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White Paper

Complex Molecular Genetic Testing Made Simple

Accelerating the CTA to CDx Journey with Bio-Techne's AmplideX® Platform for Streamlined Amplification of GC-rich and Other Repetitive DNA Sequences

Contents

Overview

Many genetic diseases are associated with DNA mutations that are highly difficult to robustly detect using conventional methods[1]. While progress has been made in characterizing these dark genomic regions in a research setting, the testing approaches employed for amplification and sequencing are often complex and inefficient. These limitations impose serious barriers to using these tests in clinical trials or as companion diagnostics (CDx).

For more than a decade, Asuragen, a Bio-Techne brand, has advanced testing methods and helped overcome major challenges in reliably amplifying GC-rich and other repetitive DNA sequences. This was accomplished by developing a novel PCR platform that can be coupled with capillary electrophoresis (CE) technology or long read sequencing. Originally developed for genetic testing of fragile X syndrome (*FMR1*)[2] and frontotemporal dementia/amyotrophic lateral sclerosis (*C9orf72*)[3], the AmplideX platform has been extended to several other repeat disorders like myotonic dystrophy (*DMPK*)[4], Huntington's disease (HD) (*HTT*)[5], and through exploratory prototypes for Friedreich's ataxia (*FXN*) and spinocerebellar ataxia (*ATXN1, ATXN3, ATXN7, ATX2, CANA1A*)*.

Since then, this technology has been utilized to solve other genetic testing issues, such as disorders requiring copy number resolution like spinal muscular atrophy (SMA) or a high degree of multiplexing and detection of heterogeneous mutations as in cystic fibrosis (CF)[7]. More recently, AmplideX PCR has been coupled with long read sequencing to address even greater challenges, such as large inversions as in hemophilia A (*F8* introns) and pseudogenes as in congenital adrenal hyperplasia (CAH). This broad range of in vitro nucleic acid amplification kits and assays across multiple diseases demonstrates innovation with reduced and optimized lab workflows, purpose built software, and high quality performance.

*Note that the PCR/CE kits for *C9orf72* and *HTT* referenced here are for research use only. Not for use in diagnostic procedures. See Table 1 for additional information.

Asuragen has extensive knowledge and expertise in rapidly developing clinical trial assays (CTAs), as well as navigating regulatory processes to successfully achieve clearance. By partnering with Asuragen for CTA development, pharmaceutical companies can streamline patient enrollment and stratification into trials, focus on their therapeutic programs, and confidently move toward approval of a CDx.

This white paper describes how AmplideX products can serve as CTAs and/or CDx to therapies developed for genetic diseases and highlights how this technology can be expanded for other challenging genetic disorders with unmet testing needs.

Key Learnings

- AmplideX technology offers innovation for enabling amplification and detection of challenging gene regions, including nucleotide repeat expansions, high homology targets, copy number variation, methylation, large inversions, pseudogenes, and indels.
- The streamlined AmplideX platform establishes innovation in 3 major areas: reduced complexity, optimized workflows, and quality performance.
- The AmplideX platform is highly versatile and can be paired with a variety of downstream detection methods, from CE to long read sequencing based technologies.
- The design, configuration, and integration of reagents and software for AmplideX CTAs provide a clear path for CDx development for future genetic disorder treatments.
- Asuragen's experience with obtaining clearance of AmplideX kits can provide critical guidance through the regulatory pathway for CDx.
- Partnership between Wave Life Sciences and Asuragen enabled development of a CTA targeting the *HTT* gene. Analytical validation studies demonstrate its accuracy to confirm trial eligibility for Wave's investigational allele selective molecules to treat HD.

AmplideX Genetic Kits

There are multiple AmplideX commercial kits available that detect variants associated with different genetic diseases (**Table 1**). In addition to sharing one foundational approach enabling reliable amplification of repeat expansions and detection of methylation and structural variants, these kits share streamlined and scalable workflows (**Figure 1**). Conventional genetic testing approaches for detection and sizing of repeat expansions and copy number variations employ multiple and often cumbersome methods that can lead to long turnaround times and produce variable results. In some disorders with large or complex repetitive sequences, such as *FMR1* and *DMPK* alleles, Southern blot (SB) was previously recommended as a primary or confirmatory diagnostic test[8]. However, SB assays are resource intensive (labor and time), low resolution, low throughput, and require substantial amounts of DNA. In other disorders, such as spinal muscular atrophy (SMA), copy number identification of 2 different genes (*SMN1/2*) and resolution of additional variants is important.

To address these challenges, each AmplideX kit employs accurate amplification of the target region by PCR, combined with identification and reporting of variants through push button software. The AmplideX platform has optimized workflow efficiency, reduced subjective data analysis, and decreased time from sample to result, transforming testing for a range of genetic diseases (**Figure 2**).

Figure 1. Streamlined Workflow of AmplideX Kits

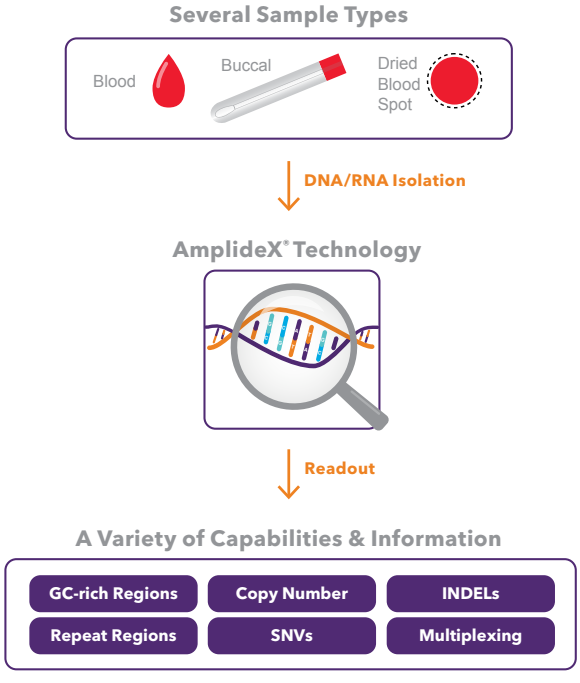


Table 1. Streamlined Workflow of AmplideX Kits

Gene(s)	Relevant Disease(s)	AmplideX Product Name	Regulatory Clearance	Challenge Addressed by AmplideX Test	Key Features of AmplideX Technology
FMR1	Fragile X	Fragile X Dx & Carrier Screen Kit[a]	US IVD	Previous *FMR1* testing relied on SB for detection of expanded alleles too large for conventional PCR and resolution of female homozygous alleles that can confound analyses of PCR data.	The Fragile X Dx & Carrier Screen Kit is the first and only genetic test for fragile X and related disorders authorized by the FDA as an aid in diagnosis and for carrier screening in adults. Both kits accurately size alleles ≤200 CGGs and detect alleles >200 CGGs, including mosaic alleles present at low allele fraction. Results are obtained in hours, with diagnostic performance superior to SB which takes several days to run. In addition, automated reporting software dramatically decreases time to result (>100 fold faster than manual analysis).
		PCR/CE *FMR1* Kit[b]	RUO, CE-IVD**		
		mPCR *FMR1* Kit[c]	RUO	Methylation status of *FMR1* is currently assessed using SB, which does not allow precise sizing across all clinical categories of alleles. Alternative PCR methods used to assess methylation status are often limited to male samples as they can fail to provide allele by allele resolution of methylation patterns.	mPCR *FMR1* is a PCR only workflow that provides more sensitive, rapid, and quantitative allele specific methylation without the need for SB analysis. Sample to result is achieved in <8 hours, a 5-fold improvement compared with SB with 50-fold less input DNA required.
		Xpansion Interpreter[d]	LDT	Standard PCR applications cannot accurately determine the number and sequence context of AGG interruptions, which provide important insights into the risk for the most common premutation carriers having children with fragile X syndrome.	As the first clinically validated LDT for *FMR1* AGG status, this kit stratifies the risk of having a child with fragile X syndrome by determining number and allelic location of AGG interruptions within the CGG repeat region of the *FMR1* gene.

Table 1. Streamlined Workflow of AmplideX Kits

Gene(s)	Relevant Disease(s)	AmplideX Product Name	Regulatory Clearance	Challenge Addressed by AmplideX Test	Key Features of AmplideX Technology
SMN1/2	Spinal muscular atrophy	PCR/CE *SMN1/2* Plus Kit[e]	RUO	Copy number variations in *SMN1* and *SMN2*, respectively, are associated with SMA onset and severity, with gene duplication and disease modifier variants associated with carrier risk and prognosis. MLPA provides copy numbers for *SMN1/2* but is more variable in performance, has a more complex workflow and has turnaround times of >24 hours. NGS provides the most comprehensive *SMN1/2* testing, but requires long and laborious workflows and specialized equipment, resulting in lengthier turnaround times compared with targeted approaches. Bioinformatic analysis is particularly challenging, as most laboratories are unable to address *SMN1/2* using NGS for technical reasons.	With a simple workflow using a single PCR, these kits integrate quantification of *SMN1* and *SMN2* copy number from 0 to 4 copies, while simultaneously detecting 2 gene duplication markers and a disease modifier SNV, with results obtained in under 4 hours, using equipment that most laboratories already have.
		SMA Plus* Kit[e]	CE IVD**		
CFTR	Cystic fibrosis	PCR/CE *CFTR* Kit[f]	RUO	Other commercial kits for *CFTR* testing are more likely to detect pathogenic variants in individuals of European descent and less likely to detect them in individuals with non European ancestry. NGS is capable of comprehensive testing of *CFTR* variants, but requires highly skilled technicians and complicated workflows, leading to lengthier turnaround times and costlier operations compared with targeted approaches.	This kit detects ~92% of *CFTR* pathogenic variants observed in the diverse US population – broader coverage than any other targeted *CFTR* assay. Results are delivered in fewer than 5 hours with fewer hands on steps than other *CFTR* assays.
C9orf72	Frontotemporal dementia Amyotrophic lateral sclerosis	PCR/CE *C9orf72* Kit[g]	RUO	Accurate quantification of hexanucleotide repeats in the *C9orf72* gene is essential to understanding familial ALS and FTD, yet manual interpretation of PCR/CE results can be laborious, time-consuming, and variable among trained users. No competitor kitted product exists for *C9orf72*.	Currently the only available commercial kit for *C9orf72*, this product accurately predicts *C9orf72* genotype following amplification of almost entirely GC rich sequences. An automated deep learning approach achieves expert level human performance while decreasing time needed for manual interpretation (200x faster than manual operators).

Gene(s)	Relevant Disease(s)	AmplideX Product Name	Regulatory Clearance	Challenge Addressed by AmplideX Test	Key Features of AmplideX Technology
DMPK	Myotonic dystrophy	*DM1 Dx* Kit[h]	CE IVD**	*DMPK* has long challenged laboratory analysis due to the presence of very large expansions (>1000 repeats) and a high degree of mosaicism. Type 1 (DM1) molecular testing requires multiple PCRs and SB assays as most laboratory developed PCR tests cannot reliably amplify >100 repeats associated with DM1.	The *DMPK* kit overcomes laboratory challenges by enabling the sensitive detection and sizing of these *DMPK* repeats, all within a single laboratory shift and without the need for SB confirmation. This kit reliably resolves normal and expanded alleles using CE for ≤200 repeats and flags >200 repeats in a single PCR tube.
HTT	Huntington's Disease	PCR/CE *HTT* Kit[i]	RUO	*HTT* genotyping is challenged by known rare polymorphisms surrounding the CAG repeat region that can cause allelic dropout. No competitor kitted product is available for *HTT* in the US.	This kit can flag very large expansions (>200 CAGs), resolve heterozygous and homozygous samples, and accommodate polymorphic regions and known SNPs. As the only commercial kit available in the US, this kit has potential to remedy concerns of false negative results with some existing *HTT* technologies and streamline clinical research in Huntington disease.

Abbreviations: ALS, amyotrophic lateral sclerosis; CE, capillary electrophoresis; DM1, myotonic dystrophy type 1; FDA, US Food and Drug Administration; FTD, frontotemporal dementia; IVD, in vitro diagnostic device; LTD, laboratory-developed test; MLPA, multiplex ligation-dependent probe amplification; mPCR, methylation PCR; NGS, next generation sequencing; RUO, research use only; SB, Southern blot; SMA, spinal muscular atrophy; SNP, single nucleotide polymorphism; SNV, single nucleotide variant. a. From Rey et al[9], b. From AmplideX PCR/CE *FMR1* Kit Product Brochure[10], c. From Grasso et al[11], d. From Nolin et al[12], e. From Milligan et al[13], f. From Asuragen[7], g. From Routsong et al[14], h. From Asuragen[4], i. From AmplideX PCR/CE *HTT* Kit Protocol Guide[5]

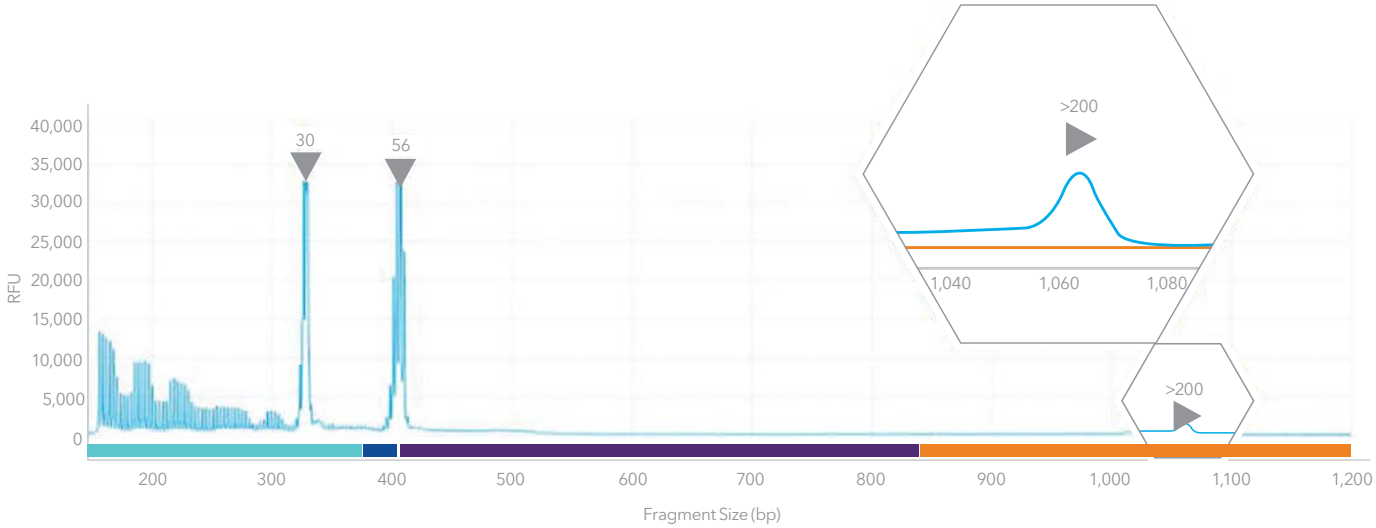
*See Instructions for Use (IFU) for specific intended use of IVD products.
**CE marked under the IVD Directive (IVDD) 98/79/EC.

Versatility and Multiplexing Capabilities of AmplideX with Emerging Analysis Technologies

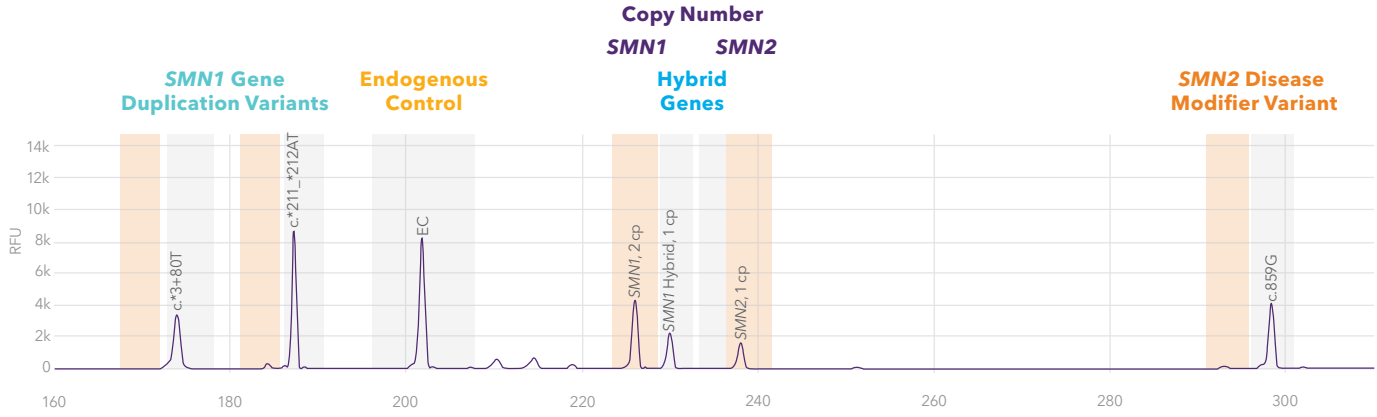
AmplideX is a powerful platform for enrichment and amplification of difficult gene regions that can be coupled with many other downstream techniques besides CE. This versatility enables its use in a wide variety of applications. For example, Asuragen has developed a multiplexed assay capable of detecting multiple high prevalence carrier genes in a single workflow to improve expanded carrier screening. The assay combines novel, long range AmplideX PCR chemistry with Oxford Nanopore Technologies' long read sequencing platform. Multiple high prevalence carrier genes are targeted in a mid range panel, such as *F8, HBA1/2* (alpha thalassemia), *GBA* (Gaucher disease), *CYP21A2, FMR1, CFTR,* and *SMN1/2*.[22] Early results indicate this prototype assay can provide uniform sequencing coverage for 29 genes and 360 amplicons in a single tube, showcasing the ability of AmplideX technology to be used for high density multiplexed PCR.

Figure 2. Overcoming Different Challenges Under the Same, Simple Workflow

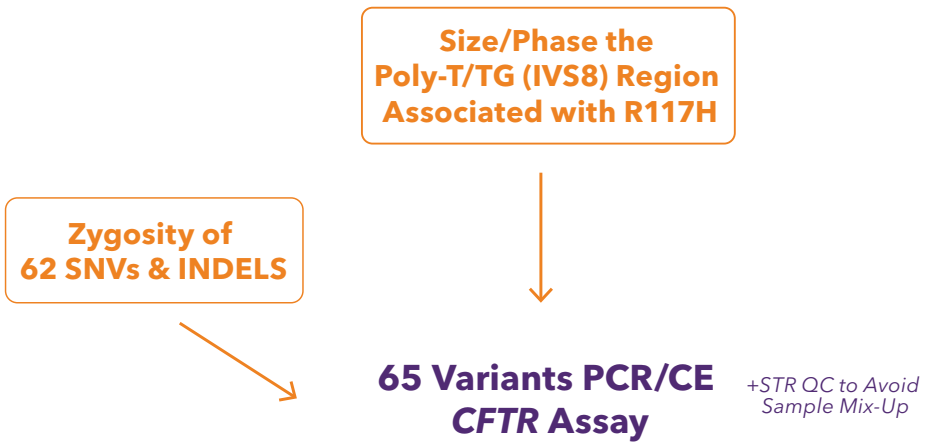
A. *FMR1* kit for detection of repeat expansions.



B. *SMN1/2* kit for copy number determination and detection of single nucleotide variants.



C. *CFTR* kit for high level multiplexing and detection of small indels.



Case Studies: Partnerships with Pharma to Develop CTAs for Advancing Clinical Trials

Asuragen has partnered with numerous pharmaceutical companies to meet their needs for robust, efficient CTAs to support their clinical trials. The AmplideX technology can be easily tailored to specific genetic testing needs: existing commercial kits can be modified to overcome a new testing challenge, or assays can be developed de novo for unexplored genetic diseases. This section highlights 3 prominent case studies where AmplideX PCR technology was successfully developed for CTA use in clinical trials.

Partnership with Wave: CTA for Huntington's Disease (HD)

Over 30,000 people in the US have Huntington's disease (HD), with approximately 150,000 more at risk of developing the disorder.[15] Huntington's disease is an autosomal dominant neurodegenerative disorder caused by expansions of ≥36 CAG repeats in exon 1 of the *HTT* gene which encodes mutant huntingtin (m*HTT*) protein. Accumulation of m*HTT* causes progressive loss of neurons in the brain, in turn leading to substantial cognitive and neurological impairments.[16] Currently, the disease is incurable.

Wave Life Sciences' Clinical Trial for HD

Certain single nucleotide polymorphisms (SNPs) are situated on the same allele as the expanded CAG repeat and thus can be targeted using allele-selective oligonucleotide therapies. Wave Life Sciences has developed antisense oligonucleotides (ASOs) as potential allele selective treatments designed to selectively silence pathogenic transcripts and leave the healthy, wild type huntingtin protein intact[17]. Wave's pipeline includes an investigational allele selective stereopure oligonucleotide WVE-003 that was designed to preferentially target the m*HTT* mRNA transcript associated with SNP3. WVE-120101 and WVE-120102, formerly in Wave's pipeline, were designed to target SNP1 and SNP2, respectively.[18]

Due to the large size of the *HTT* gene (~13.5 kb mRNA), identifying SNPs that are in phase or on the same allele as the CAG repeat expansions is a highly challenging task. The earlier phase determination approach involved a 2 month process comprised of multiple complex technologies and assays[19], including:

- Repeat primed PCR for determination of the number of CAG repeats to establish if an individual meets the requirements for an HD diagnosis
- Sanger sequencing for verification of SNP heterozygosity, which is an initial indication that an individual may be suitable for treatment
- Phasing using long read sequencing to confirm the SNP was located on the same allele as the CAG repeat expansion, confirming trial eligibility for allele selective therapies

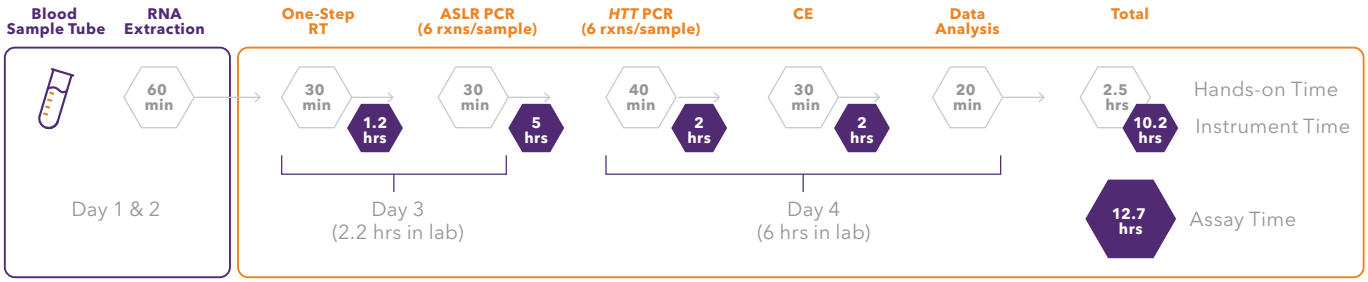
This serial testing method is cumbersome and time consuming, and thus impractical for large scale use in clinical trials and integration into clinical workflows.

Utilizing AmplideX PCR/CE *HTT* Kit in a CDx Workflow with Wave, Creating the AmplideX *HTT* SNP/Repeat Phasing CTA.

Asuragen and Wave Life Sciences entered into an agreement for the development and commercialization of a CDx for Wave's investigational allele selective therapeutic programs targeting HD. This partnership enabled development of a candidate CDx that uses a single, streamlined workflow for quantitation of CAG repeat tracts, genotyping of 3 SNPs known to have a high likelihood of being heterozygous in cases of CAG repeat expansions, and linking these SNPs to the m*HTT* allele. The resulting CTA, based on the AmplideX PCR/CE *HTT* Kit, can identify patients who may be eligible for Wave Life Sciences' allele-selective ASO, WVE-003, through the SELECT-HD Trial. This CTA enables a net turnaround time of 4-8 days, with fewer than 3 hours of hands on time needed to go from RNA to results in a streamlined workflow (**Figure 3**).

An analytical validation study was recently carried out to evaluate the performance of the CTA for diagnosis of HD positive samples, including SNPs both in phase (trial eligible) and out of phase (trial ineligible) with CAG repeats.[20] Clinical accuracy, analytical sensitivity and specificity, and intralaboratory precision, among other analytical metrics, were tested and compared with a previously validated genotyping and phasing assay. Across all the studies of analytical validation, the assay produced 96-100% positive agreement, 100% negative agreement and 98-100% overall agreement with reference values for eligibility for each SNP (**Table 2**). Zygosity agreement with results from the reference method was 98 -100% and CAG sizing agreement was 99-100% for each SNP.

Figure 3. Workflow of the AmplideX *HTT* SNP/Repeat Phasing CTA,



Key: Reverse Transcription (RT), Allele-Specific Long-Range PCR (ASLR PCR), Capillary Electrophoresis (CE)

Table 2. High Overall Accuracy During All Analytical Validation Studies of AmplideX *HTT SNP*/ Repeat Phasing CTA.

	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% (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%)

Abbreviations: CTA, clinical trial assay; SNP, single nucleotide polymorphism. Number of samples queried for each SNP are indicated in parenthesis next to the % agreement.

Collectively, analytical validation of the CTA demonstrates robust and accurate performance using a comprehensive, efficient, and integrated solution that includes controls and companion software to ensure quality and reliable performance. The AmplideX CTA improved the net turnaround time to 4-8 days to obtain results compared to 2 months with the previously validated method. The CTA is currently utilized in Asuragen's Clinical Laboratory Improvement Amendments (CLIA) certified laboratory to prescreen patients for the adaptive phase 1b/2a SELECT-HD clinical trial of WVE-003 (NCT05032196). The partnership between Asuragen and Wave highlights how existing assays can be modified and expanded to best fit the testing requirements of a trial.

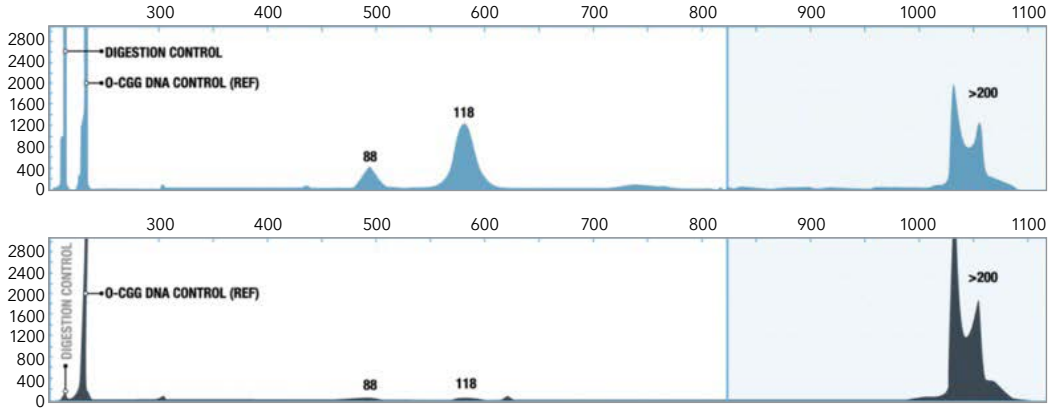
Partnership with Zynerba: Utilization of AmplideX *FMR1* Assays for Patient Screening in RECONNECT Trial for Fragile X Syndrome

As the global frontrunner for fragile X testing, Asuragen was selected to partner with Zynerba Pharmaceuticals, a developer of an investigational drug being studied in fragile X syndrome. Zynerba is conducting a confirmatory pivotal Phase 3 trial, RECONNECT, to evaluate the efficacy and safety of ZYN002*, a pharmaceutically manufactured cannabidiol transdermal gel, for treating behavioral symptoms of fragile x in adolescents, children and young adults (NCT04977986). To support the trial, Asuragen is providing CTAs, CLIA testing, and data analysis.

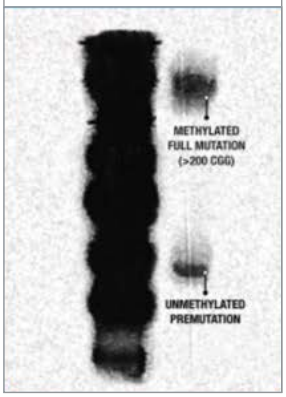
Zynerba chose to partner with Asuragen as the RECONNECT trial requires genetic testing to confirm patients have full mutation fragile X syndrome as well as the determination of the methylation status of the *FMR1* gene for each patient. Conveniently, 2 existing AmplideX based assays were well suited for use as "off the shelf" CTAs, providing several advantages over SB such as ease of use, shortened turnaround times, and increased sensitivity (**Figure 4**). In addition, testing is conducted in Asuragen's CLIA laboratory which provides guidance for data analysis in the context of the trial. This partnership provides an example of existing products already fit for use as a CTA, providing the best answers with optimal workflows and allowing time to be spent delivering actionable insights rather than searching for them.

*ZYN002 is an experimental treatment. This means that it is not approved by government regulatory bodies, including the United States Food and Drug Administration (FDA) and must be tested to see if it is an effective and safe treatment.

Figure 4. AmplideX mPCR *FMR1* Kit Enables Detection of Methylation Status Consistent with SB Analysis



Southern Blot



Methylation PCR		Calculation Methylation		
Digestion Control (% Digested)		Allele 1 88 CGG	Allele 2 118 CGG	Allele 3 >200 CGG >200 CGG
MF24	99% Fully Digested	1% Unmethylated	0% Unmethylated	100% | 100% Unmethylated

Partnership with Zinfandel and Takeda: Use of AmplideX *TOMM40* Assay to Support Screening in the TOMMORROW Trial for Alzheimer Disease

The prohibitive cost associated with the large study sizes and long durations needed to detect efficacy in a general elderly population is a serious challenge for clinical trials in Alzheimer disease (AD) prevention. Timely identification of individuals at near term risk of cognitive symptom onset can substantially mitigate costs and improve feasibility of clinical studies for a disease with extremely high unmet medical need. Sequence variants in *TOMM40* and *APOE* are 2 genetic risk predictors of AD. However, due to the AT rich character of *TOMM40*, this sequence is difficult to genotype using conventional PCR. To address these limitations, Asuragen developed the AmplideX PCR/CE *TOMM40* Kit to provide accurate CE sizing of alleles with up to 60 poly-T repeats using a rapid single tube PCR workflow. A prototype *APOE* assay was also developed for use with this kit for simultaneous genotyping of both genetic markers.

The performance of this multimodal approach for genotyping was compared with the CTA used in the TOMMORROW study, a phase 3 clinical trial (NCT01931566) for AD delay of onset sponsored by Takeda and Zinfandel.[21] For this study, a biomarker algorithm comprised of *APOE* and *TOMM40* genotypes was developed for trial enrichment. Strong concordance of results was observed between the Asuragen assays and the CTA used in the TOMMORROW study. This study exemplifies how novel CTAs can be developed based on AmplideX technology to advance clinical trials and research.

Asuragen's AmplideX Platform Can Accelerate Your Drug Development Journey

The AmplideX platform can be easily adapted for amplification and detection of challenging genetic targets, such as GC and AT rich regions, other repetitive DNA sequences, and copy number variation, using strategies parallel to those previously employed for assay development (**Table 1**).

As a global diagnostics product company, Asuragen delivers powerful genetic testing solutions that provide new insights into complex clinical questions. The development trajectory established for AmplideX products can be broadly applied toward the design of high performing, efficient, and simplified kits for challenging genetic targets with unmet CTA and CDx needs. This combination of next generation diagnostics with innovative therapeutic modalities can assist in conduct of clinical trials and accelerate the journey to approval for both therapies and CDx.

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