Analytical Validation of a PCR/CE Assay that Phases SNPs with CAG-Expanded Alleles for Selecting Huntington Disease Patients for Allele-selective Treatments

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Summary

- Huntington disease (HD) is an autosomal dominant neurological disorder that is caused by expansions of ≥36 CAG repeats in exon 1 of the *HTT* gene.
- Single nucleotide polymorphisms (SNPs) on the mutant *HTT* (mHTT) allele carrying the expanded CAG tract can be targeted in patients using antisense oligonucleotides (ASOs) as a potential allele-selective treatment.
- Asuragen's AmplideX® *HTT* SNP/Repeat Phasing Clinical Trial Assay* (CTA) quantifies CAG repeats and genotypes three distinct SNPs in phase with the repeat tract to identify patients eligible for clinical trials evaluating Wave's allele-selective ASOs.
- Analytical validation of the CTA demonstrates robust and accurate performance using a comprehensive, streamlined and integrated solution that includes controls and companion software to assure the reliability of results.

Introduction

More than 30,000 people in the US have HD and another approximately 200,000 are at-risk of developing the disorder. Expansions of ≥36 CAG repeats in exon 1 of the *HTT* gene are responsible for encoding mutant huntingtin (mHTT) protein whose accumulation causes progressive loss of neurons in the brain[1]. Specific SNPs situated on the same allele as the repeat expansions can be targeted in patients using allele-selective oligonucleotides. Wave Life Sciences has developed stereopure ASOs designed to selectively silence pathogenic *mHTT* transcripts and spare healthy, wild-type huntingtin (wtHTT) protein that may be neuroprotective and necessary for normal brain functioning[2,3]. Wave's pipeline includes an investigational allele-selective stereopure oligonucleotide WVE-003, which was designed to preferentially target the mutant huntingtin (*mHTT*) mRNA transcript associated with SNP3. WVE-120101 and WVE-120102, formerly in Wave's pipeline, were designed to target SNP1 and SNP2, respectively[4].

The previous phase determination reference method required combining multiple technologies and assays: determining the number of CAG repeats via repeat-primed (RP) PCR, confirming SNP heterozygosity with Sanger sequencing, and achieving phasing using a long-read sequencing assay on the PacBio Sequel. We developed a more unified and rapid assay that can quantitate CAG repeat tracts, genotype SNP1, SNP2 and SNP3, and link these SNPs to the expanded *mHTT* allele to identify patients that may be eligible for clinical trials evaluating with Wave's allele-selective ASOs[5].

Materials and Methods

HD positive samples representing the most clinically relevant genotypes of heterozygous and homozygous SNPs in phase (i.e. trial eligible) or out of phase (i.e. trial ineligible) with expanded CAG repeats were selected for this study (Table 1). The validation study used 52 unique residual clinical RNA samples isolated from HD positive patients' whole blood and four contrived RNA obtained from HD cell lines spiked into leukodepleted blood and extracted, to evaluate analytical sensitivity and specificity, RNA extraction method, RNA input ranges, interfering substances, clinical accuracy, and intra-laboratory precision. The assay also includes two control samples designed by spiking HD positive Coriell cell lines with different genotype combinations for each SNP, into leukodepleted blood to mirror clinical samples in RNA extraction method and performance.

Whole blood from HD patients was collected in PAXgene® RNA blood tubes. Total RNA extracted using the PAXgene™ Blood RNA Kit was assessed for purity and integrity before subjecting to reverse transcription, since intact human *HTT* mRNA is ~13.5kb in length. Allele-specific long-range (ASLR) PCR amplification of ~7 kb to ~10 kb region between the SNPs and CAG repeats followed by a repeat-primed (RP) PCR to resolve the CAG repeat size was used to generate phased SNP/CAG repeat amplicons. The products from two PCR reactions per SNP were run on an Applied Biosystems 3500xl Capillary Electrophoresis (CE) instrument (ThermoFisher). Fragment analysis was completed by directly analyzing the FSAs with the analysis module in the AmplideX® Reporter software developed using machine learning, which provides quality control checks and automatically determines SNP/repeat phasing and eligibility, SNP zygosity, and CAG repeat length. Automated results were also compared to results obtained from manual inspection of CE trace in GeneMapper.

The binary outcomes of eligibility and SNP zygosity were assessed for concordance to a reference assay, while the acceptable CAG sizing precision was based on ACMG HD testing guidelines (within ± 2 when <50 rpts; within ± 3 from 50 to 75 rpts; within ± 4 when >75 rpts). Assay validation testing was performed at Asuragen's CLIA laboratory.

The assay performance was evaluated for Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), and Overall Percent Agreement (OPA) for eligibility compared to the reference method. The acceptance criteria for primary analysis for each study was set to two-sided 95% confidence interval for eligibility agreement for each SNP contain 95%. For OPA, no more than three total incorrect calls, with at most two false positive or two false negative calls observed. Comparisons of zygosity and CAG sizing agreement associated with each SNP was also included as part of exploratory secondary analysis.

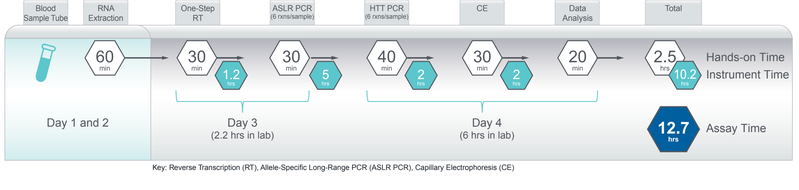

Figure 1. Workflow for the AmplideX® *HTT* SNP/Repeat Phasing CTA. With net turn around time of 4-8 days, and less than 3 hours of hands-on time from RNA to results, the assay and its assay-specific AmplideX® Reporter software offers a streamlined sample-to-answer workflow. This is estimated for a typical run with all 3 SNPs for 5 clinical samples, 2 controls and 1 NTC and total of 2 injections on a 24-capillary CE.

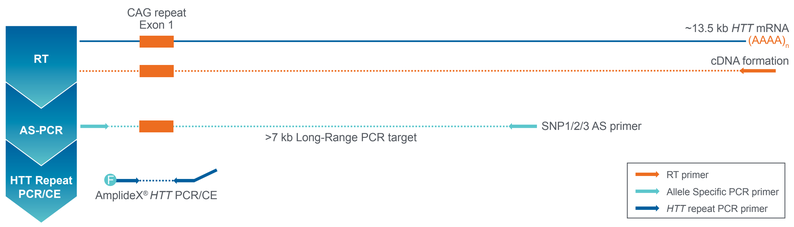

Figure 2. Design of the AmplideX *HTT* SNP/Repeat Phasing Clinical Trial Assay. Total RNA undergoes reverse transcription (RT) reaction to form full length *HTT* cDNA (~13.5kb). cDNA is subjected to ASLR PCR providing specificity during the allele enrichment process. Two PCR products per SNP are then used as input into two separate AmplideX *HTT* PCR/CE reactions. The resulting output ascertains phase genotyping; only the allele that is in-phase with the particular SNP 1/2/3 location and status will be amplified in the ASLR PCR reaction and detected in the *HTT* PCR/CE assay output. No signal will be detected if the allele is out of phase. Results are generated using automated AmplideX Reporter software.

Results

The assay performance was evaluated for PPA, NPA and OPA for eligibility compared to the reference method. The acceptance criteria for primary analysis for each study was set to ≥95% agreement for eligibility within two-sided 95% confidence interval for each SNP. Number of samples queried for each SNP are indicated in parenthesis next to the observed % agreement. 95% CIs values are indicated within parenthesis below agreement percentage values in the tables 2 to 6. Throughout the data, HET and HOM are used as abbreviations for heterozygous and homozygous, respectively. Comparisons of zygosity and CAG sizing agreement associated with each SNP was also included as part of exploratory secondary analysis.

Category #	HD Diagnosis	SNP Zygosity	Eligible	SNP1 (n=56)	SNP2 (n=56)	SNP3 (n=52*)
1	Yes	HET-E	Yes	29	29	23
2		HET-I	No	1	4	3
3		HOM-T/A	No	1	18	26
4		HOM-C/G	No	25	5	0

Table 1. Samples Analyzed in Validation Studies as Represented by Eligibility and Across SNP Genotype Categories. A total of 56 unique samples were used in the different studies during analytical validation. The number of samples containing each SNP at the indicated zygosity and corresponding eligibility status is shown. For all the samples, RNA integrity (RIN values) ranged between 6.9 and 9.9, extracted RNA concentration ranged between 31 and 277 ng/µl, and CAG repeat number across both alleles ranged between 15 and 69. Heterozygous eligible (HET-E), heterozygous ineligible (HET-I), homozygous with T or A (HOM-T/A) and homozygous with C or G (HOM-C/G). *Information not available for SNP3 in 4 samples

	Eligibility PPA (95% CI)	Eligibility NPA (95% CI)	Eligibility OPA (95% CI)	Zygosity Percent Agreement (95% CI)	CAG Length Percent Agreement (95% CI)
SNP1	100% (112/112) (96.8% - 100%)	100% (134/134) (97.3% - 100%)	100% (246/246) (98.5% - 100%)	100% (246/246) (98.5% - 100%)	100% (492/492) (99.3% - 100%)
SNP2	99.1% (108/109) (95.0% - 100%)	100% (150/150) (97.6% - 100%)	99.6% (258/259) (97.9% - 100%)	99.6% (258/259) (97.9% - 100%)	100% (518/518) (99.3% - 100%)
SNP3	96.1% (99/103) (89.1% - 98.4%)	100% (145/145) (97.5% - 100%)	98.4% (244/248) (95.4% - 99.3%)	98.4% (244/248) (95.4% - 99.3%)	99.6% (510/512) (98.6% - 100%)

Table 2. High Overall Accuracy During All the Studies of Analytical Validation. Across all the studies, the assay produced 96-100% positive agreement, 100% negative agreement and 98-100% overall agreement with reference values for eligibility for each SNP. Note: Three SNP3 eligibility samples (from Studies 1 and 2) that were discordant with the reference values were from the same cell line-derived RNA sample. Zygosity agreement with reference results was 98-100% and CAG sizing agreement was 99-100% for each SNP.

	Eligibility Percent Agreement (95% CI)	Zygosity Percent Agreement (95% CI)	CAG Length Percent Agreement (95% CI)
SNP1	100% (38/38) (90.7% - 100%)	100% (38/38) (90.7% - 100%)	100% (76/76) (95.3% - 100%)
SNP2	100% (38/38) (90.7% - 100%)	100% (38/38) (90.7% - 100%)	100% (76/76) (95.3% - 100%)
SNP3	97.4% (37/38) (86.2% - 99.9%)	97.4% (37*/38) (86.2% - 99.9%)	100% (76/76) (95.3% - 100%)

Table 3. Agreement in Repeatability Study Using 4 Samples x 10 Replicates x 1 Operator/CE. The assay demonstrated 97-100% eligibility and zygosity agreement, and 100% CAG size call agreement with the sample mode for each SNPs in all the samples.
*SNP3 expected HET, observed HOM

	Eligibility Percent Agreement (95% CI)	Zygosity Percent Agreement (95% CI)	CAG Length Percent Agreement (95% CI)
SNP1	100% (18/18) (81.5% - 100%)	100% (17/17) (80.5% - 100%)	100% (35/35) (90.0% - 100%)	100% (35/35) (90.0% - 100%)	100% (70/70) (94.9% - 100%)
SNP2	95.2% (20*/21) (76.2% - 99.9%)	100% (18/18) (81.5% - 100%)	97.4% (38/39) (86.5% - 99.9%)	97.4% (38/39) (86.5% - 99.9%)	100% (78/78) (95.4% - 100%)
SNP3	93% (13*/14) (59.5% - 98.3%)	100% (20/20) (82.4% - 100%)	97% (33/34) (80.3% - 99.3%)	94.1% (32/34) (80.3% - 99.3%)	100% (68/68) (94.7% - 100%)

Table 4. Agreement in Intermediate Precision Study Using 4 Samples x 5 Days x 2 Operators x 2 CE Instruments. The assay demonstrated 98.5-100% eligibility and zygosity agreement, and 99.2-100% CAG size call agreement with the reference genotypes for each SNP in all the samples.
*SNP3 expected HET, observed HOM on both CE instruments
^SNP3 expected 18|41, observed 18|38 on both CE instruments

	Eligibility Positive Percent Agreement (95% CI)	Eligibility Negative Percent Agreement (95% CI)	Eligibility Overall Percent Agreement (95% CI)	Zygosity Percent Agreement (95% CI)	CAG Length Percent Agreement (95% CI)
SNP1	100% (18/18) (81.5% - 100%)	100% (17/17) (80.5% - 100%)	100% (35/35) (90.0% - 100%)	100% (35/35) (90.0% - 100%)	100% (70/70) (94.9% - 100%)
SNP2	95.2% (20*/21) (76.2% - 99.9%)	100% (18/18) (81.5% - 100%)	97.4% (38/39) (86.5% - 99.9%)	97.4% (38/39) (86.5% - 99.9%)	100% (78/78) (95.4% - 100%)
SNP3	93% (13*/14) (59.5% - 98.3%)	100% (20/20) (82.4% - 100%)	97% (33/34) (80.3% - 99.3%)	94.1% (32/34) (80.3% - 99.3%)	100% (68/68) (94.7% - 100%)

Table 5. Agreement in Accuracy Study Using 49 Samples. 100% OPA for SNP1, 97.4% for SNP2, and 97% for SNP3 for eligibility. Zygosity calls were similarly matched in percent agreement by SNP. CAG size calls were in 100% agreement with reference genotypes for all specimens in the accuracy study.
*SNP3 expected HET, observed HOM

A

	Eligibility Overall Percent Agreement and Zygosity Percent Agreement
SNP1	100% (21/21) (9 eligible +12 ineligible) (83.9% - 100%)
SNP2	100% (24/24) (6 eligible +18 ineligible) (85.8% - 100%)
SNP3	100% (24/24) (6 eligible +18 ineligible) (85.8% - 100%)

B

	Eligibility Overall Percent Agreement
SNP1	100% (8/8) (4 eligible + 4 ineligible) (63.1% - 100%)
SNP2	100% (8/8) (2 eligible + 6 ineligible) (63.1% - 100%)
SNP3	100% (8/8) (2 eligible + 6 ineligible) (63.1% - 100%)

C

	Eligibility and Zygosity Percent Agreement
SNP1	100% (13/13) (75.3% - 100%)
SNP2	100% (16/16) (79.4% - 100%)
SNP3	100% (16/16) (79.4% - 100%)

Table 6. Measured Eligibility, Zygosity and CAG Sizing Agreement Across Analytical Sensitivity A), Analytical Specificity B) and Interfering Substances Studies C). A) Analytical sensitivity study using 4 samples x 2 replicates x 3 inputs x 1 operator/CE tested at 500-750 ng RNA input. **B)** Analytical specificity study using 4 samples x 2 replicates x 1 inputs x 1 operator/CE tested at 750 ng RNA input. **C)** Testing the effect of interfering substances using 2 samples x 2 replicates x 4 conditions x 1 operator/CE (all samples were ineligible for all SNPs). 100% agreement for eligibility, zygosity and CAG sizing was observed for each SNP in all the samples. Number of eligible and ineligible genotypes queried for each SNP are indicated in parenthesis.

Conclusions

- In accuracy studies, the CTA demonstrated 100% OPA for SNP1, 97.4% for SNP2, and 97% for SNP3 for eligibility calls. Zygosity calls were similarly matched in percent agreement by SNP, and CAG size calls were in 100% agreement with reference genotypes.
- The assay also demonstrated 97-100% PPA, NPA, and OPA for each SNP as assessed for repeatability, reproducibility, sensitivity, specificity and effects from common interfering substances.
- The AmplideX *HTT* SNP/Repeat Phasing CTA improves the net turn-around time to 4-8 days from up to 2 months using the previously validated genotyping and phasing assay.
- The CTA is currently utilized in Asuragen's CLIA laboratory to pre-screen patients for an adaptive Phase 1b/2a SELECT-HD clinical trial of WVE-003.
- The design, configuration and integration of reagents and software for this assay offers a path for development into a companion diagnostic for future allele-selective, repeat expansion disease treatments.

References

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This test is a Clinical Trial Assay used to inform the management of clinical trial subjects; it has not been cleared or approved by the US FDA. The laboratory is regulated under CLIA as qualified to perform high-complexity testing.
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