QUANTIFYING MUTANT HUNTINGTIN PROTEIN (MHTT) – TWO INDEPENDENT VALIDATIONS OF AN MHTT ASSAY TO SUPPORT THE CLINICAL DEVELOPMENT OF MHTT-TARGETING THERAPIES IN HUNTINGTON'S DISEASE

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Background

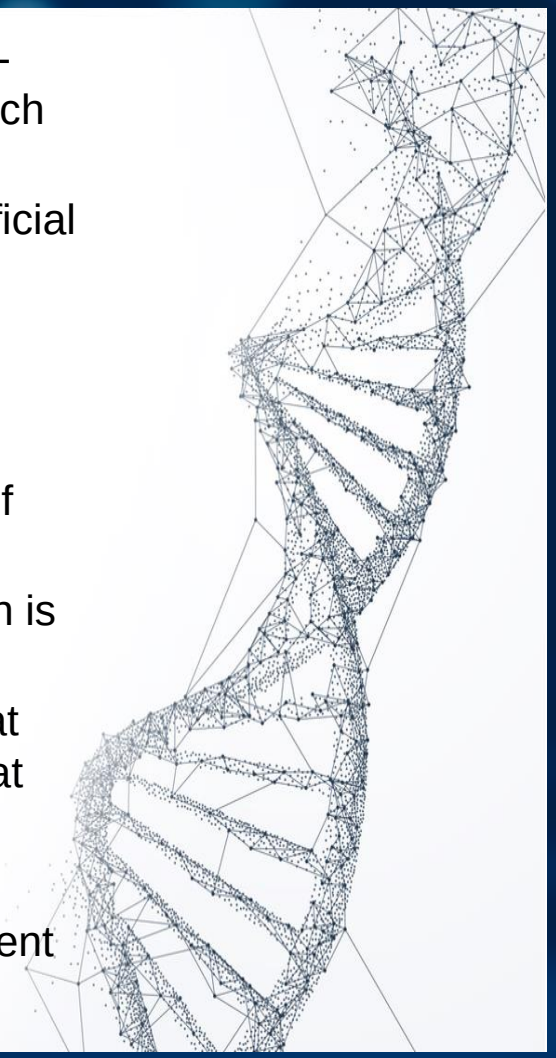
- HD is a rare, genetic, neurodegenerative disease caused by a CAG repeat expansion in the huntingtin gene, resulting in the production of toxic mHTT[1–3]
- Quantification of mHTT in the CSF of patients with HD using a method validated as per international guidelines is critical to support the clinical development of mHTT-targeting therapies
- Here we report the results of two independent validations of the same bioanalytical method for the quantification of relative mHTT levels in human CSF

CAG, cytosine adenine guanine; CSF, cerebrospinal fluid; HD, Huntington's disease; mHTT, mutant huntingtin protein.

1. Bates GP, et al. *Nat Rev Dis Primers*. 2015; 1:15005; 2. Roos RA. *Orphanet J Rare Dis.* 2010; 5:40; 3. Saudou F, et al. *Neuron.* 2016; 89:910–926.

Methods

- All results were generated in regulated bioanalytical environments (i.e. by Good Clinical Practice-trained personnel in Good Laboratory Practice-certified laboratories) using a bead-based sandwich ligand-binding assay with Single Molecule Counting detection on the SMCxPRO™ (Merck)
- The ultra-sensitive assay employs the antibody pair 2B7/MW1 for capture and detection and artificial CSF as a surrogate matrix
  - Capture antibody 2B7 binds to the N17 region of HTT (i.e. binds to mHTT and wtHTT) and conjugates to magnetic particles via biotin coupling
  - Detection antibody MW1 is specific to the polyQ chain present in mHTT
- This biomarker assay is relatively quantitative; the heterogeneous length of polyQ in the mHTT of patients results in variability in assay response
- A 599 amino acid-long recombinant HTT fragment containing a Q46 amino acid-long polyQ chain is used as reference standard (HTT Q46)
  - Frozen storage stability at –70°C of reference standard in artificial CSF was demonstrated for at least 85 days. Bench-top stability of reference standard in artificial CSF was demonstrated for at least 4 hours
- Assay validation followed international guidelines adapted to the context of use
- The assay was optimised in a single laboratory (Roche) before its transfer to a second independent laboratory (PRA Health Sciences)



CSF, cerebrospinal fluid; HTT, huntingtin protein; mHTT, mutant HTT; Q, glutamine; wtHTT, wild-type HTT.

Analysis results of CSF samples from patients with HD are highly reliable across multiple independent runs within a laboratory as well as across independent laboratories

Table 1. Inter-assay precision in the CSF of patients with HD; combined outputs from Roche and PRA Health Sciences

	Found mHTT concentration (pg/mL) in patient CSF									
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
Run ID	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA
Run 1	1.98	3.36	2.60	3.16	3.92	4.26	3.42	5.73	6.83	7.22
Run 2	2.05	2.55	2.55	3.03	3.75	3.83	3.25	3.90	6.28	7.21
Run 3	2.18	3.02	2.82	3.20	4.21	4.18	4.20	4.19	6.40	6.22
n	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Mean conc. (pg/mL)	**2.07**	**2.98**	**2.66**	**3.13**	**3.96**	**4.09**	**3.62**	**4.61**	**6.50**	**6.88**
Precision (%CV)	**4.9**	**13.7**	**5.4**	**2.9**	**5.9**	**5.5**	**14.0**	**21.4**	**4.4**	**8.3**

CSF, cerebrospinal fluid; CV, coefficient of variation; HD, Huntington's disease; mHTT, mutant huntingtin protein.

HTT Q46 calibrators prepared with reference standard spiked in surrogate matrix performed well during the method validations and enabled full recovery of frozen QC samples

- The assay has high sensitivity, with a calibration range between 1.63 pg/mL (LLOQ – Roche)/0.655 pg/mL (anchor point – PRA [validated LLOQ: 1.64 pg/mL]) and 400 pg/mL (ULOQ) HTT Q46 in surrogate matrix

Table 2. Precision and accuracy of calibration standards. Back-calculated mHTT concentrations (pg/mL) for calibration standards in surrogate matrix

	Nominal HTT Q46 concentrations in surrogate matrix (pg/mL)													
	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA
	1.63	1.64	4.08	4.10	10.2	10.2	25.6	25.6	64.0	64.0	160	160	400	400
Mean	1.62	1.67	4.16	4.07	10.3	10.3	24.8	25.2	65.5	67.1	161	156	402	409
Precision (%CV)	1.5	6.5	4.3	4.7	3.0	4.3	4.6	4.5	4.8	6.9	6.0	6.9	4.4	6.8
Relative error/bias (%)	–0.6	1.7	2.0	–0.7	1.0	1.0	–3.1	–1.4	2.3	4.8	0.6	–2.6	0.5	2.3
n	18	24	18	25	18	25	18	25	18	25	18	25	18	25

Acceptance criteria

- Accuracy of the calibration standards (determined after calibration curve fitting) including LLOQ and ULOQ should be within 70–130%
- At least 75% and a minimum of six non-zero calibrator levels within the dynamic range should meet the above criteria in each validation run

CV, coefficient of variation; HTT, huntingtin protein; LLOQ, lower limit of quantification; mHTT, mutant HTT; ULOQ, upper limit of quantification.

Intra- and inter-assay accuracy and precision in artificial CSF matched the extended acceptance criteria

Table 3. Intra- and inter-assay accuracy and precision in spiked surrogate matrix

	LLOQ (pg/mL)		LQC (pg/mL)		MQC (pg/mL)		HQC (pg/mL)		ULOQ (pg/mL)	
	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA	Roche	PRA
Intra-assay										
Intra-assay accuracy (%)	84.7	100.3–119.7	71.8	100.2–115.5	79.0	97.4–120.2	73.3	80.5–115.8	79.3	80.8–120.0
Intra-assay precision (%CV)	14.1	14.1	12.1	5.6	6.2	4.0	4.1	6.2	6.7	12.9
Inter-assay										
Inter-assay accuracy (%)	93.3	109.5	77.6	106.2	84.8	106.9	84.0	99.3	83.8	93.3
Inter-assay precision (%CV)	11.5	14.1	9.5	7.3	5.4	8.5	9.4	12.7	7.8	18.5

Acceptance criteria

- The determined mean concentration at each level including LLOQ and ULOQ should be within 70–130%
- The precision of the mean concentration determined at each level should be ≤30% CV

CSF, cerebrospinal fluid; CV, coefficient of variation; HQC, high-quality control; LLOQ, lower limit of quantification; LQC, low-quality control; MQC, mid-quality control; ULOQ, upper limit of quantification.

Good parallelism was observed during experiments involving CSF of patients with HD, and demonstrated the absence of a matrix effect

Table 4. Roche data: Parallelism (recovery data) for mHTT in CSF of patients with HD. Serial dilution of samples with surrogate matrix

Sample ID and Q-repeat length	Sample pre-dilution (excluding on-plate dilution)	Sample pre-dilution (including on-plate dilution)	Results without dilution factor correction (pg/mL)	Dilution-adjusted result (pg-eq/mL)	Recovery based on undiluted sample (%)
1 (44 Q repeats)	Undiluted	1:1.1	4.26	4.68	100.0
	1:2	1:2.2	2.40	5.29	113.0
	1:3	1:3.3	1.57*,†	5.19	110.9
	1:4	1:4.4	1.56	6.87	146.8
	1:6	1:6.6	0.601	3.96	84.6
	1:8	1:8.8	0.403	3.55	75.9
2 (50 Q repeats)	Undiluted	1:1.1	5.07	5.57	100.0
	1:2	1:2.2	2.53	5.57	100.0
	1:3	1:3.3	1.79	5.90	105.9
	1:4	1:4.4	1.11	4.86	87.3
	1:6	1:6.6	0.762	5.03	90.3
	1:8	1:8.8	0.521*	4.58	82.2
3 (51 Q repeats)	Undiluted	1:1.1	5.20	5.72	100.0
4 (51 Q repeats)	1:2	1:2.2	2.45	5.40	94.4
	1:3	1:3.3	1.55*	5.12	89.5
	1:4	1:4.4	0.856	3.77	65.9
	1:6	1:6.6	0.749	4.94	86.4
	1:8	1:8.8	0.454	3.99	69.8
3 (51 Q repeats)	Undiluted	1:1.1	5.73	6.30	100.0
4 (51 Q repeats)	1:2	1:2.2	2.83	6.23	98.9
	1:3	1:3.3	2.20	7.27	115.4
	1:4	1:4.4	1.84	8.11	128.7

Recovery values out of acceptance criteria are underlined.

***** Lowest concentration for each sample providing a parallel response (i.e. recovery within 70–130%). †$LLOQ_P$=1.57 pg/mL (1.73 pg/mL in 100% CSF taken into account sample on-plate dilution factor of 1.1).
CSF, cerebrospinal fluid; HD, Huntington's disease; $LLOQ_p$, lower limit of quantification determined on parallelism; mHTT, mutant huntingtin protein.

Good parallelism was observed during experiments involving CSF of patients with HD as well as artificial CSF matrices, and demonstrated the absence of a matrix effect

Table 4 (continued). Roche data: Parallelism (recovery data) for mHTT in CSF of patients with HD. Serial dilution of samples with surrogate matrix

Sample ID (Q repeat length)	Sample pre-dilution (excluding on-plate dilution)	Sample pre-dilution (including on-plate dilution)	Results without dilution factor correction (pg/mL)	Dilution-adjusted result (pg-eq/mL)	Recovery based on undiluted sample (%)
4 (51 Q repeats)	1:6	1:6.6	0.905	5.97	94.8
	1:8	1:8.8	0.633*	5.57	88.4
5 (48 Q repeats)	Undiluted	1:1.1	8.58	9.44	100.0
	1:2	1:2.2	4.21	9.26	98.1
	1:3	1:3.3	2.57	8.48	89.8
	1:4	1:4.4	2.14	9.42	99.8
	1:6	1:6.6	1.20	7.91	83.8
	1:8	1:8.8	1.05*	9.26	98.1
6 (48 Q repeats)	Undiluted	1:1.1	5.95	6.54	100.0
	1:2	1:2.2	2.73	6.01	91.9
	1:3	1:3.3	NV	NA	NA
	1:4	1:4.4	1.41	6.20	94.8
	1:6	1:6.6	0.915	6.04	92.4
	1:8	1:8.8	0.574*	5.05	77.2

Recovery values out of acceptance criteria are underlined.

***** Lowest concentration for each sample providing a parallel response (i.e. recovery within 70–130%).
CSF, cerebrospinal fluid; CV, coefficient of variation; HD, Huntington's disease; mHTT, mutant huntingtin protein; NA, not available; NV, duplicate well CV>20%.

Good parallelism was observed during experiments involving CSF of patients with HD as well as artificial CSF matrices, and demonstrated the absence of a matrix effect (continued)

Table 5. PRA data: Parallelism for mHTT in CSF of patients with HD. Serial dilution of clinical study samples with surrogate matrix

Sample ID	Dilution factor	Back-calculated concentration (pg/mL)	Undiluted concentration (pg/mL)	Bias (%)
1 (Run 1)	1	4.86	4.86	0.0
	2	1.97	3.95	–18.9
	3	1.53	4.59	–5.7
	4	1.10	4.39	–9.7
	6	0.646	3.88	–20.3
	8	0.803	6.43	32.1
1 (Run 2)	1	3.50	3.50	0.0
	2	1.60	3.20	–8.3
	3	1.01	3.04	–13.1
	4	0.800	3.20	–8.5
	6	1.168	7.01	100.5
	8	0.410	3.28	–6.2
2	1	5.50	5.50	0.0
	2	2.63	5.26	–4.3
	3	1.64	4.91	–10.7
	4	1.69	6.78	23.2
	6	0.718	4.31	NV
	8	0.167	1.33	–75.7

Recovery values out of acceptance criteria are underlined.

CSF, cerebrospinal fluid; CV, coefficient of variation; HD, Huntington's disease; mHTT, mutant huntingtin protein; NV, duplicate well CV>20%.

Good parallelism was observed during experiments involving CSF of patients with HD as well as artificial CSF matrices, and demonstrated the absence of a matrix effect (continued)

Table 5 (continued). PRA data: Parallelism for mHTT in CSF of patients with HD. Serial dilution of clinical study samples with surrogate matrix

Sample ID	Dilution factor	Back-calculated concentration (pg/mL)	Undiluted concentration (pg/mL)	Bias (%)
3	1	4.25	4.25	0.0
	2	1.91	3.81	–10.2
	3	1.46	4.39	3.3
	4	1.39	5.57	31.3
	6	0.681	4.08	–3.8
	8	0.408	3.26	–23.1
4	1	6.96	6.96	0.0
	2	3.45	6.89	–1.0
	3	1.60	4.79	–31.1
	4	1.72	6.89	–0.9
	6	1.02	6.11	–12.1
	8	0.625	5.00	–28.1
5 (Run 1)	1	4.24	4.24	NV
	2	2.69	5.38	0.0
	3	1.77	5.32	–1.2
	4	1.47	5.89	9.4
	6	0.699	4.20	–22.1
	8	0.384	3.07	NV

Recovery values out of acceptance criteria are underlined.

CSF, cerebrospinal fluid; CV, coefficient of variation; HD, Huntington's disease; mHTT, mutant huntingtin protein; NV, duplicate well CV>20%.

Good parallelism was observed during experiments involving CSF of patients with HD as well as artificial CSF matrices, and demonstrated the absence of a matrix effect (continued)

Table 5 (continued). PRA data: Parallelism for mHTT in CSF of patients with HD. Serial dilution of clinical study samples with surrogate matrix

Sample ID	Dilution factor	Back-calculated concentration (pg/mL)	Undiluted concentration (pg/mL)	Bias (%)
5 (run 2)	1	6.96	6.96	NV
	2	2.29	4.59	0.0
	3	1.36	4.08	–11.2
	4	0.983	3.93	–14.4
	6	0.579	3.47	–24.3
	8	0.299	2.39	–47.9

Recovery values out of acceptance criteria are underlined.

CSF, cerebrospinal fluid; CV, coefficient of variation; HD, Huntington's disease; mHTT, mutant huntingtin protein; NV, duplicate well CV>20%.

Appropriate microplate homogeneity was demonstrated at the LLOQ level

Table 6. Microplate homogeneity of mHTT in artificial CSF. Accuracy of LLOQ samples analysed over the plate

	mHTT concentration found (pg/mL)		Accuracy (%)	
Sample ID	Roche	PRA	Roche	PRA
1	1.54*	1.63	94.5	99.6
2	1.74	1.38	106.7	84.1
3	1.68	1.19	103.1	72.5
4	1.73	1.55	106.1	94.3
5	1.84	1.37	112.9	83.7
6	1.70	1.79	104.3	109.2
7	1.61*	1.69	98.8	103.0
8	1.35*	1.57	82.8	95.5
9	1.42*	1.77	87.1	107.8
10	1.93	1.68	118.4	102.5
11	1.72	1.53	105.5	93.1
12	1.58*	1.77	96.9	107.8
13	1.82	1.47	111.7	89.9
14	1.78	1.53	109.2	93.4
15	1.56*	1.55	95.7	94.7
16	1.99	1.42	122.1	86.3
17	1.50*	1.79	92.0	109.2
18	1.85	NA	113.5	NA

Acceptance criteria

- ≥80% of the LLOQ samples should show accuracies within 70–130%

* RO7234292 concentration extrapolated.
CSF, cerebrospinal fluid; LLOQ, lower limit of quantification; mHTT, mutant huntingtin protein; NA, not available.

Data showed no relevant interferences of wtHTT in the assay

Table 7. Interference of wtHTT on the determination of mHTT in CSF

		Accuracy (%)	
Spiked HTT Q46 concentration (pg/mL)	Spiked HTT-Q23 1-3144 concentration (pg/mL)	Roche	PRA
Unspiked	200	BLQ	BLQ
		BLQ	BLQ
		BLQ	BLQ
	20.0	BLQ	BLQ
		BLQ	BLQ
		BLQ	BLQ
	0.00	BLQ	BLQ
		BLQ	BLQ
		NA	BLQ
1.63 (Roche) 1.64 (PRA)	200	129.4	105.9
		118.4	<u>136.3</u>
		<u>141.1</u>	121.2
	20.0	124.5	90.1
		<u>133.7</u>	115.6
		118.4	97.2
	0.00	100.6	98.9
		106.7	104.5
		117.8	103.2
400	200	100.3	95.5
		92.3	<u>54.4</u>
		91.3	81.4
	20.0	95.0	78
		88.8	91.3
		95.0	84.4
	0.00	98.5	108.9
		98.0	81.9
		99.8	108.5

Acceptance criteria

- At least 66.7% of the blank matrix aliquots (without reference standard) should show mean assay signals below LLOQ
- At least 66.7% of the spiked matrix samples should show accuracies within 70–130%

Underlined values are out of acceptance criteria.
BLQ, below limit of quantification; CSF, cerebrospinal fluid; HTT, huntingtin protein; LLOQ, lower limit of quantification; mHTT, mutant HTT; NA, not available; wtHTT, wild-type HTT.

Interference of blood differed between the two independent validations, suggesting blood contamination may require close attention when using the assay

Table 8. Interferences of blood. Accuracy of specificity samples in surrogate matrix spiked with haemolysed full blood

		Accuracy (%)	
Spiked HTT Q46 concentration (pg/mL)	**Spiked full blood (v/v) (%)**	**Roche**	**PRA**
0	1	BLQ	BLQ
		BLQ	BLQ
		BLQ	BLQ
	0.1	BLQ	BLQ
		BLQ	BLQ
		BLQ	BLQ
	0	BLQ	BLQ
		BLQ	BLQ
		BLQ	BLQ
1.63 (Roche) **1.64 (PRA)**	1	<u>138.0</u>	482.3
		116.6	636.4
		126.4	482.1
	0.1	106.1	115.4
		120.2	116.6
		110.4	108.7
	0	95.7	142.6
		90.2	97.7
		128.2	116.3

Underlined values are out of acceptance criteria.

Acceptance criteria

- At least 66.7% of the blank matrix aliquots (without reference standard) should show mean assay signals below LLOQ
- At least 66.7% of the spiked matrix samples should show accuracies within 70–130%

BLQ, below limit of quantification; HTT, huntingtin protein; LLOQ, lower limit of quantification.

Interference of blood differed between the two independent validations, suggesting blood contamination may require close attention when using the assay (continued)

Table 8 (continued). Interferences of blood. Accuracy of specificity samples in surrogate matrix spiked with haemolysed full blood

		Accuracy (%)	
Spiked HTT Q46 concentration (pg/mL)	**Spiked full blood (v/v) (%)**	**Roche**	**PRA**
400	1	<u>147.5</u>	98.9
		100.3	94.9
		121.3	95.1
	0.1	105.0	95.4
		110.8	116.7
		94.3	121.9
	0	94.0	103.8
		99.0	70.7
		123.3	91.3

Underlined values are out of acceptance criteria.

HTT, huntingtin protein.

Conclusions

- The independent assay validations demonstrate that this ultra-sensitive assay can be replicated and transferred, making it a reliable and broadly relevant tool for generating biomarker data in registrational clinical trials for mHTT-lowering therapies

We would like to thank the patients and families who have participated and who are currently still participating in our research, and the ongoing partnership of the whole HD community

mHTT, mutant huntingtin protein.