Highly Selective Monoclonal Antibodies Targeting the HTT Exon1 Neo-Epitope

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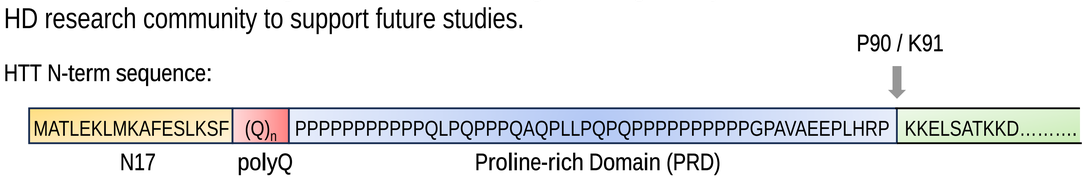
Introduction

More than two decades of research have been invested in the study of the *N*-term HTT Exon1 protein fragment and its association with HD pathology. Early studies focused on identifying the protease responsible for production of Exon1 by proteolytic cleavage of HTT. Later it was shown that Exon1 can be produced directly from *HTT1a* RNA through an alternative splicing mechanism. Conclusions were based in part on immunoassays using HTT *N*-term antibodies that had not been produced specifically for the detection of the Exon1 C-term neoepitope.

To address this gap and support future studies of Exon1 by ICC/IHC and high specificity immunoassays, we have produced novel monoclonal antibodies directed to the Exon1 P90 *C*-term neo-epitope (Q23 numbering) as well as the putative K91 *N*-term neo-epitope. We present the strategy for production of these antibodies through rabbit immunization and B-cell FACS sorting followed by a clone selection process based on ELISA, SPR, and WB. The resulting recombinant mAbs have been fully validated using recombinant proteins produced specifically for this project, as well as cell lysates from transient expression of various *N*-term HTT fragments. CHDI is making these rigorously validated novel mAbs available to the HD research community to support future studies.

HTT N-term sequence:



Rabbit mAb Production

For this project, we engaged a commercial antibody supplier – ABclonal (Woburn, MA) – that offers a de novo rabbit monoclonal antibody production platform using B-cell FACS sorting to identify individual antigen-reactive B-cells that are sequenced, cloned, and transiently expressed in mammalian cells to produce recombinant mAbs:

Antigen design, Immunization, ELISA selection

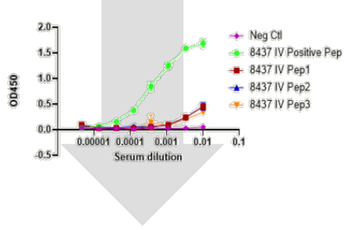
To identify neo-epitope specific mAb clones, we employed a peptide antigen strategy with epitopes selected for human-mouse sequence homology. Antigen were conjugated to KLH (Keyhole Limpet Hemocyanin) carrier protein for immunization:

P90 *C*-term neo-epitope immunogen:
KLH conjugate – C-AEEPLHRP(OH)

ELISA screen:

Positive Screening Peptide	biotin-C-AEEPLHRP-OH
Counterscreening peptide 1	biotin-C-AEEPLHRPKKELSAT-amide
Counterscreening peptide 2	biotin-C-AEEPLHRPK-amide
Counterscreening peptide 3	biotin-C-AVAEEPLHR-amide

Rabbit 8437 final bleed

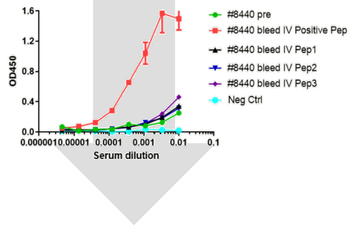


K91 *N*-term neo-epitope mmunogen:
NH2-KKELSATK-C – KLH conjugate

ELISA screen:

Positive Screening Peptide	NH2-KKELSATK-PEG2-K(biotin)-amide
Counterscreen peptide 1	acetyl-AEEPLHRPKKELSAT-PEG2-K(biotin)-amide
Counterscreen peptide 2	acetyl-RPKKELSATK-PEG2-K(biotin)-amide
Counterscreen peptide 3	acetyl-KELSATKKEL-PEG2-K(biotin)-amide

Rabbit 8440 final bleed



Antigen-positive B-Cells were collected by FACS sorting (up to 96 clones per rabbit spleen) and cultured to produce mAbs on small scale. Unpurified cell culture supernatant was screened/counter-screened by ELISA as described above. A subset of clones were then selected to be sequenced and converted to recombinant mAbs on ~100 µg scale, which afforded sufficient mAb to conduct SPR and WB studies to support the next stage in the selection process.

mAbs and Proteins for Selection and Validation

mAbs used as comparators - epitope map:

N17-Qn------PQLPQPPPQAQPLLPQPQP-------GPAV**AEEPLHRP$_{90}$K$_{91}$KELSATK**KD-----Q$_{115}$SVRNSPEFQKLLGIAME$_{129}$-----

mAb 4C9 ***MW8*** ***MAB5490***

Recombinant protein standards:

P90 *C*-term neo-epitope standard HTT Q23 Exon1 (tag free)[a]:

A$_2$TLEKLMKAFESLKSF(Q)$_{23}$PPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAEEPLHRP$_{90}$

K91 *N*-term neo-epitope standard HTT 91-171 *C*-term MBP tag:

K$_{91}$KELSATKKDRVNHCLTICENIVAQSVRNSPEFQKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMD SNLPRLQLE$_{171}$- MBP

Full length HTT standard (FL Q23 HTT *C*-term TEV-FLAG)[b]:

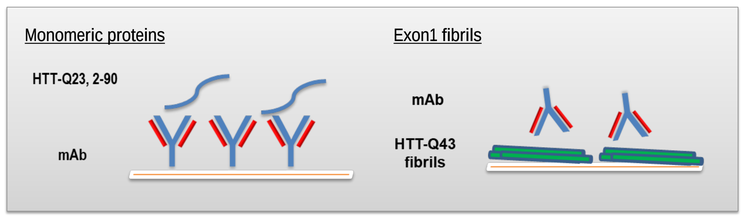
ATLEKLMKAFESLKSF(Q)$_{23}$PPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAEEPLHRPKK ELSATKKDRVNHCLTICENIVAQSVRNSPEFQKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMDSNL PRLQLE----------HKVTTC$_{3144}$- TEV-FLAG

a. Produced by Curia according to the method of Reif, et al *J. Vis. Exp*. (136), e57506 (2018).
b. Pace, et al *J. Vis. Exp*. (178), e63190 (2021).

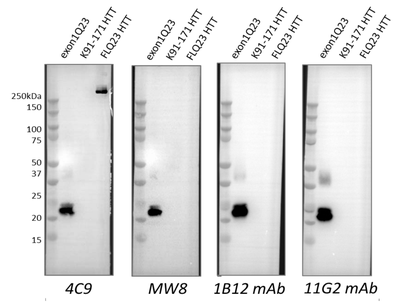
mAb Characterization and Validation

SPR, Western Blot, Dot Blot

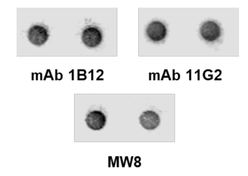
Binding affinity (K_D) was evaluated by Surface Plasmon Resonance (SPR, Biacore T200). For monomeric Exon1, HTT 1-171, and FL HTT analytes the antibody was captured on sensorchip. For Exon1 fibrils, the orientation was reversed:

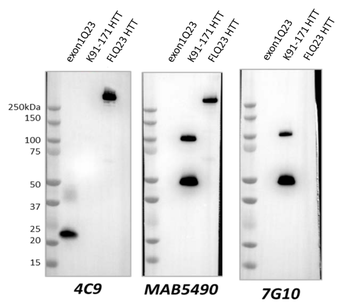


mAb	K_D monomer	K_D fibril	K_D FL HTT
11G2	27 nM	0.8 nM	No binding
1B12	26 nM	0.4 nM	No binding
MW8	700 nM	14.2 nM	No binding
7G10	3 nM	n.t.	No binding



Dot Blot HTT Q43 Exon1 fibrils:



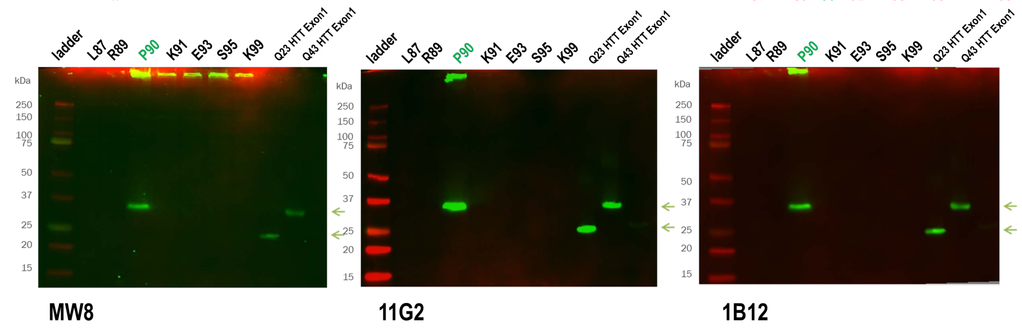


HTT comparator antibodies: mAb 4C9 (human PRD); MW8 (HTT Exon1 aggregate); MAB5490 (HTTaa 115-129).
Recombinant proteins: HTT Q23 Exon1 (monomeric); HTT Q43 Exon1 (fibrils); HTT 91-171 *C*-term MBP; FL Q23 HTT.

HTT N-term fragment over-expression studies

To evaluate HTT *N*-term fragment length specificity, comparison fragments with *C*-term at L87, R89, P90, K91, E93, S95, and K99 were expressed in mammalian cells (HEK). Cell lysates were characterized by WB using MW8 in comparison to 11G2 and 1B12. [Lanes labeled according to the *C*-term aa. Recombinant, purified, HTT Exon1 (Q23 and Q43) included as positive controls.]

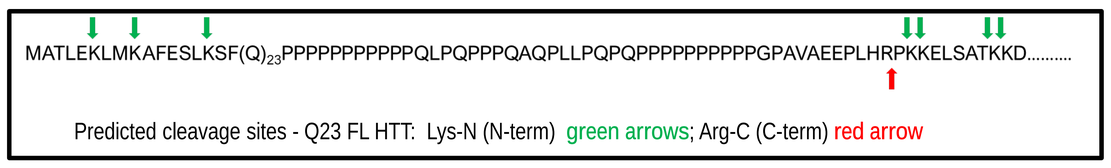
N17 - (Q)$_n$ - PPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAEEPL$_{87}$HR$_{89}$P$_{90}$K$_{91}$KE$_{93}$LS$_{95}$ATKK$_{99}$

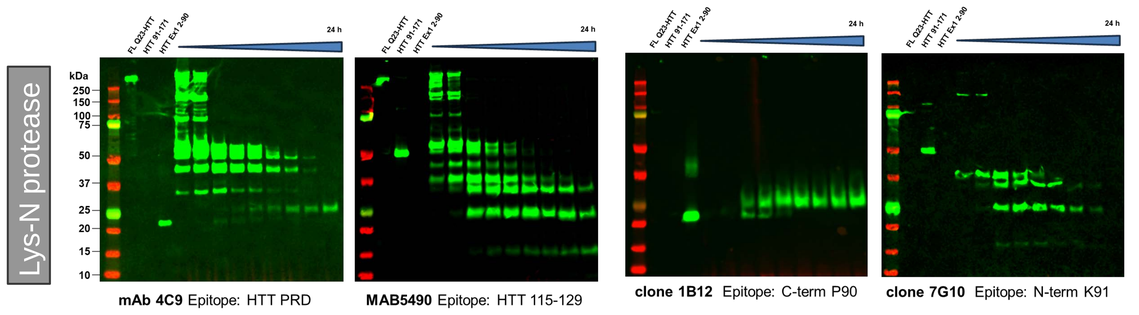


- MW8 reacts with *C*-term P90 fragment, both as the "monomer" and as the aggregate trapped in the stacking well. MW8 also reacts with longer fragment aggregates in the stacking well (*C*-term K91, E93, S95, K99).
- 11G2 and 1B12 are reactive only with the monomeric and aggregated *C*-term P90 fragment.

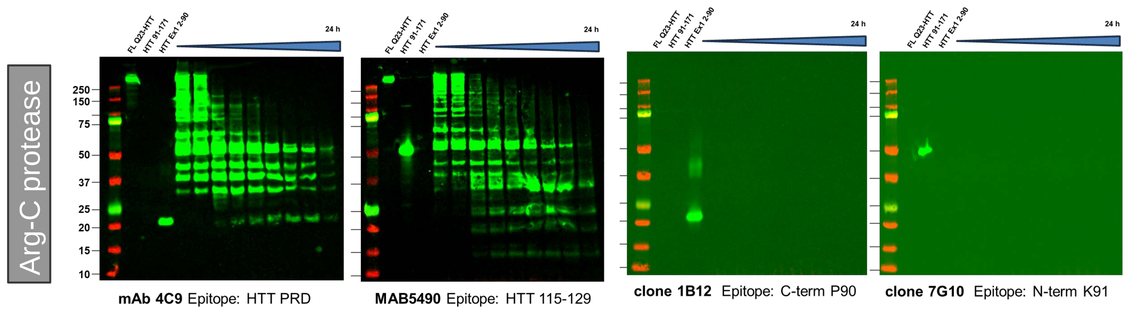
Proteolysis time course WB studies

To further interrogate neo-epitope specificity, we conducted proteolysis studies on recombinant full length HTT protein using commercial proteases: Lys-N – predicted to cleave at the *N*-term of K91 (P90/K91); and Arg-C – predicted to cleave at the *C*-term of R89 (R89/P90).





Time course shows expected fragmentation pattern for Lys-N proteolysis – *P90 and K91 mAb reactive fragments as predicted*



Time course shows expected fragmentation pattern for Arg-C proteolysis – *no P90 or K91 mAb reactive fragments*

Conclusions

mAbs are available through the Coriell HD Community Biorepository (HDCB - catalog.coriell.org) –
clone 1B12 = CHDI-90004290; clone 11G2 = CHDI-90004291; clone 7G10 = CHDI-90004394.

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