MULTI-SITE EVALUATION OF A SINGLE-TUBE LONG-READ PCR ASSAY FOR THE RELIABLE DETECTION AND CHARACTERIZATION OF C9orf72 HEXANUCLOTIDE REPEAT EXPANSIONS

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SUMMARY

• A (Gc)n repeat expansion in the noncoding region of the C9orf72 gene represents the first genetic link between amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).

• We developed sensitive and robust single-tube, 3-primer C9orf72 PCR reagents that can flag expanded samples irrespective of length and provide accurate sizing up to ~145 repeat units when resolved by capillary electrophoresis.

• A gene-specific, 2-primer configuration of this PCR can detect low-level mosaicism and reproduce reliably generate products with at least ~300 hexanucleotide repeats.

• Assay performance was evaluated at two sites (Asuragen, Inc. and the University of Pennsylvania) using a large set of ALS cell-line derived DNA samples from the Coriell repository and a diverse set of residual clinical samples, including brain, blood, and saliva.

INTRODUCTION

An intrinsic (Gc)n repeat expansion in the C9orf72 gene has been observed in the general population with a frequency of ~1% and is present in ~10% of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) cases. Fewer than 30 repeats are considered normal whereas the GC-rich repeat poses formidable challenges to routine PCR-based fragment sizing methods, and currently requires analysis using multiple short-range PCR reactions for each sample, followed by Southern blot analysis of expanded samples. Here we describe a two-site evaluation of a single-tube, highly streamlined PCR assay that both detects C9orf72 expansions and sizes expanded alleles with 100’s of repeats.

MATERIALS AND METHODS

Genomic DNA was extracted from blood, saliva, fresh-frozen or formalin-fixed paraffin-embedded FFPE) brain tissue of patients with ALS and FTD, or ALS/FTD by using the QuickGene 610L (Autogen); QIAamp DNA mini kit (Qiagen) or QIAamp DSP DNA FFPE Tissue Kit (Qiagen), respectively, using the manufacturer's protocols. The Ampliseq® PCRiCE C9orf72 Kit (Asuragen) was used to amplify and detect C9orf72 hexanucleotide repeat expansions. Reagents were sized on a 3500xL or 3130xl Genetic Analyzer (Thermo Fisher), and/or SeaKem® LE agarose gel electrophoresis (AES; Lonza) after amplification with only the flanking gene-specific primers and modified PCR conditions. The assay was evaluated at both the University of Pennsylvania (Site 1) and Asuragen (Site 2). Site 1 evaluated a subset of NNIND ALS samples (n=50) and an independent set of 166 ALS and FTD patient-derived samples from peripheral blood (n=114), saliva (n=28), and brain (n=28) and compared results to both Southern blot analysis and a PCR lab-developed test (LDT).

RESULTS

Across both evaluation sites, C9orf72 repeat repeat numbers were resolved in agreement with previous annotations for all but a single ambiguous sample. Fragment sizing using capillary electrophoresis (CE) was limited to 145 repeats by the sizing constraints of the CE liquid polymer, and not by PCR amplification. Compatibility with blood, saliva, fresh-frozen, and FFPE brain DNA at 40 ng inputs was demonstrated at Site 1, and samples with expansions, size mosaicism and/or alleles could be identified from the repeat-primed CE traces. Site 2 further demonstrated sensitivity to as few as 1 ng input of cell-line DNA. Both sites also showed proof-of-concept for agarse gel sizing of PCR amplicons from expansions with up to 800 repeats.

CONCLUSIONS

• Ampliseq® PCR technology can amplify C9orf72 repeat expansions that are more than 10-fold larger than conventional assays.

• A 3-primer RP-PCR design provides robust performance across multiple sample types (including challenging FFPEs), high sensitivity and accuracy up to 145 repeats on CE, and clear indication of both size mosaicism and sequence variations.

• A gene-specific, 2-primer PCR format identified low-level size mosaicism and produced amplicons consistent with up to at least ~800 hexanucleotide repeats in expanded samples when resolved on agarose gel.

• This simple, single-tube PCR technology has potential to advance clinical research and emerging diagnostic, therapeutic, and screening applications for the C9orf72 marker in the context of ALS, FTD and other late-onset neurodegenerative disorders.

References


Figure 1. A novel PCR technology for the amplification of the hexanucleotide repeat element within the C9orf72 gene. A) Schlieren representation of the C9orf72 gene showing the position 11 repeats over 1,700 bp. B) The novel Ampliseq PCRiCE C9orf72 design, with multiplexed forward (F) and reverse (R) primers to amplify the repeat region. The primers can be designed to target repeat number and distance for the differential amplification of expand and non-expanded alleles into the assay. C) A non-expanded allele (left) and expanded allele (right) of C9orf72. D) A modular design can accommodate both PCRiCE and PCRiCE® assays, meaning that ~20-25 samples can be run per batch. E) The described PCRiCE approach offers a rapid, streamlined C9orf72 molecular test appropriate to a standard PCR workflow.

Figure 2. Performance characterization of the Ampliseq PCRiCE C9orf72 Kit®. A) A dosage ranging of up to 154 repeats (900d) is a simple CE profile for expanded samples to be detected using 3-primer PCR. The multiplicity of gels generated across the repeat primer enables scalable mutation. B) A ladder of dilutions spanning wild type through to ~1500 repeats was detected as primer expansion as the separation of an open peak off of the ladder (900d). C) A critical amplification parameter was the multi-component lar-ger PCRiCE® PCR mix (1200–2000%) to capture expansion to ~1000d and intermediate (500–800d) repeats. D) A normal hexanucleotide repeat profile was observed, as expected, for 1 ng input of non-expanded C9orf72 and intermediate (500–800d) repeats in cell-line DNA. E) A normal repeat profile was observed for 1 ng input of non-expanded C9orf72 and intermediate (500–800d) repeats in cell-line DNA.

Figure 3. 2 3-primer comparison of the 2-primer C9orf72 PCRiCE assay for detection of expanded alleles up to ~800 repeats. Modified thermocycling conditions were used to achieve PCRs of up to ~30 kb expansions in C9orf72 by using 3-primer (Asuragen) and 4-primer (UPenn) designs. A) The 145 repeat threshold for both sites was observed using 3-primer design. B) The 145 repeat threshold for both sites was observed using 3-primer design. C) The 145 repeat threshold for both sites was observed using 3-primer design. D) The 145 repeat threshold for both sites was observed using 3-primer design. E) The 145 repeat threshold for both sites was observed using 3-primer design.

Table 1: Genotype concordance of C9orf72 alleles in FTD and ALS residual samples between the Ampliseq PCRiCE C9orf72 Kit® and Infinium C9orf72 ASP Chip. All samples were verified as positive expansions, some were not detected on the ASP chip. Genotype assignments were further confirmed using Southern blot analysis. All sample results, except for a single negative repeat expanded sample, generated across the assays. B) Detection of C. European Huntington's Chorea (CHC); C. Huntington's disease (HD).