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Methods and Protocols

Edited by

Nicholas T. Potter

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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

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I

Quantitative PCR

1

Determination of Gene Dosage

Utilization of Endogenous and Exogenous Internal Standards

Thomas W. Prior

1. Introduction

There are currently several screening methods for the detection of point mutations, such as single-stranded conformation polymorphism, heteroduplex analysis, denaturing gradient gel electrophoresis, and chemical cleavage. These are powerful tools for the identification of small sequence changes, but fail to detect heterozygous deletions or duplications of exons, genes or chromosomes. There are many genetic disorders where the primary defect is either owing to allelic deletions (Duchenne muscular dystrophy, spinal muscular atrophy, alpha thalassemia, growth hormone deficiency, familial hypercholesterolemia, and so on) or duplications (Charcot-Marie-Tooth, Klinefelter syndrome, Down syndrome, and so on). Furthermore, for the determination of the carrier state, for disorders such as Duchenne muscular dystrophy and spinal muscular atrophy, the accurate determination of heterozygous deletions is essential. This chapter will describe two methods for the determination of gene dosage, using Duchenne muscular dystrophy and spinal muscular atrophy as examples.

1.1. Duchenne Muscular Dystrophy Dosage Testing Utilization of an Endogenous Internal Standard

Duchenne muscular dystrophy is an X-linked neuromuscular disease characterized by progressive muscular weakness and degeneration of skeletal muscle. Approximately 60% of the DMD and BMD patients have deletions of the dystrophin gene *(1–3)*. Originally, in order to identify female carriers in Duchenne muscular dystrophy, one performed gene dosage using quantitative Southern blot analysis, whereby one determines whether the female at risk exhibits no reduction (noncarrier status) or 50% reduction (carrier status) in hybridization intensity in those bands that are deleted in the affected male *(4,5)*. The dosage determinations permit direct carrier analysis and eliminates the inherent problems of the restriction fragment length polymorphism (RFLP) technique (recombinations, noninformative meioses, unavailability of family members, and sporadic mutations). To further increase the accuracy of the dosage analysis, the autoradiographic bands can be scanned with a densitometer *(6)*.

Although dosage analysis has significantly improved carrier studies, particularly in the isolated cases of the disease, there are technical limitations. Dosage analysis of

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Southern blots requires optimal conditions; very good quality blots are necessary, with even transfer and hybridization, and low background. In order to obtain this high quality we have found that approx 20% of the Southerns have to be repeated, resulting in increased time and labor. Rather than directly comparing single bands, band ratios are calculated as a means of decreasing the error caused by differences in the amount of DNA in each lane. The normal control ratio is established by comparing a band lacking against a band present in the patient (which serves as an internal control) in an unaffected female. When this ratio in a female (at risk) is approx half the control ratio, this indicates that she has a single copy of the band deleted in the patient and therefore is a carrier. Depending on the extent of the deletion, the restriction fragments involved in the deletion, and the specific cDNA probe that identifies the deletion, one may be extremely limited as to what bands are used in the control ratio. We have found that bands greater than 10 kb and less than 0.5 kb typically result in weaker intensities and are not always adequate for scanning purposes. Mao et al. stated that for deletions in the center of the gene (cDNA 8 hybridizations), they prefer to make a statement of the carrier status only if at least one of the strong hybridizing fragments (7, 3.8, 3.7, or 3.1 kb) is deleted in the patient *(7)*. Furthermore the difference between one or two copies is relatively easy to detect but differences between two and three copies, or sometimes three or four copies, in the case of a duplication or comigrating bands can be very difficult. Lastly, due to the extent of a deletion in an affected individual, no hybridizing bands may be detected with a cDNA probe and comparison of hybridization bands within a lane is not possible in these cases.

The determination of carrier status has significantly improved by using the polymerase chain reaction (PCR). Since the extension product of each primer serves as a template for the other primer, each cycle essentially doubles the amount of the DNA product produced in the previous PCR cycle. This results in the exponential accumulation of the specific fragment, up to several millionfold in a few hours. However, to obtain quantitative results, the PCR products must be estimated during the exponential phase of the amplification process, because it is during the exponential phase where the amount of amplified products is proportional to the abundance of starting DNA *(8)*. This occurs when the primers, nucleotides, and Taq polymerase are in a large excess over that of the template concentration. In our experience, after the completion of an adequate number of cycles (25–30) to visualize the PCR products on an ethidium-bromide-stained gel, the PCR reaction is no longer in the exponential quantitative range. Therefore the gene dosage-PCR is accomplished by amplifying the genomic DNA at lower cycle numbers (before visualization by ethidium bromide), running the products out on an agarose gel, Southern transferring the products, and hybridizing the amplicons with a radiolabeled probe. We have found that linearity is well-maintained within 10–15 cycles and hybridization band intensity is still strong *(9)*.

A case study using quantitative PCR is shown in **Fig. 1**. A DMD patient was found to have a molecular deletion for exons 8–19. This was an isolated case of the disease and the mother and two daughters were tested for carrier status. Therefore exons 19 and 50 in the mother, daughters, proband, and a normal female control were amplified for 12 cycles, hybridized with the corresponding cDNA probes, and the autoradiogram is shown in **Fig. 1**. Exon 50 serves as an endogenous internal control, because this is an exon that is not deleted in the patient. The endogenous internal standard is coamplified

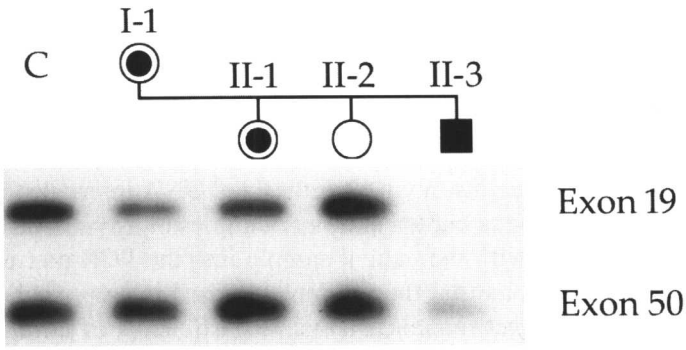

Fig. 1. Duchenne muscular dystrophy carrier determination by gene dosage using an endogenous internal standard. The affected son is deleted for exon 19. Exon 50 is the endogenous internal standard, since the affected son is not deleted for this exon. The mother (I-1) and daughter (II-1) show a 50% reduction in the exon 19/50 ratio compared to the C (noncarrier female control). Daughter II-2 is a noncarrier since her exon 19/50 is equivalent to C.

with the target of interest (deleted exon) and serves as a control for several factors: differences in initial template concentrations between different samples, sample-to-sample variations in the PCR, the extent of any DNA degradation, and differences in the amounts of amplicon loaded onto the gel. Thus, rather than directly comparing single bands, band ratios are calculated. The 19/50 exon ratios in the mother and daughter (II-1) were approx half the normal control ratio, and the ratio in daughter (II-2) was the same as the control. The ratios were confirmed by densitometer. Therefore, the mother, daughter (II-1) are carriers and daughter (II-2) is a noncarrier of the exon 19. Dosage determinations permit direct carrier analysis and eliminates the inherent problems of the RFLP technique (recombinations, noninformative meioses, unavailability of family members, and spontaneous mutations). This is important since unlike the affected males, the heterozygous females are generally asymptomatic and creatine kinase (CK) is only elevated in approx 50–60% of known carriers ***(10)***.

1.2. Spinal Muscular Atrophy Dosage Testing: Utilization of an Exogenous Internal Standard

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration of the motor neurons in the spinal cord, resulting in symmetrical limb and trunk paralysis. With a prevalence of about 1 in 10,000 live births, and a carrier frequency of approx 1 in 50, SMA is the second most common fatal autosomal recessive disorder after cystic fibrosis. The survival motor neuron gene (SMN) has been shown to be deleted in approx 95% of patients with SMA ***(11)***. Although direct diagnostics have significantly improved by the identification of homozygous SMN gene deletions, carrier detection for the determination of a single copy of the gene is a technical challenge. This is mainly due to the fact that the SMA region is characterized by the presence of many repeated elements. The SMN gene itself is present in two almost identical copies, a telomeric (SMN1) and a centromeric copy (SMN2). The two genes differ in exons by only two base pairs, one in exon 7 and one in exon 8, and it is SMN1 that is deleted in cases of SMA. Although the centromic and telomeric copies can be readily

separated by a restriction enzyme digestion, using the centromeric copy as the internal endogenous standard will not be accurate since it is not constant. In the normal population we have observed the following: approx 10% are homozygously deleted, 40% have one copy, and 50% have two copies of the SMN2 gene, respectively.

Dosage determination of the SMN copy number is performed by a competitive PCR method using an exogenous in vitro synthesized DNA internal standard *(12)*. In the competitive PCR method, a known number of copies of a synthetic mutated internal standard is introduced with the patient sample into the PCR mixture. The major advantage of this technique is that the internal standard is amplified with the same primers that amplify the target sequence. Thus, the efficiency of the amplification of the patient DNA and the internal standard DNA should be very similar and allow one to accurately determine the gene copy number. The internal standard is synthesized using the same forward specific primer. However, the reverse primer has now been moved 50 bases upstream from the original reverse primer and is tagged at it 5' end with the original reverse primer sequence *(13)*. The resulting PCR product will thus be identical to the original specific PCR product, but will lack 50 base pairs and thus be distinguished from the endogenous sequence by size (**Fig. 2A**). The internal standard is quantitated by UV spectrophotometry, and diluted to an appropriate concentration. The quantitative PCR dosage assay then consists of spiking a known amount of the internal standard to the patient sample and amplifying the sample with the original forward and reverse primers that are against common sequences (**Fig. 2B**). One of the primers is ^{32}P-end-labeled. With this approach, two products will be generated: one derived from the patient and a second 50 bp smaller product from the internal standard. The PCR products are then diluted in loading buffer, electrophoresed on a 6% denaturing gel, and autoradiography is performed.

Our dosage assay also uses an exon from the cystic fibrosis transmembrane regulator (CFTR) as an internal standard *(14)*. Thus, multiple ratios can be utilized for the accurate determination of carrier status and, most importantly, changes in the SMN1 dosage as a result of fluctuations in the SMN2 copy number are avoided. Furthermore, the use of two internal standards (SMN-IS and CFTR-IS) allows one to monitor the efficiency of the PCR reaction and ensures that equal amounts of target DNA is added to each tube. Similar quantitative PCR approaches have been used successfully to identify deletions in the insulin receptor gene *(15)*, to detect duplications in Down syndrome patients *(15)*, and to quantitate oncogene amplification *(16)*.

Figure 3 shows several carriers and noncarriers identified using the competitive PCR with the exogenous internal standards. As shown, although the SMN2 copies varies from 0–2 copies, the dosage ratios are maintained. Furthermore, multiple ratios can be used in determining carrier status and thereby improve the overall quality assurance of the assay. Our present protocol utilizes the SMN1/CFTR ratio.

2. Materials

2.1. Genomic Isolation

1. Genomic DNA was extracted from leukocytes harvested from whole blood anticoagulated with EDTA using a salting out procedure *(17)*. DNA concentrations were determined using a spectrophotometer, as well as by monitoring the intensity of ethidium bromide staining on a test gel.