Evaluation of Single-tube Combined Amplicon-length and Repeat-primed Long-read PCR Assay for Clinical Detection and Characterization of *C9orf72* Hexanucleotide Repeat Expansion

[1]Cristiane M. Ida, [1]Patrick A. Lundquist, [2]Eran Bram, [1]Malinda L. Butz, [2]Gary Latham, [1]W. Edward Highsmith

[1]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA,

and [2]Asuragen, Inc, Austin, TX, USA

Introduction

A non-coding $(GGGGCC)_n$ hexanucleotide repeat expansion of the *C9orf72* gene has been recognized as the most common genetic alteration associated with familial and sporadic amyotrophic lateral sclerosis (ALS) and frontotemporal dementia[1,2]. Although the exact cut-off for pathogenicity has yet to be established, fewer than 20 repeats are generally considered normal (non-expanded) whereas definite pathogenic *C9orf72* expansions have 100's to 1000's of repeats. The large size and high GC content of pathogenic *C9orf72* repeat expansions represent a challenge to routine PCR-based fragment sizing and repeat-primed methods, and Southern blot analysis has been recommended as a confirmatory test in clinical diagnostic setting. Southern blot is a labor-intensive, time-consuming, low throughput technique that requires significant amounts of genomic DNA, and a PCR-based assay that may confidently identify large repeat expansions (in the 1000's) and resolve shorter repeat expansions (in the 100's) is warranted.

Herein, we evaluated the performance of AmplideX® PCR/CE *C9orf72* Kit, a commercially available single-tube, combined amplicon-length and repeat-primed, long-read PCR assay that can identify *C9orf72* expanded cases and size expanded alleles up to approximately 145 repeats. Our evaluation consisted of an initial two-site assessment of non-clinically tested samples and a subsequent side-by-side comparative assessment of clinical samples previously tested using a validated laboratory-developed test (LDT) consisting of an amplicon-length PCR assay with reflex to Southern blot.

Materials and Methods

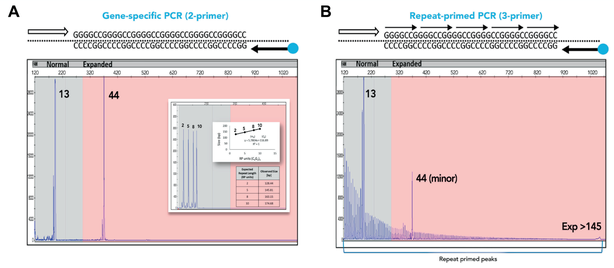
For the purposes of this evaluation, cases with fewer than 30 repeats were considered non-expanded, whereas cases with 100 repeats or greater were considered expanded; there were no cases within the 31-99 repeat range. The AmplideX® PCR/CE *C9orf72* Kit was initially evaluated at both the Mayo Clinic (Site 1) and Asuragen, Inc. (Site 2) using non-clinically tested DNA samples from peripheral blood-derived immortalized cell lines (n-CS; n=9) and Coriell repository ALS cell lines (CR; n=5). Subsequently, site 1 independently evaluated the AmplideX® PCR/CE *C9orf72* Kit performance on a set of peripheral blood DNA samples (CS; n=47) previously tested clinically by the LDT assay.

AmplideX® PCR reagents were optimized for the amplification of *C9orf72* hexanucleotide repeats. The AmplideX® PCR/CE *C9orf72* Kit relies on a combination of repeat-primed strategy with gene-specific primers for amplification and allele sizing in a single-tube reaction. At site 1, amplicons were sized on an ABI 3730xL genetic analyzer (Thermo Fisher Scientific, Waltham, MA) and allele size was determined using GeneMarker v2.4 software (Soft Genetics, State College, PA). At site 2, amplicons were sized on an ABI 3500xL instrument (Thermo Fisher Scientific, Waltham, MA) and data were analyzed using GeneMapper® V4.1 software (Thermo Fisher Scientific, Waltham, MA) using size and mobility conversion factors on site 2 (**Figure 1**).

The LDT amplicon-length PCR assay consists of a genotyping PCR in which the *C9orf72* hexanucleotide repeat region is amplified using published fluorescently-labeled primers flanking the repeat region[1] and can resolve alleles with up to about 30 repeats. Amplicons were sized on an ABI 3730xL and data was analyzed using GeneMarker v2.4 software [G_4C_2 repeat size = (bp size – 116)/ 6]. Follow-up Southern blot analysis using XbaI and HindIII restriction digests and a ^{32}P-labeled oligonucleotide probe to detect a region immediately upstream of the hexanucleotide repeat region was performed for any homozygous samples. Gel images were captured using PhosphorImager instrument (Typhoon FLA 7000 control software, GE Healthcare) and analyzed with ImageQuant LAS4000 digital imaging system software (GE Healthcare). Repeat expansion was estimated based on comparison to a pre-defined repeat size approximation of the XbaI digest using an end-labeled 1kb ladder.

Targeted Sanger sequencing using PCR primers with 5-prime universal sequencing primer tails designed to flank the LDT forward and reverse primer sequences was performed to further investigate discordant cases. Template PCR was done using the KAPA2G PCR system (KAPA Biosystems, Woburn, MA). The resulting PCR product was purified using AMPure XP DNA purification reagent (Agencourt, Beverly, MA) and amplicons were sequenced bidirectionally using universal forward and reverse sequencing primers and the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). Sequencing reaction products were purified using the CleanSEQ purification reagent (Agencourt, Beverly, MA). Diluted purified sequencing products were detected on the ABI 3730xL. Sequencing traces were analyzed using the Mutation Surveyor software (Soft Genetics, State College, PA).

Figure 1



AmplideX® PCR/CE *C9orf72* Kit: A combined 2-primer (A) and 3-primer (B) FAM-labeled PCR design, with representative capillary electrophoresis profiles with either gene-specific peaks only or gene-specific peaks overlaid with a sawtooth repeat profile (Coriell sample ND06769). A mix of cell-line DNA is used to generate a size calibration curve that converts size in base pairs to repeat number and corrects for the differential mobility of GC-rich DNA on capillary electrophoresis (A inset).

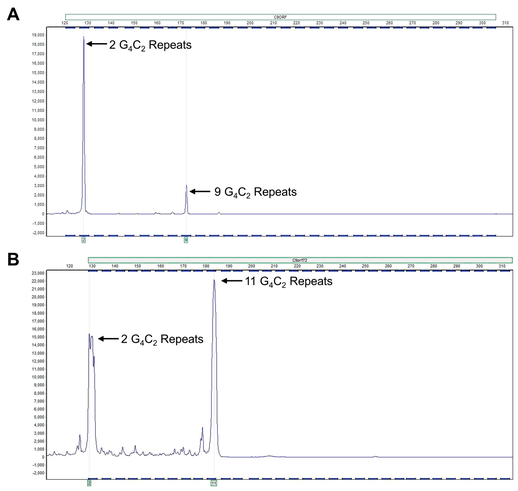
Results

Repeat sizes were resolved for all 61 samples, with agreement of results in 14 of 14 (100%) non-clinically tested (**Table 1**) and in 46 of 47 (98%) clinically tested cases (**Table 2**). The single discordant case (CS29) was sized as 2 and 9 repeats by the LDT and as 2 and 11 by AmplideX® PCR/CE *C9orf72* Kit (**Table 2, Figure 2**). Targeted Sanger sequencing of this discordant sample was consistent with alleles containing 2 and 9 repeats in addition to a rare heterozygous 14-base pair duplication of the 9-repeat allele involving the 3-prime binding region of the LDT reverse primer (**Figure 3**). This duplication appeared to result in decreased amplification efficiency for the 9-repeat allele by the LDT assay and in slight overestimation of the repeat size by the AmplideX® PCR/CE *C9orf72* Kit since its reverse primer flanks the duplicated region. The 14-base pair duplication has not been previously reported and the discordant test result would not have significant clinical impact, as the 2 and 11-repeat genotype would still be consistent with a negative result. Of note, the AmplideX® PCR/CE *C9orf72* Kit was able to accurately resolve zygosity (non-expanded homozygous versus expanded heterozygous cases) and identify the presence of *C9orf72* repeat expansion in all cases that had been reflexed to Southern blot analysis. (**Table 2, Figure 4**).

Table 1

Sample	Asuragen AmplideX® PCR/CE *C9orf72* Kit Site 1 Result (# G_4C_2 repeats)	Asuragen AmplideX® PCR/CE *C9orf72* Kit Site 2 Result (# G_4C_2 repeats)
n-CS1	2,2	2,2
n-CS2	5,10	5,10
n-CS3	2,2	2,2
n-CS4	2,2	2,2
n-CS5	2,2	2,2
n-CS6	5,7	5,7
n-CS7	2,5	2,5
n-CS8	2,5	2,5
n-CS9	2,8	2,8
CR1	2,expansion	2,expansion
CR2	13,44,expansion	13,44,expansion
CR3	8,expansion	8,expansion
CR4	11,expansion	11,expansion
CR5	11,28	11,28

Figure 2



Side-by-side comparative assessment of previously tested clinical samples: The single discordant case (CS29) was sized as 2 and 9 repeats by Mayo Clinic *C9orf72* LDT assay (A) and as 2 and 11 by AmplideX® PCR/CE *C9orf72* Kit (B).

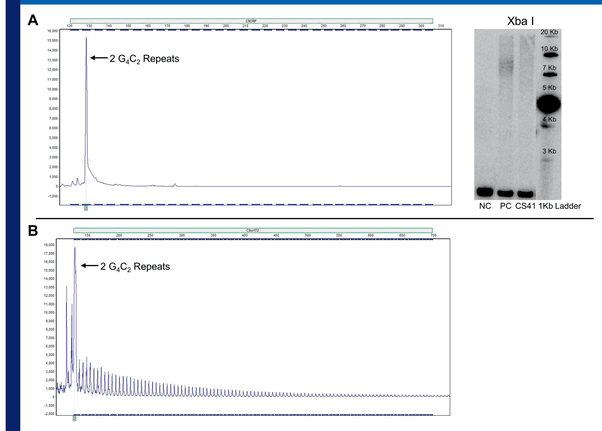
Table 2

Sample	Mayo Clinic *C9orf72* Laboratory-Developed Test Result (# G_4C_2 repeats)	Asuragen AmplideX® PCR/CE *C9orf72* Kit Result (# G_4C_2 repeats)
CS1	6,expansion (~ >2000)	6,expansion
CS2	2,7	2,7
CS3	2,24	2,24
CS4	2,2	2,2
CS5	19,expansion (~ >1000)	19,expansion
CS6	6,15	6,15
CS7	3,expansion (~ >2000)	3,expansion
CS8	4,12	4,12
CS9	2,9	2,9
CS10	4,5	4,5
CS11	5,expansion (~ >2000)	5,expansion
CS12	5,expansion (~ >2000)	5,expansion
CS13	4,4	4,4
CS14	2,11	2,11
CS15	10,expansion (~ >3000)	10,expansion
CS16	10,expansion (~ >3000)	10,expansion
CS17	8,14	8,14
CS18	2,2	2,2
CS19	8,8	8,8
CS20	5,5	5,5
CS21	4,4	4,4
CS22	8,21	8,21
CS23	8,expansion (~ >900)	8,expansion
CS24	2,6	2,6
CS25	2,2	2,2
CS26	5,8	5,8
CS27	5,8	5,8
CS28	2,8	2,8
CS29	2,9	2,11
CS30	2,10	2,10
CS31	2,5	2,5
CS32	5,expansion (~ >500)	5,expansion
CS33	2,expansion (~ >1500)	2,expansion
CS34	2,10	2,10
CS35	5,5	5,5
CS36	2,10	2,10
CS37	2,4	2,4
CS38	2,2	2,2
CS39	2,8	2,8
CS40	2,5	2,5
CS41	2,expansion (~ >3000)	2,expansion
CS42	5,expansion (~ >2000)	5,expansion
CS43	2,8	2,8
CS44	2,expansion (~ >1500)	2,expansion
CS45	2,expansion (~ >2000)	2,expansion
CS46	2,expansion (~ >2500)	2,expansion
CS47	2,8	2,8

Figure 3

Targeted Sanger sequencing of the single discordant sample: Allele sizes were consistent with 2 and 9 repeats (A). A heterozygous 14-base pair duplication located in the 3-prime binding region of the LDT reverse primer in the 9-repeat allele (B).

Figure 4



Side-by-side comparative assessment of clinical samples: Example of a sample (CS41) with apparently homozygous result by LDT amplicon-length PCR (left) in which Southern blot analysis disclosed an expanded allele in addition to the non-expanded allele (right); NC=negative control; PC=positive control (A). AmplideX® PCR/CE *C9orf72* Kit resolved zygosity (expanded heterozygous rather than non-expanded homozygous) and identified the *C9orf72* repeat expansion with the characteristic sawtooth pattern of the repeat-primed peaks for this clinical sample (B).

Conclusions

- Evaluation of the AmplideX® PCR/CE *C9orf72* Kit, a single-tube, combined amplicon-length and repeat-primed long-read PCR technology confirms the robustness of this PCR assay for allele sizing and large repeat expansion detection.
- In our hands, the AmplideX® PCR/CE *C9orf72* Kit showed reliable detection of *C9orf72* repeat expansions, with 98% agreement for previously clinically tested cases; the single discordant case was caused by interference of a rare duplication in which the discordant test result would not have significant clinical impact.
- This innovative assay relies on a combined workflow in a single reaction that allows faster overall turnaround time, reduced hands-on-time and reagent use in comparison to our laboratory-developed test (amplicon-length PCR assay with reflex to Southern blot), and has potential utility for clinical diagnostic testing.

References

1. DeJesus-Hernandez M et al. Expanded GGGGCC hexanucleotide repeat in noncoding region of *C9ORF72* causes chromosome 9p-linked FTD and ALS. *Neuron* 2011, 72:245-256
2. Renton AE et al. A hexanucleotide repeat expansion in *C9ORF72* is the cause of chromosome 9p21-linked ALS-FTD. *Neuron* 2011, 72:257-268