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# Improved PCR based methods for detecting *C9orf72* hexanucleotide repeat expansions

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ABSTRACT

Due to the GC-rich, repetitive nature of *C9orf72* hexanucleotide repeat expansions, PCR based detection methods are challenging. Several limitations of PCR have been reported and overcoming these could help to define the pathogenic range. There is also a need to develop improved repeat-primed PCR assays which allow detection even in the presence of genomic variation around the repeat region. We have optimised PCR conditions for the *C9orf72* hexanucleotide repeat expansion, using betaine as a co-solvent and specific cycling conditions, including slow ramping and a high denaturation temperature. We have developed a flanking assay, and repeat-primed PCR assays for both 3′ and 5′ ends of the repeat expansion, which when used together provide a robust strategy for detecting the presence or absence of expansions greater than ~100 repeats, even in the presence of genomic variability at the 3′ end of the repeat. Using our assays, we have detected repeat expansions in 47/442 Scottish ALS patients. Furthermore, we recommend the combined use of these assays in a clinical diagnostic setting.



## 1. Introduction

A hexanucleotide repeat expansion (HRE) of a noncoding GGGGCC repeat within the Chromosome 9 open reading frame 72 (*C9orf72*) gene has been identified as a major cause of amyotrophic lateral sclerosis (ALS, MIM: 612069) and frontotemporal lobar degeneration (FTLD, MIM: 600274) [1,2]. In the UK population, 7.5% of patients with FTLD and 8.1% of patients with ALS have *C9orf72* expansions greater than 32 repeats [3].

The threshold size range of pathogenic alleles has not been well defined, and often relies on the technical cut-off of detection by PCR based assays (30–50 repeats) [2,4]. There is one report of a stable 70 repeat allele in an unaffected individual expanding in his offspring, but further studies are required to determine whether anticipation is associated with this repeat expansion [5]. To ascertain the minimal pathogenic repeat size, it is necessary to detect and accurately measure repeat sizes in small expansion carriers.

Historically, Southern blotting has been regarded as the gold standard method for detecting and sizing large repeat expansions such as in Fragile X syndrome. However, improvements in PCR based methods, particularly repeat-primed (RP-) PCR [6], has meant that clinical diagnosis can now be made using PCR methods alone. RP-PCR uses a locus-specific flanking primer along with a paired repeat primer that amplifies from multiple sites within the repeat, generating a characteristic ladder of fragments after capillary electrophoresis. In *C9orf72*, somatic mosaicism for repeat length in blood samples has been reported, and this can make accurate interpretation of Southern blots challenging, as well as making it difficult to predict any genotype-phenotype correlations with varying repeat size [1,3,7,8]. For this reason, developing reliable and robust RP-PCR methods is important, and others agree that Southern blot results should be interpreted in conjunction with RP-PCR [3].

Within both research and diagnostic settings, it is desirable to have high-throughput, rapid PCR based tests which are highly accurate and do not require large amounts of input DNA. The challenges of PCR amplification of the 100% GC rich *C9orf72* HRE have

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been highlighted by a blinded international study which showed a wide variability in results obtained by different research laboratories using PCR methods [9]. Furthermore, the presence of variable deletions and insertions at the 3′ end of the HRE [10], can adversely affect the reliability of PCR assays targeting this region [11].

There are various ways in which PCR can be enhanced such as the addition of co-solvents such as dimethyl sulfoxide and betaine, modified Taq polymerase and alteration of cycling conditions [12]. Heat-pulse extension (HPE) PCR has been reported to successfully allow amplification of repetitive GC-rich sequences similar to *C9orf72* HRE, and so in this study we used these cycling conditions as a starting point to then optimise for these amplicons [12].

The objectives of this study were to develop a conventional flanking PCR assay which could amplify repeat alleles beyond the 50–70 limit reported in the literature, and to optimise RP-PCR assays for both ends of the repeat to ascertain whether there were greater than 100 repeats present. We also wanted to overcome the issues of the Renton et al. assay where the expansion is not detected by RP-PCR in cases with genomic variability adjacent to the HRE [11]. These assays were then used to screen for *C9orf72* HRE in ALS patients from the Scottish population.

## 2. Materials and methods

### 2.1. Patients and DNA samples

442 consecutive DNA samples obtained from patients with ALS who donated blood for research to the Scottish Regenerative Neurology Tissue Bank, and were phenotyped as part of the Scottish Motor Neurone Disease (MND) Register (between 1989 and 2015) were analysed. The diagnostic criteria used by the Scottish MND Register were the Modified World Federation of Neurology (1989–1994) or 'El Escorial' (1995 onwards) [13,14]. Clinical diagnostic samples received to the South East Scotland Genetics Service for *C9orf72* testing from 2013 to 2016 were also used for assay development. In addition, positive control DNA samples derived from lymphoblast cell lines were obtained from Coriell Cell Repositories. The Institute of Neurology (UCL, Queen Square, London) shared positive control DNA derived from blood, from two short expansion (60–120 repeats) carriers.

### 2.2. Ethics

Ethical approval for research analysis of the Scottish Regenerative Neurology Tissue Bank samples affiliated to the Scottish MND register was obtained from the East of Scotland Research Ethics Service. NHS clinical diagnostic samples were consented for assay development.

### 2.3. Molecular testing

DNA was extracted from whole blood samples by phenol-chloroform, manual salting out, the Nucleon BACC3 genomic DNA kit (Tepnel Life Sciences), or Chemagic DNA blood kit (Perkin Elmer).

PCR reactions, in a total volume of 20 μl consisted of 0.8× Optimized DyNAzyme™ EXT buffer, 0.16 mM dATP, 0.16 mM dTTP, 0.56 mM dCTP, 0.56 mM dGTP, 1.8 M Betaine and 0.12 U/μl DyNAzyme™ EXT DNA Polymerase (ThermoFisher Scientific). For flanking PCR, primers were at 1.25 μM and 20 ng DNA was added. For RP-PCR, primer concentrations were: FAM labelled flanking, 0.5 μM; repeat, 0.25 μM and Tail R; 0.75 μM, and 200 ng DNA was added. PCR primers are listed in Table 1. PCR amplification was carried out on a Veriti® thermal cycler (Life Technologies). Cycling conditions are shown in Table 2.

PCR products were separated by capillary electrophoresis using an ABI 3130xL with a 50 cm array (Life Technologies) with either Genescan™ LIZ600 or LIZ1200 size standard (Life Technologies). Data was analysed using GeneMarker® software v2.4.0 (Soft Genetics). Alternatively, PCR products were separated on 0.8% UltraPure agarose (ThermoFisher Scientific) gels in TBE buffer with 100 bp DNA ladder (Promega) and 1 kb DNA extension ladder (Invitrogen).

For Sanger sequencing, either flanking PCR or an alternative 3′ RP-PCR was used (Table 1). PCR products were purified using Agencourt Ampure XP (Beckman Coulter), as per the manufacturer's instructions, using a Biomek® NX robot (Beckman Coulter). Sequencing was then performed using R6 primer and BigDye® Terminator v3.1 (Life Technologies). Agencourt CleanSeq (Beckman Coulter) was used, according to the manufacturer's instructions, to clean-up sequencing products prior to capillary electrophoresis on an ABI 3130xL (Life Technologies). Data was analysed using Mutation Surveyor® software v4.0.8 (Soft Genetics).

## 3. Results

### 3.1. C9orf72 HRE frequency in the Scottish ALS population

We tested 442 archival DNA samples from the Scottish Regenerative Neurology Tissue Bank, linked to the Scottish MND Register, collected from 1989 to 2015, using flanking PCR to assess the sizes of normal alleles. 157 cases which gave a homozygous result on this assay were then tested using both 3′RP-PCR and 5′RP-PCR, which led to detection of *C9orf72* expansions in 47 patients (10.6%), and gave one equivocal result which could not be resolved due to insufficient DNA. The repeat sizes that were obtained are shown in Fig. 1, which shows a similar distribution to the UK population [3].

### 3.2. Optimal conditions for flanking PCR

We developed a PCR assay using primers flanking the *C9orf72* HRE and applied the HPE PCR conditions developed for Fragile X syndrome [12]. HPE PCR involves multiple heat pulses during the extension phase of the cycling protocol to temporarily destabilize GC rich structures which may otherwise lead to replication stalling [12]. These conditions permitted superior amplification to that achieved with Qiagen Multiplex PCR kit or Roche Fast Start High Fidelity PCR system with standard cycling conditions (data not shown). We then varied cycling conditions to determine the annealing temperature, and whether high denaturation, slow ramping or heat-pulse extension were required, and also the optimal extension time. We found that the slow ramp from annealing to extension phase and high denaturation temperature were the most important features, and in this case the heat pulses during extension were of no benefit (data not shown). The optimised conditions gave relatively balanced amplification of normal alleles, as highlighted in the series of samples with alleles ranging between 2 and 26 repeats (Fig. 2a–c). The Institute of Neurology, UCL, Queen Square, London sent us two samples with 'short' expansions. The first was estimated as having 60 repeats, with mosaicism for a large expansion (James Polke, personal communication), and another with 90 repeats in blood estimated by Southern blotting [15]. These alleles had not been amplified using existing PCR methods by ourselves or the Institute of Neurology (data not shown). Using our method, we could detect alleles of approximately 70 and 80 repeats, and revealed a high level of mosaicism in both cases (Fig. 2d–f). The largest repeat size we detected in blood was ~120 repeats, although we did note that a large smear was present in a number of samples with expansions present (data not shown). To determine the upper size range of