

POLICY

Best practice guidelines and recommendations on the molecular diagnosis of myotonic dystrophy types 1 and 2

Erik-Jan Kamsteeg¹, Wolfram Kress², Claudio Catalli³, Jens M Hertz⁴, Martina Witsch-Baumgartner⁵, Michael F Buckley¹, Baziel GM van Engelen⁶, Marianne Schwartz⁷ and Hans Scheffer^{*,1}

Myotonic dystrophy is an autosomal dominant, multisystem disorder that is characterized by myotonic myopathy. The symptoms and severity of myotonic dystrophy type 1 (DM1) ranges from severe and congenital forms, which frequently result in death because of respiratory deficiency, through to late-onset baldness and cataract. In adult patients, cardiac conduction abnormalities may occur and cause a shorter life span. In subsequent generations, the symptoms in DM1 may present at an earlier age and have a more severe course (anticipation). In myotonic dystrophy type 2 (DM2), no anticipation is described, but cardiac conduction abnormalities as in DM1 are observed and patients with DM2 additionally have muscle pain and stiffness. Both DM1 and DM2 are caused by unstable DNA repeats in untranslated regions of different genes: A (CTG)_n repeat in the 3'-UTR of the *DMPK* gene and a (CCTG)_n repeat in intron 1 of the *CNBP* (formerly *ZNF9*) gene, respectively. The length of the (CTG)_n repeat expansion in DM1 correlates with disease severity and age of onset. Nevertheless, these repeat sizes have limited predictive values on individual bases. Because of the disease characteristics in DM1 and DM2, appropriate molecular testing and reporting is very important for the optimal counseling in myotonic dystrophy. Here, we describe best practice guidelines for clinical molecular genetic analysis and reporting in DM1 and DM2, including presymptomatic and prenatal testing.

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MYOTONIC DYSTROPHY TYPES 1 AND 2

Myotonic dystrophy (DM) is an autosomal dominant, multisystem disorder that is characterized by myotonic myopathy with associated abnormalities in other organs.¹ DM is clinically heterogeneous, and at least two types can be distinguished: DM type 1 (DM1; Steinert disease), which can be subdivided into four clinical subgroups, and DM type 2 (DM2; proximal myotonic myopathy (PROMM), or Ricker syndrome). DM1 is the most common form of muscular dystrophy in adults with an estimated incidence of 1:8000.² The incidence for DM2 seems to be much lower than for DM1 and appears to be population dependent, reaching a higher incidence in Germany and Finland³ with an incidence similar to that of DM1. Despite the comparable core diagnostic features and multi-organ involvement, there are specific clinical features of DM1 and DM2, which enable them to be distinguished.

Myotonic dystrophy type 1

There is a wide range of symptoms in DM1, ranging from mild features, such as baldness and cataracts, to very severe features including muscle weakness and cardiac conduction defects. Many of the severely affected patients die of cardiac failure or respiratory distress. The cardiac involvement in DM1 may range between asymptomatic ECG abnormalities to sudden cardiac death.⁴

From a clinical perspective DM1 may be classified into four different subtypes: (i) mild; (ii) classical; (iii) juvenile and (iv) congenital types: (i) mild DM1: mildly symptomatic patients may have premature cataracts and baldness as the sole clinical features. A late-onset myopathy may develop and myotonia may only be detectable by electromyography. Cardiac conduction abnormalities may arise and result in a shorter life span. (ii) Classical or adult-onset DM1: The age of onset is typically in the second or third decade of life. The most frequent symptoms are distal weakness, involving the long finger flexors of the arms and the dorsiflexors of the legs, leading to symptoms relating to the strength of hand grasping and an increased incidence of stumbling. In addition patients may have cataracts, baldness and cardiac conduction abnormalities as in minimal DM1. Clinical myotonia, gastro-intestinal symptoms and fatigue may also occur. Apathy, lack of initiative, daytime sleepiness and experience fatigue can be distressing to DM1-patients, their partners and other family members. These features have a significant impact on quality of life. (iii) Juvenile DM1: this form resembles the classical form of myotonic dystrophy. However, it is more clearly associated with cognitive and behavioral abnormalities, for example, difficulties in learning and socialization at school. Muscle involvement may be minimal in the juvenile presentation. (iv) Congenital DM1. Polyhydramnios and poor fetal movements precede the birth of an

¹Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; ²Institute of Human Genetics, University of Würzburg, Biozentrum, Würzburg, Germany; ³Department of Biopathology and Diagnostic Imaging, Tor Vergata University, Rome, Italy; ⁴Department of Clinical Genetics, Aarhus University Hospital, Aarhus, Denmark; ⁵Department of Medical Genetics, Molecular and Clinical Pharmacology Medical University Innsbruck, Innsbruck, Austria; ⁶Neuromuscular Centre Nijmegen, Department of Neurology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; ⁷Department of Clinical Genetics, University Hospital Rigshospitalet, Copenhagen, Denmark

*Correspondence: Dr H Scheffer, Department of Human Genetics, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands. Tel: 31 243617757; Fax: 31 243616658; E-mail: H.Scheffer@gen.umcn.nl

infant with congenital DM1. The affected parent is nearly always the mother and congenital DM1 occurs in a quarter of offspring of affected DM1 mothers.² The infant is typically a floppy baby with failure to thrive and severe respiratory distress. Facial and jaw muscles are weak and produce a tented upper lip with difficulties in feeding and suckling. Clubfeet and joint contractures are also common. Myotonia is typically absent clinically, and may be difficult to detect initially even on EMG. Mental retardation and developmental delay are common. There is a high mortality rate in the perinatal period. Muscle biopsies of patients with congenital DM1 may reveal only variability in fiber size and centralization of nuclei. However, none of the characteristics found in muscle biopsies of patients with classical or adult-onset DM1 myotonic dystrophy are present. Therefore, in order to confirm a clinical suspicion of congenital DM1, the diagnosis can only be established by DNA analysis.^{1,2}

Myotonic dystrophy type 2

The DM2 phenotype has some similarities to that of DM1 (myotonia, cataract and muscle weakness), but also some differences. The typical clinical presentation of DM2 is that of predominantly proximal muscle weakness, with muscle pain but no atrophy (PROMM phenotype).⁵ Myotonia is variable (both clinically as well as on EMG). However, in its mildest form, DM2 can be difficult to recognize. Conversely, DM2 may also present with severe proximal muscle involvement with remarkable muscle wasting without clinical myotonia.^{6,7} The mean age of onset for DM2 is in the third and fourth decades of life. Unlike DM1, no congenital form of DM2 has been described.

In summary, the clinical challenge in DM1 lies in supplying optimal care for this multisystem disease, whereas in DM2 it is also a diagnostic challenge to recognize the disease. Detection of a repeat expansion in the *DMPK* gene and the *CNBP* gene is fast, inexpensive and reliable. Both the clinical sensitivity and the clinical specificity are >99% (Eurogentest clinical utility gene cards: <http://www.eurogentest.org>).

MOLECULAR GENETIC DEFECTS IN MYOTONIC DYSTROPHY TYPES 1 AND 2

DM1 (MIM #160900) is caused by a (CTG)_n repeat expansion in the 3'-untranslated region of the *DMPK* gene located within chromosome band 19q13.3.^{8–10} DM2 (MIM #602668) is caused by a large (CCTG)_n repeat expansion in intron 1 of the *CNBP* (formerly known as *ZNF9*) gene, at chromosome 3q21.^{11–13}

The number of CTG-repeat units in stable nonpathogenic *DMPK* alleles ranges from 5 to 35.^{1,14} (In previous publications, we have used 37 as limit of the largest normal allele size. Because of the use of 35 by other bodies, such as 'The International Myotonic Dystrophy Consortium (IDMC),¹ this was adapted for congruity. As repeats of 36 and 37 have not been detected in a large group of individuals,¹⁴ this adaptation is not anticipated to have any consequences for past or future reporting). *DMPK* alleles containing over 35 CTG-repeat units demonstrate a length-dependent risk of instability on transmission. Alleles containing a CTG-repeat with a length of 51–150 may be either asymptomatic or may give rise to minimal or classical DM1. A more severe DM1 phenotype is associated with *DMPK* alleles with sizes >150 CTG-repeat units. An overview of the general phenotype–genotype correlation^{1,14–16} is provided in Table 1.

Thus, the severity of the disease and the age of onset is correlated with the number of CTG-repeat units within the expanded DNA

Table 1 Size ranges of CTG repeats in the *DMPK* gene and corresponding most common phenotypes

Number of repeats	Stability of repeat	DM phenotype
5–35 (normal range)	Stable	No DM
36–50	May be unstable	No DM
51–150	Unstable	No, minimal or classical DM
> 150	Unstable	Classical, juvenile or congenital DM

Abbreviation: DM, myotonic dystrophy.

sequence, although the individual variability is high.^{15–19} An increasing number of CTG-repeat units within the *DMPK* gene in successive generations in a family is associated with an earlier onset of the disorder. This phenomenon is termed 'anticipation'.²⁰ For DM1 parental sex has an important role in determining the level of intergenerational instability once the repeat size exceeds 150 repeats.¹⁵ Congenital DM1 is almost exclusively transmitted by the mother, a phenomenon that is not well understood.

In less than 5% of DM1 patients, the otherwise pure CTG-repeat tract is interrupted by GGC, CCG or CTC repeats.^{21,22} Despite the reported unusual disease characteristics in some of these patients, the clinical relevance of such interruption remains to be established.

The *CNBP* gene harbors a complex-repeat tract (TG)_n(TCTG)_n(CCTG)_n, and expansions of the CCTG-repeat array within this complex repeat are the cause of DM2.¹¹ Nonpathogenic alleles contain to 26 CCTG-repeat units. In DM2 patients, the range of repeat units is extremely wide, ranging from about 75 to over 11 000 units, with a mean of 5000.¹¹ Unlike in DM1, no significant association between the age of onset and the number of CCTG-repeat unit has been detected in DM2.²³ Likewise, anticipation has not been observed in DM2 families.

MOLECULAR DIAGNOSTIC ANALYSIS IN DM1 AND DM2

The first step in the molecular analysis of DM1 or DM2 is to analyze whether an individual has two alleles with a low number of repeats that can easily be detected by conventional PCR and fragment-length analysis. If only one allele size is detected, subsequent techniques such as repeat-primed PCR and or Southern blotting of genomic DNA or long-range PCR products are used to detect possible repeat expansions. PCR primers used by different laboratories are listed in Table 2.

In prenatal DM1 testing, analysis of DNA from the mother is also required to exclude maternal contamination in the fetal samples. The combination of these data allows interpretation and reporting. The delivery of diagnostic molecular genetics services for pre/post-natal DM requires in-house validation, on-going quality control processes and the supervision by a clinical molecular geneticist.

Molecular diagnostic tests in DM1

PCR and fragment-length analysis. In the lower range of DM1 expansions, the best analytical method is PCR. Alleles containing between 5 and 100 to 125 CTG-repeat units can be detected and characterized using synthetic fluorescently-labeled primers flanking the CTG-repeat region, followed by direct analysis of the length of the amplified products by capillary electrophoresis.

Triplet-repeat Primed (TP)-PCR. The TP-PCR is a PCR with three primers, where one lies outside the repeat, one within the repeat (which is added in limiting amounts) that also has a sequence-tail

Table 2 Primer sequences used for myotonic dystrophy tests

Gene	Location	Purpose	Center	Sequence (5'-3')
<i>DMPK</i>	Forward	PCR	C	cttccaggcctgcagttgccatc
	Reverse	PCR	C	gaacgggctcgaaggctcctgtagc
	Forward	PCR	N	ctcgaagggtccttgtagcc
	Forward	TP-PCR	N	gaagggtccttgtagccgggaa
	Forward	LR-PCR	N	ccgttgggaagactgagtg
	Reverse	PCR	N	tgacaagaagccttgacac
	Reverse	TP-PCR	N	tacgcatcccagttgagac <u>gcagcagcagcagcag</u>
	Universal	TP-PCR	N	tacgcatcccagttgagacg
	Reverse	LR-PCR	N	ctggccgaagaagaaatg
	<i>CNBP</i>	Forward	PCR	W
Forward		PCR	N	ttgacttggaaatgagtgaatg
Forward		LR-PCR	N/W	ggccttataacctgcaaatg ^a
Reverse		(LR)-PCR/ QP-PCR	W/R	gcctaggggacaagtgag(a) ^a
Reverse		PCR	N	agccgagatcataccactgc
Reverse		LR-PCR/ QP-PCR	N	agcctaggggacaagtgag
Forward		QP-PCR	N/W/R	tacgcatcccagttgagac <u>gcctgctgctgctgctgctgctg</u>
Universal		QP-PCR	R	tacgcatcccagttgagacg

Abbreviations: C, Copenhagen; LR, long-range; N, Nijmegen; QP, quadruplet-repeat primed; R, Rome; TP: triplet-repeat primed, W, Würzburg

Repeat sequences are underlined.

^aThese primers amplify the CL3N58 marker, which is defined as a CAGG repeat and is the reverse complement of the CCTG repeat in the *CNBP* gene.

(a) A rare polymorphism affecting this base was detected on a *CNBP* allele that also harbors a CCTG expansion (E Ormshaw, SW Thames Molecular Genetics Diagnostic Laboratory, London, UK, personal communication).

complementary to the third, universal, and fluorescently-labeled primer.²⁴ This will result in a mixture of PCR fragments of different sizes that can be analyzed by capillary electrophoresis. Although expansions in all size ranges can be detected by TP-PCR, no reliable information about the length of the expanded repeat will be obtained because of extinction of the signal in the higher size region. In DM1 expansion analysis (see Figure 1a for an example), the TP-PCR has proven to be an accurate technique. Nevertheless, the presence of rare interruptions of the otherwise pure CTG repeat^{21,22} may result in aberrant patterns or failure to detect expansions with TP-PCR.²⁵ Therefore, to exclude false-negative reports, an additional test (such as a TP-PCR at the other repeat end or Southern blotting) is strongly recommended.

Southern blotting of long-range PCR-products. This Southern blot method (for an example, see Figure 1b) requires precise PCR conditions to ensure the amplification of larger fragments, monitored by using appropriate positive and negative (healthy) controls, followed by probing with an end-labeled $(CTG)_x$ (where x is 5 or more) probe.²⁶ On these Southern blots, expanded *DMPK* alleles will appear in virtually all cases as smears or multiple fragments, which is (in part) owing to somatic heterogeneity. Also, as with the TP-PCR, interrupted repeats may remain undetectable under certain conditions. Therefore, conditions that ensure the amplification of CG-rich and long templates is recommended.

Southern blotting of genomic DNA. Southern blotting of genomic DNA (for an example, see Figure 1c), digested with an appropriate restriction enzyme (*EcoRI*, *BamHI*, *NcoI* or *BglII*), has been the gold standard for the detection of *DMPK* alleles containing 100 CTG-

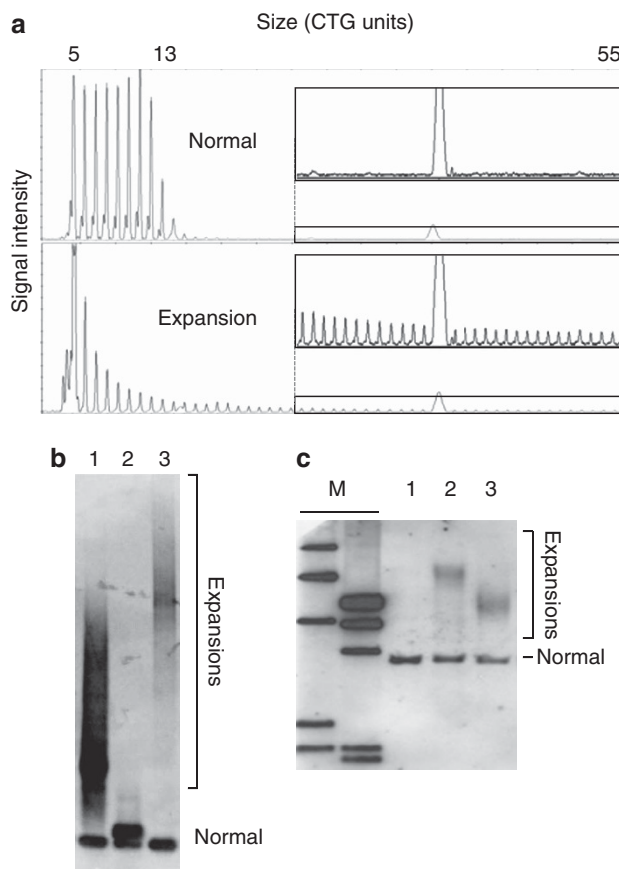


Figure 1 Molecular diagnostic tests in myotonic dystrophy type 1. (a) Fragment-length analysis of TP-PCR products of the CTG repeat in the *DMPK* gene. Fluorescently-labeled PCR products of a healthy individual having two normal alleles (5 and 13 CTG repeats; top panel) and an affected individual (bottom panel) with one normal allele (5 repeats) and one expanded allele were separated by capillary electrophoresis. (b) Southern blotting of long-range PCR-products of the CTG repeat in the *DMPK* gene. Long-range PCR fragments were subjected to agarose gel electrophoresis and capillary transfer to a nylon membrane. Subsequently, the membrane was hybridized with a labeled $(CAG)_5$ probe. Visualized repeats were from a healthy control (lane 2), a patient with a heterozygous expansion in the size range of 51–150 repeats (lane 1), and a patient with a heterozygous expansion in the size range over 150 repeats (lane 3). (c) Southern blotting of genomic DNA probed for the *DMPK* gene. Genomic DNA samples were fragmented using *BglII* and subjected to electrophoresis and capillary blotting to a nylon membrane. Subsequently, the membrane was hybridized with a ^{32}P -labeled probe for the *DMPK* gene. Visualized fragments were from a healthy control (1), and patients with one normal and one expanded allele (2 and 3). Estimation of the lengths of the repeat expansions can be done using the size markers (M). Normal sized alleles and expansions are indicated.

repeat units and over since the identification of the *DMPK* gene.⁸ Several different probes have been reported for hybridization.^{8,9,27,28} However, this procedure is time-consuming and has been replaced by TP-PCR and/or by Southern blotting of long-range PCR-products in most diagnostic centers.

Indirect DNA analysis. Though direct detection of expansions in the *DMPK* gene is the most straightforward technique, indirect DNA analysis is useful in some cases where confirmation of result is

required by a separate technique. Polymorphic markers surrounding the *DMPK* gene that can be used for such an indirect analysis are outlined in Table 3.

Molecular diagnostic tests in DM2

Owing to the extreme expansion sizes (to >40 kb) that may be present in DM2 patients,¹¹ it is important to ensure isolation of high molecular weight DNA from peripheral blood.

PCR and fragment-length analysis. In the lower range of DM2 repeat sizes, the best analytical method is PCR. Such alleles can be detected and characterized using synthetic, fluorescently-labeled primers flanking the complex-repeat region (also known as the CL3N58 repeat marker), followed by direct sizing of the amplified products by capillary electrophoresis. Because of the variability of the different units of this complex (TG)_n(TCTG)_n(CCTG)_n repeat, the precise length of the pathogenic CCTG unit within this repeat can only be determined by sequencing. However, as the difference between normal alleles (repeats to 26 CCTG units) and disease-associated

alleles (75 units and more, with a mean of 5000) almost always is evident, exact sizing is not routinely performed.

Quadruplet-repeat primed (QP)-PCR. This technique, is similar to the TP-PCR used for DM1, but now with a primer in the tetra-nucleotide repeat,²⁹ see Figure 2a for an example). In theory, the QP-PCR will detect expansions independent of their lengths. Although expansions in all size ranges can in theory be detected by QP-PCR, no reliable information about the length of the expanded repeat will be obtained because of extinction of the signal in the higher size region.

Southern blotting of long-range PCR-products. This Southern blot method (see Figure 2b for an example) requires precise PCR conditions to ensure the amplification of larger fragments, monitored by using appropriate positive and negative (healthy) controls. Southern blotting of these fragments is followed by probing with an end-labeled (CCTG)_{5or6} probe.³⁰ On these Southern blots, expanded *CNBP* alleles will appear in virtually all cases as smears or multiple fragments, which is (in part) due to somatic heterogeneity. Using this technique in DM2 diagnostics, one should be aware of false positives, probably occurring because of background signals of total genomic DNA after overexposure of the blot signals. Therefore, negative controls (healthy subjects) should be free of smears on the blot.

Southern blotting of genomic DNA. Direct analysis by Southern blotting (for an example, see Figure 2c), after digestion of genomic DNA with *EcoRI*, *TaqI* or alternative enzymes, followed by hybridization using the CL3N58 probe, was the first method used for studying the DM2 mutation.¹¹ Owing to the extreme size of the expansion and the level of somatic instability as well as cross hybridizations of the probe, only a very heterogeneous smear can be visualized, limiting the sensitivity of this test to ~80% of known carriers when applied without additional precautions. Several improvements of the basic

Table 3 Markers flanking the *DMPK* gene

Marker	Location in bp (Human RefMap)	Position in cM (Genethon)	Allele size (bp)
D19S217	40,713,572–40,713,794	67.8	219–233
D19S574	41,555,837–4,1556,038	69.20	164–202
D19S918	NA	69.50	140–182
D19S219	42,423,170–42,423,347	69.90	160–190
<i>DMPK</i>	42,701,722–42,714,534	70.14	
D19S902	44,758,758–44,758,960	76.20	199–217

Abbreviation: NA, not applicable.

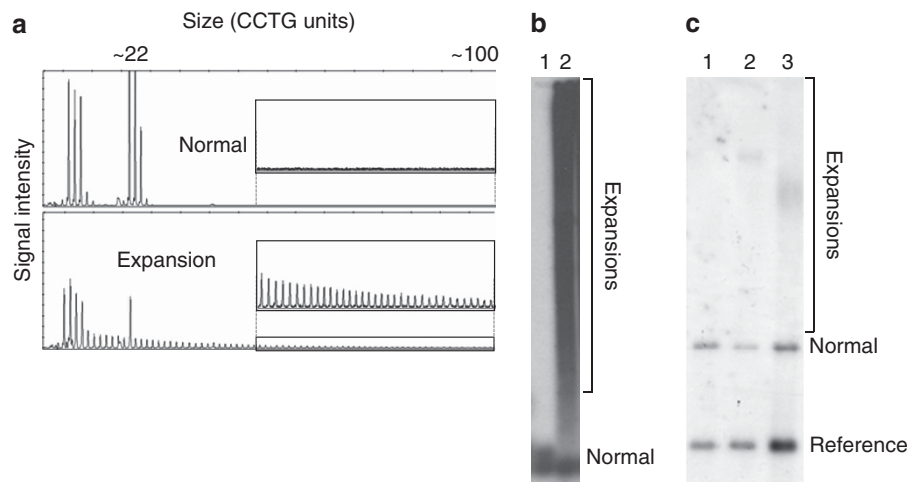


Figure 2 Molecular diagnostic tests in myotonic dystrophy type 2. (a) Fragment-length analysis of QP-PCR products of the CCTG repeat in the *CNBP* gene. Fluorescently-labeled PCR products of a healthy individual (top panel) and an affected individual (bottom panel) with one normal allele and one expanded allele (> 100 repeats) were separated by capillary electrophoresis. (b) Southern blotting of long-range PCR products of the CCTG repeat in the *CNBP* gene. Long-range PCR fragments were subjected to agarose gel electrophoresis and capillary transfer to a nylon membrane. Subsequently, the membrane was hybridized with an end-labeled (CAGG)₅ probe. Visualized repeats were from a healthy compound heterozygous individual (lane 1) and a patient (lane 2) with one normal and one expanded allele. (c) Southern blotting of genomic DNA probed for the *CNBP* gene. Genomic DNA samples were fragmented using *EcoRI* and subjected to electrophoresis and capillary blotting to a nylon membrane. Subsequently, the membrane was hybridized with a mixture of the CL3N58 (within the *CNBP* gene) and a reference probe (both ³²P-labeled). Visualized fragments were from a healthy control (lane 1), and patients with one normal and one expanded allele (lanes 2 and 3). Note the decrease in intensity of the normal allele in the patients with an expansion, when compared with the control probe signal. Normal sized *CNBP* alleles and expansions, as well as the reference probe, are indicated.

protocol can be adopted in order to minimize possible sources of false-negative results, such as the inclusion of a reference probe to allow quantification of the non-expanded alleles.³¹ However, like in DM1 analysis, this procedure is time-consuming and has been replaced by QP-PCR or by Southern blotting of long-range PCR-products by most diagnostic laboratories.

INTERPRETATION AND REPORTING

Myotonic dystrophy type 1

There is an association between CTG-repeat size and the age of onset and severity of symptoms (see Table 1). However, there is a large inter-individual variability and one should be very cautious with phenotypic predictions in individual cases.^{15–19} Therefore, the boundaries in repeat lengths of the four groups in Table 1 should not be interpreted too rigidly.

In DM, it is sufficient to report the size range of a repeat rather than an exact repeat length, that is, 'a normal (CTG)_n repeat length of ($n = 5–35$)' or 'a large expansion of the (CTG)_n repeat ($n > 150$)'. Whenever an exact allele size is reported it should be accompanied by a statement of the testing laboratory's uncertainty of measurement in the sizing.

For the reporting of exact normal allele sizes ($n = 5–35$), which are determined by PCR and subsequent capillary electrophoresis, internal control standards from sequence-verified samples should be included. Alternatively, exact allele sizes can be obtained by DNA sequence analysis. For larger expansions, a range of repeat lengths should be estimated based on comparisons with molecular weight standards and/or characterized control samples.

The result of the appropriate tests will lead to one of the following possibilities and recommended reporting guidelines. (i) No expansion (homozygous or compound heterozygous for a normal allele size): This excludes the diagnosis of DM1. When it concerns a fetus, it is not affected. (ii) A heterozygous expansion in the size range of 36–50 repeats: (a) This excludes the diagnosis of DM1; when it concerns a fetus, it is not affected, (b) repeats of this length may or may not expand in future generations and (c) relatives (including offspring) of the counselee may be at risk of developing DM1. Therefore, they should be offered counseling. An offer of repeat-length analysis to those relatives at risk is warranted. (iii) A heterozygous expansion in the size range of 51–150 repeats: (a) when symptoms are evident, the diagnosis of DM1 is confirmed, (b) When symptoms of DM1 are not evident (asymptomatic family member or fetus), the individual is at risk of developing DM1, although individuals with a repeat expansion of this size may also remain symptomless, (c) counselees of reproductive age, and especially women, are at risk of having children with the congenital form of DM1 and (d) relatives (including offspring) of the counselee may be at risk of developing DM1. Owing to anticipation in DM1, offspring may be more severely affected. Relatives should therefore be offered counseling. An offer of repeat-length analysis to those relatives is warranted. (iv) A heterozygous expansion with a size over 150 repeats: (a) when symptoms are evident, the diagnosis of DM1 is confirmed, (b) when symptoms of DM1 are not evident (asymptomatic family member), the individual is at risk of developing DM1, although individuals with a repeat expansion of this size may also remain symptomless, (c) when it concerns a fetus, it is very likely to be affected and has a high risk to be more severely affected than the affected parent, (d) counselees in the reproductive age, and especially women, are at risk of having children with the congenital form of DM1 and (e) relatives (including offspring) of the counselee may be at risk of developing DM1. Because of

anticipation in DM1, the offspring may be more severely affected. Therefore, relatives should be offered counseling. An offer of repeat-length analysis to those relatives is warranted.

A tabular format of the reporting guidelines is available as Supplementary Data.

Myotonic dystrophy type 2

As variability in the sizes of the different subunits of the complex repeat, (TG)_n(TCTG)_n(CCTG)_n, in the *CNBP* gene exists, the precise length of the pathogenic CCTG unit within this repeat can only be determined by DNA sequencing. However, as repeats to 26 CCTG units are considered to be nonpathogenic, whereas disease-associated alleles contain 75 units and more, exact repeat sizing is not routinely performed. Experiences from several laboratories suggest that most frequently encountered pathogenic repeats reside in the high range (with a mean of 5000 repeats). Alleles between 26 and 75 CCTG units (gray area) are very rare but have been reported.³² Their clinical relevance, however, is not well characterized.

If reporting allele sizes (rather than a range), estimates of the uncertainty of measurement in the sizing in the testing laboratory should be reported. For allele sizes determined by PCR and subsequent capillary electrophoresis, a deviation of two repeat units may be present. For (larger) expansions, a range of repeat lengths should be estimated on basis of markers and/or control samples. However, in the reports of most laboratories the exact length of the repeat is not specified. The result of appropriate testing leads to one of the following possibilities and subsequent recommended reporting guidelines: (i) no expansion (homozygous or compound heterozygous for a normal allele size): this excludes the diagnosis of DM2. (ii) A heterozygous expansion of over 75 CCTG-repeat units: (a) the diagnosis of DM2 is confirmed and (b) relatives (including offspring) of the counselee may be at risk of developing DM2. Therefore, they should be offered counseling. An offer of repeat-length analysis to those relatives is warranted.

A tabular format of the reporting guidelines is available as Supplementary Data.

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