EVALUATION OF A NOVEL PCR TECHNOLOGY FOR THE QUANTIFICATION OF *C9orf72* HEXANUCLEOTIDE REPEAT EXPANSIONS

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SUMMARY

- A $(G_4C_2)_n$ hexanucleotide 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 highly sensitive and robust, single-tube, 3-primer *C9orf72* PCR reagents that can flag all expanded samples irrespective of length and provide accurate sizing up to ~145 repeat units using capillary electrophoresis.
- A gene-specific, 2-primer configuration of this PCR can detect low-level mosaicism and reproducibly generate products with up to ~800 repeats from expanded samples.
- Assay performance was evaluated across two sites including Asuragen, Inc. and the University of Pennsylvania.

INTRODUCTION

Hexanucleotide repeat expansions in the noncoding region of the *C9orf72* gene are present in ~7% of all clinical cases of ALS and FTD. These expansions are of considerable interest for both clinical research and potential diagnostic and screening applications. The GC-rich repeat element is recalcitrant to PCR and consequently, a combination of multiple PCR assays and Southern blot (SB) are used to profile this region. Here we describe the performance of an innovative PCR technology with capabilities that exceed current amplification assays.

METHODS

PCR reagents were optimized for the amplification of G_4C_2 hexanucleotide repeats (AmplideX® PCR/CE *C9orf72* Kit, Asuragen, Inc.). Amplicons were sized using capillary electrophoresis (CE) on a 3500xL/3130xL Genetic Analyzer (Thermo Fisher) or agarose gel electrophoresis (AGE; Lonza). A total of 101 genomic DNA samples from the Coriell ALS sample repository (30 unexpanded and 71 expanded) were assessed at Asuragen. The assay was also independently evaluated at the University of Pennsylvania (UPenn) using a subset of these samples as well as residual patient specimens.

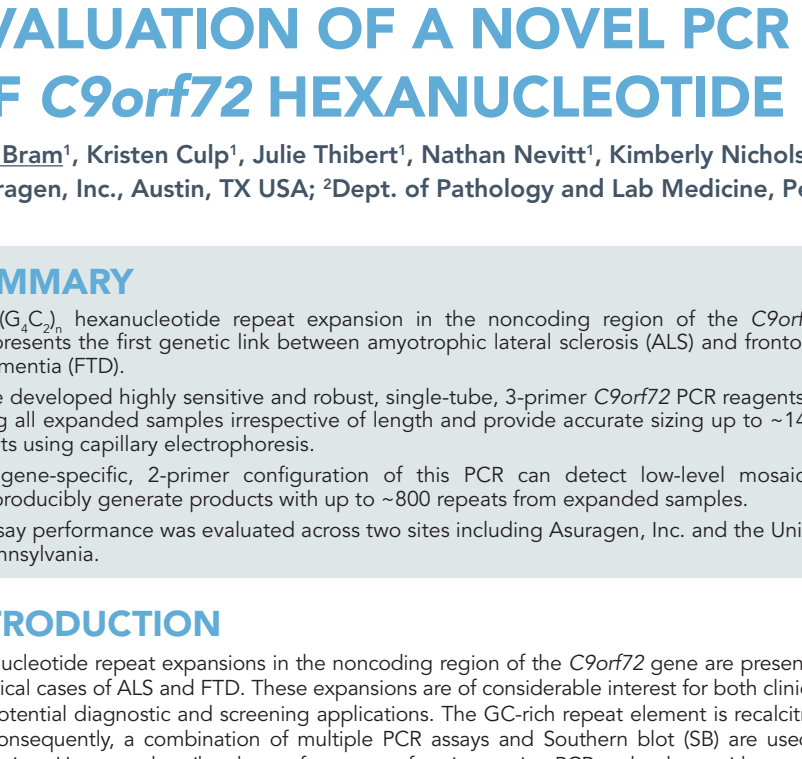

Figure 1. A novel PCR for the amplification of a hexanucleotide (G_4C_2)$_n$ repeat element in the *C9orf72* gene. (A) Schematic representation of the *C9orf72* gene structure denoting predicted 11 exons (boxes) and location of the intronic hexanucleotide repeat expansion (vertical lines). **(B)** A 2-primer (left) or 3-primer (right) FAM-labeled PCR design, with representative CE profiles with either gene-specific (GS) peaks only or GS peaks overlaid with a consistent repeat (RP) profile (Coriell sample ND06769). The 2-primer PCR products can also be resolved by AGE (left inset). **(C)** A modular design can accommodate both PCR/CE and PCR/AGE assays, requiring ~6 hr or ~9.5 hr to complete, respectively, with less than 1.5 hours of hands-on-time per 24 sample batch. **(D)** A simpler and more informative *C9orf72* sample analysis workflow incorporating PCR technology.

RESULTS

Across both lab sites, repeat values were resolved consistent with previous annotations[1] for all unexpanded samples and up to 145 repeats for all expanded samples (limited only by the sizing range of CE). Expanded samples, irrespective of repeat size, were categorically identified in each case within the same PCR reaction. AGE further confirmed these expansions, resolving high molecular weight products consistent with up to ~800 repeats along with extensive size mosaicism. Heterogeneity in expanded repeat lengths could be detected below a 5% mass fraction. The assay was robust over a 2-log range of inputs and provided clear indication when 3′ indels were present.

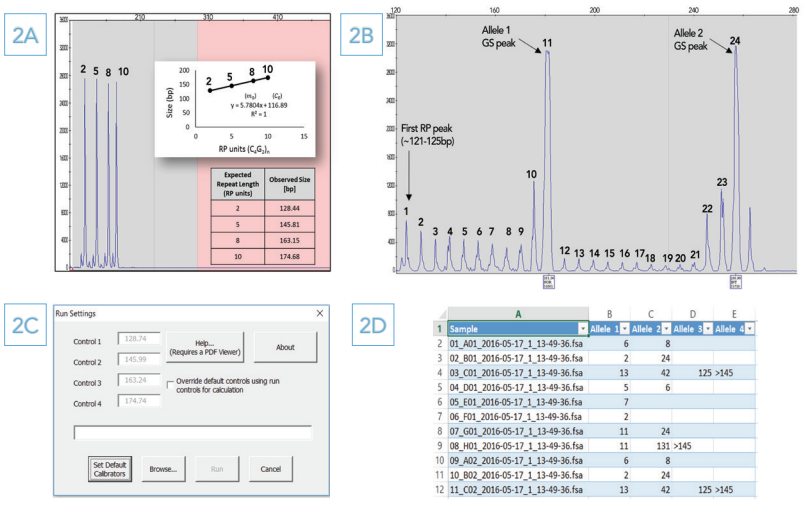

Figure 2. Assay design enables complementary repeat sizing methods (A) A mix of cell-line DNA can be used to generate a size calibration curve (inset) that converts size (in bp) to repeat number and corrects for differential mobility of GC-rich DNA on CE. **(B)** Sizing by direct counting of repeat peaks. **(C)** An Excel macro was developed to support automation of the calibration curve approach, producing a genotype report **(D).**

Research Use Only – Not For Use In Diagnostic Procedures
Preliminary research data. The full performance characteristics of this assay have not yet been established.
Presented at ICFTD 2016

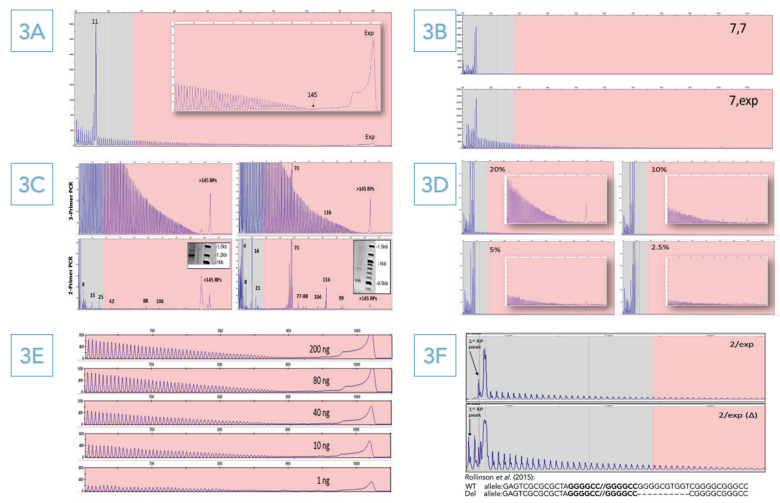

Figure 3. Performance characteristics of the AmplideX PCR/CE *C9orf72* Kit. (A) Accurate sizing of up to 145 repeats (RP) with a distinct RP profile for expanded samples, which enables zygosity resolution **(B). (C)** Unique low-level minor alleles are detected in the 2-primer assay configuration on both CE and AGE (inset), consistent with as low as 5% mass fraction in the background of 95% normal alleles **(D). (E)** PCR demonstrates robust performance across a 200-fold gDNA input range down to 1 ng/reaction and also provides clear indication for the presence of 3′ indels[2] in a representative patient sample **(F).**

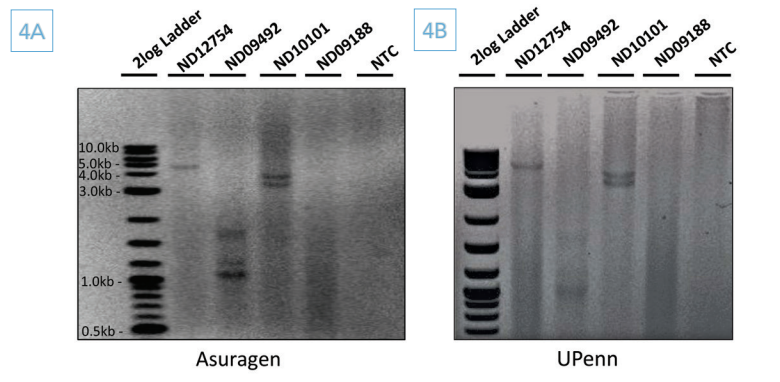

Figure 4. A 2-site comparison of the 2-primer *C9orf72* PCR/AGE assay for detection of expanded alleles of up to ~800 repeats. A modified PCR profile was used for PCR amplification of large repeat fragments (up to ~5kb) in Coriell ALS gDNA samples using Asurgen's *C9orf72* PCR reagents, at both Asuragen **(A)** and UPenn **(B).** Note several samples carry multiple bands at variable intensities. ND09188 is a normal (non-expanded) sample.

A

Asuragen PCR \ Reported genotype[1]	Normal (0-19)	Intermediate (20-29)	Expansion (≥30)
Normal (0-19)	24	0	0
Intermediate (20-29)	0	6	0
Expansion (≥30)	0	0	71

B

Sample ID	Allele 1	Allele 2	Category
ND07738	2	2	Normal
ND07995	2	2	Normal
ND08470	2	2	Normal
ND08987	2	2	Normal
ND09487	2	4	Normal
ND10209	2	4	Normal
ND04055	2	5	Normal
ND06176	2	5	Normal
ND10767	2	6	Normal
ND06186	2	7	Normal
ND10802	2	7	Normal
ND04051	2	8	Normal
ND12094	2	11	Normal
ND10710	2	16	Normal
ND09191	2	19	Normal
ND04052	4	5	Normal
ND07475	5	6	Normal
ND09694	5	7	Normal
ND10937	5	10	Normal
ND06177	6	8	Normal
ND08619	7	7	Normal
ND14685	7	13	Normal
ND12120	8	12	Normal
ND09188	12	14	Normal
ND12070	2	21	Intermediate
ND06593	2	24	Intermediate
ND11382	2	25	Intermediate
ND09180	11	24	Intermediate
ND12028	11	28	Intermediate
ND10518	14	23	Intermediate
+ND07489	2	Expanded repeat	Expanded
ND07669	2	Expanded repeat	Expanded
ND09204	2	Expanded repeat	Expanded
+ND09375	2	Expanded repeat	Expanded
+ND09407	2	Expanded repeat	Expanded
ND09492	2	Expanded repeat	Expanded
ND09508	2	Expanded repeat	Expanded
+ND09576	2	Expanded repeat	Expanded
ND09607	2	Expanded repeat	Expanded
ND09996	2	Expanded repeat	Expanded
+ND10284	2	Expanded repeat	Expanded
+ND10773	2	Expanded repeat	Expanded
ND10905	2	Expanded repeat	Expanded
ND11081	2	Expanded repeat	Expanded
ND11252	2	Expanded repeat	Expanded
ND11548	2	Expanded repeat	Expanded
ND11554	2	Expanded repeat	Expanded
+ND11583	2	Expanded repeat	Expanded
+ND11668	2	Expanded repeat	Expanded
ND11749	2	Expanded repeat	Expanded
+ND11755	2	Expanded repeat	Expanded

Sample ID	Allele 1	Allele 2	Category
ND11933	2	Expanded repeat	Expanded
ND12099	2	Expanded repeat	Expanded
ND12100	2	Expanded repeat	Expanded
ND12277	2	Expanded repeat	Expanded
ND12402	2	Expanded repeat	Expanded
ND12438	2	Expanded repeat	Expanded
ND12455	2	Expanded repeat	Expanded
ND12754	2	Expanded repeat	Expanded
ND12909	2	Expanded repeat	Expanded
ND13216	2	Expanded repeat	Expanded
ND13682	2	Expanded repeat	Expanded
+ND13803	2	Expanded repeat	Expanded
ND13939	2	Expanded repeat	Expanded
ND14186	2	Expanded repeat	Expanded
ND14442	2	Expanded repeat	Expanded
ND14596	2	Expanded repeat	Expanded
ND14954	2	Expanded repeat	Expanded
+ND08957	4	Expanded repeat	Expanded
+ND10023	5	Expanded repeat	Expanded
ND10689	5	Expanded repeat	Expanded
ND10808	5	Expanded repeat (minor allele @ 86)	Expanded
ND11680	5	Expanded repeat	Expanded
ND11917	5	Expanded repeat	Expanded
+ND12200	5	Expanded repeat	Expanded
+ND13828	5	Expanded repeat	Expanded
ND13944	5	Expanded repeat	Expanded
+ND14339	5	Expanded repeat	Expanded
ND14911	5	Expanded repeat	Expanded
+ND08078	6	Expanded repeat	Expanded
ND12089	6	Expanded repeat	Expanded
ND12161	6	Expanded repeat	Expanded
+ND12199	6	Expanded repeat (minor allele @ 46)	Expanded
ND12904	6	Expanded repeat	Expanded
ND12102	7	Expanded repeat	Expanded
ND06751	8	Expanded repeat	Expanded
ND10101	8	Expanded repeat	Expanded
+ND11374	8	Expanded repeat	Expanded
ND11494	8	Expanded repeat	Expanded
ND11836	8	Expanded repeat	Expanded
ND10966	9	Expanded repeat	Expanded
ND10973	9	Expanded repeat	Expanded
ND09373	10	Expanded repeat	Expanded
ND10000	10	Expanded repeat	Expanded
ND09438	11	Expanded repeat	Expanded
ND06769	13	Expanded repeat	Expanded
ND08544/A	2	Expanded repeat	Expanded
ND08544/B	2	Expanded repeat	Expanded
+ND15362	8	Expanded repeat	Expanded
+ND15686	2	Expanded repeat	Expanded
ND15814	2	Expanded repeat	Expanded
+ND20142	2	Expanded repeat	Expanded

Table 1. Genotyping of 101 Coriell NINDS ALS samples. (A) Genotype concordance across 101 samples from the Coriell NINDS ALS collection. **(B)** Full genotype (in RP units) for tested 101 samples. Samples in bold were genotyped at both Asuragen and UPenn. Samples with + had a 3′-deletion in expanded alleles. Last 6 samples lack reference genotype for normal allele.

CONCLUSIONS

- This novel PCR technology can amplify repeat expansions that are more than 10-fold larger than conventional assays.
- A 3-primer RP-PCR design provides robust performance, high sensitivity and accuracy up to 145 repeats on CE, and clear indication of both size mosaicism and 3′ sequence variations.
- A gene-specific, 2-primer PCR format identified known *C9orf72*-expanded samples and produced amplicons consistent with up to at least ~800 hexanucleotide repeats.
- 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 age-onset neurodegenerative disorders.

References
1. Rutherford N.J., et al., *C9orf72* hexanucleotide repeat expansions in patients with ALS from the Coriell Cell Repository. Neurology, 2012. 31;79(5):482-3.
2. Rollinson S., et al., A small deletion in *C9orf72* hides a proportion of expansion carriers in FTLD, Neurobiol Aging. 2015 Mar; 36(3): 1601.e1–1601.e5.

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