A Robust PCR/CE Assay Using AmplideX® Technology for Rapid and Accurate Genotyping of CAG Repeat Expansions in *HTT*

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Summary

- Huntington's disease (HD) is a neurodegenerative disorder characterized by uncontrollable movements, mood alterations and cognitive decline.
- Huntingtin (*HTT*) genotyping is challenged by the requirement for high resolution, and known rare polymorphisms surrounding the CAG repeat region that can cause allelic dropout.
- We leveraged AmplideX® PCR technology to resolve CAG expansions at the *HTT* gene locus with single-repeat resolution across multiple operators, days and gDNA concentrations.
- The data demonstrate concordant *HTT* genotypes across cell-line gDNA samples using the rapid prototype AmplideX® PCR/CE *HTT* Kit*.

Introduction

Huntington's disease (HD) is caused by expansions of an unstable, highly polymorphic trinucleotide CAG repeat in exon 1 of the huntingtin (*HTT*) gene. Determination of the number of CAG repeats is critical for molecular diagnosis of HD and is typically accomplished using PCR-based methods. However, polymorphisms near the CAG tract, such as the non-clinically relevant CCG repeat region located 3' of the CAG expansion, can lead to mis-priming and inaccuracies in allele sizing[1]. Here we describe a robust, rapid and accurate PCR assay that can resolve *HTT* zygosity and enable reliable repeat quantification even in the presence of known gene polymorphisms.

Materials and Methods

Cell-line gDNA reference samples were acquired from the National Institute of Standards and Technology (NIST) (6 HD) and the Coriell Institute for Medical Research with *HTT* alleles ranging from 15 to 250 CAG repeats. Additionally, Ultramer® DNA Oligonucleotides (Integrated DNA Technologies) were synthesized as controls containing well-characterized *HTT* SNPs between the polymorphic CAG and CCG regions associated with allele dropout in other *HTT* PCR based detection methods. Samples were amplified using prototype AmplideX® reagents (Asuragen) that enlist two co-incident priming modes, effectively combining two complementary amplification reactions in a single-tube that accommodate known polymorphisms and resolve zygosity in unexpanded homozygous and expanded heterozygous samples via a robust repeat peak pattern. Post-PCR allelic detection of the fluorescently-labelled products were resolved by capillary electrophoresis (CE) on the 3500xL Genetic Analyzer (Applied Biosystems). Genotypes were determined from the mobility of the target amplicon in combination with the repeat-primed peak pattern.

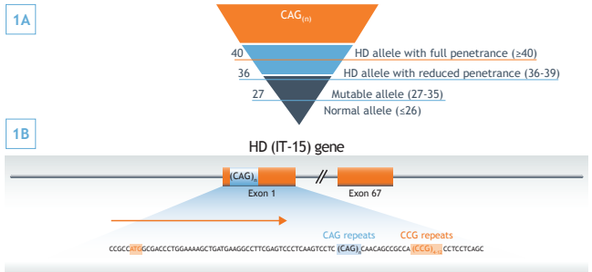

Figure 1. *HTT* Gene and Risk Alleles. A) CAG repeat length and associated HD boundary categories: normal allele (≤26 CAGs), mutable allele (27-35 CAGs), reduced penetrance (36-39 CAGs) and full penetrance (≥40 CAGs)[2]. **B)** Diagram of the *HTT* gene located on exon 1 with the outlined location of the polymorphic CAG and CCG regions with the interjecting 12-bp spacer sequence.

Figure 2. Time-Motion for the AmplideX® PCR/CE *HTT* Kit Workflow. An overview of the workflow, highlighting the rapid turnaround time of <6 hours from gDNA to answer. Total hands on time is ~65 min for 24 samples with one CE injection on the 3500xL Genetic Analyzer.

*This product is under development. Future availability and performance cannot be ensured.

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Results

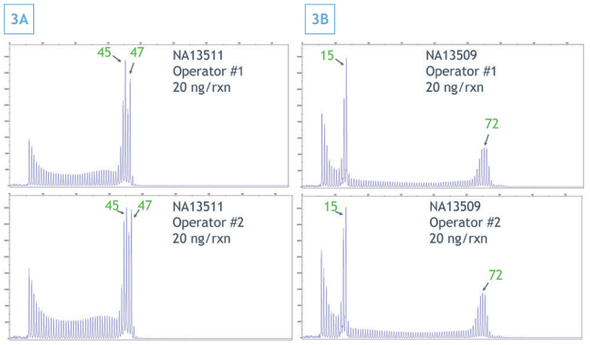

Figure 3. The AmplideX® PCR/CE *HTT* Kit Produces Reproducible CAG Expansion Genotypes Across Multiple Operators and Days. DNA samples (20 ng per reaction) with 15-72 CAGs were assessed by two operators across two different days. All results obtained from the 3500xL Genetic Analyzer were in agreement with single-repeat resolution among operators and days and within ACMG proficiency guidelines[2]. Representative electropherograms are shown for **A)** NA13511, a sample of 45/47 CAG repeat units and **B)** NA13509, a sample of 15/72 CAG repeat units.

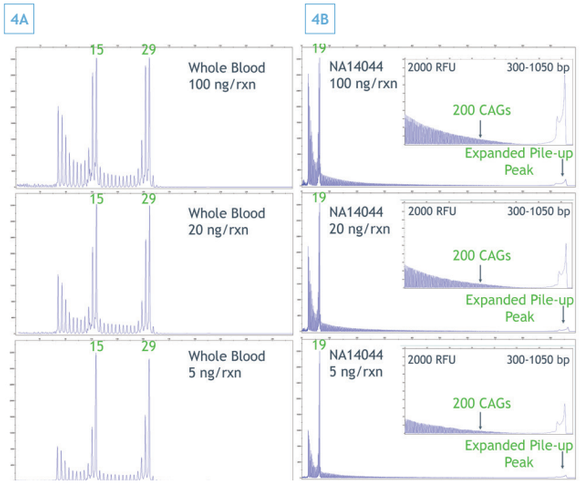

Figure 4. Broad DNA Inputs are Accommodated by the AmplideX® PCR/CE *HTT* Kit. Genomic DNA sample titrations of 100, 80, 40, 20 and 5 ng per reaction were evaluated in the prototype AmplideX PCR/CE *HTT* Kit. Representative electropherograms from 100, 20 and 5 ng per reaction are shown for **A)** a 15/29 whole blood sample isolated at Asuragen using the QIAamp DNA Blood Mini Kit (Qiagen) and **B)** NA14044, a sample of 19/250 repeat units. Countable repeat-primed peaks extend to >200 CAG expansions along with the production of the highly expanded pile-up peak at the CE instrument's upper resolution.

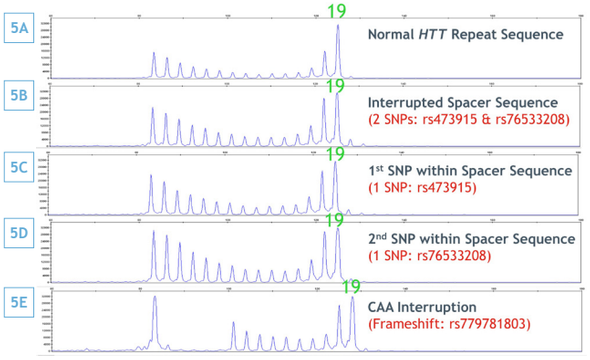

Figure 5. Successful Amplification and Accurate Genotyping of Synthetic Sequences Containing Well-Characterized *HTT* SNPs. Ultramer® DNA Oligonucleotides were synthesized as controls containing well-characterized *HTT* SNPs[1] located between the polymorphic CAG and the non-clinically relevant CCG repeat regions. Corresponding electropherograms for **A)** normal ultramer with 19 CAGs showed accurate genotyping with a synthetic sequence, **B)** two SNPs (rs473915 A->G & rs76533208 A->G) interrupting the 12-bp spacer region located 3' of the CAG expansion region did not alter the accurate genotyping of 19 CAGs, **C & D)** discrete SNPs (rs473915 A->G or rs76533208 A->G respectively) within the spacer did not alter the 19 CAG output and **E)** CAA frameshift interruption (rs779781803) did not hinder a successful amplification and accurate genotype with the last repeat peak located at 19 CAGs.

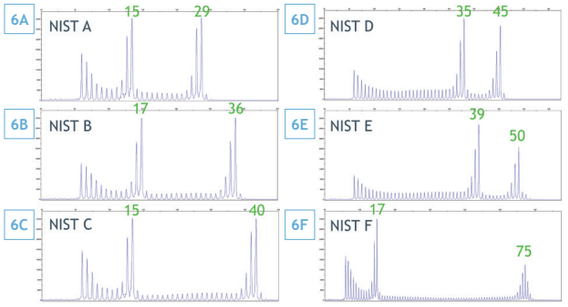

Figure 6. Accurate Genotyping of NIST CAG Repeat Length Mutations in Huntington's Disease Controls. Two microliters of NIST controls (SRM 2393) at 16-24 ng gDNA per reaction were used in the prototype AmplideX PCR/CE *HTT* Assay. Representative electropherograms are presented for **A)** Control A 15/29, **B)** Control B 17/36, **C)** Control C 15/40, **D)** Control D 35/45, **E)** Control E 39/50 and **F)** Control F 17/75 repeat units.

Sample ID	Coriell Institute *HTT* Genotype		CE Calibrator Determined CAG Repeat Length	
	Allele 1	Allele 2	Allele 1	Allele 2
NA20245	15	15	15	15
NA20206	17	18	17	18
NA20207	19	21	19	21
NA20246	15	24	15	24
CD00022	18	31	18	31
NA20248	17	36	17	36
NA20249	22	39	22	39
NA20250	15[3]	40[3]	15	40
NA13510	15	44	15	44
NA13512	16	44	16	44
NA13503	17	45	17	45
NA20208	35	45	35	45
NA13504	16	46	16	46
NA13511	45	47	45	47
NA20209	45	47	45	47
NA13506	17	48	17	48
NA13513	15	49	15	49
NA13505	22	50	22	50
NA20251	39	50	39	50
NA13514	15	52	15	52
NA13507	15	55	15	54
NA13508	22	58	22	57
NA03620	18	60	18	60
NA13515	16	66	16	65
NA20252	22	66	22	66
NA13509	15	70	15	72
NA20210	17[3]	74[3]	17	75
NA20253	22[3]	100[3]	22	100
NA14044	19	250	19	>200
NA09197	Juvenile Onset; ≥40		18	177

Table 1. AmplideX® PCR/CE *HTT* Kit is Capable of Resolving Zygosity in Unexpanded Homozygous and Expanded Heterozygous Samples Through Concordant *HTT* Genotypes Across 30 Cell-Line gDNA Samples. Evaluation of the prototype AmplideX PCR/CE *HTT* Kit with 30 Coriell cell-line gDNA samples resulted in full agreement with Coriell or peer-reviewed literature[3] CAG repeat numbers, within ACMG 2014 technical guidelines[2] for acceptable ranges (<50 CAG repeats ±2 repeats; 50-75 repeats ±3 repeats and >75 repeats ±4 repeats). An *HTT* CE Calibrator (NA2025, 39/50 and NA20210, 17/75 repeat units) slope and intercept was used for extrapolation of CAG expansion numbers for sample genotyping following CE analysis. Overall, accurate and unambiguous CAG repeat determination was achieved for >100 repeats.

Conclusions

- We describe a robust, rapid and accurate PCR assay that can resolve *HTT* zygosity and enable reliable repeat quantification to at least 100 CAG repeats, and flag expansions with >200 repeats.
- The assay design accommodates known polymorphisms near the CAG repeat and may mitigate, if not eliminate, false-negative results that challenge current *HTT* repeat technologies.
- The assay enables reproducible results, single-repeat resolution, and unambiguous data interpretation with ~1 hour of hands-on-time and <6 hours from gDNA to answer.

References

1. Goldberg, YP. et al. Increased instability of intermediate alleles in families with sporadic Huntington disease compared to similar sized intermediate alleles in the general population. Human Molecular Genetics; 10. 1911-8 (1995)
2. Bean, L. et al. American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories, 2014 editions: technical standards and guidelines for Huntington disease. Genetics in Medicine; 16, e2 (2014).
3. Kalman, L. et al. Development of genomic reference materials for Huntington disease genetic testing. Genetics in Medicine; 9, 719-723 (2007).

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