normal, he has paraplegia and has difficulty walking long distances. His mother (patient 3M) is intellectually normal. After graduating from college she married and works as housewife. She has dozens of café-au-lait spots and cutaneous neurofibromas. The mother of patient 3M also has NF1 and, accordingly, has prominent large cutaneous neurofibromas on her face and trunk.

Family 4 (patient 4M, 4S). Patient 4S is a 3-year-old boy who first visited our hospital at the age of 6 months for examination prompted by the suspicion of developmental delays. He has a few small café-au-lait spots, no Lisch nodules, a large head (+2.5SD) and a depressed nasal bridge. His development progressed slowly and, at 28 months, has just learned to walk without assistance. He speaks a few words, communicates to others using simple gestures and is able to understand simple orders. Café-au-lait spots stand out gradually with age and, at the age of 3, he now has dozens. His mother (patient 4M) has hundreds of café-au-lait spots and multiple cutaneous neurofibromas. She has mild mental retardation and has lived with her mother following her divorce. She is the first patient with NF1 in this family.

Family 5 (patient 5). Patient 5 is a another sporadic case with NF1. This 21-year-old woman has thousands of café-au-lait spots all over the body. She also has Lisch nodules and multiple nodular plexiform neurofibromas palpable under the skin. Her facial appearance shows

hyperterolism and a flat nasal bridge with hypoplasia of the nasal cartilage. She has mild mental retardation, but communication problems are not apparent. Brain MRI results were normal.

A summary of the clinical characteristics of these patients is given in Table 1. After an explanation of this study, written informed consent was obtained from all of the subjects. This study was approved by the ethical board of facilities.

2.2. *NF1 Pseudogene check and NF1-specific PCR primer construction*

It is well known that *NF1* has several pseudogenes and these highly homologous sequences interfere with mutation analysis. However, previous studies have not provided the number of pseudogenes that exist in the human genome and, subsequently, identified which of these pseudo sequences are homologous to each of the *NF1* exons. Therefore, before designing *NF1*-specific PCR primers, we analyzed every *NF1* coding exon sequence (exons 1 to 58) using Human BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and identified all of the *NF1* pseudogene sequences in the human genome. In this process, we found a total of 15 *NF1* pseudogenes. While 12 of these were previously noted in the literature, the remaining 3, LOC101930488, LOC102724666 and MED15P6, are newly recognized NF1 pseudogenes. Each pseudogene contains extremely similar sequence of *NF1* exon 9 to 36 in various conbination with spearing exon 12, 14, 30 and 31 sequences (Supplementary Table 1).

To make *NF1* specific PCR primers for these pseudogene-positive exons, we curated previously reported *NF1* PCR primers from several papers by manual aligning primer sequences to *NF1* and *NF1* pseudogene sequences. We also performed Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) analysis to predict whether these previous PCR primers specifically amplify the *NF1* gene. As a result, we have adopted PCR primers

previously described in four different studies for axons 9 - 11, 13, 15, 19 - 22, 25, 27, 28, 32 - 36 and have modified them in three different studies for exons 18, 24 and 26. *NF1* primers for other pseudogene-positive exons (exon 16, 17, 23 and 29) and all pseudogene negative exons were originally designed for this study using the program Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) [19]. These primer sequences are listed in Supplementary Table 2, and the alignment of primer sequences for *NF1* and *NF1* pseudogenes are represented in Supplementary Fig. 1 with respect to each exon. Finally, all PCR products of normal control DNA with these PCR primers were sequenced and the absence of a pseudogene sequence was confirmed. The *NF1* genomic DNA sequences, mRNA sequences, and the amino acid sequences in this study are given according to GenBank accession numbers NG_009018.1, NM_000267.3 (mRNA variant 2) and NP_000258.1 (protein isoform 2) respectively. Mutation nomenclature are provided according to the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/) [20].

2.3. CHIPS Analysis

Peripheral blood leukocyte DNA was extracted by rapid method [21] and diluted to a concentration of 50 ng/μl by 1xTE (10mM Tris-HCl, 1mM Na_2EDTA at pH 7.8). CHIPS analysis was performed as described in our previous publications [16,17] with the exception of the modification of PCR reagents and cycle parameters required for *NF1* gene analysis. The only instance in which a unique protocol and reagents was required was exon 1 of *NF1* which was located in a GC rich region. We used KOD -Plus- Ver 2. polymerase (Toyobo) and the temperature cycle parameters were set to 94° C for 5 min, followed by 30 cycles of 98° C for 20 sec, 58° C for 30 sec and 68° C for 30 sec. The PCR product was determined by direct

sequencing without CHIPS screening.

For exons 9, 10, 11, 12, 13, 15, 19, 20, 21, 24, 32, 33, 34, 36, 38 and 48, PCR reagents were used: 50 ng of template DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM MgCl$_2$, 0.4 μM of both forward and reverse PCR primers, 200 μM dNTPs mix and 0.2 unit Taq DNA polymerase (Roche) in a 10 μl reaction volume. The PCR temperature cycle parameters of these exons were held at 94° C for 5 min (initial denaturation), followed by 7 cycles of 97° C for 10 sec and 65° C for 10 sec with the annealing temperature being decreased at a rate of 1° C per cycle (touch down cycles), followed by 30 cycles of 96° C for 10 sec, 58° C for 10 sec (amplification cycles), and the final extension where the temperature was held at 72° C for 2 min. For other exons, the following PCR reagents were used: 50 ng of template DNA, 0.4 μM of each primer, 200 μM dNTPs mix and 0.25 units of Blend Taq -plus- (Toyobo) with 1x manufacture's supplied buffer in a 10 μl reaction volume. Temperature cycle parameters were 94° C for 5 min followed by 7 cycles of 97° C for 10 sec, 67° C for 10 sec the annealing temperature decreasing at a rate of 1° C per cycle, then 30 cycles of 96° C for 10 sec, 60° C for 10 sec, and the final extension of 72° C over a 2 min period.

After completion of the PCR cycles, heteroduplex DNA was successively produced in a thermal cycler and 1 μl of heteroduplex substrate was digested by SURVEYOR Nuclease S (Transgenomic) as previously described [16, 17]. Also, 1 μl of heteroduplex substrate was used for undigested control, and these samples were run on 10% polyacrylamide gels and developed using a modified optimized silver staining method [16, 22]. Mismatch cleavage positive samples were sequenced bidirectionally using the remaining heteroduplex PCR products as templates.

2.4.   Reverse transcription PCR (RT-PCR)

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-anticoagulated blood by Ficoll-Hypaque gradient centrifugation. RNA was isolated using TRIzol Reagents (Life Technologies) according to the manufacturer's protocol. Next, single strand cDNA was synthesized using standard methods with a mixture of oligo-dT primer, a random primer (9 mer) (Toyobo) and RevaTra Ace (reverse transcriptase) (Toyobo). PCR was performed with Blend Taq -plus- (Toyobo) and temperature cycle parameters were 94° C for 2 min followed by 35 cycles of 96° C for 10 sec, 60° C for 10 sec, 72° C for 60 sec, and a final extension of 72° C over a 3 min period. RT-PCR primers used in this study were also originally designed by the Primer 3 program on website (Supplementary Table 3). PCR products were separated on either 2% agarose gel or 10% polyacrylamide gels (PAGE) and developed using ethidium bromide and silver staining, respectedly. All RT-PCR products were sequenced bidirectionally and the entire *NF1* mRNA coding sequence was determined in this study.

2.5.   DNA sequencing and cloning

Direct DNA sequencing was performed with the BigDye Terminator v3.1 cycle sequencing kit and the ABI PRISM 3130xl Genetic analyzer (Applied Biosystems).
The RT-PCR products of patients 2, 3M and 3S showing aberrant splicing by mutation were cloned into pT7Blue T-Vector (EMD Chemicals) according to the manufacturer's protocol. A sufficient number of colonies for analysis was acquired from each sample, amplified with the same PCR primers and sequenced.

2.6.   SNP Array

In order to screen for and identify large deletions in the *NF1* region, microarray

analyses of families 4 and 5 were performed using an Affymetrix Genome-Wide Human single nucleotide polymorphism (SNP) 6.0 array (Affymetrix) according to manufacturer's protocol. For microarray analysis, peripheral blood DNA of the patients were extracted using a QIAamp DNA Mini Kit (Qiagen).

3.   Results

3.1.   DNA level analysis

CHIPS analysis of entire *NF1* coding exons was performed on the probands of all families (patient 1D, 2, 3S, 4S and 5). CHIPS revealed three causative *NF1* mutations in patients 1D, 2 and 3S (Fig. 1). It was determined that patient 1D has the c.625C>T (p.Gln209*) nonsense mutation in *NF1* exon 6 (Fig. 1A), and her mother (patient 1M) also confirmed to have the same mutation without somatic mosaicism. This nonsense mutation has not previously been described in the database and, therefore, seems to be a new mutation. Patient 2 has the splicing mutation in the single base substitution at intron 21 splicing donor site, c.2850+1G>T (Fig. 1B). This mutation has not been reported in previous studies but the same mutation was found in the LOVD v.2.0 - Leiden Open Variation Database, Mendelian genes Neurofibromin 1(NF1) (http://www.LOVD.nl/NF1) Patient data (#0060821). It was determined that patient 3S has c.4402A>G in exon 34 and is considered to carry the missense mutation p.Ser1468Gly as previously described [23] (Fig. 1C). Furthermore, the mother of this patient (patient 3M) was confirmed to have same mutation. In each of these three patients, CHIPS gels detected clear heteroduplex cleavage bands and direct sequencing readily confirms these mutations. For patients 4S and 5, CHIPS analysis detected no mutations or polymorphisms throughout the

gene.

During the CHIPS analysis, we noticed that ladder bands appeared in exons 7, 14, 25 and in 42 PCR products. These PCR products include poly T or A stretch, making it difficult to distinguish true polymorphisms from artificial PCR slippage errors. After it was confirmed that the entire *NF1* gene deletion was localized on a single allele, we used a sample from patient 5 as a control to solved this problem. Because the DNA from patient 5 never made a heteroduplex of *NF1*, the appearance of ladders and enzymatic cleavage occurring in patient 5 was determined to be an artifact. Comparing the CHIPS results of patients 1D and 5 in with respect to their exons, we concluded that exons 7, 25 and 42 were caused by PCR slippage, while exon 14 is a true polymorphism. This PCR slippage could not be overcome by changing the DNA polymerase or the cycle parameters in this experiment. Furthermore, these intrinsic poly T/A stretches are very close to exon intron boundary and are impossible to avoid when designing a PCR primer set. Interestingly, CHIPS detected a true polymorphism in patient 1D in the artifact background in the PCR products of exon 7 (Supplementary Fig.2E). In conclusion, we should better to confirm exons 7, 25 and 42 sequences when no mutation was detected in other exons by CHIPS screening. Although CHIPS is highly sensitive, it may possible that the background obscures the true mutation bands in these exons.

3.2. RNA-level analysis

To explore the transcription effects of three mutations detected by DNA analysis, we also performed RT-PCR and sequencing analysis (Fig. 2). In patients 1M and 1D, the mutation allele (T) was detected in the sequence histogram (Fig. 2A), which demonstrates that the effect of nonsense-mediated decay is small for nonsense mutation Gln209*. For patient 2, the effects

of c.2850+1G>T on *NF1* transcription were more complicated. PAGE results from the RT-PCR products (primer set e) showed shorter bands of different sizes. This feature was not observed in the control sample, suggesting a complex splicing abnormality. RT-PCR products were cloned to the T-vector and the sequences for 24 clones were determined. Of these 24 clones, 16 were wild-type, 2 were classified as 90 bp in-frame deletions (mutation allele 1 in Fig. 2B) and 6 were classified as 144 bp in-frame deletions (mutation allele 2 in Fig. 2B). Each mutation allele uses different GT sequences in exon 21 as aberrant splicing donor sites and is spliced with the normal intron 21/exon 22 splicing acceptor site. As a result, each mutation allele has a different deletion length in exon 21. These deletions are both in-frame and result in 30 and 48 amino acids deletions in the protein levels, respectively.

In patients 3M and 3S, the PAGE of the RT-PCR products (primer set h) unexpectedly showed transcriptions of different sizes. The RT-PCR products were cloned to the T-vector and 15 of those clones were sequenced. 7 of these clones were characterized as wild-type and 8 of the clones had shorter, aberrantly spliced sequences. It has been interpreted that c.4402A>G creates a new splicing acceptor site (AG) in exon 34. In this case, the mutant allele is spliced between exon 33/interon 33 at the normal splicing donor site and at the newly generated splicing acceptor site in exon 34 while skipping the normal intron 33/exon 34 splicing acceptor site. The consequence is a 35 bp deletion in the mRNA, resulting in a frame-shift and the creation of a stop codon immediately following the aberrant splicing site (Fig. 2C). In conclusion, c.4402A>G is not considered to be a missense mutation like p.Ser1468Gly but, rather a nonsense mutation at p.Phe1457* with respect to protein levels.

In families 1 and 3, the difference in clinical severity is not caused by somatic mosaicism or a difference in the transcription of the mutant allele. We consider the possibility

that RNA editing of the *NF1* gene plays a role in the diversity of clinical symptoms. RNA editing is a post-transcriptional modification of the mRNA and creates a stop codon without changing the DNA sequence. RNA editing of the *NF1* exon 29 Arg1306* is well understood [12]. To confirm the hypothesis, we also sequenced each of the RT-PCR products. Although we determined the entire *NF1* mRNA coding sequence for each patient, no evidence of RNA editing was found.

3.3.   DNA microarray analysis

In families 4 and 5, no mutations for polymorphisms were detected by our initial CHIPS analysis and RT-PCR sequencing. Next, we performed DNA microarray analysis under the suspicion of finding a *NF1* large deletion (Fig. 3). 1.37 Mb deletions, including the entire *NF1* gene, were detected in patients 4M and 4S. Furthermore, a 1.29 Mb deletion spanning nearly the same region but associated with a different proximal break point was detected in patient 5. A clearly decreasing Log2 ratio of signal intensity demonstrated that patient 4M (mother of 4S) carries this large deletion without somatic mosaicism. Furthermore, microarray results were identical for both the mother and the child in this family and the break point of this *NF1* large deletion was generationally conserved. According to previous studies conducted in Europe, two recurrent microdeletion types are found in most cases. These microdeletions have break points located in paralogous regions flanking *NF1* (proximal *NF1-REP-a* and distal *NF1-REP–c* for the 1.4 Mb type-1 microdeletion in about 80% of *NF1* large deletion, and *SUZ12P1* and *SUZ12* for the 1.2 Mb type-2 microdeletion in about 10%). [24]. The proximal break point of the type-1 microdeletion is located between *LRRC37BP1* and *SUZ12P1* and the distal break point is located on the telomere side of *LRRC37B* [25], consistent with the deletion

range of family 4. Interestingly, in patient 5, *SUZ12P1* served at the proximal break point for the type-2 microdeletion, while *NF1-REP–c* served at the distal break point for the type-1 microdeletion instead of *SUZ12* for regular type-2 microdeletion. This variety of type-1 and type-2 hybrid deletion has not been reported in European and US studies as far as we know. This new type microdeletion has an intermediate size (1.3 Mb) of the type-1 (1.4 Mb) and type-2 (1.2Mb) microdeletion. (Fig. 3B).

4.   Discussion

To date, thousands of *NF1* mutations have been reported and the distribution of the *NF1* mutation spectrum in patients has been studied in both Europe and the United States. For example, more than 1,000 different *NF1* mutations are listed in the Human Gene Mutation Database (http://www.hgmd.org)[26]. Approximately the half of these mutations (55%) lead to a truncated form of neurofibromin. 13% of these mutations fall under the category of nonsense mutations, 42% as frame shift mutations, 10% are missense mutations, 25% are RNA splicing abnormalities, and 10% are gross deletions. Because, HGMD counts recurrent point mutations as single entries so the actual ratio of *NF1* gross deletions is smaller. A summary of the previous studies shows more than 90% of these patients have *NF1* small sequence variants thoroughout the *NF1* gene, 5% have partial or whole gene deletions and less than 1% have chromosomal rearrangements detected by cytogenetic analysis [27].

It has not yet been confirmed whether the European/US *NF1* mutation distribution is same in the Japanese population. In this study we found two *NF1* large deletions in five separate families. This ratio is higher than the previous report above. Of course we cannot draw the

conclusion from these small sample numbers that the Japanese *NF1* mutation cohort has a different percentage of large deletions. Notably, one of the large *NF1* deletions detected in this study has a hybrid break point for type-1 and type-2 microdeletions, something that has not been previously reported in Europe and the US. This result may suggest the existence of a unique mechanism of *NF1* deletion in the Japanese population. A similar situation exists for Sotos syndrome (SOTOS1 [MIM 117550]). In Japan, about 50% of patients with Sotos syndrome have the 5q35 microdeletion encompassing the *NSD1* detected by FISH [28]. In non-Japanese patients, however, large deletions detected by FISH occur less than 10% and most *NSD1* mutations are small sequence variants. It is believed that the *NSD1* deletion is mediated by non-allelic homologous recombination of low-copy repeats that are centromeric and telomeric to *NSD1* [29] and that this mechanism might be responsible for an ethnic difference between mutation cohorts.

Interpretations of *NF1* point mutations are often difficult. The effects of DNA substitution on mRNA or protein levels cannot always be correctly predicted. In this study, c.2850+1G>T of patient 2 unexpectedly contained two splicing mutations, both of which are responsible for in-frame deletion of neurofibromin. We were also concluded that the c.4402A>G substitution in family 3 was a nonsense mutation p.Phe1475* in the protein level caused by aberrant mRNA splicing, rather than a missense mutation p.Ser1468Gly as previously described [23]. These results clearly indicate that both DNA and RNA level analysis are required to understand the consequences of *NF1* mutation.

Our results indicate that there is poor correlation between the specific mutation and disease phenotype in patients with NF1. As with families 1 and 3 in this study, members of the same family typically show different manifestations of the disorder. It is also known that

unrelated individuals with identical *NF1* mutations may show different manifestations and levels

of severity. Moreover, mutation types are not correlated with clinical manifestations and one can

not know if missence or in-frame *NF1* mutations cause a milder phenotype. Actually, the

mutation in patient 2, the most severe phenotype of NF1 found in this study, is a set of in-frame

deletions of neurofibromin. It has been reported that whole *NF1* gene deletion is associated with

a higher incidence of learning disabilities (80.0% vs. 51.9%) and facial dysmorphism (51.9% vs.

10.9%), but not significantly different with respect to cutaneous and plexiform neurofibroma,

café-au-lait spots, Lisch nodules and optic gliomas. Patients with large *NF1* deletions also have a

tendency toward childhood overgrowth [24]. In this study, we detected whole *NF1* gene

deletion in three patients by DNA microarray screening. Patients 4M and 5 have mild mental

retardation and patient 4S has severe mental retardation. It is noteworthy that the patients with

NF1 large deletion do not always present with severe mental retardation and familial cases exist.

In these patients, cutaneous neurofibromas are of average severity and no symptoms of

overgrowth were found. As mentioned previously, these patients have distinct dysmorphic facial

features. Specifically, a wide nose with marked depressed nasal bridge have been prominent in

all of these patients and discreet in patients with *NF1* point mutations. Families 1, 3 and 4 are

mother and child pairs with NF1. In these families, mothers have a milder phenotype than their

child, the cause of which is not explained by somatic mosaicism, RNA editing, or differences in

efficiencies of aberrant mRNA splicing of the mutation allele. Some of the cutaneous

neurofibromas have LOH and their developments are affected by chance and duration (age of

the patient), so the individual variability between manifestations is reasonable to some degree.

However, the mechanism of intellectual disability and brain dysfunction with NF1 is still

unknown and individual variability in the same family for those patients that have same *NF1*

mutation is still unclear. In the literature, the high correlation of the severity of manifestations

seen in monozygotic twins suggests that there is a strong genetic component to the variability.

However, the relatively low correlation seen in distant relatives indicates that the specific

mutation of *NF1* plays a relatively minor role in the disease severity, and the involvement of

unknown modifier genes other than *NF1* has been considered [2].

Through this pilot study, we were able to establish a strategy towards practical and

inexpensive *NF1* mutation screening methodologies. We proved that the combination of

NF1-specific CHIPS and RT-PCR can comprehensively analyze *NF1* point mutations. Some

researcher choose to screen RT-PCR first due to the advantage of reducing the number of

screening PCR amplicons. However, the effect of nonsense mediated decay and the difficulty in

understanding aberrant mRNA sequences as demonstrated by this study is worrisome.

Technically, *NF1* mRNA expression levels in peripheral blood mononuclear cells (PBMC) is

small and, in order to obtain a sufficient quantity of amplification products, the appearance of the

PCR sub-products was unavoidable. Unfortunately, these PCR sub-products interfere with

CHIPS analysis. When adapting CHIPS technology to *NF1* RT-PCR, PCR primers should be

designed to amplify smaller products (300~400 bp) in order to increase efficiency with

increasing numbers of primer sets. Alternatively PBMC could be cultured with mitogens such as

phytohemagglutinin (PHA) or EBV transformed lymphblastoid cell lines could be established to

yield larger amounts of *NF1* mRNA expression. In order to accomplish the former, the effort

required is similar to that for DNA based CHIPS analysis. For the latter, extra time and effort are

necessary. Taking this information and our experimental results into account, we concluded that

DNA-CHIPS is the most convenient and reliable screening method, thereby rendering this

method the first screening tool for *NF1* mutation analysis. Furthermore, CHIPS detects not only

mutations but polymorphisms simultaneously. When a *NF1* polymorphism is found in an exon but no disease causative mutation is present, the allelic expression of the polymorphism site can be easily checked by RT-PCR. Because wild-type and mutated cDNA are amplified competitively via the same PCR primer set, the results are quantitative. It is possible to detect a reduction in the expression of one allele by mutation of the regulatory regions. When DNA screening reveals no causative mutation, one must consider the possibility of a splicing mutation due to deep intronic sequence alternations which could be determined by full set RT-PCR or *NF1* large deletion.

If further analysis is pursued in the future, we may find that a significant percentage of Japanese patients with NF1 have a gross gene deletion. In this case, as with Sotos syndrome, FISH is preferred over DNA-CHIPS as the primary screening test . In this case, it would be preferable to design *NF1* FISH probes by removing the region in which *NF1* pseudogene exists: exon 9 to 36. Also, because intragenic *NF1* deletions are not detected by these methods, long PCR or MLPA must be employed as needed.

As advancements in our understanding of NF1 are made, this genetic disease is now recognized as a PI3K/AKT/mTOR pathway disease and subsequent attempts to treat it with mTOR inhibitors have begun. However, it is not clear how the *NF1* germ line or the second hit mutation affect the efficiency of mTOR inhibitor treatment and, further, if there is a genotype-phenotype correlation at this level. On the other hand, novel genotype-phenotype correlations of NF1 has finally been revealed; It is reported that *NF1* mutations located in a third of the 5' side show a significant correlation with the development of optical pathway gliomas (OPG). These gliomas are mainly found in young children and sometime result in visual impairment [30]. Because the clinical diagnosis of NF1 in young children is difficult (it

frequently manifests in café-au-lait spots only) access to *NF1* genetic testing is of great

importance. For these reasons, the need for reliable *NF1* genetic testing methodologies has

increased significantly. Through this pilot study, preparation for the clinical application of *NF1*

genetic testing in our institute has been completed. By increasing the analysis cases future, we

would like to elucidate the nature and distribution of *NF1* gene mutations among the Japanese

population.

Acknowledgment

References

[1] Guttmann DH, Aylsworth A, Carey JC, Korf B, Marks J, Pyeritz RE, et al. The diagnostic evaluation and multidisciplinary management of neurofibromatosis 1 and neurofibromatosis 2. JAMA 1997;278:51-7.

[2] Yohay KH. The genetic and molecular pathogenesis of NF1 and NF2. Semin Pediatr Neurol 2006;13:21-6.

[3] Hirbe AC, Gutmann DH. Neurofibromatosis type 1: a multidisciplinary approach to care. Lancet Neurol 2014;13:834-43.

[4] Jones KL, Jones MC, del Campo M, editors. Smith's Recognizable patterns of human malformation. Philadelphia: Elsevier Saunders; 2013

[5] Neurofibromatosis. Conference statement. National Institutes of Health Consensus Development Conference. Arch Neurol 1988;45:575-8.

[6] Yoshida Y, Kubota Y, Kaneda M, Tsuchida T, Matsunaga K, Nakagawa H, et al. Treatment guidelines and diagnostic criteria for neurofibromatosis type 1 (von Recklinghausen's disease) (in Japanese). Nihon Hifuka Gakkai Zasshi (Tokyo) 2008;118:1657-66.

[7] Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, et al. Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three NF1 patients. Science 1990;249:181-6.

[8] Franz DN, Weiss BD. Molecular therapies for tuberous sclerosis and neurofibromatosis. Curr Neurol Neurosci Rep 2012;12:294-301.

[9] Hua C, Zehou O, Ducassou S, Minard-Colin V, Hamel-Teillac D, Wolkenstein P, et al.

Sirolimus improves pain in NF1 patients with severe plexiform neurofibromas. Pediatrics 2014;133:e1792-7.

[10] Brinckmann A, Mischung C, Bässmann I, Kühnisch J, Schuelke M, Tinschert S, et al. Detection of novel *NF1* mutations and rapid mutation prescreening with Pyrosequencing. Electrophoresis 2007;28:4295-301.

[11] Osborn MJ, Upadhyaya M. Evaluation of the protein truncation test and mutation detection in the *NF1* gene: mutational analysis of 15 known and 40 unknown mutations. Hum Genet 1999;105:327-32.

[12] Cappione AJ, French BL, Skuse GR. A potential role for NF1 mRNA editing in the pathogenesis of NF1 tumors. Am J Hum Genet 1997;60:305-12.

[13] Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, Kücükceylan N, et al. Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790-818.

[14] Ko JM, Sohn YB, Jeong SY, Kim HJ, Messiaen LM. Mutation spectrum of *NF1* and clinical characteristics in 78 Korean patients with neurofibromatosis type 1. Pediatr Neurol 2013;48:447-53.

[15] Balla B, Árvai K, Horváth P, Tobiás B, Takács I, Nagy Z, et al. Fast and robust next-generation sequencing technique using ion torrent personal genome machine for the screening of neurofibromatosis type 1 (NF1) gene. J Mol Neurosci 2014;53:204-10.

[16] Tsuji T, Niida Y. Development of a simple and highly sensitive mutation screening system by enzyme mismatch cleavage with optimized conditions for standard laboratories. Electrophoresis 2008;29:1473-83.

[17] Niida Y, Kuroda M, Mitani Y, Okumura A, Yokoi A. Applying and testing the conveniently optimized enzyme mismatch cleavage method to clinical DNA diagnosis. Mol Genet Metab 2012;107:580-5.

[18] Niida Y, Ozaki M, Inoue M, Takase E, Kuroda M, Mitani Y, et al. CHIPS for genetic testing to improve a regional clinical genetic service. Clin Genet 2014. doi: 10.1111/cge.12463. [Epub ahead of print]

[19] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. Nucleic Acids Res 2012;40:e115.

[20] Taschner PE, den Dunnen JT. Describing structural changes by extending HGVS sequence variation nomenclature. Hum Mutat 2011;32:507-11.

[21] Lahiri DK, Schnabel B. DNA isolation by a rapid method from human blood samples: effects of MgCl$_2$, EDTA, storage time, and temperature on DNA yield and quality. Biochem Genet 1993;31:321-8.

[22] Ji YT, Qu CQ, Cao BY. An optimal method of DNA silver staining in polyacrylamide gels. Electrophoresis 2007;28:1173-5.

[23] Upadhyaya M, Osborn MJ, Maynard J, Kim MR, Tamanoi F, Cooper DN. Mutational and functional analysis of the neurofibromatosis type 1 (*NF1*) gene. Hum Genet 1997;99:88-92.

[24] Pasmant E, Sabbagh A, Spurlock G, Laurendeau I, Grillo E, Hamel MJ, et al. *NF1* microdeletions in neurofibromatosis type 1: from genotype to phenotype. Hum Mutat 2010;31:E1506-18.

[25] López-Correa C, Dorschner M, Brems H, Lázaro C, Clementi M, Upadhyaya M, et al. Recombination hotspot in *NF1* microdeletion patients. Hum Mol Genet

2001;10:1387-92.

[26]   Stenson PD, Ball EV, Mort M, Phillips AD, Shaw K, Cooper DN. The Human Gene

Mutation Database (HGMD) and its exploitation in the fields of personalized genomics

and molecular evolution. Curr Protoc Bioinformatics 2012;Chapter 1:Unit1.13.

[27]   Kluwe L, Siebert R, Gesk S, Friedrich RE, Tinschert S, Kehrer-Sawatzki H, et al.

Screening 500 unselected neurofibromatosis 1 patients for deletions of the *NF1* gene.

Hum Mutat 2004;23:111-6.

[28]   Kurotaki N, Harada N, Shimokawa O, Miyake N, Kawame H, Uetake K, et al. Fifty

microdeletions among 112 cases of Sotos syndrome: low copy repeats possibly mediate

the common deletion. Hum Mutat 2003;22:378-87.

[29]   Kurotaki N, Stankiewicz P, Wakui K, Niikawa N, Lupski JR. Sotos syndrome common

deletion is mediated by directly oriented subunits within inverted Sos-REP low-copy

repeats. Hum Mol Genet 2005;14:535-42.

[30]   Sharif S, Upadhyaya M, Ferner R, Majounie E, Shenton A, Baser M, et al. A molecular

analysis of individuals with neurofibromatosis type 1 (NF1) and optic pathway gliomas

(OPGs), and an assessment of genotype-phenotype correlations. J Med Genet

2011;48:256-60.

Figure legends

Fig.1.

NF1 mutation analysis results of DNA level. CHIPS gel (left) and sequencing histogram (right) are shown in each panel. p.Gln209* was detected in patient 1D (A). A splicing donor site mutation, c.2850+1G>T was detected in patient 2 (B). One base pair substitution seemeds to have a missense mutation. p.Ser1468Gly was detected in patient 3S (C). Cleaved heteroduplex bands are indicated by black arrowheads. Ct, control DNA; Pt, patient DNA; U, undigested control of PCR product; C, cleaved PCR product with SURVEYOR Nuclease S.

Fig. 2.

NF1 mutation analysis results of the mRNA level. RT-PCR products are separated on 10% polyacrylamide gel and developed by silver staining. Each mutant allele is cloned and sequenced. Mutation alleles (T) are detected relatively low peak in both patients 1M and 1D (A). c.2850+1G>T results in two different aberrant splicing mutations of mRNA. Each splicing mutation use different GT nucleotide in exon 21 as the aberrant splicing donor site and created different in-frame deletions at protein levels (B). c.4402A>G creates new aberrant splicing acceptor sites and mutated alleles resulted in the p.Phe1457* nonsense mutation (C). M, molecular weight marker; Ct, control DNA; Pt, patient DNA; Mut, mutation; WT, wild-type.

Fig. 3.

Results of DNA microarray analysis. Patient 4M, 4S and 5 have a 1.3~1.4Mb microdeletion in

chromosome 17q11.2, indicated by arrow (A). A close up of microdeletion site shows that patients 4M and 4S have a type-1 microdeletion, but patient 5 has a type-2 proximal boundary and type-1 distal boundary. CN, copy number.

Fig. 1.
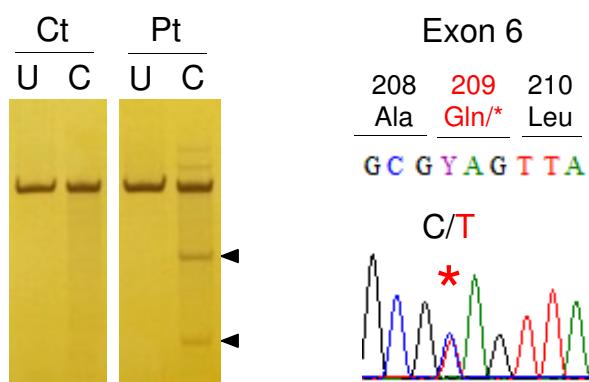
A Patient 1D

B Patient 2
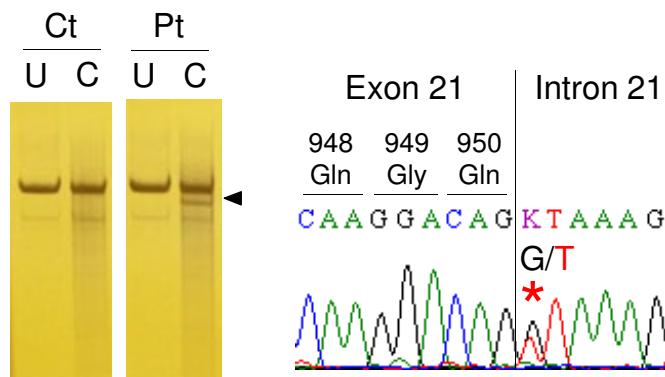
C Patient 3S
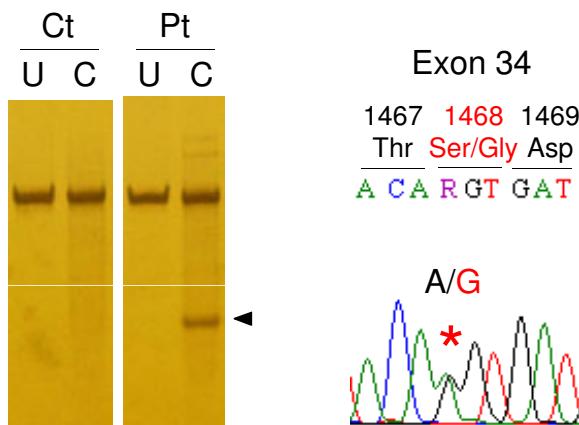
Fig. 2.

A Patient 1M and 1D

208 Ala — 209 Gln/* — 210 Leu
G C G Y A G T T A

Pt 1M — C/T — *

G C G Y A G T T A

Pt 1D — C/T — *

WT Allele

Exon 21
T C C T T A A T G T G T G T A A C C A T G A G A A A G T G G G A C T T C A A A T A C G G A C C A A T G T T A A G G A T C T G G T G G G T C T A G A A T T G A G T
900 Ser 901 Leu 902 Met 903 Val 904 Cys 905 Asn 906 His 907 Glu 908 Lys 909 Val 910 Gly 911 Leu 912 Gln 913 Ile 914 Arg 915 Thr 916 Asn 917 Val 918 Lys 919 Asp 920 Leu 921 Val 922 Gly 923 Leu 924 Glu 925 Leu 926 Ser

C C T G C T C T G T A T C C A A T G C T A T T T A A C A A A T T G A A G A A T A C C A T C A G C A A G T T T T T T G A C T C C C A A G G A C A G G T T T T A T T G
927 Pro 928 Ala 929 Leu 930 Tyr 931 Pro 932 Met 933 Leu 934 Phe 935 Asn 936 Lys 937 Leu 938 Lys 939 Asn 940 Thr 941 Ile 942 Ser 943 Lys 944 Phe 945 Phe 946 Asp 947 Ser 948 Gln 949 Gly 950 Gln 951 Val 952 Leu 953 Leu
Exon 21 | Exon 22

B Patient 2

1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
M Ct Pt

Splice Mut 1

Splice Mut 2

918 Lys 919 Asp 920 Leu 951 Val 952 Leu 953 Leu
A A G G A T C T G G T T T T A T T G
Exon 21 | Exon 22

900 Ser 901 Leu 902 Met 903 Val 904 Leu 905 Leu
T C C T T A A T G G T T T T A T T G

C Patient 3M and 3S

M Ct Pt 3M Pt 3S
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
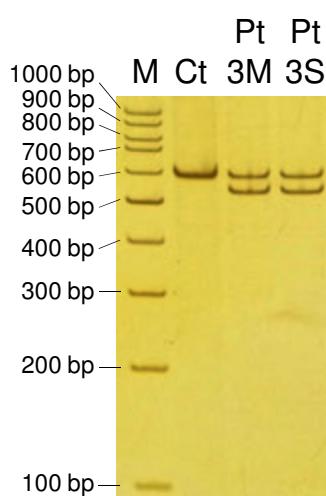
Mut Allele

1453 Ala 1454 Ala 1455 Arg 1456 Arg 1457 *
G C A G C A C G C A G G T G A T G C A G T

1453 Ala 1454 Ala 1455 Arg 1456 Arg 1457 Phe 1458 Phe 1459 Leu 1460 Asp 1461 Ile 1462 Ala 1463 Ser 1464 Asp 1465 Cys 1466 Pro 1467 Thr 1468 Ser 1469 Asp 1470 Ala 1471 Val 1472 Asn
G C A G C A C G C A G G T T T T T C C T T G A T A T A G C A T C T G A T T G T C C T A C A A G T G A T G C A G T A A A T
Exon 33 | Exon 34

A G

WT Allele

Fig. 3.
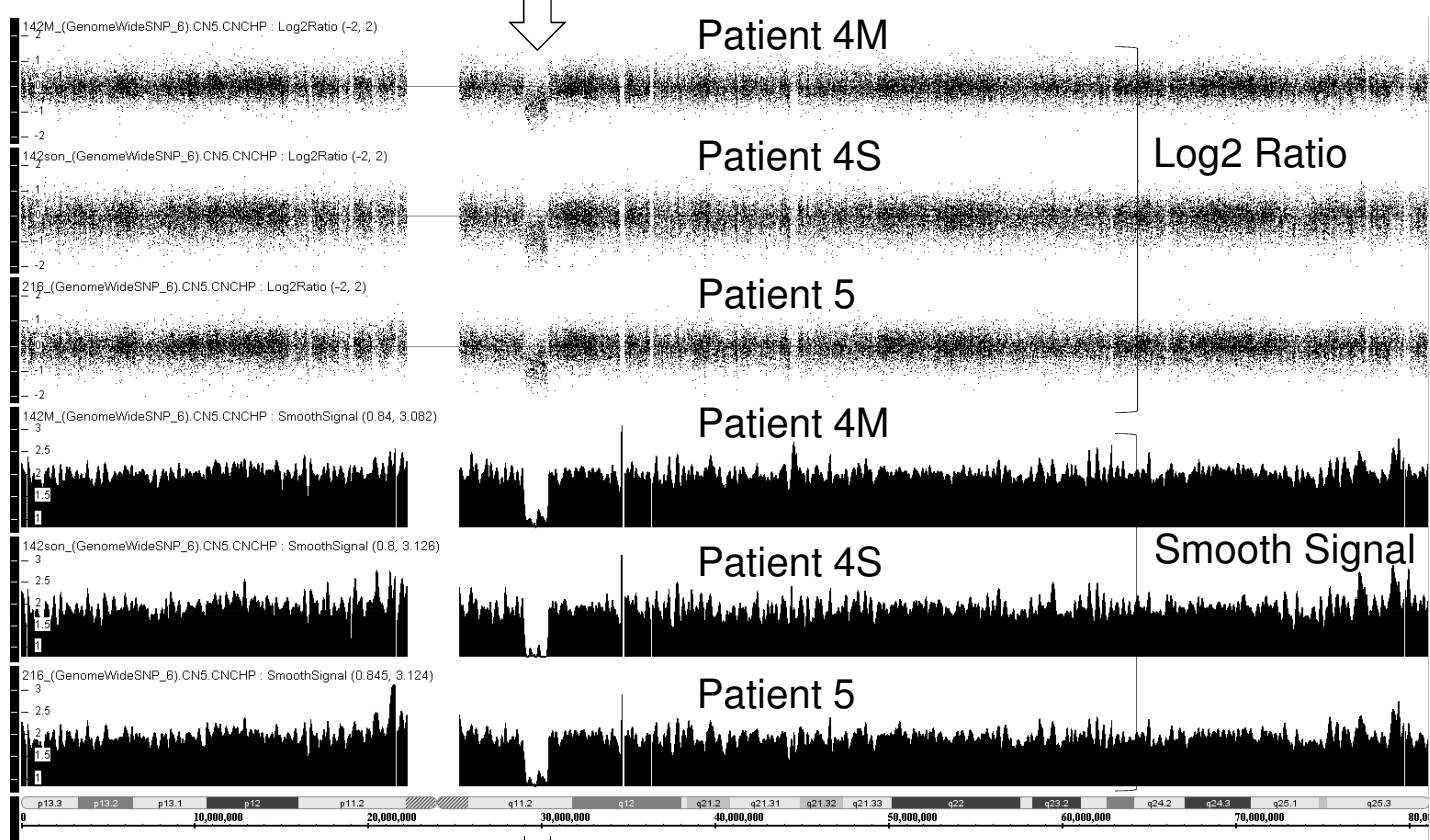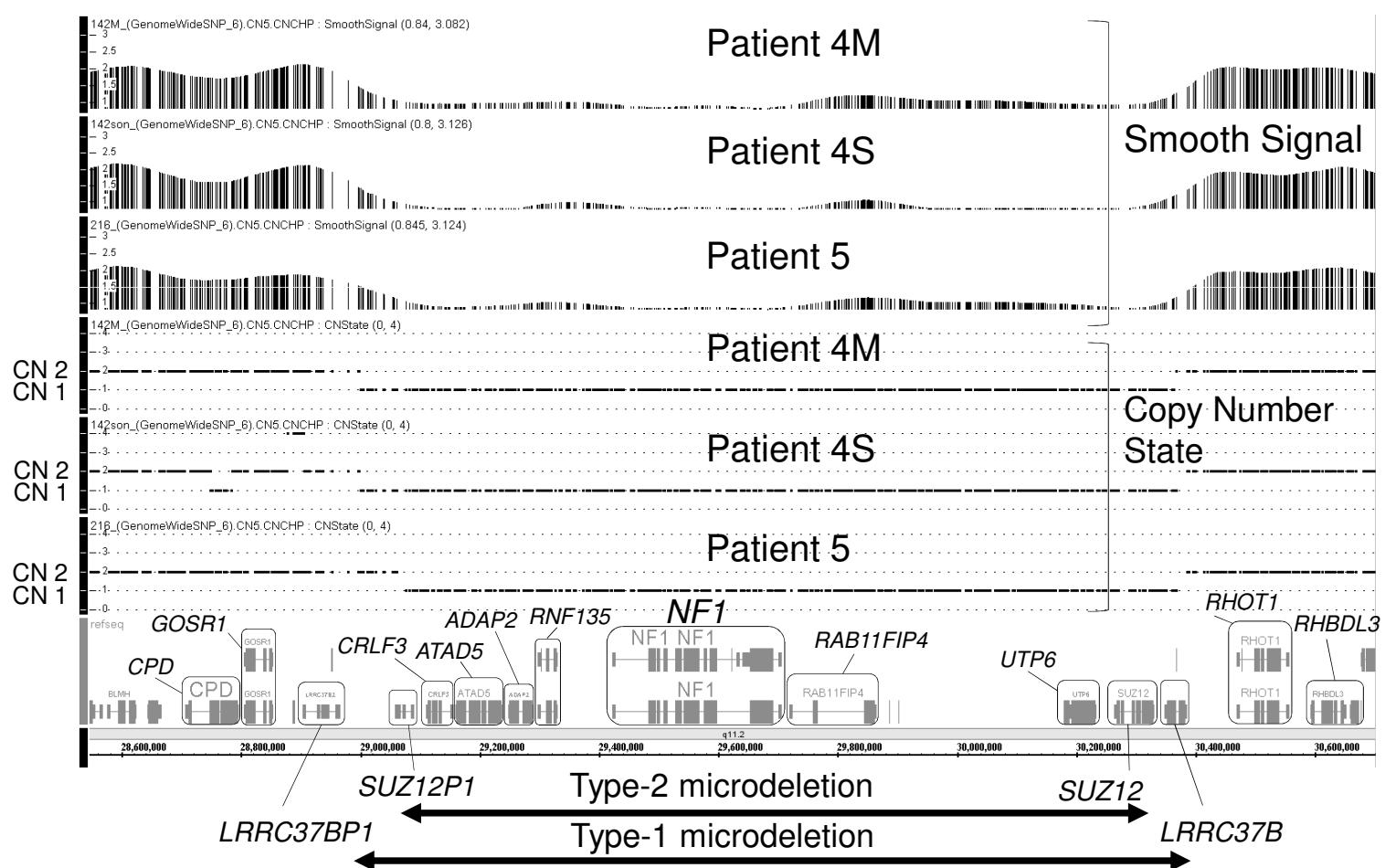
Table 1
Summary of clinical characteristics of the patients with NF1.

Trait	Family 1 Patient 1M	Patient 1D	Family 2 Patient 2	Family 3 Patient 3M	Patient 3S	Family 4 Patient 4M	Patient 4S	Family 5 Patient 5
Age at examined/sex	56 years/female	30 years/female	16 years/female	43 years/female	12 years/male	28 years/female	3 years/male	21 years/female
Mental retardation	No	Moderate	Severe	No	Severe	Mild	Severe	Mild, FIQ65 (WAIS-III)
Language	Normal	Speak, not	Few words	Normal	No words	Normal	Few words	Speak and write
Education	University graduation	Graduation from high school course	High school course of SSES	College graduation	Junior highschool course of	High school graduation	Preschool course of SSES	Dropping out of collage
Marige	Yes	No		Yes		Yes		No
Work	Nurse of the hospital	Vocational aid center	Student	Housewife	Student	Housewife	Student	Domestic help
Social interaction	Normal	Normal	Autism	Normal	Autism	Normal	Normal	Normal
Aggressive behabior	No	No	Yes	No	Yes	No	No	No
Epilepsy	No	Complex partial seizure, monthly	Generalized seizure, controlled	No	Generalized seizure, controlled	No	No	No
Drug	No	CBZ	VPA, LTG, RSP	No	VPA, LTG, LEV, RSP	No	No	No
Café au leit spots	Dosens	Dosens	Hundreds	Dosens	Dosens	Hundreds	Dosens	Thousands
Freckling	No	No	Yes	Yes	No	Yes	No	Yes
Cutaneous neurofibron	Yes	No	No	Yes	No	Yes	No	Yes
Nodural plexiform neurofibroma	No	No	No	No	No	No	No	Yes
Lish nodules	NT	Yes	Yes	NT	No	NT	No	Yes
Facial appearance	hyperterolism, downslanting of palpeblar fissure	hyperterolism, downslanting of palpeblar fissure	hyperterolism, flat nasal bridge	hyperterolism, downslanting of palpeblar fissure	hyperterolism, downslanting of palpeblar fissure	hyperterolism, wide nose with marked flat nasal bridge	Macrocephaly, hyperterolism, flat nasal bridge	hyperterolism, wide nose with marked flat nasal bridge
Skeletal system	Normal	Scoliosis	Scoliosis	Normal	Normal	Nasal cartirage hypoplasia	Scoliosis	Nasal cartirage hypoplasia
Brain MRI	Normal	Normal	Abnormal[a]	NT	Normal	NT	Normal	Normal

[a] Unidentified bright objects (UBOs) in bilateral globus pardus and moyamoya disease
CBZ, carbamazepin; LEV, levetiracetam; LTG, lamotorigine; NT, not tested; SSES, special support education school; VPA, valproic acid
WAIS, Wechsler adult intelligence scale

Table 2
Summary of detected mutations and polymorphisms.

Patient	Disease causative mutation					Polymorphism			
	Position	DNA level	RNA lavel	Protein level	Reference	Position	DNA level	Protein level	Reference SNP
1D	Exon 6	c.625C>T	r.625c>u	p.Gln209*	New	Intron 3	c.288+41G>A		rs2952976
						Intron 4	c.480-90C>T		rs2905807
						Exon 7	c.702G>A	p.Leu234=	rs1801052
						Intron 13	c.1528-35T[8]		
						Exon 18	c.2034G>A	p.Pro678=	rs2285892
						Intron 26	c.3496+33C>A		rs2066736
						Intron 48	c.7126+37C>G		rs7405740
1M	Exon 6	c.625C>T	r.625c>u	p.Gln209*		NT			
2	Intron 21	c.2850+1G>T	r.[2761_2850del; 2707_2850del]	p.[Gly922_Val951del; Cys904_Val951del]	LOVD v.2.0[a]	Intron 43	c.6579+45T>A		rs17883614
						Exon 56	c.8088G>A	p.Pro2696=	rs2285895
3S	Exon 34	c.4402A>G	r.4368_4402del	p.Phe1457*		Intron 3	c.288+41G>A		rs2952976
						Exon 7	c.702G>A	p.Leu234=	rs1801052
						Intron 13	c.1528-35T[8]		
					Upadhyaya et al. [25][b]	Exon 18	c.2034G>A	p.Pro678=	rs2285892
						Intron 26	c.3496+33C>A		rs2066736
						Intron 37	c.5205+23T>C		rs9894648
3M	Exon 34	c.4402A>G	r.4368_4402del	p.Phe1457*		NT			
4M	Whole	46,XX.arr[hg19] 17q11.2(29,000,019-30,368,308)x1				ND			
4S	NF1 gene	46,XY.arr[hg19] 17q11.2(29,000,019-30,368,308)x1				ND			
5	deletion	46,XX.arr[hg19] 17q11.2(29,076,347-30,368,308)x1				ND			

NT, not tested; ND, not detected by CHIPS analysis

[a] LOVD v.2.0 - Leiden Open Variation Database, Mendelian genes Neurofibromin 1(NF1) (http://www.LOVD.nl/NF1) Patient data (#0060821)

[b] Reported as Ser1468Gly

Supplementary data

Supplementary figure legends

Suppl. Fig. 1.

NF1 pseudogenes and *NF1* specific PCR primers. *NF1* and *NF1* pseudogenes are aligned by each exon. Exon sequences are indicated by gray and PCR primer sequences are indicated by yellow. Nucleotides of *NF1* pseudogenes same to *NF1* are indicated by dots, and only different nucleotides in *NF1* pseudogenes are described.

Suppl. Fig. 2.

NF1 mutation analysis of patient 1D. Peripheral mononuclear cell derived cDNA are amplified by 14 sets of PCR primers (A). Two different size bands are seen in primer set g, because of mRNA alternative splicing. *NF1* mRNA variant 1 includes exon 31, but mRNA variant 2 does not include exon 31. Ex1 of *NF1* gene is located in GC rich sequence and required specific PCR protocol (B). CHIPS analysis of $60\,^{\circ}\mathrm{C}$ annealing temperature series (C) and $58\,^{\circ}\mathrm{C}$ annealing temperature series (D). Cleaved heteroduplex bands are indicated by black arrow heads and shifted bands by heteroduplex formations are indicated by white arrow heads. To distinguish PCR slippage error by poly T/A stretch from real polymorphism, compared CHIPS results to patient 5 (large *NF1* deletion) for exon 7, 14, 25 and 42. Unique cleavage bands (*) are detected in exon 7 and 14, but not in exon 25 and 42. M, molecular weight marker; U, undigested control of PCR product; C, cleaved PCR product with SURVEYOR Nuclease S.

NF1 Ex9 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
                10        20        30        40        50        60        70        80        90       100
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    AATATAAATTATGCATTCTTTATAGTATGAGTTTTAGAGGCTGTTAATTTGCTATAATATTAGCTACATCTGGAATAGAAGAAACTTCATATATTATCTT
NF1P3  .............C..........................T..............C......G..A...............................
NF1P5  .............C..........................T..............C......G..A............................A....

               110       120       130       140       150       160       170       180       190       200
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    ATCGCTATATTTGAATTCTGTAGAAGTTATTTCTGGACAGTCTACGAAAAGCTCTTGCTGGCCATGGAGGAAGTAGGCAGCTGACAGAAAGTGCTGCAAT
NF1P3  ..T....................G...............T.G...................C.........................C....T......
NF1P5  .......................G....C..........T....................C.........................A....T......

               210       220       230       240       250       260       270       280       290       300
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    TGCCTGTGTCAAACTGTGTAAAGCAAGTACTTACATCAATTGGGAAGATAACTCTGTCATTTTCCTACTTGTTCAGTCCATGGTGGTTGATCTTAAGGTA
NF1P3  .......C.............................C..........G...................................C...
NF1P5  .......C.............................G...

               310       320       330       340       350       360       370       380       390       400
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    ACATGCTTATTCTTTCTCTACTACAAACTTTAAGAAAATTAAATGAATTTTCTAGCATAAGTATTATGTCAAAGATAATTGCTAACATTAAAGTTCTGAC
NF1P3  ...........................G...............T.........A.................G.....................T
NF1P5  .T.........................G...............T...........................G.....................T
```

NF1 Ex10 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
                10        20        30        40        50        60        70        80        90       100
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CTGCCATTTGTGTGGGTAATGTGTTGATGTTATTACATGTTAGTAAAGAAATACTGCATGGGTATTTAAAGGCTTTTGTTTTCTGTTGGGGTTTTTATAG
NF1P3  ......C.A.....A...................A................................................................
NF1P5  ......C.A.A...A...................A................................................................

               110       120       130       140       150       160       170       180       190       200
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    AACCTGCTTTTTAATCCAAGTAAGCCATTCTCAAGAGGCAGTCAGCCTGCAGATGTGGATCTAATGATTGACTGCCTTGTTTCTTGCTTTCGTATAAGCC
NF1P3  ..........................G..........................T...........................C.....G......
NF1P5  ...................G......G..........................T...........................C.....G......

               210       220       230       240       250       260       270       280       290       300
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CTCACAACAACCAACACTTTAAGGTGAGAGCATTGGTTTTTATCTAACTATATTTACTGATGCTGTTATCCTTTATAAACAAAAAGACTATAGAGATTAA
NF1P3  ..............G.........G...C...................G........C.................GG.......G..G...-.
NF1P5  ..............G........G...................G........CA.............GG.......G..G...-.
```

NF1 Ex11 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
              10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      —ATAAACAAAAAGACTATAGAGATTAATAGGTTCACTTTTATCGGTATTTCTCACTATTATGTATTGATGTTCGTTTCAAGACCTTAAAAACTTAGT
NF1P3    CTTT......GG.......G..G...—..C........C.C......C..............C.........A..........AAAA..........
NF1P5    CTTT......GG.......G..G...—..C........C.C......C..............C.........A..........AAAA..........

             110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      GTTTTTTTTTTAAACTTTCTATTTGCTGTTCTTTTTGGCTTCATTTGTATTACTGAGTATTTTTCTCATAGAAATAATCTGCTTTTTTTTTTCTTTTTCT
NF1P3    .......AAA—.........G............A................................................—......
NF1P5    .......AAA—.......................A..............................................—......

             210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      ATAGATCTGCCTGGCTCAGAATTCACCTTCTACATTTCACTATGTGCTGGTAAATTCACTCCATCGAATCATCACCAATGTAAGTCCAAAAGGTATTGCT
NF1P3    ............TG.........A.............................A.................T...C.....
NF1P5    ....T.....TG.........A.............................A.................T...C.....

             310       320       330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      AAATTACTAAAAAAATTTTTTT—CTTTCTTTTCTTTGCGTATTTCTTTTTAAGAAATGCACTCTTGGTTTTCAAAAAGGTTCTGAATTTAGATGTATGAA
NF1P3    ...C...........AAAA...—.A..A..........A............C...C..G.........................C.G.
NF1P5    ...C...........AAAA...T.A..A..........A............C...C.........................C.G.
```

NF1 Ex13 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
`NF1 Exon`

```
              10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      AAATAGCTTTTTTCAAAGGTTTTTAATAATAAATTTCTTTTAAAAGGTTGGATAGCTATTATCCTGAGTCTTATGTCTGATACCATGTTTTTGTTTTGTT
NF1P4    ..-.........T...C.C...............................TCCTGAGTCTTATGTCTGATACCATG.........................
NF1P6    ..-.........T...C.C...............................TCCTGAGTCTTATGTCTGATACCATG.........................
NF1P7    ..-.........T...C.C...............................TCCTGAGTCTTATGTCTGATACCATG.........................
NF1P8    ..-.........T...C................................TCCTGAGTCTTATGTCTGATACCATG...............G...
MED15P6  ..-.........T...C.C...............................TCCTGAGTCTTATGTCTGATACCATG.........................

             110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      TTTAGAGTCTTACATTTAAAGAAAAAGTAACAAGCCTTAAATTTAAAGAAAAACCTACAGACCTGGAGACAAGAAGCTATAAGTATCTTCTCTTGTCCAT
NF1P4    .............T...............................................G..................G.....................
NF1P6    .............T...............................................G.T................G.....................
NF1P7    .............T...............................................G..................G.....................
NF1P8    ......C.....................................................C..G..................G..................
MED15P6  .............T...............................................G.........C........G.....................

             210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      GGTGAAACTAATTCATGCAGATCCAAAGCTCTTGCTTTGTGTAAGTATTTTTTTTATGAAATGTCTCAAAATTATCACACTAAGTTAATTGGGTTTAGCTG
NF1P4    ......................G...................G....................G.........C..........C..T.....A...
NF1P6    ......................G...................G....................ACG.......C..........C..T.....A...
NF1P7    ......................G...................G.........G.........C..........C..T.....A...
NF1P8    .........................................G.............................C..........C..T....GA...
MED15P6  ................................................

             310       320       330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1      AAACGCCAAGACCTTGAGGATAATTTTTAGCTCAAGAGCACTTACTGATCCTTTCTGATCATACCTGAAAACTAAGACGTCTCTAAGAAAACTAATGTAT
NF1P4    ...................G..................................A....................C.........
NF1P6    ...................G..........................G..A....................C.........
NF1P7    ...................G..................................A....................C.........
NF1P8    ....A..............G..................................A................TC.........
MED15P6  ----------------------------------------------------------------------------------------
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NF1 Ex15 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
            10        20        30        40        50        60        70        80        90       100
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    TGATTTCATTCTGTCTGTATTATTCCCTAGAGGTTTGTGTTCACCAGAGTTTCTCTCTTTTTACCTTTTACTATATATTGAAACTACAAATGAAAGAGCT
NF1P3  ....A.........A...................................A.................G...G.................T....
NF1P5  -...A.........A...................................A...G..............G...................T....

           110       120       130       140       150       160       170       180       190       200
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CAATTTCTTAGCATTTATAAAATAAGTACTCCAGTGTTATGTTTACCAAAAATGTTTGAGTGAGTCTTCTCTTTGTCTTTCTCTTTTTTAAAAAA-TTCA
NF1P3  .................C.G...........C.............C.....................C...........A......-....
NF1P5  .................C.....A.......C.............C.....................C...........A.....A....

           210       220       230       240       250       260       270       280       290       300
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    GGCTCTGCTGGTTCTTCATCAGTTAGATAGCATTGATTTGTGGAATCCTGATGCTCCTGTAGAAACATTTTGGGAGATTAGGTATATGTACTTTTATTTT
NF1P3  ..............G.............................G...........................C.
NF1P5  ..............G.............................G..............................

           310       320       330       340       350       360       370       380       390       400
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    TTAAATTCAACTTTTAAATTTTATTTTGTATTTTT-GTCTTGAAATATTAACTCTGTAGTACTTAGTACATTGTAAAGCTTACACTTCCAAAGGTTTTAT
NF1P3  .....................................T...................A....T.....T..........T................
NF1P5  .....................................T...................A....T.....T..........T................
```

NF1 Ex16 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
NF1 Exon

```
              10        20        30        40        50        60        70        80        90       100
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    GTTTATTGTTTCTTCTTATGT--CTACTACAAGGATGAGATACATGGATAGAAACACTGGGAGTTTAATGAAACATAGGGGGCTTATAGATAAATACTTG
NF1P4  -G.A...T..AGC..AAGA.CACT....GATCCTT.CT...-..AAC.GA....TAA.ACGTC.CT.A.....C..AT.------..-...G.....GA
NF1P6  -G.A...T..AGC..AAGA.CACT....GATCCTT.CT...-G.AAC.GA....TAA.ACGTC.CT.A.....C..AT.------..-...G.....GA
NF1P7  -G.A...T..AGC..AAGA.CACT....GATCCTT.CT...-..AAC.GA....TAA.ACGTC.CT.A.....C..AT.------..-...G.....GA
NF1P8  -G.A...T..AGC..AAGA.CACT....GATCCTT.CT...-..AAC.GA....TAA.ACGTC.CT.A...T.C..AT.------..-...G.....GA

              110       120       130       140       150       160       170       180       190       200
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CTTTTTTTCTATAACCTGTAGACT--TATTTCCTTTGATTATAATGCTATTGACACTTTGATAACTGTTTCTCTAAA--ACCTTACAAGAAAAACTAAG
NF1P4  TA.AA..AGATC.GAT.C.TA.T.GCCC..A..A..TC...AC..TTCTC.TT.TTC..A..TT.C..A..AT.C..CTG..A.GCTT.AC.T......
NF1P6  TA.AA..AGATC.GAT.C.TA.T.GCCC..A..A..TC...AC..TTCTC.TT.TTC..A..TT.C..A..AT.C..CTG..A.GCTT.AC.T......
NF1P7  TA.AA..AGATC.GAT.C.TA.T.GCCC..A..A..TC...AC..TTCTC.TT.TTC..A..TT.C..A..AT.C..CTG..A.GCTT.AC.T......
NF1P8  TA.AA..AGATC.GAT.C.TA.T.GCCC..A..A..TC...AC..TTCTC.TT.TTC..A..TT.CC.G..AT.C..CTA..A.GCTT.AC.T......

              210       220       230       240       250       260       270       280       290       300
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CTTCTCTAAACTTGTATTCATTATGGGAGAATGCCATTCTTATGTCTGGTTATATCTGCATTAGGTTATTGATGATGCTAGTAACAATGAACTTTATGTT
NF1P4  ......C.........................................................................T....G.........A.T.....
NF1P6  .........G..G..................G................................................T........G...A.T.....
NF1P7  ......C.........................................................................T....G.........A.T.....
NF1P8  ......C.........................................................................AT..................

              310       320       330       340       350       360       370       380       390       400
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    ACTGCAGCTCACAAATGCTTTTTTTACATCTGCAAGAAATTAACTAGTCATCAAATGCTTAGTAGCACAGAAATTCTCAAGTGGTTGCGGGAAATATTGAT
NF1P4  ..........G..TA...................T..............................................A......T...
NF1P6  ..........G..TA...................T..............................................A......T...
NF1P7  ..........G..TA...................T..............................................A......T...
NF1P8  ..........TA..............G...................................................A......T...C

              410       420       430       440       450       460       470       480       490       500
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    CTGCAGGAATAAATTTCTT CTTAAAAATAAGGTAAGCAAAATGACATATTTAAAAAATGGAAGAATATTTGGAATGGTAATGGTGAGAGATTACTAAAG
NF1P4  ...................T.................A..G.........C...........AG.................G....G.........AG....
NF1P6  ..............T..T............A...A..G.........C...........G.................G....G.........AG....
NF1P7  ...................T.................A..G.........C...........AG.................G....G.........AG....
NF1P8  ...................CT.........................C...........G.................G....G.........AG....

              510       520       530       540       550       560       570       580       590       600
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1    TGTTTTATAGCCAAATTAGGTTCTATTTCAGCTTCTCCTTCCTCCCAATGTTCTCAAAAGGAAATATGTATGCAGAGGACAATGACTGGCAAATCAGCAT
NF1P4  ................T.........C.......C....G............T...A.G..........G.G........T.....
NF1P6  ................T.........C.......C....G............T...A.G..........G.G........T.....
NF1P7  ................T.........C.......C....G............T...A.G..........G.G........T.....
NF1P8  ................T.........C.......C....G........T.C..A.G..........G.G........T...C
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NF1 Ex17 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
NF1 Exon

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                10        20        30        40        50        60        70        80        90       100
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           -GAAACTGAAAGAAATTTTTAAAGA-ATTAAGTAAACCTTGTTTGTTCTAATGGGTTTCTAGTGAATCTCCTTCAAGTTGGGGCATAGAGATTGAGAGGAG
NF1P4         -..........C......G...A..A.............................T.........G.....TG..............
NF1P6         -..........C......G...A..A.............................T.........G....................
NF1P7         -..........C......G...A..A.............................T.........G....TG..............
NF1P8         T..........C......G...A..A.............................T.........A...................G....
LOC102724666  -..........C......G...A..A.............................T.........G....................

                110       120       130       140       150       160       170       180       190       200
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           AGGTTTTTTAGGAGAGTCTCAAACAGGAAGACAACTCAAATAAGTGTTTATTCCTCTTGGTTGTCAGTGCTTCAGTAAAGCTTATTTATTTATTTTTTTC
NF1P4         .........C....................CG.T....................TG..........C...CC..-C...
NF1P6         .........C....................CG.T....................G...........C...CC..-C...
NF1P7         .........C....................CG.T....................TG..........C...CC..-C...
NF1P8         .........C....................C..C..T.................A...........C...-C...
LOC102724666  .........C....................CG.T....................G...........C...CC..-C...

                210       220       230       240       250       260       270       280       290       300
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TAGCAGGCAGATAGAAGTTCCTGTCACTTTCTCCTTTTTTACGGGGTAGGATGTGATATTCCTTCTAGTGGAAATACCAGTCAAATGTCCATGGATCATG
NF1P4         ...........A...............T.A........C...........A..........G....
NF1P6         ...........A...............T.A........C...........A..........G....
NF1P7         ...........A...............T.A........C...........A..........G....
NF1P8         ..........A................T.A........C...............G...G..T.
LOC102724666  ...........A...............T.A........C...........A..........G....

                310       320       330       340       350       360       370       380       390       400
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           AAGAATTACTACGTACTCCTGGAGCCTCTCTCCGGAAGGGAAAAGGGAACTCCTCTATGGTCAGCTTCTTCTGTACTTTTTCTGTATCATTTTATGTGCT
NF1P4         ...TG.C....................A..................................T....A....C..........C.CG.....
NF1P6         ...TG.C....T...............A.........................T.....T....A....C..........C.CG.....
NF1P7         ...TG.C....................A.................................T....A....C..........C.CG.....
NF1P8         ...TG.C...G.A..............A.................................T....A....C..........C.CG.....
LOC102724666  ...TG.C....T...............A.................................T....A....C..........C.CG.....

                410       420       430       440       450       460       470       480       490       500
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           CTGTTTGTTTTCTGAATGAAATTTGGTAAATTTCATCTAGGTAATATAGTGTAATAGTGAAAAACTGCTTAGAATCTAGTTCTATTACTGCTCTTTGTGG
NF1P4         ..A...........C.......C.......A..............C.........C........A...
NF1P6         ..A...........C.......C.......A..............C.........C........A...
NF1P7         ..A...........C.......C.......A..............C.........C........A...
NF1P8         ..A...........C.......C...............C.........C........A...
LOC102724666  ..A...........C.......C.......A..............C.........C........A...

                510       520       530       540       550       560       570       580       590       600
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           AACTTTGGGCAAGTTCTTAACATCCTTGTTCCTCAGTTCCTCAACTTTAAAATGGGGACAGTAATAGTTCCTGCCCCAGAGTTGTTAAAGAGGGTTAAAT
NF1P4         ...............................A.............A....A.......T.........GT-..........
NF1P6         .T.............................A.............A....A.......T.........GT-..........
NF1P7         ...............................A.............A....A.......T.........GT-..........
NF1P8         ...........A......C.........A....GT....A....A.......T.........GT-A.........
LOC102724666  .T.............................A.............A....A.......T.........GT-..........
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NF1 Ex18 PCR Primers and Pseudogenes

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                  10        20        30        40        50        60        70        80        90       100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            AATCTCATTTCTCATTTGGACAAGATATTTTGGGGTTTGAAAAATTCCTATAATGATTAAAGGAAAAAGCTCTTGTGAGTTATTGTATGCGGAGACACAC
NF1P1          .............................GA.................G..G.........G...A...................T...........
NF1P2          .............................GA.................G..G.........G...A...................T...........
NF1P4          .......................A................A...........G.........A.................T...T.T.T
NF1P6          ........................................A...........G.........A.................T...T.T.T
NF1P7          .......................A................A...........G.........A.................T...T.T.T
NF1P8          ...................................................G.....................C......T....T.T.T
LOC101930150   .............................GA.................G..G.........G...A...................T...........
LOC102724666   ........................................A...........G.........A.................T...T.T.T
MED15P6        ........................................A...........G.........A.................T...T.T.T

                 110       120       130       140       150       160       170       180       190       200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            ACACACACACACACACACACACACACACACACA-------CACAGTTTATTGCATTGTTAGATTTTATACATAAAATTACCCAAGTTGCAAATATATGTCTTC
NF1P1          ...................------------------G.................G.G.......................................A
NF1P2          ...................-----------------.G.................G.G.......................................A
NF1P4          GTGG.............CACACA.G.................G.G.....................................A.
NF1P6          GTGG.............G.................G.G.....................................A.
NF1P7          GTGG.............CACAC.G.................G.G.....................................A.
NF1P8          .TGG................G..T----.G.CA..G.G...T.....G...C...................C...A.
LOC101930150   ...................-----------.................G.G.......................................A
LOC102724666   GTGG.............CACACA.G.................G.G.....................................A.
MED15P6        GTGG.............G.................G.G.....................................A.

                 210       220       230       240       250       260       270       280       290       300
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            CACCCTTGACTCTCAGGATAGTGCAGCAGGATGCAGCGGAACC          CCCCCGATTTGCCGACAAGCCCAGACCAAACTAGAAGTG  G
NF1P1          ..A.T.................A....C....GCCCCCGCCCCCCTT.............A.....GG.................TG.
NF1P2          ..A.T.................A..T.C....GCCCCCGCCCCCCTT.............A.....GG.................TG.
NF1P4          .............................................T..C....T.......G................
NF1P6          .............................................T..C....T.......G.....GT..........
NF1P7          .............................................T..C....T.......G................
NF1P8          ...........................G.......C.......G.....GT..........
LOC101930150   ..A.T.................A..T.C....GCCCCCGCCCCCCTT.............A.....GG.................TG.
LOC102724666   .............................................T..C....T.......G................
MED15P6        .............................................T..C....T.......G.....GT..........

                 310       320       330       340       350       360       370       380       390       400
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            CCCTGTACATGTTTCTGTGG                    AACCCTGACACTGAAGCTGTTCTGGTTGCCATGTCCTGTTTCCGCCACCTCTG
NF1P1          .G.................................G.........................
NF1P2          .G.................................G.........................
NF1P4          ...............C..............................................
NF1P6          ...............C..............................................
NF1P7          ...............C..............................................
NF1P8          ...............CA.......................................T.....
LOC101930150   .G.................................G.........................
LOC102724666   ...............C..............................................
MED15P6        ...............C..GAAGTGGCCCTGTACATGTTTCTGCGG................
```

NF1 Ex19 PCR Primers and Pseudogenes

```
                 10        20        30        40        50        60        70        80        90       100
              |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
NF1           GGGAATAGCTATTTTATTCTGTGGACATTGTAGAGTCCAAGATAAGTACCTTTCCTGTGAGGTTAGTGAAAGGAAGTTTTTGGCTTTATCATTTGAAGCA
NF1P1         .........G.G.............TT..........................................................................G...
NF1P2         .........G.G..............T..........................................................................G...
NF1P4         ..........G..............T...........................................C...............................G...
NF1P6         ..........G..............T...........................................C...............................G...
NF1P7         ..........G..............T...........................................C...............................G...
NF1P8         .....G....G.G............T...........................................C................C..............G...
LOC101930150  ..........G.G............T...........................................G...............................G...
LOC102724666  ..........GT.............T...........................................CA..............................G...
MED15P6       ..........G..............T...........................................C...............................G...

                110       120       130       140       150       160       170       180       190       200
              |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
NF1           TTTGCTCTGCTCTTCCTACTC--CTTTTGGGTGGAGCTTATCAGGTTCTCCATTGGCAGGCAGGGCTCTAAGTGCAGTAACTTGATTTGCTGTTGTATT
NF1P1         ..........C........TGC........A.G......G.........G...............G.........G......
NF1P2         ..........C........TGC........A.G......G.........G...............G.........G......
NF1P4         ..........C........TGC........G...................................................G.
NF1P6         ..........C........TGC........G-........G.........................................G.
NF1P7         ..........C........TGC........G...................................................G.
NF1P8         ..........C........TGC........G...............................G.........G.........G.
LOC101930150  ..........C........TGC........A.G......G..........................G.........G......
LOC102724666  ..........C........TGC........G-........G.........................................G.
MED15P6       ..........C........TGC........G-........G.........................................G.

                210       220       230       240       250       260       270       280       290       300
              |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
NF1           TGCTTAGGAAGAGCAGCACTTCAGAAAAGAGTGATGGCACTGCTGAGGCGCATTGAGCATCCC              ACTGCAGGAAACACTGAGGTA
NF1P1         ........C.....................G.......C.T..........                   .........................G
NF1P2         ........C.....................G.......C.T..........                   .........................G
NF1P4         ........C.C...................C.........                            .........................G
NF1P6         ........C.C.......C...........C.........                            .........................G
NF1P7         ........C.C...................C.........                            .........................G
NF1P8         ........C.....................C.........                            .........................G
LOC101930150  ........C.....................G.......C.T..........                   .........................G
LOC102724666  ........C.......C.............C.........                            .........................G
MED15P6       ........C.C.......C...........C.......GCGCACTGAGCATCCC.............G

                310       320       330       340       350       360       370       380       390       400
              |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
NF1           TGCCCTTAGCAACAGAAACACCCCTCCCAGGCGCCCACCCTCAATTTGGAAGCCTCTTGTTACATATGTGTGATCAGGAATAGCTTTTGAAGTAAATCC
NF1P1         ...T........G...........T..............T...A..A...........A.....................
NF1P2         ...T........G...........T..............T...A..A...C.............A.....................
NF1P4         ...T........G.........................G..T.......................G.....................
NF1P6         ...T.A.....A.....T.....A....G..G..T............................................
NF1P7         ...T........G.........................G..T.....................G.....................
NF1P8         ..................T..................T.........................G.....................
LOC101930150  ...T........G...........T..............T...A..A...C.........A.....................
LOC102724666  ......A.....A....T.....A..G..G..T.............................
MED15P6       ...T........G.........................G..T.....................G.....................
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NF1 Ex20 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
NF1 Exon

```
              10        20        30        40        50        60        70        80        90       100
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TCTGAGTATTTAATATACATCAAGTTTGAAACTTGGCTGTAGCTGATTGATGTTTAGCTCTAGACT--AAGTTGCTTTCAAGTGATAATTGCCTTCATTT
NF1P1         .......C...T..............CA.T.........C...........TA.................
NF1P2         .......C...T..............CA.T.........C...........TA.................
NF1P4         .......C.....T.......G.........T..................TA...........C..T...
NF1P6         .......C.......T.....G.........T..................TA...........C..T...
NF1P7         .......C.......T.....G.........T..................TA...........C..T...
NF1P8         .......C.......T....C.G.........T..................TA...........C..T...
LOC101930150  ------------------------------------------------------------.................
LOC102724666  .......C.....T......G.........T..................TA...........C..T...
MED15P6       .......C.....T......G.........T..................TA...........C..T...

             110       120       130       140       150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TAGGCTTGGGAAGATACACATGCAAAATGGGAACAAGCAACAAAGCTAATCCTTAACTATCCAAAAGCCAAAATGGAAGATGGCCAGGTAAGTCTGTAAA
NF1P1         ........................................A.......T..............T..CA...............
NF1P2         ........................................A.......T..............T..CA...............
NF1P4         ...A....................................A.......G...........C.......................
NF1P6         ...A....................................A.......G...........C.......................
NF1P7         ...A....................................A.......G...........C.......................
NF1P8         ........................................A.......G...........C..G.................T.
LOC101930150  ........................................A.......T..............T..CA...............
LOC102724666  ...A....................................A.......G...........C.......................
MED15P6       ...A....................................A.......G...........C.......................

             210       220       230       240       250       260       270       280       290       300
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GTTGACTTTTGTCTGTTAACTGATCTGCTAAATATATGTACTTCACTTTGATAATCTTTCAAGAGTCGCTCAGTAAAGTAAACATATAGCTGTGTGAAGA
NF1P1         ......................A...............C....T....G......C..C.....A.........G.......T..C.....
NF1P2         ......................A...............C....T....G......C..C.....A.........G.......T..C.....
NF1P4         ......................A...............C....T....G......C.......A.........G.......T...C.....
NF1P6         ......................A...............C....T....G......C.......A.........G.......T...C.....A.
NF1P7         ......................A...............C....T....G......C.......A.........G.......T...C.....
NF1P8         ....C.................A.........  ............G.........C......A.....A..G.......T...C.....
LOC101930150  ......................A...............C....T....G...C..C.....A.........G.......T...C.....
LOC102724666  ......................A...............C....T....G......C.....A.........G.......T...C.....A.
MED15P6       ......................A...............C....T....G......C.....A.........G.......T...C.....
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NF1 Ex21 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
NF1 Exon

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                    10        20        30        40        50        60        70        80        90       100
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TTT-TGTACTTTTGTCATGGAAGAAATGTTGGATAAAGCATAATTTGTCAAGTCTCAACTAATTAAGGTTTAATTCATGCTTTGCACAAAAATTTTGTGT
NF1P1         ...-..........................AG...................GT...........................C...............
NF1P2         ...-..........................AG....................T............................C...............
NF1P4         ..G.....G.......C.............AG....................T...........................................
NF1P6         ...T....G.......C.............AG....................T...........................................
NF1P7         ...G....G.......C.............AG....................T...........................................
NF1P8         ...T........C...C............C.AG...................T...........................................
LOC100418983  .C.............................AGG.................T...................A..TG.............
LOC101929972  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
LOC101930150  ...-..........................AG....................T............................C...............
LOC102723592  ...-..........................AG....................T............................C...............
LOC102724666  ...T...........C.............AG....................T...........................................

                   110       120       130       140       150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TTAGGCTGCTGAAAGCCTTCACAAGACCATTGTTAAGAGGCGAATGTCCCATGTGAGTGGAGGAGGATCCATAGATTTGTCTGACACAGACTCCCTACAG
NF1P1         ............T.......T.......G..T.........C.............CG.GC...................
NF1P2         ............T.......T.......G..T......A..C.............CG.GC...................
NF1P4         .......T.............................C...............
NF1P6         .......T.............................C...............
NF1P7         .......T.............................C...............
NF1P8         .......T.............................C.......C.......
LOC100418983  ..............................C.........G................T...
LOC101929972  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
LOC101930150  ............T.......T.......G..T......A..C.............CG.GC...................
LOC102723592  ............T.......T.......G..T.........C.............G.GC...................
LOC102724666  .......T.............................G...........C...................

                   210       220       230       240       250       260       270       280       290       300
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GAATGGATCAACATGACTGGCTTCCTTTGTGCCCTTGGGGGAGTGTGCCTCCAGCAGAGAAGCAATTCTGGCCTGGCAACCTATAGCCCACCCATGGGTC
NF1P1         ..............................................T.......................
NF1P2         ..............................................T.......................
NF1P4         .C............................................G...............................G.....
NF1P6         .C.................A..........................G...............................
NF1P7         .C............................................G.............................G.....
NF1P8         .C............................................T...........C................G.....
LOC100418983  .........................................A..T.......................
LOC101929972  NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNG..............................G.....
LOC101930150  ..............................................T.......................
LOC102723592  ..............................................T.......................
LOC102724666  .C..C.........................................G...............................G.....
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                    310       320       330       340       350       360       370       380       390       400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          CAGTCAGTGAACGTAAGGGTTCTATGATTTCAGTGATGTCTTCAGAGGGAAACGCAGATACACCTGTCAGCAAATTTATGGATCGGCTGTTGTCCTTAAT
NF1P1        .........T..C........C..............C................G.............G.........A........
NF1P2        .........T..C........C..............C................G.............G.........A........
NF1P4        .G......A........C..............C.....................A.....A..........G...
NF1P6        .G......A........C..............C.....................A.....A..........G...
NF1P7        .G......A........C..............C...........G..A.....A..........G...
NF1P8        .G......A........C..............C.....................A..A..C...G...
LOC100418983 .......T..........C.......T...................G......A...
LOC101929972 .G......A........C..............C.....................A........A...
LOC101930150 .......T..C........C..............C...........G.....G.......
LOC102723592 .......T..C........C..............C...........G.....G.......
LOC102724666 .G......A........C..............C.....................A..A..........G...

                    410       420       430       440       450       460       470       480       490       500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GGTGTGTAACCATGAGAAAGTGGGACTTCAAATACGGACCAATGTTAAGGATCTGGTGGGTCTAGAATTGAGTCCTGCTCTGTATCCAATGCTATTTAAC
NF1P1        .........................G......C..........T..TG.
NF1P2        .........................G......C..........T..TG.
NF1P4        ..........GT...........A.C..........T..TG.
NF1P6        ..........G.T...........G.C..........T..TG.
NF1P7        ..........GT...........A.C..........T..TG.
NF1P8        ...........T.......C...A.C..........T..TG.
LOC100418983 AC........G.......C...............A....T..T..TG.
LOC101929972 ..........G.T...........A..........T..TG.
LOC101930150 ......................G...........T..TG.
LOC102723592 ................A.G.......A..........T..TG.
LOC102724666 ..........T...........A.C..........T..TG.

                    610       620       630       640       650       660       670       680       690       700
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          AAATTGAAGAATACCATCAGCAAGTTTTTTGACTCCCAAGGACAGGTAAAGTGTTCTCTTATT---TTTCACCTTTCTCTATGAATAGAGTGACTTGT
NF1P1        ...C.......G.....................G.......T.TT....A.CT..TT.GA.A.
NF1P2        ...C.......G.....................G.......T.TT....A.CT..TT.GA.A.
NF1P4        .....G..C..........T...C....G...T.TATT......C.......
NF1P6        .....G..G..........T......G...T.TATT......C.......
NF1P7        .....G..C..........T......G...T.TATT......C.......
NF1P8        .....G..C..........T......G...T.TATT......C.......
LOC100418983 ...C.................GT....T.TATT......C.......
LOC101929972 .................T......G...T.TATT......C.......
LOC101930150 ...C.......G.............G.......T.TT....A.CT..TT.GA.A.
LOC102723592 ...C.......G.............G.......T.TT....A.CT..TT.GA.A.
LOC102724666 .....G..G..........T....G..T..T..TATT......C.........C.......

                    710       720       730       740       750       760       770       780       790       800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TTGAAA---TAAGCCTTTTTCTTTCAGATTATTTAAATTAGGTACTCACAGTTTTTAAAAATTTCCAAAAAAATTGCAGAAAGAAGAGTCATCTCAATGT
NF1P1        ..G---G..G......CA......G.........G.........
NF1P2        ..G---G..G......CA......G.........G.........
NF1P4        ..TAATG................GT.....T......A........
NF1P6        ..TAATG................GT.....T......A........
NF1P7        ..TAATG................GT.....T......A........
NF1P8        ..TAATG...G..G......G......GT.....T......A........
LOC100418983 ..TAAGG..A....C.......G......G.........
LOC101929972 ..TAATG................GT.....T......A........
LOC101930150 .G---G..G......CA......G.........G.........
LOC102723592 .G---G..G......CA......G.........G.........
LOC102724666 ..TAATG................GT.....T......A........
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NF1 Ex22 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

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                10        20        30        40        50        60        70        80        90       100
          ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          CTTAGGAACTCTGGTGTGTATGTGTGCCTAAGGGTATACGTGCCCTGTGTATGGGTACGAGTGTCTGC-GTATATCTGTATGCT-TATTTGGCTCTATGC
NF1P1        ........................G..........C....T.............T.........-A.G.............G......G.G...
NF1P2        ........................G..........C....T.............T.........-A.G.............G......G.G...
NF1P4        ...........A............G.........T..A.....T.........T-.........G...C-G......GCG...
NF1P6        ...-.......A............G.........T..A.....T.........T-.........G...C-G......GCG...
NF1P7        ...........A............G.........T..A.....T.........T-.........G...C-G......GCG...
NF1P8        ...........A............G.........T..A.....T.........T-.........G...C-G...A..GCG...
LOC100418983 ......................G...A...A....T.........T.......-A........C..G......G.G...
LOC101929972 ...........A............G.........T..A.....T.........T-.........G...C-G......GCG...
LOC101930150 A.C.AAT.AAA..AAC.AA....AA.T..G.AAAC.ATAT.TATT.T.A.GA.AAC.TACACAC..T.T.GT..C...AC..TCAC.CCCTAG.T.G..T
LOC101930488 ........................G..........C....T.............T.........-A.G............-G......G.G...
LOC102723592 ........................G..........C....T.............T.........-A.G.........-.G......G.G...
LOC102724666 ...-.......A...C........G..........T..A.....CT.........T-.........G...C-G......GCG...

               110       120       130       140       150       160       170       180       190       200
          ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          CTGTGGGTGCACTTAC---TCTGTGTGTTTAGATCAGTCAGTTTCATCTCTCTAGGGGGTCTGTCTTCTGGGCATTGATGGCAAATCATTAATGTATTTG
NF1P1        ..............CTGTG.T...........G...G........A...-............G....A.....
NF1P2        ..............CTGTG.T...........G...G........A...-............G....A.....
NF1P4        .........TG.CTCTA.T.................A-...............A.....
NF1P6        .........TG.CTCTA.T.................A-...............A.....
NF1P7        .........TG.CTCTA.T.................A-...............A.....
NF1P8        .........TG.CTCTA.T.................A-...............A.....
LOC100418983 ....C......G.CTCTG.T........TG.......A...............T...........A.G....
LOC101929972 .........TG.CTCTA.T.................A-...............A.....
LOC101930150 GCAGTAAA.A.TGGCAA-A.TAT.T.A.CA.TTA.TAC..A.A...ATGTGTA..A..----.T.T.....AT.TC.T.AA.T.TG.TTC.GC.AC....C
LOC101930488 ..............CTGTG.T...........G...G........A...-............G....A.....
LOC102723592 ..............CTGTG.T...........G...G........A...-............G....A.....
LOC102724666 .........TG.CTCTA.T.................A-...............A.....

               210       220       230       240       250       260       270       280       290       300
          ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TTCTTTCTTTAGGTTTTTATTGACTGATACCAATACTCAATTTGTAG AACAAACCATAGCTATAATGAAGAACTTGCTAGATAATCATACTGAAGGCAGC
NF1P1        ........A............G.........C...........G............
NF1P2        ........A..T.......TG.........C...........G............
NF1P4        ....C...A.................................
NF1P6        ....C...A......................GG.....C......T............
NF1P7        ....C...A...............C.................
NF1P8        ....C...A......................G.....C...A.............
LOC100418983 C.......A.................................G............
LOC101929972 ....C...A......................GG.....C......T............
LOC101930150 AGTAGAACGCTTACC.G.CCAGA....A.TC.T.GGT.CAA.GCT.G.TTG..C...A.G.TCA..GCTG.C.T.AGT..G..T..CTA.C.A.TTCT
LOC101930488 ........A.................G.........C...........G............
LOC102723592 ........A.................G.........C...........G............
LOC102724666 ....C...A.................................
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                    310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             TCTGAACATCTAGGGCAAGCTAGCATTGAAACAATGATGTTAAATCTGGTCAGGTAAGCATTCTACTGAA—ATGTAGCAGAAACAT——————TTT
NF1P1           .........................C.............T...........G...........................AC————...
NF1P2           .........................C.............T...........G...........................AC————...
NF1P4           ..............A..........................C...........—.................ACTTTAAGA...
NF1P6           ..............A..........................C...........—.................ACTTTAAGA...
NF1P7           ..............A....................G.....C...........—.................ACTTTAAGA...
NF1P8           C.............A..........................C...........—.................ACTTTAAGA...
LOC100418983    ........................C..G.T.....T.....T.....G....................C...AC————...
LOC101929972    ..............A..........................C...........—.................ACTTTAAGA...
LOC101930150    ..ATT.T.G...T..TTT...C T.CAA.TTG.G..T..G....CATCA.TAA..A.TA.AG.AA...CAA.....TT.ATC.TT.GCCATCAATGCC
LOC101930488    .........................C.............T...........G...........................AC————...
LOC102723592    .........................C.............T...........G...........................AC————...
LOC102724666    ..............A..........................C...........—.................ACTTTAAGA...

                    410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             AAGAGATAAGAAAAACCTCTTACACACTGATACTGGTAGTAATTGATAAAATAACTGGCCATTCTTTACTGCACACAAACTAGGGTGTGACAGTAAGGTA
NF1P1           ......C.................T....T...........T.T...........................C.....
NF1P2           ......C.................T....T...........T.T...........................C.....
NF1P4           ......C.................T....T..A.........T.T.....................C....AC.....
NF1P6           ......C.........G.C.....T....T..A.........T.T.....................C....AC.....
NF1P7           ......C.................T....T..A.........T.T.....................C....AC.....
NF1P8           ......C.........C.......T....T..A.........T.T................—......A.A.....
LOC100418983    .......................T....T............T.T.T............A.......A.....
LOC101929972    ......C.........C.......T....T..A.........T.T.....................C....AC.....
LOC101930150    C...————..C.G......AGAGAGA..CA..C.ACT.ATC.AA.C.C.CA...ACAGA.G.GCAC..CA..GG..C..AGCCAAACA.GCA..CA.AT
LOC101930488    ......C.................T....T...........T.T...........................C.....
LOC102723592    ......C.................T....T...........T.T...........................C.....
LOC102724666    ......C.........C.......T....T...........T.T...........—.....AC.....
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NF1 Ex23 PCR Primers and Pseudogenes

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                    10        20        30        40        50        60        70        80        90       100
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GTAATTGATAAAATAACTGGCCATTCTTTACTGCACACAAACTAGGGTGTGACAGTAAGGTAGCCAGAAGTTGTGTACGTTCTTTCTAAATAAATATCT
NF1P1        ...........T.T..................................C.........A......G......T....CA.AA.                  --
NF1P2        ...........T.T..................................C.........A......G......T....CA.AA.                  --
NF1P4        ...........T.T.................C....AC.........A......A.......T.C...CC.A.............G...
NF1P6        ...........T.T.................C....AC.........A......A.......T.C...CC.A.............G...
NF1P7        ...........T.T.................C....AC.........A......A.......T.C...CC.A.............G...
NF1P8        ...........T.T......................A.A.......A......A.......T.C...CC.AC.
LOC101929972 ...........T.T.................C....AC.........A......A.......T.C...CC.A.............G...
LOC101930150 ...........T.T.................C.........A......G......T....CA.AA.                  --
LOC101930488 ...........T.T.................C.........A......G......T....CA.AA.                  --
LOC102723592 ...........T.T.................C.........A......G......T....CA.AA.
LOC102724666 ...........T.T.....................AC.........A......A.......T.C...CC.A.............

                   110       120       130       140       150       160       170       180       190       200
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TATTGTTTTCAAACTTACATTTAATTCGTTTTACTTGATGACTAAAGTATTTAGAATGCCTTCTCTTTTGTCTATATCTGATAATTTTTTTATTGTTTCT
NF1P1        --.........G.........G..G...A.....T.........C..T....T..................................C..C...
NF1P2        --.........G.........G..A.....T.........C..T....T..................................C...
NF1P4        ..C.........GA...G.......T.....GT.......T.....A...G....G.................................C...
NF1P6        ..C.........G...G..A.....T.....GT.......T.....A...G....G.................C..............C...
NF1P7        ..C.........GA...G.......T.....GT....C.T.......A...G....G...............................C...
NF1P8        ..C.........G.......T.....T.......T.....A...A....G..................................C...
LOC101929972 ..C.........G...G.......T.....GT.......T.....A...G....G.........C..............C...
LOC101930150 --.........G.........G..A.....T.......C..T....T..............................C...
LOC101930488 --.........G.........G..A.....T.......C..T....T..............................C...
LOC102723592 --.........G.......G..G..A.....T.......C..T....T..........................C..C...
LOC102724666 ..C.........TG....GC......T.......GT.......T......A...G....G..............................C...

                   210       220       230       240       250       260       270       280       290       300
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          ATGTCTATATAGGTATGTTCGTGTGCTTGGGAATATGGTCCATGCAATTCAAATAAAAACGAAACTGTGTCAATTAGTTGAAGTAATGATGGCAAGGAGA
NF1P1        G.....G.....................................................G..G.............C..................
NF1P2        G.....G..........A..........................................G..G.............C..................
NF1P4        ......G......C...T..........................................G..G.............C..................
NF1P6        ......G......C...T..........................................G..G.............C..................
NF1P7        ......G......C...T..........................................G..G.............C..................
NF1P8        ......G......C...T..........................................G..G.............C..................
LOC101929972 ......G......C...T..........................................G..G.............C..................
LOC101930150 G.....G.....................................................G..G.............C..................
LOC101930488 G.....G.....................................................G..G.............C..................
LOC102723592 G.....G.....................................................G..G.............C..................
LOC102724666 G.....G.....................................................G..G.............C..................
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                310       320       330       340       350       360       370       380       390       400
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GATGACCTCTCATTTTGCCAAGAGATGA                              AATTTAGGTGAGTTCTCAAAAGAGCAATGTAGGGTCTTGTAAATCTTAA
NF1P1        .............T.............                              ..................................................
NF1P2        .............T.............                              ..................................................
NF1P4        .........G.......G.......                              ..................................................
NF1P6        .................G.......                              ..................................................
NF1P7        .........G.......G.......                              ..............................................TT
NF1P8        .................G.......CCTCTCATTTTGCCAAGAGATAA........                                        G
LOC101929972 .................G.......                              ..................................................
LOC101930150 .............T.............                              ..................................................
LOC101930488 .............T.............                              ..................................................
LOC102723592 .............T.............                              ..................................................
LOC102724666 .................G.......            ...G..            ..................................................

                410       420       430       440       450       460       470       480       490       500
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TATGTCCAATGAAGTACAGAAAAAGAGTAGATATGCGGTTATTGGTAGAAAGGAGGACATGAAAAGAGAGCAATTTACATGTTTGTTTTTCTCTACATCT
NF1P1        .T...TG...........T.........CT........G.....A....A...............C......G........G...
NF1P2        .T...TG...........T.....A..CT........G.....A....A...............G........G...
NF1P4        .T...TG.........TG.......CT........G.....A....A.........G..............GTG...
NF1P6        .T...TG...........T.......CT........G...A.A....A.........G..............GTG...
NF1P7        .T.T.TTTT.TTTT.TTTTTTTTT..AC.GAG.CTC.C..C..TCGCCC...CT.GAG..C.GT.GCG.G.TC.CG───.C.CAC.GCAAG..C.GC..
NF1P8        .T...T.............T.......CT.....C........A....A................TG...
LOC101929972 .T...TG...A.......T.......CT........G...A.A....A.........G..............GTG...
LOC101930150 .T...TG...........T.......CT........G.....A....A.................G........G...
LOC101930488 .T...TG...........T.......CT........G.....A....A.................G........G...
LOC102723592 .T...TG...........T.......CT........G.....A....A............C.....G........G...
LOC102724666 .T...TG.........T...C.....CT....C.........A....A.........G..............GTG...

                510       520       530       540       550       560       570       580       590       600
       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          CTTCTCAAATTTCCCTAAGCTTTGTGCCTGTGACAATGCTCCCTTTTTCTAAAACTGTG─────CTATACTTGAGCTAAG──AATTTGATTCTATTTCCA
NF1P1        ..C..........G.............G...GC.................─.G...........G....
NF1P2        ..C..........G.............G...GC.................─.G...........G....
NF1P4        ..C........................G...GC...........C.─────.G.............C.G....
NF1P6        ..C........................G...GC...........C.────.G...........G....
NF1P7        ─.C.GGG..CA.GCC.TTC.CC....─.GCCTC..AAG.AG..GGG...ACAGGCGC.CGCCAC.AC..CCG.CT....TT..G....TT
NF1P8        ..C........G...............G...GC.................─.G...........G....
LOC101929972 ..C........................G...GC...........C.────.G...........G....
LOC101930150 ..C..........G.............G...GC.................─.G...........G....
LOC101930488 ..C..........G.............G...GC.................─.G...........G....
LOC102723592 ..C..........G.............G...GC....A...........─.G...........G....
LOC102724666 ..C........................G...GC...........C.────.G...........C.G....
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NF1 Ex24 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
NF1 Exon

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              10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1       TGATGGAGGATAAATATCCATGTTGCTTGTTCCCTTCTGGCTTTTATGTCTGTGATAGCAGTATCTCTTTTATAAAGTCGTCATGTCACTTAGGTTATCT
NF1P1     ..T.....T.........C......................C.........C.T...................................C.G.........
NF1P2     ..T.....T.........C......................CA....C.T.........C..........A..........C.GA.........
NF1P4     ........C.G.....C...................CA...A...................................C.G.........
NF1P6     ........C.G.....C...................-...CA...A...............................C.G.........
NF1P7     ........C.G.....C...................-...CA...A...............................C.G.........
NF1P8     ...........C...................-...CA...A...............................C.G.........
LOC101929972 ......C.G.....C...................CA...A...............................C.G.........
LOC101930150 ..T.....T.........C..................C.........C.T...................C.GA.........
LOC101930488 ..T.....T.........C..................CA........C.T...................C.GA.........
LOC102723592 ..T.....T.........C..................C.........C.T...................C.G.........
LOC102724666 ...........C.G.....C...................-...CA...A...............................C.G.........
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              110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1       GGCAAATTATTTGCACTATAAGAAATCTTACGTGACTAAAGGTGTGTGTGTG--------GCTTCAAAAACATTGTTTGCTGTTTCTCTTTTCTC
NF1P1     .C.............G.........ACA.T...............TGTG--------..........A.....G.................
NF1P2     .C.............G.........ACA.T...............TGTG--------..........A.................
NF1P4     .C..........C.....T.....A....G.............GGGG--GGTATGA.....G...A.................
NF1P6     .C..........C.....T.....A.C...G.............GGGG--GGTATGA.....G...A........G........
NF1P7     .C..........C.....T.....A....G.............GGGG--GGTATGA.....G...A.................
NF1P8     .C.............G...T.....A....G.............GGGGTGAGGTGTGA.......A.................
LOC101929972 .C..........C.....T.....A....G.............GGGG--GGTATGA.....G...A.....G........
LOC101930150 .C.............G.........ACA.T...............TGTG--------..........A.................
LOC101930488 .C.............G.........ACA.T...............TGTG--------..........A.................
LOC102723592 .C.............G.........ACA.T...............TGTGT-------G.......A.......G........
LOC102724666 .C..........C.....T.....A....G.............GGGG--GATATGA.....G...A.................
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              210       220       230       240       250       260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1       CACCATTCTATAGGAATAAGATGGTAGAATACCTGACAGACTGGGTTATGGGAACATCAAACCAAGCAGCAGATGATGATGTAAAATGTCTTACAAGGTA
NF1P1     .G.......T..........T...........T...............................A.........
NF1P2     .G.......T..........T...........T...............................A.........
NF1P4     .........................................................................
NF1P6     .........................................................................
NF1P7     .........................................................................
NF1P8     ...................................G.....................................
LOC101929972 .........................................................................
LOC101930150 .G.......T..........T...........T...............................A.........
LOC101930488 .G.......T..........T...........T...............................A.........
LOC102723592 .G.......T..........T...........T...............................A.........
LOC102724666 ......C..................................................................
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                    310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             AAAAA-AGA-ATGACCTTCAAGTATTAGTGGGTTTTACTGTGAGAGTTATAACTACTTAATTACAGCTTTATACTTGTATTTTGTGTGTATTTAAACTTT
NF1P1           ....G...-.....................C...A...A.....T....C.........................A.................T...
NF1P2           ....G...-.....................C...A...A.....T....CC........................A.................T...
NF1P4           ....A...---...T.....A..................A........C.........................A......................
NF1P6           ....A...---...T.....A..................A........C.........................A......................
NF1P7           ....A...---...T.....A..................A........C.........................A......................
NF1P8           ....A...--.T..C.A..................A..G.........C.........................A......................
LOC101929972    ....A...--....T.....A..................A........C.........................A......................
LOC101930150    ....G...-.....................C...A...A.....T....CC........................A.................T...
LOC101930488    ...G...-.....................C...A...A.....T....CC........................A.................T...
LOC102723592    ....G...-.....................C...A...A.....T....C.........................A.................T...
LOC102724666    ....A.A.A.....T.....A..................A........C.........................A......................

                    410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             TGAGATGTCAAACTTTTGTGTTTGAAATATGTAAAGATGCTAATCTTTATTACTGCTTTTTTTTGACTGATAGACTTTCAGTAAAATTAAATGTGAAAGA
NF1P1           .T...............G.................A..............A.................T......A.........CG...
NF1P2           .T...............G.................A..............A.................T......A.........CG...
NF1P4           .T.....A.........CA.......CA..........A.....C.....C...............G....A.....A..CG...
NF1P6           .T.....A.........CA.......CA..........A.....C.....C...............G....A.......CG...
NF1P7           .T.....A....C....CA.......CA..........A.....C.....C...............G....A.......CG...
NF1P8           .T.....A.........CA.......CA..........A.....C.....C...............G....A.......CG...
LOC101929972    .T.....A.........CA.......CA..........A.....C.....C...............G....A.......CG...
LOC101930150    .T...............G.................A..............A.................T......A.........CG...
LOC101930488    .T...............G.................A..............A.................T......A.........CG...
LOC102723592    .T...............G.................A..............A.................T......A.........CG...
LOC102724666    .T.....A.........CA.......CA..........A.....C.....C...............G....A.......CG...
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NF1 Ex25 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

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                    10        20        30        40        50        60        70        80        90       100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            AATGAAATAGGTAGTTCCTAAGGTTTATATCTGTTAGTAAGAGGTTTATTTGAGGGGAAGTGAAAGAACTTGAAAGATTCATGGTCTCTAAATTTTTTTT
NF1P1          ......C.............A.............A..............A.........T...C.........T..........
NF1P2          ......C.............A.............A..............A.........T...C.........T..........
LOC101930150   ......C.............A.............A..............A.........T...C.........T..........
LOC101930488*  ......C.............A.............A..............A.........T...C.........T..........
LOC101930488#  .G.A..T.TCAGTCAC.AA..AACAC.A.G.AAAAGCA..T.AAGCCCCCC.NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
LOC102723592   ......C.............A.............A..............A.........T...C.........T..........

                   110       120       130       140       150       160       170       180       190       200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            TTTTTTTTTTTTTTTTTTTCAGAGATTTGGACCAGGCAAGCATGGAAGCAGTAGTTTCACTTCTAGCTGGTCTCCCTCTGCAGCCTGAAGAAGGGAGATGGT
NF1P1          ------.............................................................G.........G............
NF1P2          ------.............................................................G.........G............
LOC101930150   ------.............................................................G.........G............
LOC101930488*  -----.............................................................G.........G............
LOC101930488#  ----NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN.......G.........G............
LOC102723592   ------.............................................................G.........G............

                   210       220       230       240       250       260       270       280       290       300
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            GTGGAATTGATGGAAGCCAAAT  CACAGTTATTTCTTAAGTAAATTTCAGTCACCAAAAAACATAAAGCAAAAGCAAATAAAGCCCCCCAC
NF1P1          .....................GGGGA........................C...................G.CGCCA
NF1P2          .....................GGGGA........................C...................G.CGCCA
LOC101930150   .....................GGGGA........................C...................G.CGCCA
LOC101930488*  ....................GGGGA........................C...................GNNNNNN
LOC101930488#  ....................GGGGA........................C...................G.CGCCA
LOC102723592   .....................GGGGA........................C...................G.CGCCA
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*LOC101930488 (648692 to 648812)
#LOC101930488 (648468 to 648540)

NF1 Ex26 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

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                    10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TACTAGGCTATATCAGGTAAAATCATGTCCAACATAGCACACTTCATAATAAGCCACCCTGGCTGATTATCGCGAGAGAGGAGAGAAACAGTTAACCCAG
NF1P1         .G...........T......C.....C...TG.........G..T................CT.............G......T....
NF1P2         .G...........T......C.....C...TG.........G..T................CT.............G......T....
LOC101930150  .G...........T......C.....C...TG.........G..T................CT.............G......T....
LOC101930488  .G...........T......C.....C...TG.........G..T................CT.............G......T....
LOC102723592  .G...........T......C.....C...TG.........G..T................CT.............G......T....

                   110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GGCCATTCACACCATGCACATATGATTGTTTTGGAATGTCTGGTTAGCTTTCTAGTTGATACGGCCTTCACTATGTAAAGGTCAGTCTTTTTATTTCTCA
NF1P1         .....G......G......G....A...............................TG............................
NF1P2         .....G......G......G....A...............................TG............................
LOC101930150  .....G......G......G....A...............................TG............................
LOC101930488  .....G......G......G....A...............................TG............................
LOC102723592  .....G......G......G....A...............................TG............................

                   210       220       230       240       250       260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GATACTTCACATTATTTATGAACCTTTTGAATGACTGCAGTGAAGTTGAAGATGAAAGTGCGCAAACAGGTGGCAGGAAACGTGGCATGTCTCGGAGGCT
NF1P1         ......C...............G.............C.................A...........................T.
NF1P2         ......C...............G.............C.................A...........................T.
LOC101930150  ......C...............G.............C.................A...........................T.
LOC101930488  ......C...............G.............C.................A...........................T.
LOC102723592  ......C...............G.............C.................A...........................T.

                   310       320       330       340       350       360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GGCATCACTGAGGCACTGTACGGTCCTTGCAATGTCAAACTTACTCAATGCCAACGTAGACAGTGGTCTCATGCACTCCATAGGTGAGATCAAATGAAAG
NF1P1         .........G.........G.A.................T.G....G.A....G....................
NF1P2         .........G.........G.A...............G....G.A....G....................
LOC101930150  .........G.........G.A...............G....G.A....G....................
LOC101930488  .........G.........G.A...............G....G.A....G....................
LOC102723592  .........G.........G.A...............G....G.A....G....................

                   410       420       430       440       450       460       470       480       490       500
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TTTCATATAGAAATACAAAACCTAGAGAACTGGCATGTAAGAGAAGCAAAAATTACTTCAGCAAGGCCATGTTAGTAAATTTGCATCTGTTTGTCCACAT
NF1P1         ...............C.G.........T.....T.........T.........CA.........
NF1P2         ...............C.G.........T.....T.........T.........CA.........
LOC101930150  ...............C.G.........T.....T.........T.........CA.........
LOC101930488  ...............C.G.........T.....T.........T.........CA.........
LOC102723592  ...............C.G.........T.....T.........T.........CA.........
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NF1 Ex27 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

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               10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          ATCACTGAGGCACTGTACGGTCCTTGCAATGTCAAACTTACTCAATGCCAACGTAGACAGTGGTCTCATGCACTCCATAGGTGAGATCAAATGAAAGTTT
NF1P1        .....G.........G.A...............T.G.....G.A....G.....................................................
NF1P2        .....G.........G.A...............G.....G.A....G.....................................................
LOC101930150 .....G.........G.A...............G.....G.A....G.....................................................
LOC102723592 .....G.........G.A...............G.....G.A....G.....................................................

              110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          CATATAGAAATACAAAACCTAGAGAACTGGCATGTAAGAGAAGCAAAAATTACTTCAGCAAGGCCATGTTAGTAAATTTGCATCTGTTTGTCCACATTAG
NF1P1        ..................C.G.........T.......T..............T..................CA..........C..
NF1P2        ..................C.G.........T.......T..............T..................CA..........C..
LOC101930150 ..................C.G.........T.......T..............T..................CA..........C..
LOC102723592 ..................C.G.........T.......T..............T..................CA..........C..

              210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GCTTAGGTTACCACAAGGATCTCCAGACAAGAGCTACATTTATGGAAGTTCTGACAAAAATCCTTCAACAAGGCACAGAATTTGACACACTTGCAGAAAC
NF1P1        .T.....G...T.........................................TG.......C.....................................
NF1P2        .T.....G...T.........................................TG.......C.....................................
LOC101930150 .T.....G...T.........................................TG.......C.....................................
LOC102723592 .T.....G...T.........................................TG.......C.....................................

              310       320       330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          AGTATTGGCTGATCGGTTTGAGAGATTGGTGGAACTGGTCACAATGATGGGTGATCAAGGAGAACTCCCTATAGCGATGGCTCTGGCCAATGTGGTTCCT
NF1P1        G.........G...CA.............G.............G.....................G.
NF1P2        G.........G...CA.............G.............G..........T........G.
LOC101930150 G.........G...CA.............G.............G..........T.....AA.G.
LOC102723592 G.........G...CA.............G.............G.....................G.

              410       420       430       440       450       460       470       480       490       500
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TGTTCTCAGTGGGTAAGTGATTAGAGTAAGCGGGGAAGAAAAGTGCCTGGCACATAGCAAATCCTTCAGAATATATTTGTTCAATAAATGTTTGTTGAAT
NF1P1        .................CA.........T.........................................C..
NF1P2        .................CA.........T..............T..........................C..
LOC101930150 .................CA.........T.........................................C..
LOC102723592 .................CA.........T.........................................C..

              510       520       530       540       550       560       570       580       590       600
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GAATTGATAAAATTTCAGAGCCAGAAGAAA   GATGTTTAGTTAGGTGATTTTTCAGCTGTAGGGAAGTGGTTGGCACCACTAGACCTGACTAGTGT
NF1P1        ................T.............TCTTA...........C...............TG.........A.....
NF1P2        ................T.............TCTTA...........C...............TG.........A.....
LOC101930150 ................T.............TCTTA...........C...............TG.........A.....
LOC102723592 ................T.............TCTTA...........C...............TG.........A.....
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NF1 Ex28 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
                     10        20        30        40        50        60        70        80        90       100
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           ATTTAGCAAGTGGTTGTCAACTTTGGGTTTACATTTTTGCTACTCTTTAGCTTCCTACCTAAGAATAAAAATGGGATTGTTTGCACTAACCTGATTTTGT
NF1P1         ..........A..........TGA......C.....C....AT......G....C....----------...........
NF1P2         ..........A..........TG.......C.....C....AT......G....C....----------...........
LOC101930150  ..........A..........TG.......C.....C....AT......G....C....----------...........
LOC102723592  ..........A..........TG.......C.....C....AT......G....C....----------...........

                    110       120       130       140       150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TTTGTTCTCAGGATGAACTAGCTCGAGTTCTGGTTACTCTGTTTGATTCTCGGCATTTACTCTACCAACTGCTCTGGAACATGTTTTCTAAAGAAGTAGA
NF1P1         ..............G..T...........A...G...T.....................G...........
NF1P2         ..............G..T...........A...G...T.....................G...........
LOC101930150  ..............G..T...........A...G...T.....................G...........
LOC102723592  ..............G..T...........A...G...T.....................G...........

                    210       220       230       240       250       260       270       280       290       300
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           ATTGGCAGACT   CCATGCAGACTCTCTTCCGAGGCAACAGCTTGGCCAGTAAAATAATGACATTCTGTTTCAAGGTTTGTATCATTCATTTTGTGTG
NF1P1         ..........GACT....T.........C.T.A......T...........C...................
NF1P2         ..........GACT....T.........C.T........T...........C...................
LOC101930150  ..........GACT....T.........C.T........T...........C...................
LOC102723592  ..........ACTG......T.......C.T........T...........C...................

                    310       320       330       340       350       360       370       380       390       400
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TATGTGTGTGCTGAGGTATGTCAAGTAATGATTATGTCAGAATGTGCAGGGCTGATTGTCTTCTTTTAAGGTAAAATATATGGAGCAGGTATAATAAAC
NF1P1         _.....................................A.................G..........C....G..G
NF1P2         _.....................................A.................G..........C....G..G
LOC101930150  _.....................................A.................G..........C....G..G
LOC102723592  _.....................................A.................G..........C....G..G
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NF1 Ex29 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

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                10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TAGCTCGAGTTCTGGTTACTCTGTTTGATTCTCGGCATTTACTCTACCAACTGCTCTGGAACATGTTTTCTAAAGAAGTAGAATTGGCAGA----CTCCA
NF1P1         ─...T.........A...G....T.........................................G................CTGA.....
NF1P2         ─...T.........A...G....T.........................................G................CTGA.....
LOC101930150  ─...T.........A...G....T.........................................G................CTGA.....
LOC102723592  ─...T.........A...G....T.........................................G................CTGA.....

                110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TGCAGACTCTCTTCCGAGGCAACAGCTTGGCCAGTAAAATAATGACATTCTGTTTCAAGGTTTGTATCATTCATTTTGTGTGTATGTGTGTGCTGAGGTA
NF1P1         .T.........C..T..A.....T..................................C...........................─.......
NF1P2         .T.........C..T.........T..................................C...........................─.......
LOC101930150  .T.........C..T.........T..................................C...........................─.......
LOC102723592  .T.........C..T.........T..................................C...........................─.......

                210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TGTCAAGTAATGATTATGTACAGAATGTGCAGGGCTGATTGTCTTCTTTTAAGGTAAAATATATGGAGCAGGTATAATAAACTCCTATTCGTGCATTTCT
NF1P1         ..........................A.....................G.........C.....G..G.....C..A.........
NF1P2         ..........................A.....................G.........C.....G..G.....C..A.........
LOC101930150  ..........................A.....................G.........C.....G..G.....C..A.........

                310       320       330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GTAGGTATATGGTGCTACCTATCTACAAAAACTCCTGGATCCTTTATTACGAATTGTGATCACATCCTCTGATTGGCAACATGTTAGCTTTGAAGTGGAT
NF1P1         A............T.........G.........A.........G...........
NF1P2         A............T.........G.........A.........G...........
LOC101930150  A............T.........G.........A.........G...........
LOC102723592  A............T.........G.........A.........G...........

                410       420       430       440       450       460       470       480       490       500
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           CCTACCAGGTTTGTCATCTTTTCACATAGAACCGCTGTTTTTTGTTTTTTTTTTTTTGTTTGTTTGTTTTACTAACACTGCATGAAGCAAGGCAC─
NF1P1         .......T...A.............A..GT...C......T..........─.....................G...................C.TCATA
NF1P2         .......T...A.............A..GT...C......T..........─.....................G...................C.TCATA
LOC101930150  .......T...A.............A..GT...C......T..........─.....................G...................C.TCATA
LOC102723592  .......T...A.............A..GT...C......T..........─.....................G...................C.TCATA

                510       520       530       540       550       560       570       580       590       600
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           CTTCTCCCCTTGATCATTAAAATTAGTTTTTAATTATAAAAGTTATATACAAATACACGTTTCTTTAATGATATCTGTAATTTTTTTTTTAAGGTATCCT
NF1P1         A.......T.............T................TG.T.................─...T.....
NF1P2         A.......T.............T................TG.T.................─...T.....
LOC101930150  A.......T.............T................TG.T.................─...T.....
LOC102723592  A.......T.............T................TG.T.................─...T.....
```

NF1 Ex32 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
              10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          GATTGATTCAGAGTTTTTATGCAAAGTTTGACCTTTGAACTCTTTGTTTTCATGTCTTTATATTAATTCAAACCTTATACTCAATTCTCAACTCCTTGTT
NF1P1        ......C........G.........T.................CC....C.CC...T...................................G........ ....
NF1P2        ......C........G..G......T.................CC....C.CC...T...................................G........ ....
LOC101930150 ......C........G..G......TG................CC....C.CC...T...................................G........ ....
LOC102723592 ......C........G..G......T.................CC....C.CC...T...................................G........ ....

              110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          TTTAGGTGGTTAGCCAGCGTTTCCCTCAGAACAGCATCGGTGCAGTAGGAAGTGCCATGTTCCTCAGATTTATCAATCCTGCCATTGTCTCACCGTATGA
NF1P1        ......G.........A...........T...A.....C.....................G.......A...........T.....
NF1P2        ......G.........A...........T...A.....C.....................G.......A...........T.....
LOC101930150 ......G.........A...........T...A.....C.....................G.......AT..........T.....
LOC102723592 ......G.........AT..........T...A.....C.....................G.......A...........T.....

              210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1          AGCAGGGATTTTAGATAAAAAGCCACCACCTAGAATCGAAAGGGGCTTGAAGTTAATGTCAAAGGTGAATTATTTTTGATAATCTAGCTATCTTAAATTCC
NF1P1        ...................G................G.........................................................
NF1P2        ....................................G.........................................................
LOC101930150 ....................................G.........................................................
LOC102723592 ....................................G.........................................................
```

NF1 Ex33 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
                    10        20        30        40        50        60        70        80        90       100
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TTTTAATATGTATTTTTACATGTAAATATAATAATTATATTTGGGAAGGTTAGAAACACTACCTAAAATAATTTATAGAATGAGGAATGTTTGATTTTTA
NF1P1         .................G..........................A..A.....AA.........................----.........A...A.......-.
NF1P2         .................G..........................A..A.....AA.........................----.........A...A.......-.
LOC101930150  .................G..........................A..A.....AA.........................----.........A...A.......-.
LOC102723592  .................G..........----.........A..A.....AA.........................----.........A...A.......-.

                   110       120       130       140       150       160       170       180       190       200
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           AGTACTAGCAGAAATTATATCAATGAGAAAATTCATGTTTTTAAAGAATGTCTTAATGTATAGACTTCATACAATAAATAATCTGATTATTTATAACCCT
NF1P1         .C..................---........A..............A..A..........A...............G...........
NF1P2         .C..................---........A..............A..A..........A...............G...........
LOC101930150  .C...............---...A........A..............A..A..........A..........................
LOC102723592  .C..................---........A..............A..A..........A..........................

                   210       220       230       240       250       260       270       280       290       300
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           GTTTTATTGTGTAGATACTTCAGAGTATTGCCAATCATGTTCTCTTCACAAAAGAAGAACATATGCGGCCTTTCAATGATTTTGTGAAAAGCAACTTTGA
NF1P1         ............A..................................G...........................G......
NF1P2         ............A..................................G...........................G......
LOC101930150  ............A..................................G...........................G......
LOC102723592  ............A..................................G...........................G......

                   310       320       330       340       350       360       370       380       390       400
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1           TGCAGCACGCAGGTAATTTTCTTGCCACTTACTCAGTTGCTCTGTTTGAATCAAATATTTTCGGTTTCACATAAATCCATGTACCTGTTTTACATGAAGT
NF1P1         ......TT.A......GC.A..........T...C..........................A...................
NF1P2         ......TT.A......GC.A..........T...C..........................A...................
LOC101930150  ......TT.A......GC.A..........T...C..........................A...................
LOC102723592  ......TT.A......GC.A..........T...C..........................A...................
```

NF1 Ex34 PCR Primers and Pseudogenes

<mark>PCR Primers</mark>
<mark>NF1 Exon</mark>

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                   10        20        30        40        50        60        70        80        90       100
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            AAGCTGAAGCCGGGTATCAGAAATGGAAAGCCAACTTTCTCCTTGTCCTTTTTGCTTTGTCTAATGTCAAGTCACATTGTGTGAACAAGCCCTCCATATT
NF1P1          -.........A................................................A.................A...A.........G..................
NF1P2          -.........A................................................A.................A...A.........G..................
LOC101930150   -.........A................................................A.................A...A.....G....G..................
LOC102723592   -.........A................................................A.................A...A.........G..................

                  110       120       130       140       150       160       170       180       190       200
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            TGTAA----TCTTAGTTACTTCACAAAGTTACTTCTTATAAATTTAATTCAAACATAAGTCTGG-GTGTATCTGGTGTTGAAAATTCTAATGACTTTG
NF1P1          ....TTGTCA...C...G.................G...................................A...T....C.....................G........
NF1P2          ....TTGTCA...C...G.................G...................................A...T....C.....................G........
LOC101930150   ....TTGTCA...C...G.................G...................................A...T....C.....................G........
LOC102723592   ....TTGTCA...C...G.................G...................................A...T....C.....................G........

                  210       220       230       240       250       260       270       280       290       300
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            CATTTTTGAAGGTTTTTCCTTGATATAGCATCTGATTGTCCTACAAGTGATGCAGTAAATCATAGTCTTTCCTTCATAAGTGACGGCAATGTGCTTGCTT
NF1P1          ......CT..T.....T........G.............................................C..........GC...............C...
NF1P2          ......CT..T.....T........G.............................................C..........GC...............C...
LOC101930150   ......CT..T.....T........G.............................................C..........GC...............C...
LOC102723592   ......CT..T.....T........G...........................................C..C..........GC...............C...

                  310       320       330       340       350       360       370       380       390       400
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            TACATCGTCTACTCTGGAACAATCAGGAGAAAATTGGGCAGTATCTTTCCAGCAACAGGTAAGATTTCCCAGTCATGGGGATAGTGAACACTCTCCGTTT
NF1P1          ......A......C................C...G.....T................................A.............T.....A...
NF1P2          ......A......C................C...G.....T................................A.............T.....A...
LOC101930150   ......A......C................C...G.....T.A..............................A.............T.....A...
LOC102723592   ......A......C................C...G.....T................................A.............T.....A...
```

NF1 Ex35 PCR Primers and Pseudogenes

PCR Primers
NF1 Exon

```
                10        20        30        40        50        60        70        80        90        100
                |         |         |         |         |         |         |         |         |         |
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             GATGGGAGTTTAAATGACAGGGCATTTTAATCTTTTATGAATTATTAATGGATTGAAGTAGACATGGTCCTGAGGTCTTTTTGGTGCTGTTTACAAATCA
NF1P1           .........CC....A.........C.........................G............G..A...........
NF1P2           .........CC....A.........C.........................G............G.............
LOC101930150    .........CC....A.........C.........................G............G.............
LOC102723592    .........CC....A.........C.........................G............G.............

                110       120       130       140       150       160       170       180       190       200
                |         |         |         |         |         |         |         |         |         |
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             --------GCTGACAGTAAAAGGAAAAGCAACCAGTTACAAGTTAAAGAAATGTGTAGTGCTAAATGTGAACTGCTAATTTTTTTTCTAAGTAGT
NF1P1           ACAGGGACCACCA.........T...................................C......................C.........
NF1P2           ACAGGGACCACCA.........T.................._.................C......................C.........
LOC101930150    ACAGGGACCACCA.........T.................._.................C......................C.........
LOC102723592    ACAGGGACCACCA.........T...................................C......................C.........

                210       220       230       240       250       260       270       280       290       300
                |         |         |         |         |         |         |         |         |         |
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             TTGCTGTATCTAGGGATCATAAAGCTGTTGGAAGACGACCTTTTGATAAGATGGCAACACTTCTTGCATACCTGGGTCCTCCAGAGCACAAACCTGTGGC
NF1P1           .....A................................T...............T......C.......................
NF1P2           .....A................................T...............T......C.......................
LOC101930150    .....A................................T...............T......C.......................
LOC102723592    .....A................................T...............T......C.......................

                310       320       330       340       350       360       370       380       390       400
                |         |         |         |         |         |         |         |         |         |
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             AGATACACACTGGTCCAGCCTTAACCTTACCAGTTCAAAGTTTGAGGAATTTATGACTAGGTAAAGTACAACCTTGAAATAGTTGATTGCTTTCTTTTTG
NF1P1           .......T..C.......................A...............TG..........................A...........
NF1P2           .......T..C.......................A...............TG..........................A...........
LOC101930150    .......T..C.......................A...............TG.........................A..C........
LOC102723592    .......T..C.......................A...............TG..........................A...........

                410       420       430       440       450       460       470       480       490       500
                |         |         |         |         |         |         |         |         |         |
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1             GTTGAGAAGGAGAGTTTGCCACCAGGCCACTTGTTAGATATGATAGAAGACTATGAGGAAAGATGTATTTAATAATCACATTGCCATGTTTGGGGAATCC
NF1P1           ........C......A...T..................................C..C.......................CC.A
NF1P2           ........C......A...T..................................C..C.......................CC.A
LOC101930150    ........C......A...T..................................C..C.......................CC.A
LOC102723592    ........C......A...T..................................C..C.......................CC.A
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NF1 Ex36 PCR Primers and Pseudogenes

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                  10        20        30        40        50        60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            TTTTTCATTTTAGTATTTTATTGTTTATCCAATTATAGACTTTTTTACATACTCAGTAGACAACATAAAGCCTCATAATTACTCTGTTATTTTTCTTTTA
NF1P1          .........C.............G...G......................C.........C.G....C....C...........
NF1P2          .........C.............G...G......................C.........C.G....C....C...........
NF1P4          .....T..............................A.......................G.GG...........C.........
NF1P6          .....-..............................A.......................G.GG...........C.........
NF1P7          .....T..............................A.......................G.GG....G....C...........
NF1P8          .....-..............................A.......................G.GG...........C.........
LOC101929972   .....-..............................A.......................G.GG...........C.........
LOC101930150   .........C...............G.G..G..............C.........C.G....C....C...........
LOC102724666   .....T..............................A.......................G.GG...........C.........

                 110       120       130       140       150       160       170       180       190       200
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            GGCATCAGGTACATGAAAAAGAAGAATTCAAGGCTTTGAAAACGTTAAGTATTTTCTACCAAGCTGGGACTTCCAAAGCTGGGAATCCTATTTTTTTATTA
NF1P1          ..........TG.........GA.........A..........T..........A...........G..A.......
NF1P2          ..........TG.........GA.........A..........T..........A...........G..A.......
NF1P4          ..........C.........GA...................G..............
NF1P6          ..........C.........GA...................G..............
NF1P7          ..........C.........GA...................G..............
NF1P8          ..........CA.........G..............
LOC101929972   ..........C.........GA...................G..............
LOC101930150   ..........TG.........GA.........A..........T..........A...........G..A.......
LOC102724666   ..........C.........GA...................G..............

                 210       220       230       240       250       260       270       280       290       300
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NF1            TGTTGCACGGAGGTAAGAAATACTATGTTTTGG-GTC----TCTTAACAGAATTTTTTAAATTATAGCAAATATAGAGAGATGGCAAGTTTGGTTTTTCCC
NF1P1          ......A......C.............T....-------.........G...T.......................T
NF1P2          ......A......C.............T....-------.........G...T.......................T
NF1P4          ..C.........G.T-...TCC...C..........CG......T..............AT
NF1P6          ..C.........G.T-...TCC...C..........CG......T..............AT
NF1P7          ..C.........G.T-...TCC...C..........CG......T..............AT
NF1P8          ..C.........G.T-...TCC...C.....C...G......T........C.....AT
LOC101929972   ..C.........G.T-...TCC...C..........CG......T..............AT
LOC101930150   ......A......C...........T....-------.........G...T.......................
LOC102724666   ..C.........GCT-...TCC...C..........CG......T..............A.
```
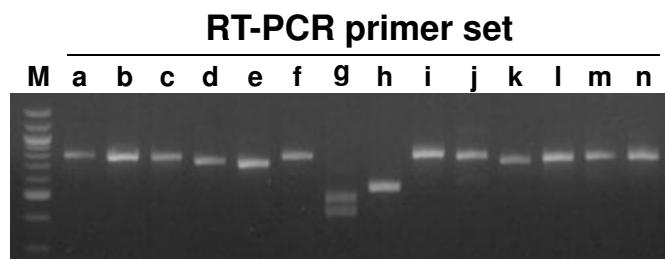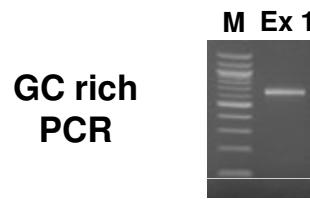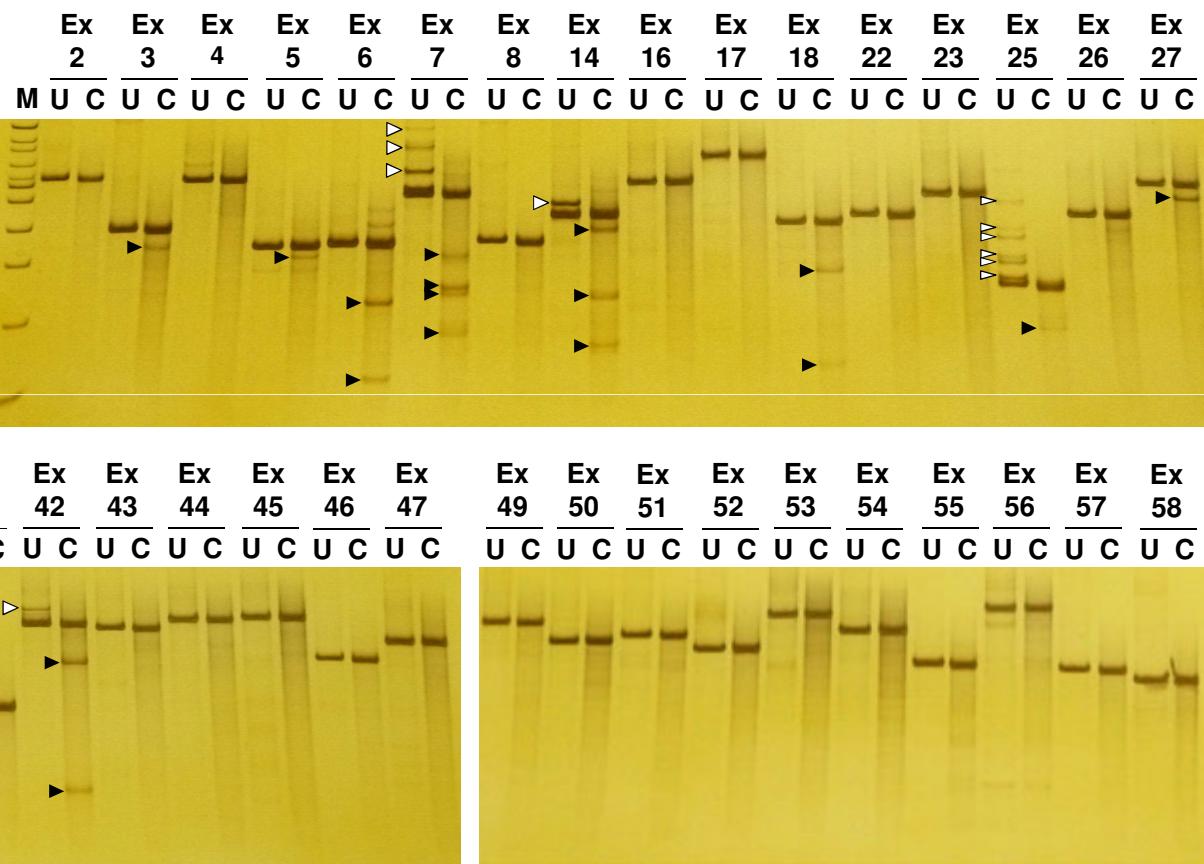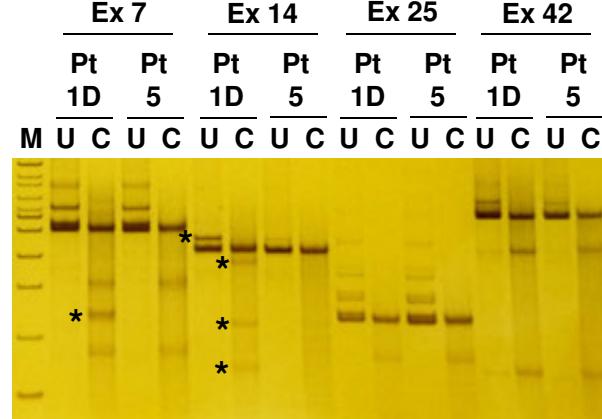
Supplement Table 1
NF1 and Pseudogenes. Human BLAST results by more dissimilar sequences (discontiguous megablast).

Gene symbol	Official Full Name/Gene description	Entrez Gene ID	Chr.	GenBank Accsession	Exon 9	Exon 10	Exon 11	Exon 12	Exon 13	Exon 14	Exon 15	Exon 16	Exon 17	Exon 18	Exon 19	Exon 20	Exon 21	Exon 22	Exon 23	Exon 24	Exon 25	Exon 26	Exon 27	Exon 28	Exon 29	Exon 30	Exon 31	Exon 32	Exson33	Exon 34	Exon 35	Exon 36
NF1	neurofibromin 1	4763	17	NC_000017.11	31200422 > 31200595	31201037 > 31201159	31201411 > 31201485	31206240 > 31206371	31214451 > 31214585	31219005 > 31219118	31221850 > 31221929	31223444 > 31223567	31225095 > 31225095	31226435 > 31226684	31227218 > 31227291	31227523 > 31227606	31229025 > 31229465	31229835 > 31229974	31230260 > 31230382	31230842 > 31230925	31232073 > 31232189	31232700 > 31232881	31233002 > 31233213	31235611 > 31235772	31235918 > 31236021	31248984 > 31249119	31252938 > 31253000	31258344 > 31258502	31259032 > 31259129	31260369 > 31260515	31261711 > 31261857	31265229 > 31265339
NF1P1	neurofibromin 1 pseudogene 1	100419006	15	NC_000015.10	x	x	x	x	x	x	x	x	x	20935435 < 20935701	20934827 < 20934899	20934509 < 20934592	20932681 < 20933120	20932183 < 20932322	20931778 < 20931900	20931231 < 20931314	20929957 < 20930077	20929197 < 20929378	20928869 < 20929074	20926607 < 20926772	20926360 < 20926463	x	x	20923777 < 20923933	20923174 < 20923265	20921785 < 20921931	20920416 < 20920557	20916690 < 20916797
NF1P2	neurofibromin 1 pseudogene 2	440225	15	NC_000015.10	x	x	x	x	x	x	x	x	x	21946418 < 21946641	21945810 < 21945882	21945492 < 21945575	21943662 < 21944101	21943164 < 21943302	21942760 < 21942881	21942213 < 21942296	21940939 < 21941059	21940179 < 21940360	21939851 < 21940056	21937589 < 21937754	21937342 < 21937445	x	x	21934754 < 21934910	21934151 < 21934242	21932762 < 21932908	21931402 < 21931543	21927655 < 21927762
NF1P3	neurofibromin 1 pseudogene 3	4764	21	NC_000021.9	14000927 > 14001098	14001542 > 14001655	14001910 > 14001984	x	x	x	14005200 > 14005279	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
NF1P4	neurofibromin 1 pseudogene 4	100419029	14	NC_000014.9	x	x	x	x	19492280 > 19492414	x	x	19492794 > 19492918	19494437 > 19494591	19495766 > 19496013	19496629 > 19496697	19496933 > 19497015	19498428 > 19498868	19499246 > 19499373	19499668 > 19499790	19500259 > 19500342	x	x	x	x	x	x	x	x	x	x	x	19501620 > 19501728
NF1P5	neurofibromin 1 pseudogene 5	4768	18	NC_000018.10	14157169 < 14157342	14156605 < 14156727	14156283 < 14156357	x	x	x	14152997 < 14153076	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
NF1P6	neurofibromin 1 pseudogene 6	644637	22	NC_000022.11	x	x	x	x	15631915 < 15632049	x	x	15631411 < 15631535	15629736 < 15629890	15628330 < 15628578	15627647 < 15627715	15627330 < 15627412	15625472 < 15625911	15624955 < 15625094	15624538 < 15624660	15623986 < 15624069	x	x	x	x	x	x	x	x	x	x	x	15622601 < 15622709
NF1P7	neurofibromin 1 pseudogene 7	100158257	14	NC_000014.9	x	x	x	x	18909539 < 18909673	x	x	18909035 < 18909159	18907362 < 18907516	18905942 < 18906189	18905258 < 18905326	18904940 < 18905022	18903087 < 18903527	18902582 < 18902709	18902165 < 18902287	18901326 < 18901409	x	x	x	x	x	x	x	x	x	x	x	18899940 < 18900048
NF1P8	neurofibromin 1 pseudogene 8	401007	2	NC_000002.12	x	x	x	x	131197873 < 131198005	x	x	131197370 < 131197493	131195689 < 131195843	131194099 < 131194345	131193519 < 131193591	131193201 < 131193284	131191343 < 131191783	131190826 < 131190965	131190419 < 131190534	131189834 < 131189917	x	x	x	x	x	x	x	x	x	x	x	131188448 < 131188556
LOC100418983	neurofibromin 1 pseudogene	100418983	12	NC_000012.12	x	x	x	x	x	x	x	x	x	x	x	x	38206754 < 38207192	38206235 < 38206374	x	x	x	x	x	x	x	x	x	x	x	x	x	x
LOC101929972	putative neurofibromin 1-like protein 4/6-like	1019929972	14	NC_000014.9	x	x	x	x	x	x	x	x	x	x	x	x	18712361 < 18712643	18711844 < 18711983	18711427 < 18711549	18710875 < 18710958	x	x	x	x	x	x	x	x	x	x	x	18709490 < 18709598
LOC101930150	neurofibromin-like	101930150	15	NC_000015.10	x	x	x	x	x	x	x	x	x	21509131 < 21509354	21508523 < 21508595	21508205 < 21508288	21506375 < 21506814	21505877 < 21506015	21505473 < 21505594	21504926 < 21505009	21503652 < 21503772	21502898 < 21503079	21502570 < 21502775	21500308 < 21500473	21500061 < 21500164	x	x	21497474 < 21497630	21496871 < 21496962	21495482 < 21495628	21494110 < 21494251	21490362 < 21490469
LOC101930488	uncharacterized LOC101930488	101930488	15	NC_000015.10	x	x	x	x	x	x	x	x	x	x	x	x	x	650917 < 651056	650513 < 650634	649966 < 650049	648692 < 648812 ; 647717 < 647898	647717 < 647898	x	x	x	x	x	x	x	x	x	x
LOC102723592	neurofibromin-like	102723592	15	NT_187382.1	x	x	x	x	x	x	x	x	x	x	x	x	233517 < 233956	233019 < 233158	232614 < 232736	232065 < 232148	230790 < 230910	230024 < 230205	229696 < 229901	227434 < 227599	227187 < 227290	x	x	224598 < 224754	223995 < 224086	222606 < 222752	221244 < 221385	x
LOC102724666	uncharacterized LOC102724666	102724666	14	NC_000014.9	x	x	x	x	x	x	x	x	x	19612191 > 19612345	19613520 > 19613767	19614378 > 19614450	19614685 > 19614767	19616177 > 19616617	19616995 > 19617122	19617416 > 19617538	19618006 > 19618089	x	x	x	x	x	x	x	x	x	x	19619371 > 19619479
MED15P6	mediator complex subunit 15 pseudogene 6	100996438	14	NC_018925.2	x	x	x	x	20078032 > 20078125	x	x	x	x	19493296 < 19493483	20082425 > 20082475	20082745 > 20082827	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

Numbers in each exon box indicates nucleotide positons of reference sequence.
x, No homologous sequence
Exon 31 is an alternatively splicing exon.

Supplymentary Table 2
NF1 DNA Primers.

Exon	Ta (°C)	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	Remarks
1	60	AGACGGCCCAGAGGAGTTA	CCTCCCCTCACCTACTCTGTC	KOD -Plus- Ver.2 for GC rich PCR
2	60	TGTGGTTGATGCAGTTTTCC	AAAAGGAGACATGGTCACAAAG	
3	60	ATGCCATTTCTGTTTGCCTTAG	TCCACATCTGTACTTTGGGACA	
4	60	TGATTATGGGGTCAGAGATGG	AGGGGCAAAAGAACTTGTCTC	
5	60	AAGTGGTCCTCCTGCCTTG	AATCCAGTTTGGTGTTCTAGTTCAG	
6	60	AGCAGACAACTATCGAGTTTTGG	ATTCAGGATGCTAACAACAGCA	
7	60	AAAATTTGCTGTTGTTAGCATCC	AAGGAAAGGAAGAGGGTTGG	
8	60	TTTAATGCCAGGGATTTTGTTC	ATGGTTCCCTGAAATACTTTGCT	
9	58	CTGTTAATTTGCTATAATATTAGC	CATAATACTTATGCTAGAAAATTC	according to Bausch et al.[a]
10	58	GTAATGTGTTGATGTTATTACATG	GTCTTTTTGTTTATAAAGGATAACA	according to Bausch et al.[a]
11	58	TTGAAGTTCGTTTCAAGACC	ACGCAAAGAAAAGAAAGAAAA	according to Han et al.[b]
12	58	ACTTTGGAAATCATGGTGTGTG	GCAGCAGGAATAGGATAAAGACA	
13	58	TCCTGAGTCTTATGTCTGATACCAT	TTGGCGTTTCAGCTAAACCC	according to Bausch et al.[a]
14	60	CAAAGTCCTGGGCTTACAGG	ACCACACACCAAAGGAACATC	
15	58	CTATATATTGAAACTACAAATGAAA	AGTTTTACAATGTACTAAGTACTAC	according to Bausch et al.[a]
16	60	CTGGGAGTTTAATGAAACATAGGG	GAGAACATTGGGAGGAAGGAG	
17	60	TCCTTCAAGTTGGGGCATAG	ACAACTCTGGGGCAGGAAC	
18	60	ACAGTTTATTGCATTGTTAGA	GCCATGTGCTTTGAGGCAGAC	modified Li et al.[c]
19	58	TGAAGCATTTGCTCTGCTCT	GTTTCAAACTTGATGTATATTAAA	according to Bausch et al.[a]
20	58	CAAGTTTGAAACTTGGCTGTA	ATATATTTAGCAGATCAGTTAAC	according to Bausch et al.[a]
21	58	GGAAGAAATGTTGGATAAAGCA	AAACAAGTCACTCTATTCATAGA	according to Bausch et al.[a]
22	60	TATCTGTATGCTTATTGGCTCTA	GTGCAGTAAAGAATGGCCAG	according to Bausch et al.[a]
23	60	GCCAGAAGTTGTGTACGTTCTTT	CAAAGCTTAGGGAAATTTGAGAAG	
24	58	TTGTTCCCTTCTGGCTTTTAT	TCTCAAAGTTTAAATACACAC	modified Bausch et al.[a]
25	60	TGAGGGGAAGTGAAAGAACT	GGCTTTATTTGCTTTTTGCT	according to Han et al.[b]
26	60	CACCCTGGCTGATTATCGC	TAATTTTTGCTTCTCTTACATGC	modified Han et al.[b]
27	60	TGGTCTCATGCACTCCATA	AACATCTTTCTTCTGGCTCTG	according to Bausch et al.[a]
28	60	TGCTACTCTTTAGCTTCCTAC	CATACCTCAGCACACACATA	according to Bausch et al.[a]
29	60	AGAATTGGCAGACTCCATGC	AAAACAGCGGTTCTATGTGAAAA	
30	60	GAAGTCTACACGTTGCACTTGG	ACCATCAGCAGCTAGATCCTTC	
31	60	TCCATTTGTGTTACATTTTATGGTG	AAGGTCAAATAGGCTGAAGTGAAG	alternative splicing exon
32	58	CTTATACTCAATTCTCAACTCC	GAATTTAAGATAGCTAGATTATC	according to Bausch et al.[a]
33	58	AATATAATAATTATATTTGGGAAGC	GAAAATATTTGATTCAAACAGAGC	according to Li et al.[c]
34	58	CTCCATATTTGTAATCTTAGTTA	GGAGAGTGTTCACTATCCC	according to Bausch et al.[a]
35	60	ATGGTCCTGAGGTCTTTTTG	CTAACAAGTGGCCTGGTGGC	according to Fahshold et al.[d]
36	58	TAGACAACATAAAGCCTCATAA	CCATCTCTATATTTGCTATA	according to Fahshold et al.[d]
37	60	GCCTTATTTCTCAGTGTCCAAAA	CTTTGAGGCCAGTCAGCAG	primer set 37.1
37	60	TTCCTGGCTTTGCTTACGAC	GATCTGCCCACCTTGACATC	primer set 37.2
38	58	CAATGGTGGGAACTCTTCCTTA	AGCAACAAACCCCAAATCAA	
39	60	GTCATAGGAGCCTCACAGTGC	TGAAAGGGTTTTCTTTGAATTCTC	
40	60	CTCCAGGCCTGATTCTAGGTAA	GTGTCTAGCGCAGTGCTTTG	
41	60	ATTGATTAGGCTGTTCCAATGAA	CCTCCTGATGATAAACAGATATGC	
42	60	ATTGGTTTCAAGCAAGGTAATCA	AGCAACTTGGTGTTAGAGCACA	
43	60	TGTAGATAAATGAAGCAAGGAGCA	ACCAATAACACAGTCCATGCAA	
44	60	GCTTTGTTGGAGATCATGGAG	TGCAGGGATGGATTATATTGG	
45	60	AGTGGACAGAACTAGCTCAAAGG	CTGCTTAGCAAAAGAGGCAAC	
46	60	ATTCATTCCGAGATTCAGTTTAGG	TTTCCTTTAGCACTGATGAGACC	
47	60	CAAACCTTGGTGACTGGATTTAG	AGCAACAAGAAAAGATGGAAGAGT	
48	58	TGTTCTGTGGTTTTCTGCAGTC	CTAGCCTCAAGTGATCCTCCTG	
49	60	TCCTTTCCTTGCAGAGTTGTTAG	TTGAACCAAAGATGACCAAGG	
50	60	CCCTGCCTACTTTGTGTTTGTTA	CTTGCCTCCATTAGTTGGAAAA	
51	60	AGGACAGCCACTTGGAAGG	TTCAAGGTCCCATTCTCATTTC	
52	60	GAAACAGAAAGCTATGGGAACAA	AAATGGCTGGAAAATAAACATGA	
53	60	GGTGGGAGAGTACATGAAAGTCA	GGGCCTCCTAAAAGTAGACTGG	
54	60	GATTTCCTCATCACAACAGCAC	CCAGTGCACATTACTGGGTAAG	
55	60	TGAAGAAATGCCCCAGAAAG	AGCGCGCATGTTAGCAA	
56	60	CGCTGTTGTAGAATGCACTGA	GAATACACACACCCCAACACC	
57	60	TGGCTTCAGATGGGGATTTA	AGGGAATTCCTAATGTTGGTGTC	
58	60	GCGACACATGACTGCAATG	GCAAACCGGATGGGTTC	

Ta, annealing temparature

[a] Bausch B, Borozdin W, Mautner VF, Hoffmann MM, Boehm D, Robledo M, et al. Germline NF1 mutational spectra and loss-of-heterozygosity analyses in patients with pheochromocytoma and neurofibromatosis type 1. J Clin Endocrinol Metab 2007;92:2784-92.

[b] Han SS, Cooper DN, Upadhyaya MN. Evaluation of denaturing high performance liquid chromatography (DHPLC) for the mutational analysis of the neurofibromatosis type 1 (NF1) gene. Hum Genet 2001;109:487-97.

[c] Li Y, O'Connell P, Breidenbach HH, Cawthon R, Stevens J, Xu G, et al. Genomic organization of the neurofibromatosis 1 gene (NF1). Genomics 1995;25:9-18.

[d] Fahsold R, Hoffmeyer S, Mischung C, Gille C, Ehlers C, Kücükceylan N, et al. Minor lesion mutational spectrum of the entire NF1 gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet 2000;66:790-

Supplymentary Table 3
NF1 RT-PCR Primers.

Primer set	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	Position[a]	Procuct size (bp	Covered exons
a	GACCCTCTCCTTGCCTCTTC	TTTCTACCCAGTTCCAAAATGC	-109~676	785	Exon 1 to 7
b	AAGAAGGTTGCGCAGTTAGC	CATTCGTATTGCTGGGTGTG	613~1386	774	Exon 7 to 12
c	TGTCACTCGGTTGAACTTCG	CAGGGCCACTTCTAGTTTGG	1300~2073	775	Exon 12 to 18
d	CTCTCTCCGGAAGGGAAAAG	TTAAGGACAACAGCCGATCC	1968~2704	737	Exon 17 to 21
e	GAGGGAAACGCAGATACACC	TGCGCACTTTCATCTTCAAC	2649~3377	729	Exon 21 to 26
f	TGATGGAAGCCAAATCACAG	GGGGAATTCTGAGGAGGAAC	3284~4074	791	Exon 25 to 30
g	GATTGGCAACATGTTAGCTTTG	GCTGCATCAAAGTTGCTTTTC	3937~4361	488, 425 (Exon 31skip)	Exon 29 to 33 (Exon 31 alternative
h	AATCCTGCCATTGTCTCACC	CCAGCTTGGTAGAAAATACTTAACG	4180~4727	548	Exon 32 to 36
i	CCAGAGCACAAACCTGTGG	CTACTAGGCAGATTTCTTCAATTTCC	4582~5320	739	Exon 35 to 38
j	ACTGCTGTCCAAGTAACTTCAGC	CACCTGTTGCACTGGTTTTG	5215~6001	787	Exon 38 to 41
k	AAAATATGGGGAAGCCTTGG	TTGGAATGCAAATCTTTGAGC	5920~6654	735	Exon 40 to 44
l	TGCTTTGACATCCTTGGAAAC	CGACTTCGAACTTCTTCAGACAC	6528~7292	765	Exon 43 to 50
m	ACTCTGGTTAACAAACACAGAAATG	AATTTGGATCTTGGCACAATG	7189~7966	778	Exon 49 to 55
n	TAGCAGAGGCCAGTGTTGTG	GCAGCATTAAATTTAGGCAAGG	7865~*183 (Stop at 8457)	775	Exon 54 to 3'UTR

[a] Nucleotide number is according to *NF1* transcript variant 2 (GenBank NM_000267.3) and A of start codon is represented as 1.