# Preface

The rapid identification and characterization of genes of neurological relevance holds great potential for offering insight into the diagnosis, management, and understanding of the pathophysiologic mechanisms of neurological diseases. This volume in the *Methods in Molecular Biology*™ series was conceived to highlight many of the contemporary methodological approaches utilized for the characterization of neurologically relevant gene mutations and their protein products. Although an emphasis has been placed upon descriptions of methodologies with a defined clinical utility, it is hoped that *Neurogenetics: Methods and Protocols* will appeal not only to clinical laboratory diagnosticians, but also to clinicians, and to biomedical researchers with an interest in advances in disease diagnosis and the functional consequences of neurologically relevant gene mutations.

To meet this challenge, more than 60 authors graciously accepted my invitation to contribute to the 32 chapters of this book. Through their collective commitment and diligence, what has emerged is a comprehensive and timely treatise that covers many methodological aspects of mutation detection and screening, including discussions on quantitative PCR, trinucleotide repeat detection, sequence-based mutation detection, molecular detection of imprinted genes, fluorescence *in situ* hybridization (FISH), in vitro protein expression systems, and studies of protein expression and function. I would like to take this opportunity to formally thank my colleagues for their effort and dedication to this work.

This book would not have been possible without the guidance and wisdom of the Series Editor, Professor John M. Walker, whose intimate knowledge of the nuances of the editorial process made my job somewhat less intimidating. I would also like to thank Thomas Lanigan, President of Humana Press, who enthusiastically embraced the book concept and my original prospectus from the very beginning, and Craig Adams, also at Humana Press, for transforming the individual chapters into their final form.

***Nicholas T. Potter***

# 2

# Semiquantitative PCR for the Detection of Exon Rearrangements in the Parkin Gene

**Christoph B. Lücking and Alexis Brice**

## 1. Introduction

### *1.1. Frequency and Type of Parkin Mutations*

Mutations in the *parkin* gene have been shown to be responsible for a substantial number of cases of autosomal recessive early onset parkinsonism (AR-JP, PARK2, OMIM 602544) worldwide ***(1–4)***. The gene on chromosome 6q25.2-27 consists of 12 coding exons with an open reading frame of 1395 bp. The gene is estimated to cover >1.5 Mb. The gene product, Parkin, functions an an E3-ubiquitin-protein ligase ***(5)***. The only known substrate to date is CDCrel-1 ***(6)*** but the existence of other substrates is likely ***(5)***.

The mutations in the *parkin* gene identified so far have been extremely varied in both location and type. About 50% of affected Caucasian sibpairs without affected parents (suggesting autosomal-recessive inheritance) in which onset occured before or at the age of 45 yr in at least one of the affecteds, have mutations in the parkin gene ***(3)***. In sporadic cases, 80% had mutations when the age at onset was ≤ 20, 25% when onset was at age 21–30, and only 2% when age of onset was 31–45. About 50% of the mutations in the Caucasian population are exon rearrangements, consisting mainly of deletions but multiplications also occur ***(3)***. In nonconsanguineous cases, these rearrangements might be present in the heterozygous state and thus escape detection by nonquantitative polymerase chain reaction (PCR) because there is still a normal copy of the deleted exon. Direct sequencing will not permit detection of such deletions either, since only the undeleted allele, normal in most cases, will be analyzed. Thus, a simple, fast, and accurate assay is needed to quantify the number of exons present in a genomic DNA sample.

### *1.2. Principle of the Semiquantitative Multiplex PCR Dosage Assay*

Quantitative PCR is considered to be superior to Southern blotting for detecting gene dosage ***(7)***. Since absolute quantification of the template DNA is not necessary, we have chosen a semi-quantitative PCR assay that compares the relative amounts of template DNA. This is sufficient to answer to the question whether the amount of template DNA corresponding to one or more exons is abnormal. This assay can be performed with any

From: *Methods in Molecular Biology, vol. 217: Neurogenetics: Methods and Protocols*
Edited by: N. T. Potter © Humana Press Inc., Totowa, NJ

fluorescence-based automated sequencer, now available in many laboratories, unlike real-time PCR analyzers that remain relatively rare.

In order to detect homozygous or heterozygous exon deletions or multiplications in the *parkin* gene, several exons amplified simultaneously in one multiplex PCR are compared, the co-amplified exons serving as internal standards for quantification. Fluorescently labeled PCR primers are used, so that the PCR product can be quantified from the height of the peak detected by the automated sequencer. The amount of PCR product is directly related to the amount of template DNA as long as PCR amplification is exponential. Each multiplex PCR for a given combination of exons results in a typical pattern of peak heights for normal control cases and thus in typical ratios between the peaks. If an exon is heterozygously deleted, for example, its peak is half as high as expected and the ratios between the deleted and the undeleted exons change. The resulting ratios are then compared to the ratios of the peaks obtained with DNA from known normal subjects that are run in parallel. If the ratios for a given exon differ from those of a control, the exon has been rearranged. In order to detect a heterozygous deletion of the entire *parkin* gene, an 328 bp PCR product (C328) of a distant gene (*transthyretin* gene on chromosome 18) is amplified as external standard in one of the multiplex PCR combinations. The fact that only ratios between peak heights are compared, renders the assay relatively independent of the quantity and quality of the template DNA. In addition, small-scale deletions and insertions within the PCR products can be detected by comparing the size of the patient's PCR product to the product of a control. Seventy percent of the parkin mutations identified by our group have been reliably detected with this technique ***(3)***.

## 2. Materials

The products used are listed below. Comparable products from other suppliers should also be effective.

### 2.1. Multiplex PCR

1. High quality genomic DNA extracted by standard procedures. Prepare a working dilution at 20 ng/µL (diluted in water) just before the experiment. It can be kept at +4°C for short periods of time, or at –20°C for long-term storage.
2. 96-well thin-walled PCR plates with appropriate lid-strips.
3. 8-well PCR r-tube strips with appropriate lid-strips.
4. TaqPolymerase (5 U/µL, Life Technologies, store at –20°C) with the supplied buffers and $MgCl_2$ (kept at 4°C).
5. 100 m*M* single dNTP solutions (Life Technologies, store at –20°C). Combine them (resulting in 25 m*M* for each dNTP), and prepare aliquots to be stored at –20°C. The working aliquot is kept at +4°C to avoid freeze-thaw-cycles.
6. HEX-labeled (*see* **Notes 1–3**) forward primers (light sensitive) and unlabeled reverse primers (Life Technologies, sequences in **Table 1**). The primers are re-suspended in water (we did not try dilution in TE) to 200 µ*M* (stock solution) and further diluted to 20 µ*M* (working dilution). Aliquots (50–200 µL) of the working dilution should be kept at –20°C in order to minimize freezing and thawing of the stock solution. Once an aliquot of the working dilution is thawed, it should be kept at 4°C and used within 4 wk.
7. "Hybaid PCR Express" PCR machine.

**Table 1**
**Primers Used for the Multiplex PCR[a]**

| Primer | Forward (5'- 3') | Reverse (5'- 3') | Product (bp) |
|---|---|---|---|
| Ex 1* | CGCGCATGGGCCTGTTCCTG | GCGGCGCAGAGAGGCTGTAC | 94 |
| Ex 2 | ATGTTGCTATCACCATTTAAGGG | AGATTGGCAGCGCAGGCGGCATG | 308 |
| Ex 3i* | AATTGTGACCTGGATCAGC | CTGGACTTCCAGCTGGTGGTGAG | 243 |
| Ex 3iDos* | as Ex 3i For | AGTCAAGCTCTGGGGCTCC | 138 |
| Ex 4o | ACAAGCTTTTAAAGAGTTTCTTGT | AGGCAATGTGTTAGTACACA | 261 |
| Ex 5 | ACATGTCTTAAGGAGTACATTT | TCTCTAATTTCCTGGCAAACAGTG | 227 |
| Ex 6 | AGAGATTGTTTACTGTGGAAACA | GAGTGATGCTATTTTTAGATCCT | 268 |
| Ex 7o | TGCCTTTCCACACTGACAGGTCT | TCTGTTCTTCATTAGCATTAGAGA | 239 |
| Ex 8 | TGATAGTCATAACTGTGTGTAAG | ACTGTCTCATTAGCGTCTATCTT | 206 |
| Ex 9 | GGGTGAAATTTGCAGTCAGT | AATATAATCCCAGCCCATGTGCA | 278 |
| Ex 10 | ATTGCCAAATGCAACCTMTGTC | TTGGAGGAATGAGTAGGGCATT | 165 |
| Ex 11 | ACAGGGAACATAAACTCTGATCC | CAACACACCAGGCACCTTCAGA | 303 |
| Ex 12 | GTTTGGGAATGCGTGTTTT | AGAATTAGAAAATGAAGGTAGACA | 255 |
| C328* | ACGTTCCTGATAATGGGATC | CCTCTCTCTACCAAGTGAGG | 328 |

[a]The primers of the exons marked with an * were designed for this assay, the others are as in Kitada et al. ***(1)***. Forward primers are 5'-fluorescently labeled (with the same fluorchrome or *see* **Note 3**).

### 2.2. Polyacrylamide Gel Electrophoresis

1. Prepare stocks of 5% denaturing polyacrylamide gels using Page-Plus 40% (Amersham) according to the manufacturer's recommendations. Keep 35-mL aliquots at 4°C and use within 4 wk.
2. An automated sequencer (e.g., ABIPRISM 377 upgraded for 96 wells by Applied Biosystems, *see* **Note 3**).
3. Internal size standard (TAMRA-500 and TAMRA-500 XL by Applied Biosystems) and its blue loading buffer.
4. Deionized formamide (Amersham).

### 2.3. Interpretation of Data

1. Fragment analysis software (e.g., Genescan 3.1 and Genotyper 1.1.1 software by Applied Biosystems).
2. Calculation software (e.g., Excel 97 by Microsoft).

## 3. Methods

### 3.1. Multiplex PCR

#### 3.1.1. For a 25 µL Reaction

1. Set up premixes (13 µL per reaction) corresponding to the first 3 exon combinations (for combination 4, *see* **Note 4**) by adding 2.5 µL of 10X buffer (final concentration 1X), 1.5 µL of 50 m*M* $MgCl_2$ (final concentration 3 m*M*), the appropriate amount of each primer (between 0.3 and 2.5 µL, final concentration between 0.4 and 2.0 µ*M*, *see* **Note 5**), 0.2 µL of 25 m*M* dNTP-Mix (final concentration 0.2 m*M* per dNTP) and complete with $H_2O$ up to 13 µl per reaction. Prepare enough premix for duplicate (or more) reactions for each case.
2. Spot 2 µL of the DNA (= 40 ng) on the wall of a 96-well PCR-plate. This is an easy way to control which well contains DNA (*see* **Note 6**).
3. Add 13 µL of the appropriate premix to the well (*see* **Note 7**). Close the wells with lid strips, vortex briefly, and spin down the contents in a centrifuge equipped for PCR plates.
4. Prepare for a semi-hotstart (*see* **Note 8**) by heating the PCR apparatus to 94°C. Open the wells carefully, add 10 µL of Taq solution (2 U/10 µL water kept on ice) using a distribution pipet, close the wells with new strips, vortex briefly, tap the plate on the lab bench to collect all liquid at the bottom of the wells, and place it on the preheated PCR block.
5. Use the following cycling conditions in "tube simulation mode" for combinations 1–3: 94°C for 5 min (initial denaturation), 23 cycles of 94°C for 30 s, 53°C for 45 s and 68°C for 2.5 min, 68°C for 5 min (final extension). For combination 4, *see* **Note 4**.

### 3.2. Polyacrylamide Gel Electrophoresis

1. Prepare the sequencing gel by adding ammonium persulfate and TEMED, following the manufacturer's recommendations, to an aliquot of the acrylamide.
2. Prepare the loading buffer by pipetting together a premix containing: 0.3 µL of TAMRA-500 XL, 0.6 µL of loading blue, and 3 µL of formamide (for sequencer with 36 lanes; *see* **Note 3**) per sample.
3. Pipet 3.5 µL aliquots of this loading premix into the wells or tubes used to prepare the samples for loading.
4. Add 2–2.5 µL of the PCR products to the premix. Close the tubes, centrifuge briefly, vortex, and centrifuge again (for sequencers with 36 lanes; *see* **Note 3**).

5. Load 1–1.5 µL of this mixture into the wells of the sequencing gel and let run it as for a standard fragment length analysis protocol.
6. Analyze the gel image with appropriate software for calculating the peak heights (e.g., Genescan 3.1 for gel analysis and Genotyper 1.1.1 for peak labeling; *see* **Note 9**). Print the results.

### 3.3. Interpretation of Data

1. Transfer the peak heights, indicated by Genotyper, to an Excel sheet that has been configured to automatically calculate all the ratios that are needed (*see* **Notes 10–12**).
2. Check the size of the PCR products in order to detect small deletions and insertions. This is done by placing a ruler over the respective peaks on the printed pages. Alternatively, Genotyper can label peak size/length and height.

### 3.4. Verification that PCR Amplification is in the Exponential Phase

Correct quantification requires that PCR amplification is in the exponential phase for each of the 4 exon combinations:

1. Prepare 50 µL PCR reactions in duplicate for each multiplex combination using DNA from a control subject as template. Ideally, use an 8-well-PCR-strip.
2. Run the aforementioned PCR programs.
3. Take 8 µL aliquots after 20, 21, 22, 23, 24, and 25 cycles by stopping ("pause") the PCR machine a few seconds before the end of the respective extension phase. Take the aliquot with a multipipet to work as quickly as possible. Continue the PCR program afterwards.
4. Collect the aliquots in an appropriately labeled 96-well plate on ice.
5. Transfer 2 µL of each aliquot into loading buffer, load the gel, and perform the fragment analysis.
6. Label and print the electrophoregrams of the exon combinations and the size marker.
7. Prepare an "Excel" sheet where you can enter the crude peak height values. The program can correct for differences in the amount of sample loaded. For this purpose, calculate the ratio of the peak height of a TAMRA-peak (e.g., 200 bp) in the first lane to the TAMRA-200-bp-peaks of the other samples on the same gel. This results in a factor of normalization by that the exon peak heights of the corresponding sample should be multiplied.
8. Finally, let "Excel" plot the corrected peak heights as a function of cycle number on a logarithmic scale. The exponential phase is shown by a straight ascending line. The lines should be parallel for all exons. Ideally, the should coincide.
9. If amplification is not exponential for one or more exons, adjust the primer concentrations (decrease the concentration for exons that amplify too rapidly and increase the concentration for exons that amplify too slowly).

## 4. Notes

1. During preliminary experiments, it was noted that exons that amplify well might negatively affect the amplification of the other exons. Thus, we partly followed the recommendations of Henegariu et al. ***(8)*** and grouped together exons that amplify to the same extent. In addition, since smaller PCR products often amplified better than longer products, we grouped together exons of similar size. Thus, the following 3 exon combinations were chosen for multiplex PCR (primers are as specified in **Table 1**):

   comb 1: Ex 4o (261 bp) + 7o (239 bp) + 8 (206 bp) + 11 (303 bp),
   comb 2: Ex 5 (227 bp) + 6 (268 bp) + 8 (206 bp) + 10 (165 bp) and
   comb 3: Ex 2 (308 bp) + 3i (243 bp) + 9 (278 bp) + 12 (255 bp) + C328
   (external control of 328 bp, derived from the transthyretine gene on chromosome 18).

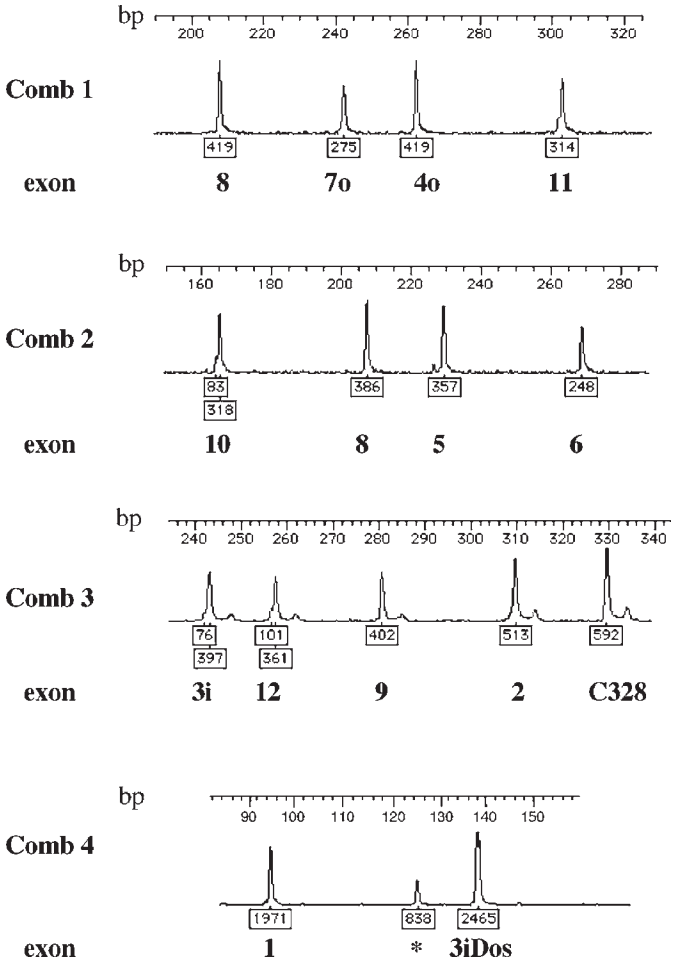


Fig. 1. Four exon combinations used in multiplex PCRs. Representative electrophoregrams obtained after multiplex PCR with HEX-labeled forward primers and separation on 5% denaturing gels on an automated Sequencer (ABI PRISM 377). Peak length (indicated in bp above each electrophoregram) and peak height (indicated below each electrophoregram) are given as calculated by Genotyper 1.1.1 software (Applied Biosystems). Exons are numbered below each peak. Please note that two peak heights are given for double peaks the sizes of which have to be added (*see* **Note 9**). *, unspecific peak.

Representative examples of electrophoregrams are given in **Fig. 1**. However, other peak patterns are also possible, as long as all exons amplify exponentially (*see* **Subheading 3.4.**). Exon 8 was amplified in comb 1 and comb 2, so that the same positive control (heterozygously deleted for exons 8 and 9) could be used in all three combinations.

2. More recently, we added a fourth exon combination: Ex1 (94 bp) + Ex3iDos (138 bp) in order to co-amplify exon 1. This is difficult, since the 5'- untranslated region of the parkin gene (containing the forward primer binding site for exon 1) is very GC-rich. We obtained the best results with the primers mentioned in **Table 1** for exon 1 and exon 3iDos. However, a nonspecific peak is also obtained (*see* **Fig. 1**) that co-amplifies exponentially with the two exons (for PCR conditions; *see* **Note 8**). Since all known exon 3 deletions were

detected with this combination, we assume that exon 1 deletions should also have been detected. However, we have not yet detected an exon 1 deletion in the patients studied.

3. In order to save space on the sequencing gel (e.g., with a 36 lanes sequencer), the exons of the first 3 combinations can be labeled with 3 different fluorchromes (e.g., comb 1 with TET, comb 2 with FAM, and comb 3 with HEX). Aliquots of the PCRs are then pooled in the loading buffer. Under these conditions, we obtained good results with 1 µL of comb 1, 1.5 µL of comb 2 and 3 µL of comb 3, added to 1.5 µL of TAMRA-500, 3 µL of formamide and 0.7 µL of loading blue. Combination 4 is run separately, since no other fluorchrome is available.
4. For multiplex combination 4, the best results were obtained with the following PCR protocol: in a final volume of 25 µL, 40 ng of DNA, 10% DMSO, 1.5 µ*M* of each primer, 0.2 m*M* of each dNTP and 2 U of *Taq* polymerase. The cycling protocol consisted of 94°C for 4 min followed by 22 cycles of 94°C for 30 s, 53°C for 30 s and 68°C for 45 s. Final elongation was performed at 68°C for 10 m.
5. Concentrations of the primers varied from 0.40 µ*M* to 2 µ*M*. The exact primer concentrations have to be determined experimentally because they vary among batches.

   In order to obtain approximately equal peak heights for the exons in each multiplex PCR combination and to be in the exponential phase for each exon, we followed in part the recommendations of Henegariou et al. ***(8)***, in particular: lowering the annealing temperature to 53°C and extension temperature to 68°C, increasing the $MgCl_2$ concentration to 3 µ*M* and the extension time to 2.5 min (*see* **Note 4** for combination 4). In addition, the primer concentrations have to be adjusted. We recommend starting with 0.8 µ*M* for each primer and adapting the concentration according to the resulting peak height (i.e., primer concentrations have to be decreased for exons that amplify well and increased for exons that amplify less well).
6. In addition to the cases, at least one normal control and a negative control (without template DNA) should be run in parallel. We also included a positive control with known heterozygous deletions of exons 8 and 9 in order to control for false results due to variation in the premix. However, if no positive control with known exon rearrangements is available, the dosage assay can still be considered to be valid as long as PCR amplification is exponential.
7. In order to facilitate mixing of the reagents, pipet the premix onto the drop of DNA (e.g., with a Gilson Distriman with Distritips) but avoid touching the DNA to prevent carryover contamination.
8. If the PCR premix is kept on ice or if a Taq Polymerase with automated hot-start is used, the TaqPolymerase could also probably be added directly to the premix, although we have not done this.
9. Peak labeling programs can determine peak length (size of the PCR product), peak height, and peak area. Peak area would seem to be the most precise reflection of the amount of PCR product, but often the algorithms used only approximate the area. We have had more consistent results using peak height. It is important to sum the peak heights when a PCR product forms a double peak with 1 bp difference, caused by the addition of an A to the PCR products by the polymerase (*see* examples in **Figs. 1–3**). Genotyper does not always distinguish double peaks, so vigilence is necessary.
10. The interpretation of the data is based on the relative peak heights obtained within a given multiplex reaction. The method is therefore only semi-quantitative and does not give absolute values that would be affected by the amount and quality of template DNA as well as slight variations in the premix. In order to establish the peak height patterns for cases and controls, the ratios of the peak heights of each exon to every other exon in a given exon combination are calculated (*see* **Tables 2** and **3**). The average ratio of the duplicate/triplicate/quadruplicate reactions are then calculated and normalized with respect to the

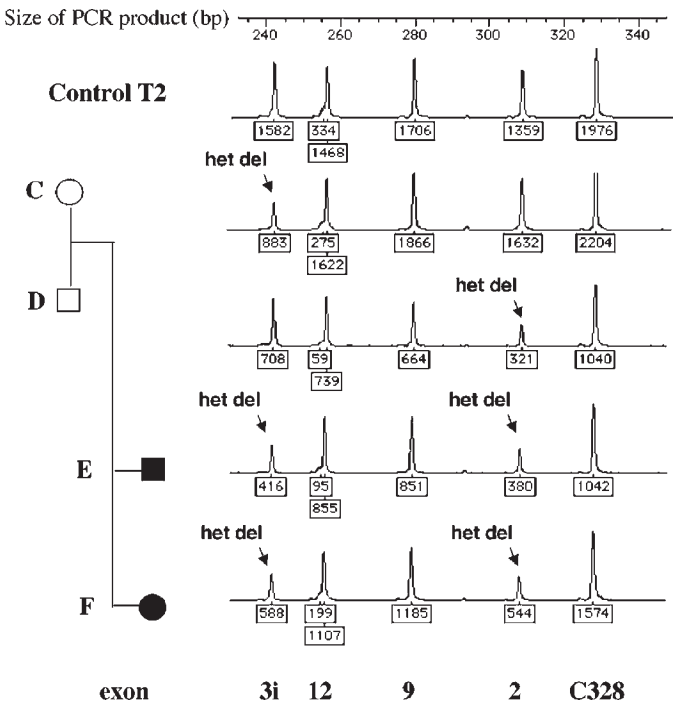


Fig 2. Compound heterozygous deletions of exon 2 and 3. Representative electrophoregrams of the cosegregation of two different exon deletions in a family compared to a control. Peak labeling is as in **Fig. 1**. For each case, one of the triplicate PCRs is shown. However, in **Table 2**, all three PCRs are used for the NR calculation. Circles, women; squares, men; filled symbols, patients; het, heterozygous; del, deletion.

control(s). The normalized ratios (NRs) obtained in different experiments (e.g., if experiments are performed several times with different batches of DNA) are therefore comparable and mean normalized ratios (MNRs) can be calculated.

In practice, a template "Excel" sheet should be established containing all the calculation instructions described below, so that only the crude peak heights need to be entered. Two examples are given in **Tables 2** and **3**, that correspond to the electrophoregrams in **Figs. 2** and **3**. In the left part of the table, the peak heights for each case are entered, e.g., the duplicate PCRs of controls 1 to 3 and the quadruplicate PCRs of the cases A and B **(Table 3)**. In the right part of the table, next to the peak heights, the program calculates the peak height ratios as indicated at the top of the tables. Below, the program calculates the mean values (in bold) and the standard deviation of the ratios, respectively. Rearrangements are deduced from the division of the mean peak height ratios of all available controls (i.e., one control in triplicate in **Table 2** and three controls in duplicate in **Table 3**) by the mean peak height ratios of the case. This results in the NRs for each exon peak height ratio (shown in bold italics for normal values or bold underlined for pathological values in the table). The NRs have a value of approx 1 (i.e., 0.8 to 1.2) for nonrearranged exons (2 copies of an exon). Values of 0.6 or less are interpreted as one copy of an exon (i.e., heterozygous deletion), values of 1.3 to 1.7 as 3 copies (i.e., heterozygous duplication), values of 1.8 to 2.3 as 4 copies (i.e., homozygous duplication or heterozygous triplication)

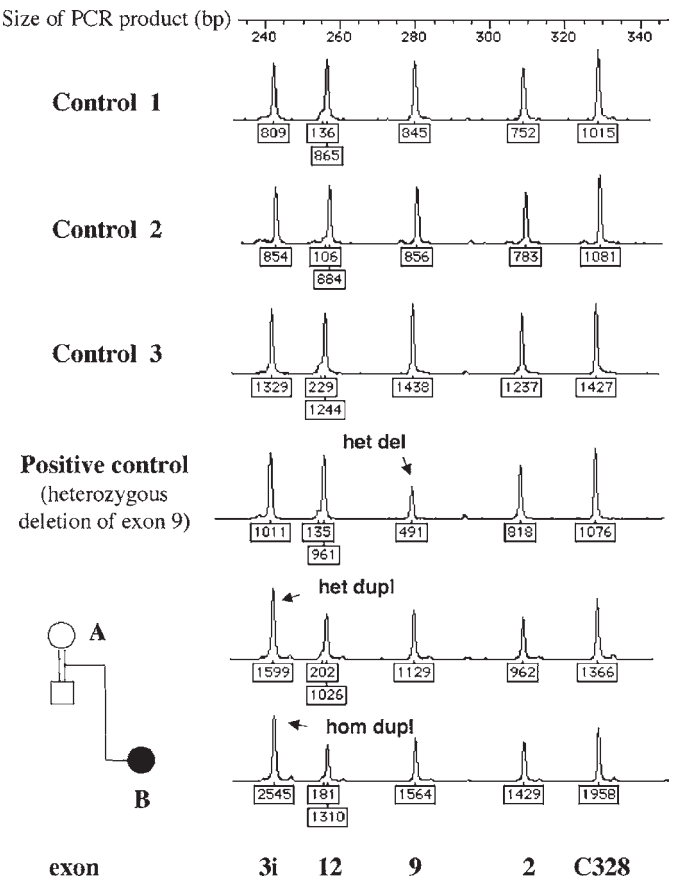


Fig 3. Homozygous duplication of exon 3. Representative electrophoregrams of the cosegregation of an exon duplication in a family compared to 3 controls. In addition, a positive control with known heterozygous deletions of exons 8 and 9 was run in parallel. All values of the duplicate or quadruplicate PCRs are shown in table 3. Peak labeling and symbols are as in **Figs.1** and **2**. Het, heterozygous; hom, homozygous; del, deletion; dupl, duplication.

and values above 2.6 as indication for 6 copies (i.e., homozygous triplication) (*see* **Fig. 4**). Please note that the NR values given above apply only if the exon under investigation is the dividing value in the exon ratio, i.e., C328/**3i**. If the ratio is Ex**3i**/12 (e.g., case C in **Table 2**), the NR becomes approx 2 (1/0.5). An exon rearrangement is confirmed only if all of the ratios concerning the exon are abnormal (*see* tables). If the results are ambiguous, the PCR should be repeated, if possible with a new DNA sample and a larger number of replicates (e.g., up to quadruplicate). In addition, several control cases can be included to obtain mean values for the control ratios.

11. Reproducibility. As seen from the tables, standard deviations are usually about 10% of the mean, and rarely exceed 20%, for both the crude exon ratios calculated from the PCR replicates and the mean NRs from several different experiments (data not shown).
12. Search for homozygous exon deletions. In addition to the detection of heterozygous exon deletions, the multiplex PCR assay also detects homozygous exon deletions that are obvious, since one or several peaks are missing.

**Table 2**
**Calculation of Exon Ratios, SD, and Normalized Ratios Corresponding to Fig. 2[a]**

| *Case* | *3i* | *12* | *9* | *2* | *C328* | *C328/3i* | *C328/12* | *C328/9* | *C328/2* | *Ex3i/12* | *Ex3i/9* | *Ex3i/2* | *Ex12/9* | *Ex12/2* | *Ex9/2* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control T2 | 1491 | 1550 | 1338 | 1143 | 2031 | 1.36 | 1.31 | 1.52 | 1.78 | 0.96 | 1.11 | 1.30 | 1.16 | 1.36 | 1.17 |
| | 1582 | 1802 | 1706 | 1359 | 1976 | 1.25 | 1.10 | 1.16 | 1.45 | 0.88 | 0.93 | 1.16 | 1.06 | 1.33 | 1.26 |
| | 1445 | 1580 | 1525 | 1379 | 1990 | 1.38 | 1.26 | 1.30 | 1.44 | 0.91 | 0.95 | 1.05 | 1.04 | 1.15 | 1.11 |
| **Mean** | | | | | | **1.33** | **1.22** | **1.33** | **1.56** | **0.92** | **1.00** | **1.17** | **1.08** | **1.28** | **1.18** |
| SD | | | | | | 0.07 | 0.11 | 0.18 | 0.19 | 0.04 | 0.10 | 0.13 | 0.07 | 0.11 | 0.07 |
| Case C | 693 | 1593 | 1606 | 1441 | 1990 | 2.87 | 1.25 | 1.24 | 1.38 | 0.44 | 0.43 | 0.48 | 0.99 | 1.11 | 1.11 |
| | 760 | 1746 | 1646 | 1435 | 2143 | 2.82 | 1.23 | 1.30 | 1.49 | 0.44 | 0.46 | 0.53 | 1.06 | 1.22 | 1.15 |
| | 883 | 1897 | 1866 | 1632 | 2204 | 2.50 | 1.16 | 1.18 | 1.35 | 0.47 | 0.47 | 0.54 | 1.02 | 1.16 | 1.14 |
| **Mean** | | | | | | **2.73** | **1.21** | **1.24** | **1.41** | **0.45** | **0.46** | **0.52** | **1.02** | **1.16** | **1.13** |
| SD | | | | | | 0.20 | 0.05 | 0.06 | 0.08 | 0.02 | 0.02 | 0.03 | 0.03 | 0.06 | 0.02 |
| **Mean T2/mean C** | | | | | | ***0.47*** | ***1.01*** | ***1.07*** | ***1.11*** | ***2.15*** | ***2.28*** | ***2.37*** | ***1.06*** | ***1.10*** | ***1.04*** |
| Case D | 657 | 762 | 757 | 276 | 862 | 1.31 | 1.13 | 1.14 | 3.12 | 0.86 | 0.87 | 2.38 | 1.01 | 2.76 | 2.74 |
| | 708 | 798 | 664 | 321 | 1040 | 1.47 | 1.30 | 1.57 | 3.24 | 0.89 | 1.07 | 2.21 | 1.20 | 2.49 | 2.07 |
| | 811 | 962 | 894 | 414 | 1278 | 1.58 | 1.33 | 1.43 | 3.09 | 0.84 | 0.91 | 1.96 | 1.08 | 2.32 | 2.16 |
| | | | | | | ***1.45*** | ***1.25*** | ***1.38*** | ***3.15*** | ***0.86*** | ***0.95*** | ***2.18*** | ***1.09*** | ***2.52*** | ***2.32*** |
| SD | | | | | | 0.13 | 0.11 | 0.22 | 0.08 | 0.02 | 0.11 | 0.21 | 0.10 | 0.22 | 0.37 |
| **Mean T2/mean D** | | | | | | ***0.88*** | ***0.97*** | ***0.96*** | ***0.49*** | ***1.11*** | ***1.10*** | ***0.56*** | ***0.99*** | ***0.51*** | ***0.51*** |
| Case E | 430 | 973 | 881 | 428 | 1333 | 3.10 | 1.37 | 1.51 | 3.11 | 0.44 | 0.49 | 1.00 | 1.10 | 2.27 | 2.06 |
| | 416 | 950 | 851 | 380 | 1042 | 2.50 | 1.10 | 1.22 | 2.74 | 0.44 | 0.49 | 1.09 | 1.12 | 2.50 | 2.24 |
| | 463 | 1024 | 943 | 427 | 1248 | 2.70 | 1.22 | 1.32 | 2.92 | 0.45 | 0.49 | 1.08 | 1.09 | 2.40 | 2.21 |
| **Mean** | | | | | | **2.77** | **1.23** | **1.35** | **2.93** | **0.44** | **0.49** | **1.06** | **1.10** | **2.39** | **2.17** |
| SD | | | | | | 0.30 | 0.14 | 0.15 | 0.19 | 0.01 | 0.00 | 0.05 | 0.02 | 0.11 | 0.10 |
| **Mean T2/mean E** | | | | | | ***0.46*** | ***0.99*** | ***0.98*** | ***0.53*** | ***2.16*** | ***2.12*** | ***1.16*** | ***0.98*** | ***0.53*** | ***0.54*** |
| Case F | 588 | 1306 | 1185 | 544 | 1574 | 2.68 | 1.21 | 1.33 | 2.89 | 0.45 | 0.50 | 1.08 | 1.10 | 2.40 | 2.18 |
| | 915 | 2078 | 1943 | 853 | 2413 | 2.64 | 1.16 | 1.24 | 2.83 | 0.44 | 0.47 | 1.07 | 1.07 | 2.44 | 2.28 |
| | 944 | 1913 | 1825 | 844 | 2428 | 2.57 | 1.27 | 1.33 | 2.88 | 0.49 | 0.52 | 1.12 | 1.05 | 2.27 | 2.16 |
| **Mean** | | | | | | **2.63** | **1.21** | **1.30** | **2.87** | **0.46** | **0.49** | **1.09** | **1.07** | **2.37** | **2.21** |
| SD | | | | | | 0.05 | 0.05 | 0.05 | 0.03 | 0.03 | 0.02 | 0.02 | 0.03 | 0.09 | 0.06 |
| **Mean T2/mean F** | | | | | | ***0.49*** | ***1.01*** | ***1.02*** | ***0.54*** | ***2.08*** | ***2.10*** | ***1.12*** | ***1.01*** | ***0.54*** | ***0.53*** |

[a]The steps of the calculation are explained in **Note 10**. Note that the exon ratios are independent of the absolute peak heights.

**Table 3**
**Calculation of Exon Ratios, SD, and Normalized Ratios Corresponding to Fig. 3**[a]

| *Case* | *3i* | *12* | *9* | *2* | *C328* | *C328/3i* | *C328/12* | *C328/9* | *C328/2* | *Ex3i/12* | *Ex3i/9* | *Ex3i/2* | *Ex12/9* | *Ex12/2* | *Ex9/2* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control 1(C1) | 809 | 1001 | 845 | 752 | 1015 | 1.25 | 1.01 | 1.20 | 1.35 | 0.81 | 0.96 | 1.08 | 1.18 | 1.33 | 1.12 |
| | 953 | 1157 | 1000 | 959 | 1344 | 1.41 | 1.16 | 1.34 | 1.40 | 0.82 | 0.95 | 0.99 | 1.16 | 1.21 | 1.04 |
| **Mean** | | | | | | **1.33** | **1.09** | **1.27** | **1.38** | **0.82** | **0.96** | **1.03** | **1.17** | **1.27** | **1.08** |
| SD | | | | | | 0.11 | 0.10 | 0.10 | 0.04 | 0.01 | 0.00 | 0.06 | 0.02 | 0.09 | 0.06 |
| ***Mean C1-3/mean C1*** | | | | | | ***0.90*** | ***0.95*** | ***0.92*** | ***0.96*** | ***1.05*** | ***1.02*** | ***1.06*** | ***0.97*** | ***1.01*** | ***1.05*** |
| Control 2(C2) | 745 | 897 | 764 | 669 | 828 | 1.11 | 0.92 | 1.08 | 1.24 | 0.83 | 0.98 | 1.11 | 1.17 | 1.34 | 1.14 |
| | 854 | 990 | 856 | 783 | 1081 | 1.27 | 1.09 | 1.26 | 1.38 | 0.86 | 0.98 | 1.11 | 1.17 | 1.34 | 1.14 |
| **Mean** | | | | | | **1.19** | **1.01** | **1.17** | **1.31** | **0.85** | **0.99** | **1.10** | **1.17** | **1.30** | **1.12** |
| SD | | | | | | 0.11 | 0.12 | 0.13 | 0.10 | 0.02 | 0.02 | 0.02 | 0.01 | 0.05 | 0.03 |
| ***Mean C1-3/mean C2*** | | | | | | ***1.01*** | ***1.02*** | ***1.00*** | ***1.01*** | ***1.01*** | ***0.98*** | ***1.00*** | ***0.97*** | ***0.99*** | ***1.01*** |
| Control 3(C3) | 1329 | 1473 | 1438 | 1237 | 1427 | 1.07 | 0.97 | 0.99 | 1.15 | 0.90 | 0.92 | 1.07 | 1.02 | 1.19 | 1.16 |
| | 1444 | 1578 | 1423 | 1146 | 1616 | 1.12 | 1.02 | 1.14 | 1.41 | 0.92 | 1.01 | 1.26 | 1.11 | 1.38 | 1.24 |
| **Mean** | | | | | | **1.10** | **1.00** | **1.06** | **1.28** | **0.91** | **0.97** | **1.17** | **1.07** | **1.28** | **1.20** |
| SD | | | | | | 0.03 | 0.04 | 0.10 | 0.18 | 0.01 | 0.06 | 0.13 | 0.06 | 0.13 | 0.06 |
| ***Mean C1-3/mean C13*** | | | | | | ***1.10*** | ***1.03*** | ***1.10*** | ***1.03*** | ***0.94*** | ***1.00*** | ***0.94*** | ***1.06*** | ***1.00*** | ***0.94*** |
| **Mean of C1-3** | | | | | | 1.21 | 1.03 | 1.17 | 1.32 | 0.86 | 0.97 | 1.10 | 1.13 | 1.29 | 1.13 |
| **SD of C1-3** | | | | | | 0.13 | 0.09 | 0.13 | 0.10 | 0.04 | 0.03 | 0.09 | 0.06 | 0.08 | 0.07 |

*(continued)*

**Table 3 *(continued)***
**Calculation of Exon Ratios, SD and Normalized Ratios Corresponding to Fig. 3[a]**

| Case | 3i | 12 | 9 | 2 | C328 | C328/3i | C328/12 | C328/9 | C328/2 | Ex3i/12 | Ex3i/9 | Ex3i/2 | Ex12/9 | Ex12/2 | Ex9/2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Positive | | | | | | | | | | | | | | | |
| Control (PC) | 937 | 1041 | 494 | 901 | 1294 | 1.38 | 1.24 | 2.62 | 1.44 | 0.90 | 1.90 | 1.04 | 2.11 | 1.16 | 0.55 |
| | 1011 | 1096 | 491 | 818 | 1076 | 1.06 | 0.98 | 2.19 | 1.32 | 0.92 | 2.06 | 1.24 | 2.23 | 1.34 | 0.60 |
| **Mean** | | | | | | **1.22** | **1.11** | **2.41** | **1.38** | **0.91** | **1.98** | **1.14** | **2.17** | **1.25** | **0.57** |
| SD | | | | | | 0.22 | 0.18 | 0.30 | 0.09 | 0.02 | 0.11 | 0.14 | 0.09 | 0.13 | 0.04 |
| ***Mean C1-3/mean PC*** | | | | | | ***0.99*** | ***0.93*** | ***0.49*** | ***0.96*** | ***0.94*** | ***0.49*** | ***0.97*** | ***0.52*** | ***1.03*** | ***1.98*** |
| CaseA | 2116 | 1634 | 1465 | 1372 | 1930 | 0.91 | 1.18 | 1.32 | 1.14 | 1.29 | 1.44 | 1.54 | 1.12 | 1.19 | 1.07 |
| | 1375 | 1252 | 1087 | 963 | 1259 | 0.92 | 1.01 | 1.16 | 1.31 | 1.10 | 1.26 | 1.43 | 1.15 | 1.30 | 1.13 |
| | 1599 | 1228 | 1129 | 962 | 1366 | 0.85 | 1.11 | 1.21 | 1.42 | 1.30 | 1.42 | 1.66 | 1.09 | 1.28 | 1.17 |
| | 2569 | 1700 | 1586 | 1377 | 1965 | 0.73 | 1.10 | 1.18 | 1.35 | 1.51 | 1.62 | 1.87 | 1.07 | 1.23 | 1.15 |
| **Mean** | | | | | | **0.85** | **1.10** | **1.22** | **1.37** | **1.30** | **1.44** | **1.62** | **1.11** | **1.25** | **1.13** |
| SD | | | | | | 0.09 | 0.07 | 0.07 | 0.05 | 0.17 | 0.15 | 0.19 | 0.04 | 0.05 | 0.05 |
| ***Mean C1-3/mean A*** | | | | | | ***1.42*** | ***0.94*** | ***0.96*** | ***0.96*** | ***0.66*** | ***0.68*** | ***0.68*** | ***1.02*** | ***1.03*** | ***1.00*** |
| Case B | 2975 | 1704 | 1545 | 1428 | 1881 | 0.63 | 1.10 | 1.22 | 1.32 | 1.75 | 1.93 | 2.08 | 1.10 | 1.19 | 1.08 |
| | 2452 | 1273 | 1144 | 991 | 1370 | 0.56 | 1.08 | 1.20 | 1.38 | 1.93 | 2.14 | 2.47 | 1.11 | 1.28 | 1.15 |
| | 2545 | 1491 | 1564 | 1429 | 1958 | 0.77 | 1.31 | 1.25 | 1.37 | 1.71 | 1.63 | 1.78 | 0.95 | 1.04 | 1.09 |
| | 2776 | 1523 | 1435 | 1107 | 1495 | 0.54 | 0.98 | 1.04 | 1.35 | 1.82 | 1.93 | 2.51 | 1.06 | 1.38 | 1.30 |
| **Mean** | | | | | | ***0.62*** | ***1.12*** | ***1.18*** | ***1.36*** | ***1.80*** | ***1.91*** | ***2.21*** | ***1.06*** | ***1.22*** | ***1.16*** |
| SD | | | | | | 0.10 | 0.14 | 0.09 | 0.03 | 0.10 | 0.21 | 0.35 | 0.07 | 0.14 | 0.10 |
| **Mean C1-3/mean B** | | | | | | ***1.93*** | ***0.92*** | ***0.99*** | ***0.98*** | ***0.48*** | ***0.51*** | ***0.50*** | ***1.07*** | ***1.05*** | ***0.98*** |

[a]The steps of the calculation are explained in Note 10. Note that the exon ratios are independent of the absolute peak heights.

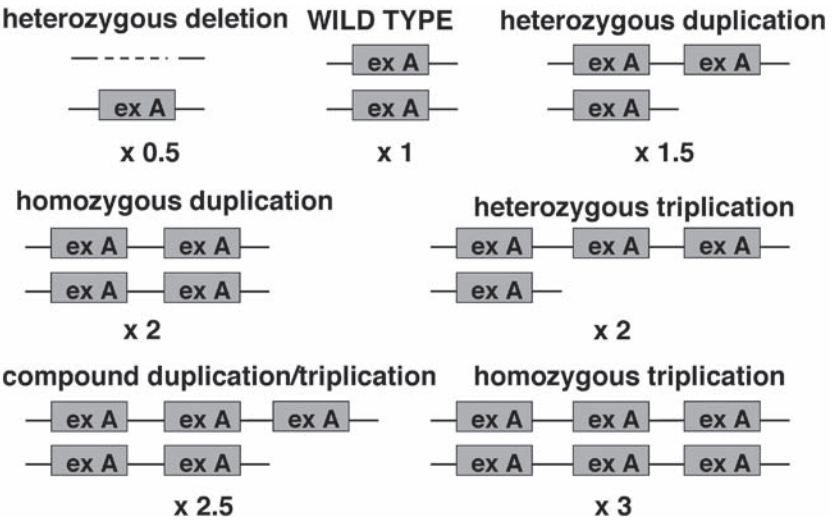


Fig 4. Schematic representation of the exon rearrangements. Below each type of rearrangement, the NR is indicated. Since the presence of two exons results in an NR of 1, the presence of one exon (heterozygous deletion) results in a NR of 0.5, more than 2 exons (as shown for the different multiplications) in NRs >1, i.e., 4 exons resulting in a NR of 2 and 6 exons in a NR of 3. The positions of the multiplied exons are theoretical, because we do not know whether they are arranged in tandem.

## Acknowledgments

This work was supported by the AP-HP (Assistance publique - Hôpitaux de Paris), the Association France-Parkinson, the European Community Biomed 2 (BMH4CT960664), and Aventis Pharma France. C.B.L. was supported by the Deutsche Forschungsgemeinschaft (DFG). We thank the family members for their participation, N. Rawal for the establishment of the multiplex combination 4, and Dr. Merle Ruberg for helpful discussions.

## References

1. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura,Y., Minoshima, S., et al. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392,** 605–608.
2. Hattori, N., Kitada, T., Matsumine, H., Asakawa, S., Yamamura, Y., Yoshino, H., et al. (1998) Molecular genetic analysis of a novel parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the parkin gene in affected individuals. *Ann. Neurol.* **44,** 935–941.
3. Lücking, C. B., Dürr , A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., et al. (2000) Association between early-onset Parkinson's Disease and mutations in the *parkin* gene. *N. Engl. J. Med.* **342,** 1560–1567.
4. Klein, C., Pramstaller, P. P., Kis, B., Page, C. C., Kann, M., Leung, J., et al. (2000) Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. *Ann. Neurol.* **48,** 65–71.

5. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., et al. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25,** 302–305.
6. Zhang, Y., Gao, J., Chung, K. K. K., Huang, H., Dawson, V. L., and Dawson, T. M. (2000) Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl. Acad. Sci. USA* **97,** 13,354–13,359.
7. Prior, T. W. (1998) Determining gene dosage. *Clin. Chem.* **44,** 703–704.
8. Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H., and Vogt, P. H. (1997) Multiplex PCR: Critical Parameters and Step-by-Step Protocol. *BioTechniques* **23,** 504–511.