

## **Myotonic Dystrophy: Accurately Scoring the Boundaries that Define Regions of Triplet Repeat Expansion Mutations**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Author PAD wrote the manuscript and performed the lab work at Auckland City Hospital. Author KH verified the data and participated in the lab work. Author DYH performed the statistical analysis and edited the manuscript. Author AA performed the lab work and author DZ verified the data at Concord Hospital and edited the manuscript and added Concord Hospital's methods. Authors ED and DRL supervised the design and execution of the study. Author DL also edited the manuscript. All authors read and approved the final manuscript.*

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### **ABSTRACT**

**Aims:** Myotonic Dystrophy type 1 (DM1) is an autosomal dominant neuromuscular multi-systemic disorder caused by a CTG triplet repeat expansion mutation in the *DMPK* gene. The clinical decision points defining the CTG repeat boundaries between normal, premutation and mild disease ranges are poorly characterised with a lack of commercially available sequenced controls. There are no US Food and Drug Administration (FDA) approved tests for DM1 so testing protocols are developed and managed by individual laboratories.

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**Study Design:** This paper presents a cross-laboratory exchange scheme between Auckland City Hospital and Concord Hospital, which took place between October 2013 and January 2014, in order to validate the scoring of CTG repeats within the *DMPK* gene and to build comprehensive allelic libraries.

**Methodology:** Seven samples ranging from 30-59 repeats, spanning the critical clinical decision points, were sequenced to confirm the “true” repeat sizes, and 19 samples were tested by both laboratories using standard and triplet repeat-primed PCR methods.

**Results:** The results showed a very strong correlation between the sequencing results and the standard PCR results for the 7 selected samples with a Pearson correlation coefficient of 0.999 and  $P = 1.20 \times 10^{-7}$ . The results from the inter-laboratory comparison also showed a very strong correlation between the diagnostic tests of the two labs with a Pearson correlation coefficient of 0.999 and  $P = 1 \times 10^{-29}$ . A paired t-test showed no significant difference between the two laboratories data with a Mean (SD) = 0.263 (0.828),  $P = .058$ .

**Conclusion:** This study provides two critical outcomes. The first is that the extrapolations that were used by each of the participating laboratories in determining the number of CTG repeats in the absence of well-characterised controls in the 35-51 repeat range were within their reported margins-of-error. The second outcome is that small regional laboratories can gain confidence in the accuracy of their reported allele calls, specifically around clinically critical decision points, with inter-laboratory exchange studies and in-house sequencing of relevant control samples.

*Keywords: Myotonic dystrophy; trinucleotide repeats; DNA repeat expansion; DNA sequencing.*

## 1. INTRODUCTION

Myotonic Dystrophy type 1 (DM1) is an autosomal dominant neuromuscular multi-systemic disorder affecting the skeletal muscles, heart, brain, eyes and endocrine system [1-3]. DM1 is the most common adult muscular dystrophy, which is caused by a CTG trinucleotide repeat expansion in the *DMPK* gene that results in a highly variable phenotype. Symptoms include myotonia, muscle wasting, cardiac conduction abnormalities and cataracts [4-8]. DM1 shows genetic anticipation, whereby the number of CTG repeats increase in successive generations decreasing the age of disease onset [9-12]. This can lead to the more severe congenital form that has additional features such as hypotonia in the newborn, respiratory distress, difficulty in sucking and swallowing, delay in muscle maturity and developmental abnormalities [13-15].

The current reference ranges of CTG repeat expansions are: 5-35 repeats for unaffected individuals with a stable repeat transmission; 36-50 repeats for unaffected individuals with a possibly unstable repeat transmission (termed the premutation range); 51-150 repeats for patients who exhibit mild or classic DM1 symptoms with unstable repeat transmission; and >150 repeats for patients who exhibit classic, juvenile or congenital DM1 symptoms with unstable repeat transmission [9,12].

There are no US Food and Drug Administration (FDA) approved tests for DM1 so testing protocols are currently developed and managed by individual laboratories [8]. Current methods for determining the number of CTG repeats largely involve PCR amplification of the CTG repeat region of the *DMPK* gene and then sizing the subsequent amplicons using capillary-based electrophoresis [9]. The lengths of the amplicons are compared against those of known controls based on a correlation curve. Unfortunately, there is a lack of a comprehensive number of characterised control samples to enable accurate estimates to be made of patients' CTG repeat lengths. As a consequence, length estimates are based on extrapolating from a limited number of characterised repeat lengths.

One such region where there is a significant lack of characterised controls at our disposal, and one of the most significant regions for the scoring of CTG repeat lengths in the *DMPK* gene, is between 35 and 51 repeats which border the clinical decision points between normal, premutation and mild DM1. Kalman et al. [8], in conjunction with The Coriell Institute for Medical Research, have characterised 10 DM1 cell lines that carry CTG repeats that lie in four of the five DM1 clinical categories (normal, mild, classical and congenital). Critically, no cell lines with repeat lengths in the premutation range, near the clinical decision points of 35-51 CTG repeats are available. This paper describes the sequencing of a range of controls within the critical region of

35-51 CTG repeats in the *DMPK* gene as well as an international cross-laboratory validation study.

## 2. MATERIALS AND METHODS

An international cross-laboratory validation study of DM1 testing was undertaken using 10 samples tested by the Diagnostic Genetics of LabPlus, Auckland City Hospital, and 9 samples tested by the Molecular Medicine Laboratory of Concord Hospital, Sydney, Australia. Patients, or their guardians, provided informed consent for molecular diagnostic assessment of the DM referrals described here.

Seven samples with alleles ranging from 30-59 repeats spanning the critical clinical decision points between normal, permutation and mild disease were selected for sequencing analysis to accurately measure the repeat lengths. Each laboratory performed testing on all 19 samples in accordance with their own current diagnostic methods using fragment analysis by standard PCR and triplet repeat-primed PCR (TRP-PCR).

PCR amplification of the CTG repeats in the *DMPK* gene for subsequent sequencing used primers that were designed to encompass the triplet repeat region as well as a sufficient buffer region to ensure that sequences directly adjacent to the triplet repeat were easily read. Each amplification used FastStart reaction buffer without MgCl<sub>2</sub> (Roche), 2 mM MgCl<sub>2</sub> (Roche), GC-rich solution (Roche), 0.4 mM dNTPs, 0.8 μM of each of the forward (P1-FAM) and reverse (P2) primers (Table 1), 1U FastStart Taq DNA Polymerase (Roche) and 50 ng of genomic DNA. The PCR conditions involved denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 30 sec, extension at 72°C for 2 min, and then a final extension at 72°C for 30 min.

The PCR products were electrophoretically separated in a 3% Nuseive Gel for up to 2 hours

at 150 volts. Selected amplicons were excised from gels and extracted using a Qiagen QIAquick Gel Extraction Kit. The DNAs were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and the primers P1-SEQ and P2-SEQ (Table 1) at a concentration of 5μM. The sequencing products were subjected to capillary electrophoresis using an Applied Biosystems model 3130xl Genetic Analyzer. The number of CTG repeats was determined from sequence traces that were viewed using Chromas Lite Version 2.0 (Technelysium Pty Ltd).

All of the 19 genomic DNA samples were extracted from peripheral blood using the Gentra Puregene Blood kit (Qiagen, Hilden, Germany). These samples were assessed by the two laboratories to determine the number of CTG repeats in the *DMPK* gene using both standard PCR and TRP-PCR. In the case of Auckland City Hospital, standard DM PCRs and TRP-PCRs were carried out as previously described [16]. Standard PCR amplification used the same PCR reagents and conditions as the Sequencing PCR (see above), but with primers P1-FAM and P2 (Table 1). Two TRP-PCRs were undertaken for each DNA sample: one uses a fluoresceinated primer upstream of the CTG repeat, and the other uses a fluoresceinated primer downstream of the CTG repeat. This approach reduces the risk of false negatives caused by interruptions in the CTG repeat sequence. Master Mix 1 (MM1) used a FAM labelled forward primer (P1-FAM) and a repeat-specific reverse primer (P4CTG); Master Mix 2 (MM2) used a FAM labelled reverse primer (P2-FAM) and a repeat-specific forward primer (P4CAG) (Table 1). The PCR cycling conditions involved denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 70°C for 30 sec, extension at 72°C for 30 sec, then a final extension at 72°C for 10 min.

**Table 1. Primer sequences**

Primer name	Sequence (5' – 3')	PCR method
P1-FAM	FAM-CTTCCCAGGCCTGCAGTTTGCCCATC	Standard PCR, TRP PCR MM1
P4CTG	GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC	TRP PCR MM1
P2	AACGGGGCTCGAAGGGTCCTTGTAGC	Standard PCR
P2-FAM	FAM-AACGGGGCTCGAAGGGTCCTTGTAGC	TRP PCR MM2
P4CAG	GGCGGTGGCGGCTGTTGCCAGCAGCAGCA GCAG	TRP PCR MM2
P1-SEQ	AGTTTGCCCATCCACGTC	Sequencing PCR
P2-SEQ	CAGCTCCAGTCCTGTGATCC	Sequencing PCR

In the case of Concord Hospital, standard DM PCRs used Taq PCR Master Mix (Affymetrix USB), 0.4  $\mu\text{M}$  of each of the DM-F and DM-R primers, and 20ng genomic DNA in a final volume of 10  $\mu\text{l}$ . The PCR cycling conditions involved denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec, then a final extension at 72°C for 5 min with a hold at 25°C. TRP-PCRs used 1X ImmoMix™ (Bioline), 0.5M Betaine (Sigma Aldrich), 1.04  $\mu\text{M}$  of each of the DM-TRPP1-F and P3 primers, 0.16  $\mu\text{M}$  of primer DM-TRPP2-CAG, and 150ng genomic DNA in a final volume of 25  $\mu\text{l}$ . The PCR cycling conditions involved denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 2 min, then a final extension at 72°C for 5 min with a hold at 25°C.

The PCR products from both the Standard PCRs and the TRP-PCRs were electrophoretically separated using an Applied Biosystems model 3130xl Genetic Analyzer and the data were analysed using Applied Biosystems GeneMapper® software version 4.0.

Initial statistical analysis compared the Auckland City Hospital sequencing results with the results from their diagnostic methods using Pearson correlation coefficient (to show correlation between the two methods). The second comparison was between diagnostic results of the Auckland City Hospital and Concord Hospital. This comparison involved determining a Pearson correlation coefficient to show the correlation between the two datasets, and a paired t-test to show the difference between the two datasets. The Pearson correlation coefficient and paired t-test analysis methods were performed using the SAS statistical analysis software (V9.3 SAS Institute., Cary, NC, USA).

### 3. RESULTS

Approximately 170 DNAs were tested at Auckland City Hospital in the period 2007-2014 to determine the number of CTG repeats in the *DMPK* gene; allele frequencies are shown in Fig. 1. No alleles were detected between 30 and 48 CTG repeats, and only 3 DNA samples carried CTG repeats between 48 and 51, which were all tested recently (2013-2014). An inter-laboratory collaboration was established between Auckland City Hospital and Concord Hospital in order to build a comprehensive allelic library within the range of 35-51 CTG repeats of that

would serve as a foundation to calculate more accurate correlation curves to deduce repeat lengths from fragment analysis data.

Panel A shows the frequency of CTG repeats detected in DM referrals in the period 2007-2014 (Auckland City Hospital). Panel B shows a truncated allelic frequency chart highlighting the critical clinical decision region containing only 3 alleles. Of the 19 combined samples, 12 samples carried CTG repeats close to the boundary regions; specifically, between the normal, premutation and mild ranges of 32-60 CTG repeats, as well as seven patients with CTG repeats near the boundary between mild and classic DM1. Sequencing of targeted amplicons was undertaken in order to accurately determine the number of CTG repeats. These targeted amplicons comprised seven alleles ranging from 32 to approximately 59 CTG repeats, as estimated by determining the length of the amplicons.

Due to slipped strand mis-pairing events that occur during *in vivo* as well as *in vitro* DNA replication, fragment analysis profiles usually comprise multiple peaks (Panel A, Fig. 2). These peaks need to be taken into consideration when scoring sequence traces. We constructed a theoretical estimate of the consequences of these slippage events on the expected sequence electropherograms (Panel B, Fig. 2). It was assumed that the highest peak in the fragment analysis profile reflected the “cognate” CTG repeat. Therefore, the strongest CATT sequence following a CAG repeat was taken to reflect the “true” end of “n” repeat lengths, which is shown in Panel C of Fig. 2. Statistical analysis of the sequencing results compared to the Auckland City Hospital diagnostic results showed a very strong level of correlation with a Pearson correlation coefficient of 0.999 where  $P = 1.20 \times 10^{-7}$ . These results confirmed the high level of accuracy of our reported repeat lengths determined by fragment analysis. The results also provided us with a well-characterised set of controls that lay at critical clinical decision points in order to create more accurate correlation curves and hence improved estimates of CTG repeat lengths.

Panel A: An example of a fragment analysis profile for a DM1 triplet repeat. “n” indicates the peak corresponding to the true number of repeats, “n-1” indicates a repeat slippage of minus 1 and “n-2” indicates a repeat slippage of minus 2.

Panel B: Theoretical estimate of the combined sequences that contribute to the sequencing results seen in Panel C.

Panel C: Truncated section of sequencing output showing the end of a DM1 triplet repeat sequence. The final CAG and bordering CATT sequence are indicated by text.

The 19 samples were shared and tested in both laboratories to cross-validate the scoring methods. Statistical analysis of all the diagnostic results from Auckland City Hospital and Concord

Hospital showed a very strong correlation with a Pearson correlation coefficient of 0.999 and  $P = 1 \times 10^{-29}$ , along with a paired t-test resulting in a Mean (SD) = 0.263 (0.828), and  $P = .058$  showing no significant difference between the two datasets. No CTG repeat scores had a difference of greater than two repeats, which is in accordance with the combined margins of error of plus or minus one repeat for each laboratory, indicating strong inter-laboratory reproducibility of results and a high level of accuracy between both laboratories.

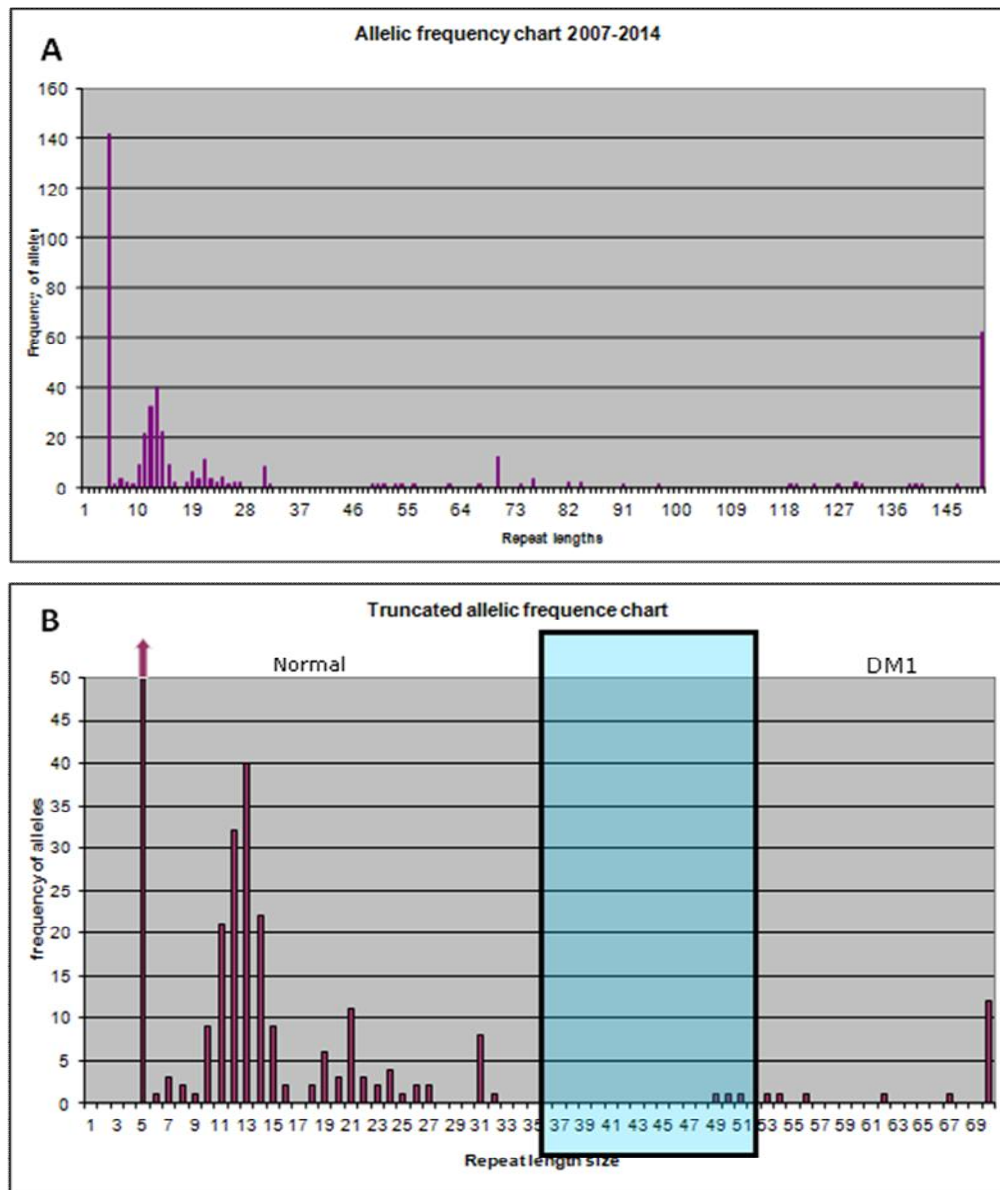
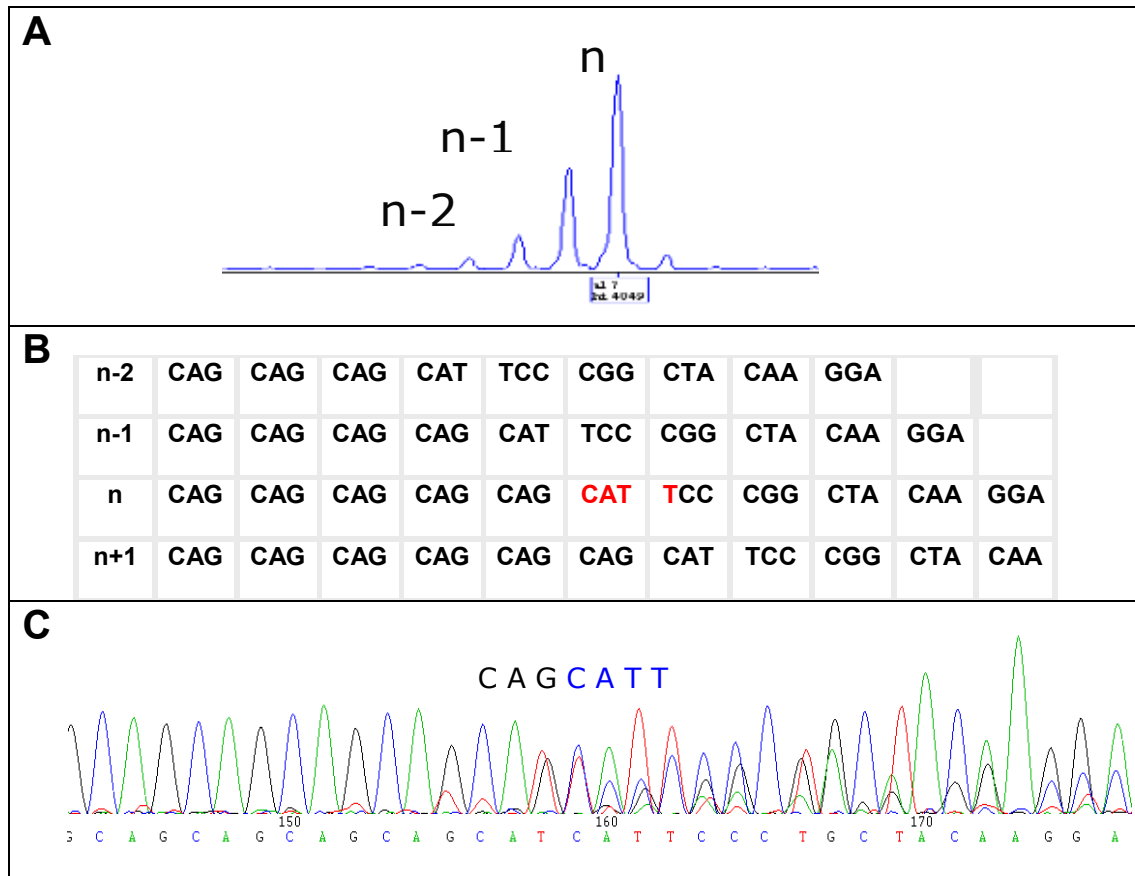


Fig. 1. Allelic frequency chart from 2007 – 2014 tested at Auckland City Hospital



**Fig. 2. Theoretical estimate of the consequences of slippage events on the expected sequence electropherograms**

#### 4. DISCUSSION

Genetic testing without FDA-approved testing methods involves each laboratory validating their own method for *in vitro* diagnostics. In the context of DM1, the low prevalence of CTG alleles near critical clinical decision points makes it challenging to gain access to well-characterised control samples. The aim of this study was to use sequencing and an inter-laboratory sample exchange process to cross-validate and improve a diagnostic method that relies on the accurate scoring of clinically critical allele lengths.

There are two principal difficulties associated with sequencing defined CTG repeat lengths of the *DMPK* gene. First, as DM1 is an autosomal dominant condition there are no samples with homozygous CTG repeats available within the ranges of interest described here, therefore gel purification of specific alleles is required. Secondly, any amplicon isolated from a gel

would carry contaminating fragments of lengths that differ by one to two repeats, hence complicating the interpretation of the sequence.

Sequencing analysis performed at Auckland City Hospital confirmed that the repeat lengths of seven alleles in the sample exchange study were within two repeats of the reported fragment lengths. This concordance reflects a high level of CTG repeat length accuracy in the reported fragment lengths in each of the participating laboratories. In addition, fragment analysis of all 19 diagnostic samples was comparable between both laboratories (within two repeats of each other), despite each laboratory using different primers flanking the CTG repeat region of the *DMPK* gene.

#### 5. CONCLUSION

This study provides two critical outcomes. The first is that the extrapolations that were used by each of the participating laboratories in

determining the number of CTG repeats in the absence of well-characterised controls in the 35-51 repeat range were within their reported margins-of-error. The second outcome is that small regional laboratories can gain confidence in the accuracy of their reported allele calls, specifically around clinically critical decision points, with inter-laboratory exchange studies and in-house sequencing of relevant control samples.

## CONSENT

Patients, or their guardians, provided informed consent for molecular diagnostic assessment of the DM referrals described here. In the case of Auckland City Hospital, the New Zealand National multi-region ethics committee has ruled that cases of patient management do not require formal ethics committee approval. In the case of Concord Hospital, patients provided informed consent for diagnostic testing.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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