A Novel Quantitative Wild-type Huntingtin (wtHTT) Protein Biomarker Method for Human Cerebrospinal Fluid

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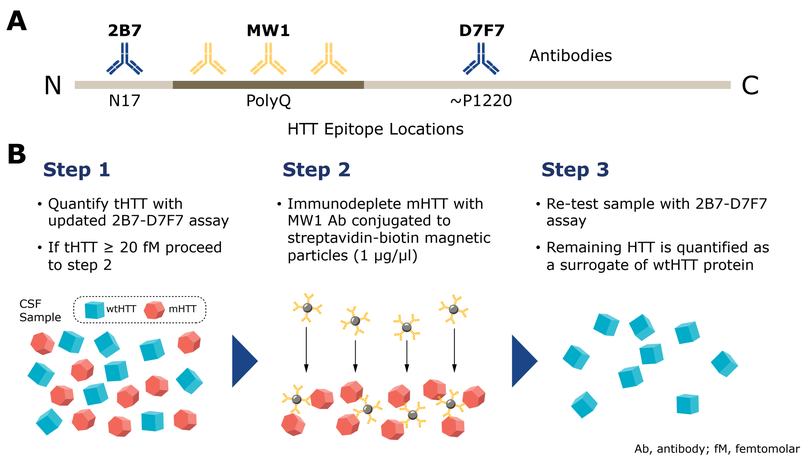
Summary

- We report the development and optimization of a novel method to quantify wild-type huntingtin (wtHTT) protein in human cerebrospinal fluid (CSF) samples.
- Our method comprises three steps:
  - First, total HTT (tHTT) protein is quantified using an optimized 2B7-D7F7 assay to confirm the concentration is within the range of acceptable assay performance.
  - Next, mutant HTT (mHTT) is immunodepleted using an antibody specific to polyQ (MW1) conjugated to streptavidin-biotin magnetic particles (MP). This step was optimized to ensure selective and efficient depletion of mHTT protein from CSF samples while leaving the wtHTT protein relatively unaffected.
  - Finally, the tHTT assay is again performed on the mHTT-depleted CSF and the value obtained is predominately wtHTT.
- Rigorous analytical qualification of our wtHTT method resulted in precise, specific, and reproducible results across a range of HTT protein concentrations in artificial CSF and human CSF samples.
- Our method performed reliably when applied to CSF from patients with Huntington's disease (HD).
- Application of this method during clinical studies can help to validate the mechanism of action for investigational therapies designed to selectively lower mHTT, including Wave Life Sciences' ongoing SELECT-HD trial (NCT05032196).
- Widespread use of this assay in the HD field would enable further optimizations and in-study qualifications. This includes utilizing homozygous HD patient CSF to investigate the performance of MW1-MP in endogenous protein scenarios.

Introduction

- A CAG trinucleotide repeat expansion (≥36) in at least one of the two alleles of the *HTT* gene leads to the expression of mHTT protein and causes HD, an autosomal dominant neurodegenerative disease.[1]
- The mHTT protein contains an elongated polyglutamine (polyQ) tract that perturbs wtHTT functions and contributes to gain of toxic activity.
- Since wtHTT supports critical functions even in adulthood[2-5], strategies to selectively lower mHTT are being explored in clinical trials for HD.
- Reliable quantification of wtHTT protein can facilitate proof of principal studies and evaluation of investigational allele-selective treatments.
- Our novel method for quantifying wtHTT protein in CSF comprises three steps (**Figure 1**) and incorporates an updated 2B7-D7F7 (tHTT) SMC assay (**Tables 1, 2**).

Figure 1. Strategy for quantifying wtHTT protein in CSF



Ab, antibody; fM, femtomolar

Results

Table 1. General specifications and qualification criteria of total HTT assay (initial and improved) to support wtHTT protein quantification method

Assay Parameters	Total HTT Assay[6] (initial)	Total HTT Assay (improved)	wtHTT Method
Sample	Human CSF	Human CSF	Human CSF
Assay analyte	Total HTT	Total HTT	Wild-type HTT
LLoQ	1.3 fM (floating)	7.3 fM	20 fM*
Assay Range	1.3 – 2000 fM	2.92 – 1500 fM	20 – 1500 fM
Intra-assay precision	CV < 20 % (30% at LLOQ and ULOQ)		CV < 25%
Inter-assay precision	CV < 20% (30% at LLOQ and ULOQ)		CV < 25%
Standard curve performance	√	√	
QC Sample accuracy and precision	×	√	
Specificity	√	√	
Selectivity	√	√	
Parallelism	√	√	
Stability	×	√	

*Robustness of wtHTT quantification demonstrated at 30 fM or higher; CV, coefficient of variation; fM, femtomolar; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

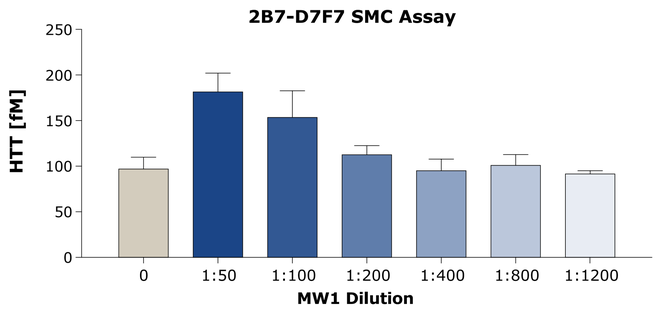
Table 2. Acceptance criteria for total HTT assay (updated) to support wtHTT protein quantification method

Quality Control (QC)	HTT (fM)	QC Type	Acceptance Criteria (CV)
Low QC	30 fM	Cal-Low	Should pass with ≤ ± 30%
High QC	200 fM	Cal-High	Should pass with ≤ ± 25%
Immunodepletion QCs			
Input QC / Mid-QC	100 fM	Cal-Mid	Should pass with ≤ ± 25%
IP QC	100 fM	(50 fM wtHTT)*	Should pass with ≤ ± 25%

- If only 2/4 passed; one of the 2 passed QC should be low-QC
- If low-QC failed, other QCs should pass

*mHTT would be removed after depletion; Cal, calibrator.

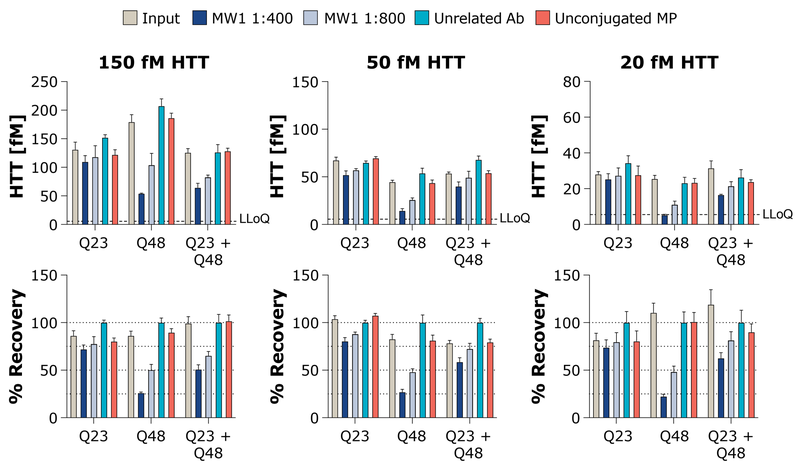
Figure 2. Free MW1 Ab concentration of 1:200 or higher minimizes interference with the 2B7-D7F7 total HTT assay



Dilutions of 1 µg/mL of free MW1 Ab stock was spiked in artificial CSF (aCSF) medium along with 150 fM recombinant full length (rFL) mHTT (48 polyQ) and incubated for one hour. Samples were then tested using the 2B7-D7F7 SMC assay. Data are mean ± SD, n=3.

- To identify a MW1 Ab dilution that maximizes mHTT depletion without interfering with the tHTT assay, a series of optimization experiments were performed (**Figure 2**).
- No interference was observed with 1:200 or greater dilution of antibody.
- Based on this data, the MW1-Strep-MPs should be diluted at 1:200 or more to avoid possible interference with the tHTT assay.

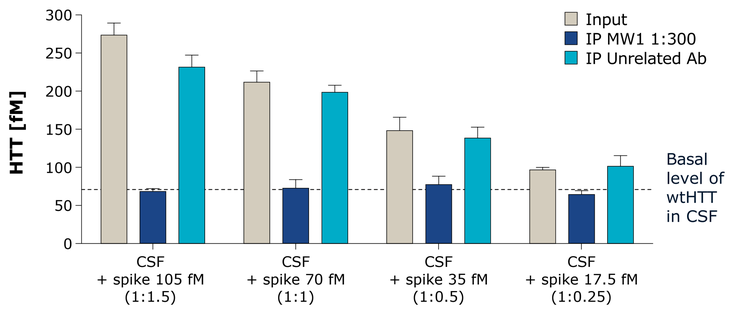
Figure 3. Immunodepletion with MW1 has an acceptable level of specificity when total HTT levels are ≥ 20 fM



Spike recovery experiments using aCSF were performed using rFL wtHTT (23Q) or mHTT (48Q) proteins. At each concentration tested (20 fM, 50 fM, and 150 fM), three groups of samples were prepared (group 1: 100% Q48, group 2: 100% Q23, group 3: 50% Q48 + 50% Q23). From each group, a set of samples was used as input, and one set each was treated with MW1-Strep-MPs at 1:400, 1:800, Strep-MP bound to unrelated Ab (control), or blocked Strep-MPs as an unconjugated Ab control. Samples tested for HTT in triplicate wells using SMC tHTT assay.

- To determine the working range of our wtHTT method, we evaluated its performance across three different concentrations of HTT protein spiked into aCSF (**Figure 3**).
- The lowering of HTT in control samples and in samples spiked with only wtHTT were relatively unchanged (≤25% to unrelated Ab) in all three HTT levels tested.
- Only in samples spiked with mHTT was HTT lowered when treated with MW1-Strep-MPs. The depletion percentages observed were concentration dependent.
- The data showed slightly lower than 50% depletion of mHTT protein at 20 fM with 1:1 ratio of wtHTT and mHTT treated with 1:400 dilution of MW1-Strep-MPs. This confirmed that detection at the lower range of the assay (≤20 fM) is possible but the percentage of mHTT depletion is not as efficient.
- Our method demonstrated acceptable variability ≤ 25% CV with three independent experiments.

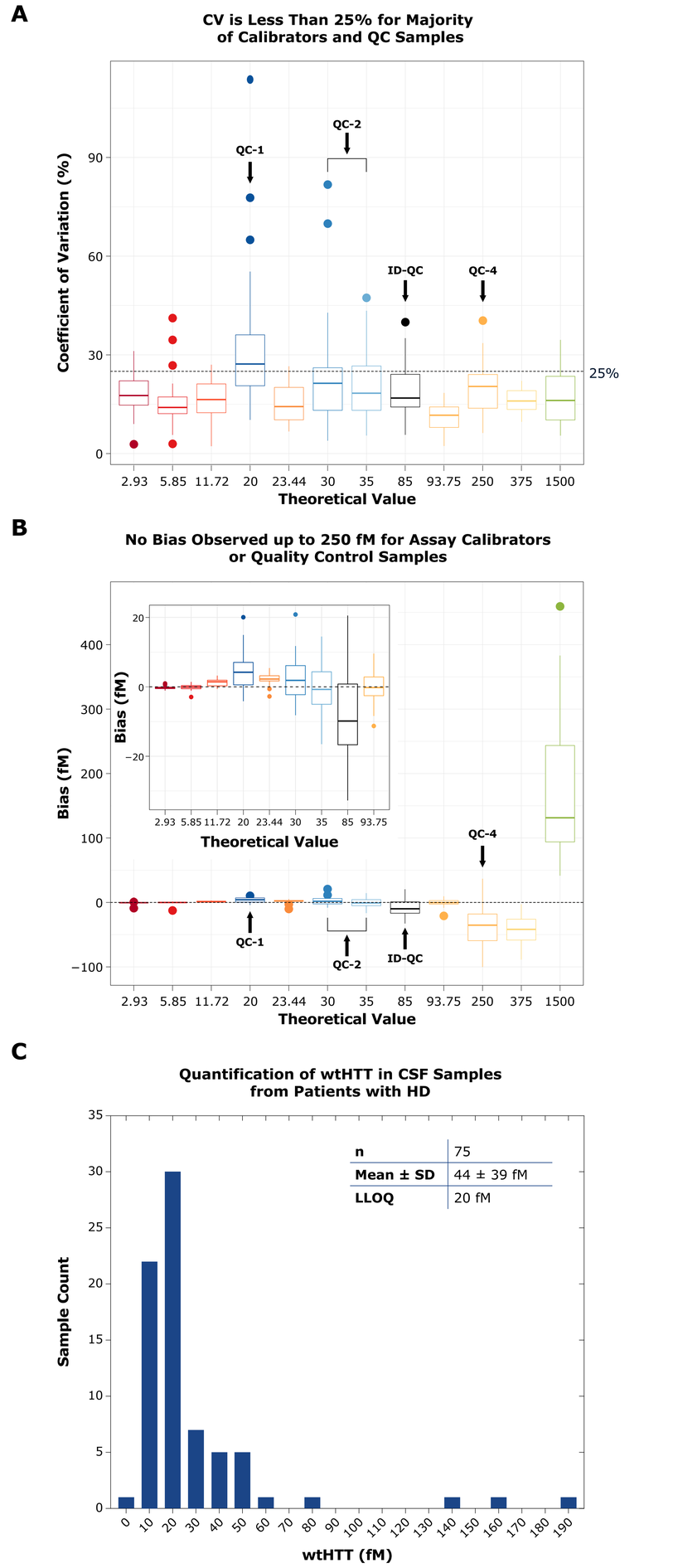
Figure 4. MW1 mediated depletion of mHTT is efficient within the m:wt ratios tested



Four groups of samples were prepared with pooled non-HD human CSF (phCSF) containing ~70 fM wtHTT that was spiked with varying amounts of rFL mHTT (48Q) to alter the wtHTT:mHTT ratio (1:1.5, 1:1, 1:0.5, or 1:0.25). The resulting concentrations of recombinant mHTT at each group were approximately 105 fM (group 1), 70 fM (group 2), 35 fM (group 3) and 17.5 fM (group 4). As controls, one set of samples from each group was used as input and another set with unrelated Ab for IP control. Data are mean ± SD, n=3.

- Next, we evaluated the specificity of mHTT protein depletion at various concentration ratios of mHTT:wtHTT to identify conditions that maximize mHTT depletion in human CSF without interfering with the endogenous wtHTT protein (**Figure 4**).
- In the control samples, HTT concentrations were relatively unchanged (≤30% to unrelated Ab) across all ratios evaluated when compared to unrelated Ab samples.
- When the samples were treated with 1:300 dilution of MW1-Strep-MPs, the HTT values were reduced to endogenous wtHTT levels, showing that the beads specifically depletion mHTT and left the wtHTT unaltered.
- The data was optimized with >90% efficiency of mHTT depletion at HTT concentrations of at least 17.5 fM in phCSF.

Figure 5. wtHTT quantification method performed reliably within the intended context of use



The QC concentrations selected ranged between the LLOQ (20 fM) and ULOQ (1500 fM). In addition, an immunodepletion with 1:1 ratio of rFL wtHTT and rFL mHTT was tested (ID-QC). (A) CV is calculated with respect to theoretical value. As shown in boxplots, CV is less than 25% for majority of the samples. (B) Boxplots of mean bias (mean of triplicate - theoretical value). Bias is negligible for QC samples with smaller theoretical values. Though absolute bias is larger for samples >250 fM, the relative bias remains small (<20%). Smaller panel within (B) shows samples <100. All data shown represents n=32 independent runs. (C) wtHTT protein levels in HD patient CSF samples* were quantified using our wtHTT method. The insert reports sample size, mean, and LLOQ which were 75, 44 fM, and 20 fM respectively.

*placebo cohort and pre-dose treatment cohorts in PRECISION-HD core studies. **mHTT assay performed with a separate vendor.

- Our assay optimized in buffer and non-HD CSF was used with the same conditions to measure wtHTT in CSF from patients with HD.
- Prior to HD sample testing, QC samples and calibrators were evaluated (**Figure 5A, 5B**).
- Standard reference curve performance and QC samples performed in line with expected LLOQ and consistent across assay plates.
- We observed that the mean of wtHTT protein in HD patient CSF is ~50% of the mean of tHTT (**Figure 5C**).

References: 1. Bates G, et al. *Huntington's Disease*, Oxford University Press. 2014; 2. Cattaneo E, et al. *Nat Rev Neurosci*. 2005 6(12):919-930; 3. Leavitt, BR et al. *J Neurochem*. 2006 96(4):1121-1129; 4. Gauthier LR, et al. *Cell*. 2004 118(1):127-138; 5. Kumar A and Ratan RR. *J Huntingtons Dis*. 2016 5(3):217-237; 6. Fodale V, et al. *Sci Rep*. 2020 10(1):22137. **Acknowledgments:** The authors thank David Howland and Doug MacDonald from CHDI for sharing the tHTT assay and their scientific inputs on the assay qualification. For development of this poster, the authors thank Brittany Mayweather (Wave Life Sciences) and Eric Smith for medical writing and graphics support, respectively.