Simple and sensitive HTRF mutant and total HTT immunoassays to speed up the discovery of new drugs against huntington's disease.

This application note demonstrates how the HTRF® soluble Mutant and Total HTT immunoassays are suitable for a sensitive and reliable measurement of HTT proteins in cell and brain tissue lysates.

Introduction

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by cognitive, motor, and psychiatric disturbance. At the molecular level, HD is caused by an autosomal dominant mutation in the huntingtin (HTT) gene. This mutation corresponds to an abnormal expansion of a trinucleotide repeat (CAG) that is translated into a toxic polyglutamine (polyQ) tract in the HTT protein (Figure 1).

The non-mutated HTT gene encodes the wild-type (WT) protein carrying a "normal" polyQ domain < 36Q. The WT HTT protein has an anti-apoptotic role crucial for neuronal survival as it is involved in selective autophagy, trafficking of organelles and vesicles, and regulation of transcription factors required for neuronal gene transcription. The mutated HTT gene produces a mutant HTT protein harboring an abnormally long polyQ tract (≥ 36Q) which causes its progressive aggregation and loss of function. At the cellular level, mutant HTT results in selective neuronal dysfunction and death through several mechanisms, including disruption of proteostasis, transcription, and mitochondrial function.

Although some treatments can reduce symptoms and improve the quality of life of people affected by HD, there is currently no therapy to effectively treat this devastating disease. It is therefore essential for scientists to have reliable tools to study the effect of new drugs on HTT in cellular/animal models. With this aim, Revvity has developed simple, robust, and highly validated HTRF immunoassays to detect the soluble forms of mutant and total HTT in cell/tissue lysates. This application note describes the possible applications, assay formats, and protocols. It also presents the different levels of validation done on recombinant proteins, cellular samples, and brain tissue samples.

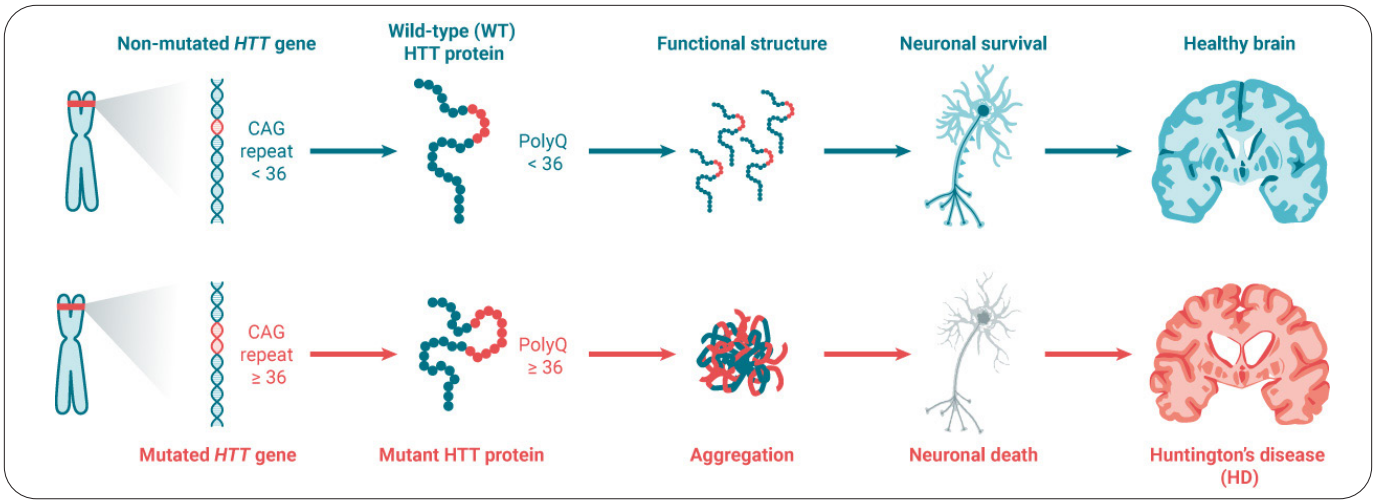

Figure 1: Molecular pathogenesis of Huntington's disease

Applications and assay formats

The assays presented here are intended for the simple and rapid detection of the soluble forms of mutant HTT and total HTT in cell/tissue lysates. Both assays are compatible with human and mouse species and can be used with cellular or animal models. After cell/tissue lysis, soluble mutant or total HTT can be detected using the corresponding kit reagents (Figure 2).

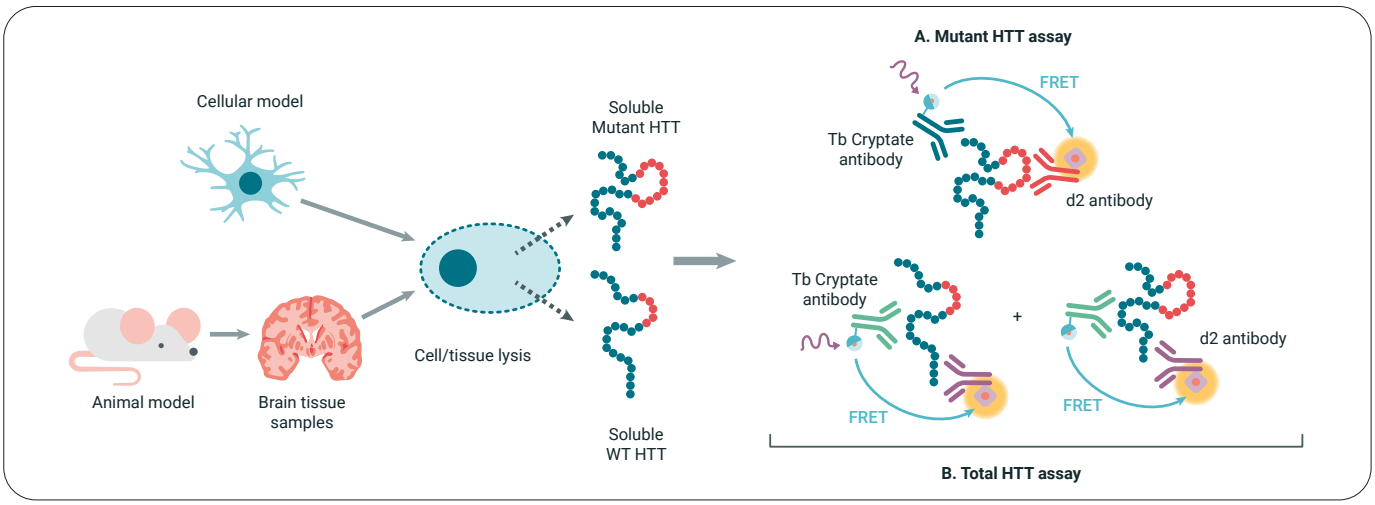

Figure 2: Applications and formats of HTRF Mutant and Total HTT assays (A. Mutant HTT assay format; B. Total HTT assay format)

The Mutant HTT assay recognizes soluble mutant HTT proteins with different polyQ repeat lengths. As illustrated in Figure 2A, mutant HTT is detected in a sandwich assay format using two specific monoclonal antibodies. One is labeled with Tb Cryptate (donor) and directed against the N-terminal part of the protein and the second is labeled with d2 (acceptor) and recognizes the mutant polyQ domain. Using a similar sandwich assay format, the Total HTT assay detects all soluble forms of WT and mutant HTT proteins by using two different monoclonal antibodies that recognize HTT independently of the size of its polyQ domain (Figure 2B).

The detection principle is based on HTRF (Homogeneous Time-Resolved Fluorescence) technology. When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The two antibodies bind to the mutant or total HTT protein present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the mutant or total HTT protein concentration.

Preliminary recommendations

Kit reagents and samples must be prepared by following the manufacturer's instructions.

Cell/tissue lysates must be generated using the kit lysis buffer (same lysis buffer in both kits).

For brain tissue sample preparation, the kit lysis buffer must be supplemented with protease inhibitors (to be purchased separately). It is recommended that a 10% or 5% (w/v) tissue homogenate is prepared using ice-cold 1X supplemented lysis buffer. After tissue lysis, insoluble fractions containing putative mutant HTT aggregates must be removed by centrifugation, and the supernatants containing soluble HTT must be collected to be directly analyzed or dispensed into aliquots for storage at -80°C. Frozen supernatants must be rapidly assayed within a few days as HTT tends to aggregate in lysates over time. For more details, please refer to the Technical Note "Best Practices For Analyzing Brain Samples With HTRF® Phospho Assays For Neuroscience".

To measure soluble HTT (and not the aggregated form), the preclinical animal models used with these assays must correspond to models of premanifest HD (before HTT aggregation has been established).

No standard is provided in the kits. Recombinant WT and mutant HTT proteins can be purchased separately (i.e., from the Coriell Institute) for absolute quantification in cell/tissue lysates.

Depending on the type of samples used, it may be necessary to dilute the lysates in the kit lysis buffer to ensure samples are within the assay linear range.

Assay protocol and data analysis

The streamlined protocol consists of transferring 10 µL of sample (cell/tissue lysate or recombinant protein used as standard) into a low-volume white detection microplate and adding 10 µL of pre-mixed detection antibodies prepared in the kit detection buffer (Figure 3). After overnight incubation at room temperature, the HTRF signal is recorded (reading at 665 and 620 nm) on an HTRF-compatible reader.

The HTRF Ratio is first calculated using the following formula: HTRF Ratio = signal 665 nm/signal 620 nm (x 10^4). The specific HTRF signal (« Delta Ratio ») is then determined: HTRF Delta Ratio = HTRF Ratio $_{\text{sample}}$ – HTRF Ratio $_{\text{negative control}}$ (negative control = sample replaced by lysis buffer).

The data presented hereafter were analyzed using the GraphPad Prism® software. The error bars represented on points and histobars correspond to the standard deviations (SD) of the means of three independent wells of sample.

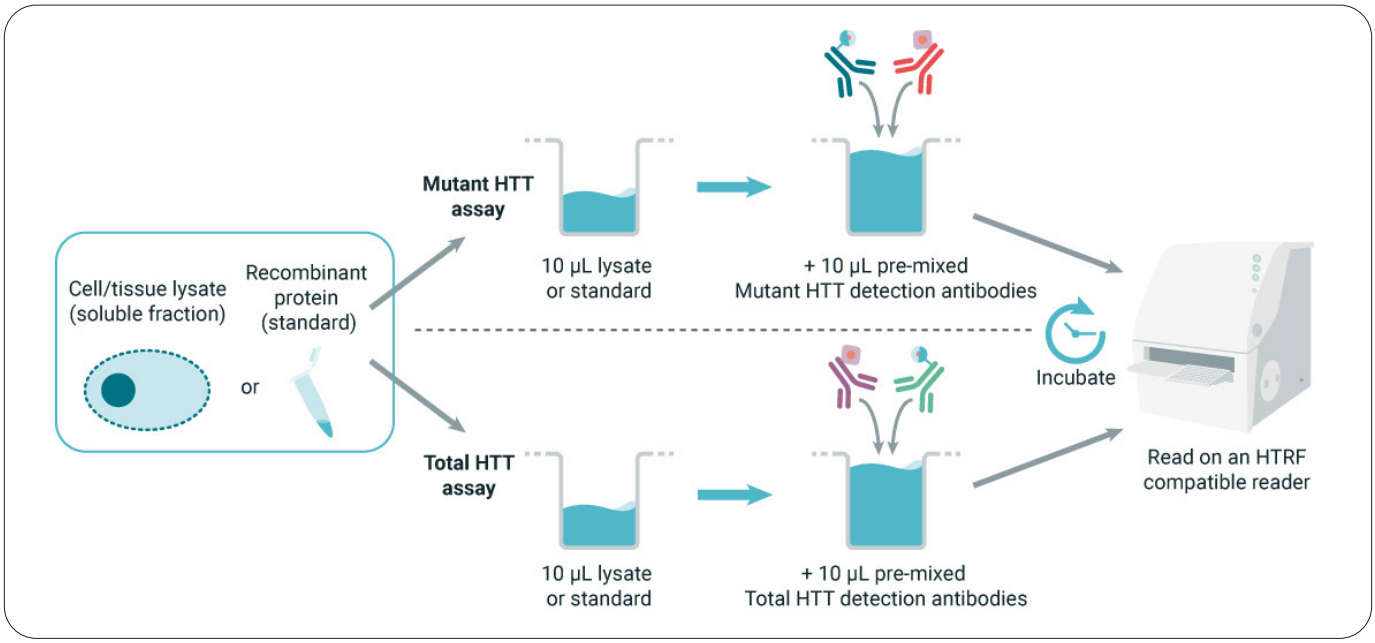

Figure 3: Assay protocols of HTRF Mutant and Total HTT assays

Assay validations

HTRF Mutant and Total HTT assays have been validated on recombinant proteins, neuroblastoma cells, and brain tissue samples. Table 1 lists all the materials and reagents used for the validations.

Table 1: Material and reagents used for the validation of HTRF Mutant and Total HTT assays

	Material / Reagent	Supplier	Part Number
HTRF kits	HTRF Human/Mouse Mutant Huntingtin (HTT) detection kit	Revvity	64HTTMPEG/H
	HTRF Human/Mouse Total Huntingtin (HTT) detection kit	Revvity	64HTTTPEG/H
	HTRF GAPDH Housekeeping detection kit	Revvity	64GAPDHPEG/H
Detection microplates	ProxiPlate-384 Plus, White 384-shallow well	Revvity	6008280/9
Recombinant proteins	WT mouse HTT-Q7	Coriell Institute	CH02324
	Mutant human HTT-Q48	Coriell Institute	CH02580
	Mutant human HTT-Q73	Coriell Institute	CH02581
Protease inhibitors	Halt™ Protease Inhibitor Cocktail, EDTA-free (100X)	ThermoFisher Scientific	78425
Tissue dissociator	GentleMACS™ Dissociator	Miltenyi Biotec	130-093-235
Cellular model	Neuro-2a	ATCC	CCL-131
Preclinical mouse model	Premanifest zQ175 knock-in mice (and WT mice as controls)		

Validation on recombinant proteins

Recombinant WT and mutant HTT proteins (WT mouse HTT-Q7, mutant human HTT-Q48 and mutant human HTT-Q73) were serially diluted in 1X kit lysis buffer and measured side- by-side using the Mutant and Total HTT detection reagents. The HTRF specific signal (Delta Ratio) was plotted as a function of the HTT concentrations tested (Figure 4).

The data presented in Figure 4A demonstrate that the HTRF Mutant HTT assay recognizes mutant proteins containing different polyQ repeat lengths (48Q and 73Q tested here), while it does not detect the WT protein. Conversely, the HTRF Total HTT assay can measure WT and mutant HTT proteins independently of the size of the polyQ domain (Figure 4B).

Both assays have good and comparable sensitivity, with a limit of detection (LoD) ranging from 1.4 to 2.1 ng/mL for the Mutant HTT assay and from 1.6 to 1.9 ng/mL for the Total HTT assay.

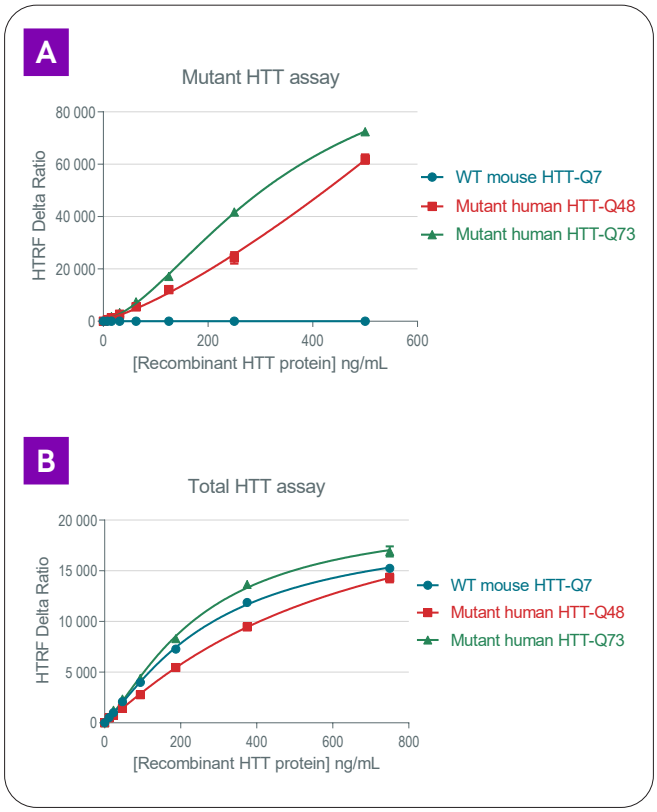

Figure 4: Validation of HTRF Mutant and Total HTT assays on recombinant proteins (A. Mutant HTT assay; B. Total HTT assay)

Validation on a cellular model

The mouse neuroblastoma cell line Neuro-2a was used to validate the assays. This cellular model contains the mouse WT HTT protein but no mutant forms.

Neuro-2a cells were cultured in a T175 flask and lysed with 3 mL of 1X kit lysis buffer. After centrifugation of the cell lysate, the supernatant was serially diluted in the same lysis buffer and measured side-by-side using the Mutant and Total HTT detection reagents.

As shown in Figure 5, the HTRF Total HTT assay easily detects the soluble mouse WT HTT protein in a cellular model of neuroblastoma. No signal was detected using the HTRF Mutant HTT kit, confirming that the assay does not cross-react with the soluble mouse WT protein.

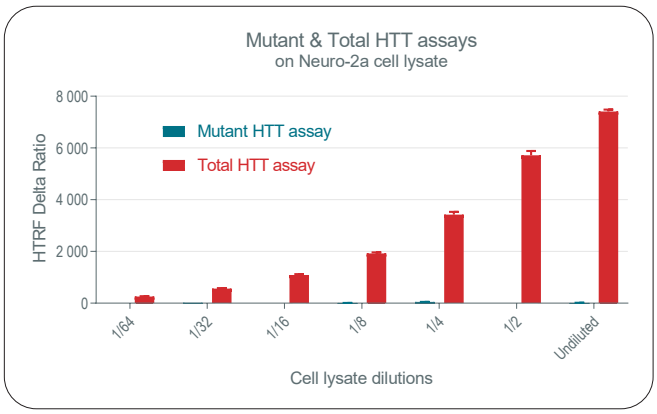

Figure 5: Validation of HTRF Mutant and Total HTT assays on mouse neuroblastoma cells Neuro-2a

Validation on a preclinical animal model

The assays were finally validated on brain tissue samples collected from premanifest zQ175 knock-in mice and WT littermates, the most extensively studied preclinical mouse model used for HD (Landles C. et al, Brain Commun. 2021; 3(1):fcaa231). In this transgenic model, exon 1 of mouse HTT is replaced with a mutated version of exon 1 of human HTT which carries a highly expanded CAG repeat (190 CAGs) and produces a highly pathogenic HTT protein.

The cortex and cerebellum tissues of three WT mice (#1, #2, and #3) and three zQ175 mice (#4, #5, and #6) were lysed using the GentleMACS™ Dissociator following the procedure described before. After centrifugation, the soluble fractions were analyzed using the HTRF Mutant and Total HTT assays. To ensure that the protein extraction was similar for each sample, the level of the protein GAPDH was also measured using the HTRF GAPDH Housekeeping assay (Figures 6A and B). To ensure that the detected analyte was assessed at a concentration compatible with the assay's linear range, the lysates were pre-diluted in 1X kit lysis buffer supplemented with protease inhibitors just before detection (1/8 for Mutant HTT assay, 1/4 for Total HTT assay, and 1/100 for GAPDH assay).

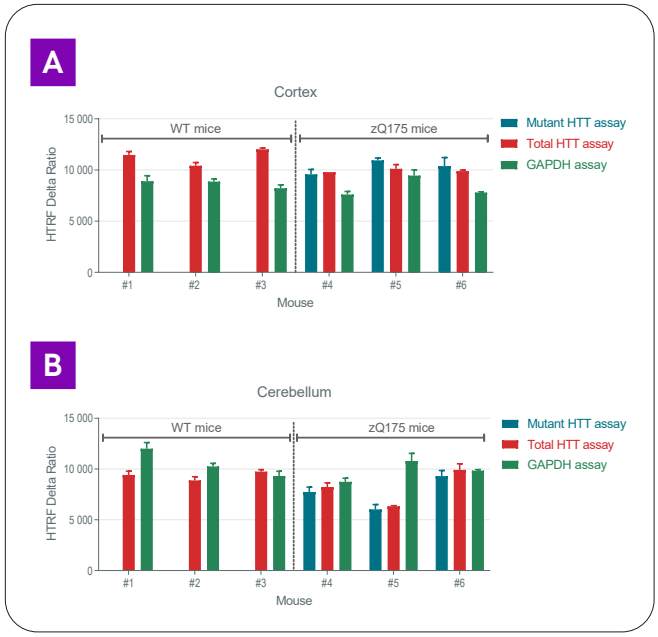

Figure 6: Validation of HTRF Mutant and Total HTT assays on brain tissues from WT and premanifest zQ175 knock-in mice (A. Validation on Cortex samples; B. Validation on cerebellum samples)

The levels of GAPDH were similar in all samples (green bars), demonstrating that the tissue lysates contained comparable total protein contents. As expected, no signal was obtained with the Mutant HTT assay on samples collected from WT mice, whereas the soluble mutant protein was clearly detected in cortex and cerebellum tissues collected from zQ175 mice (blue bars). Finally, the soluble total HTT protein (WT and mutant) was measured in all lysates (red bars), and its levels correlated well with the small variations observed for the mutant HTT protein levels.

Side-by-side comparison of HTRF and Western blot

A side-by-side comparison of HTRF Mutant and Total HTT assays and the Western blot technique was performed on several brain tissues (cortex, hippocampus, and cerebellum) collected from a WT mouse and a premanifest zQ175 mouse. Tissue lysates were prepared as previously described and the supernatants containing soluble HTT

proteins were analyzed, either by HTRF or by Western blot (Figure 7). To ensure that the detected analyte was assessed at a concentration compatible with the assay's linear range for each detection method, the lysates were pre-diluted in 1X kit lysis buffer supplemented with protease inhibitors just before detection (for HTRF: 1/8 for Mutant HTT assay and 1/4 for Total HTT assay; for Western Blot: 1/20 for both assays).

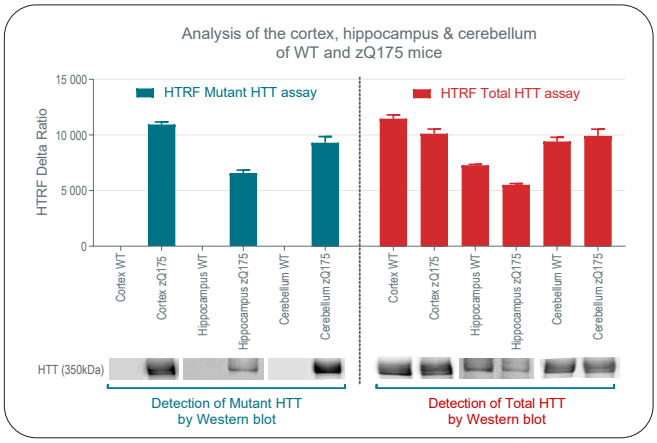

Figure 7: Side-by-side comparison of HTRF and Western blot for the analysis of soluble mutant and total HTT in brain tissues from WT and zQ175 mice

The results obtained using HTRF Mutant and Total HTT assays are correlated with those obtained by Western blotting. With both methods, no soluble mutant HTT was detected in brain tissues of WT mice, whereas the mutant protein was properly measured in all samples collected from premanifest zQ175 mice. As expected, the soluble total HTT protein (WT and mutant) was measured in all lysates and its levels correlated well with the differences observed on the mutant HTT levels for the three different brain tissues.

Conclusion

With the aim of accelerating drug discovery and finding effective treatments for patients suffering from HD, Revvity has developed simple, robust, and highly validated HTRF immunoassays to measure the soluble forms of mutant and total HTT in cellular and animal models.

As described in this application note, these kits require only 10 µL of sample and are easy-to-use (no-wash, add-and-read protocol). The different validations presented here demonstrate that both assays are sensitive, specific for the protein(s) to be detected, versatile (work on human/mouse recombinant proteins, cells, and tissues) and correlate well to the Western blot technique

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