



A Field Guide to Working with Mouse Models of Huntington's Disease



Prepared jointly by



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Preface

The objective of this field guide is to give HD researchers a broad understanding of the different HD mouse models that exist today and to provide guidance to optimize their use in preclinical research and development. An important part of CHDI Foundation's mission is to act as a 'collaborative enabler' in Huntington's disease (HD) research. Central to that is the establishment and maintenance of standardized HD model mice. CHDI has a longstanding commitment to the HD mouse model collection at The Jackson Laboratory, a centralized resource where mice bred and reared under rigorous quality-controlled conditions can be monitored by consistent practices to ensure genetic integrity and the highest quality for HD research, especially preclinical therapeutic research, and allow more meaningful comparison of research findings across the field.

In the early 2000's the High Q Foundation (an affiliate of CHDI) set out to learn how to best utilize HD mouse models in the research it was supporting, including preclinical drug testing. CHDI launched an initiative led by Allan Tobin and Ethan Signer and assembled an expert advisory group - Gillian Bates (King's College London), Michael Fanselow (University of California, Los Angeles), Dan Goldowitz (University of British Columbia), Holly Moore (Columbia University), Jenny Morton (University of Cambridge), and Jeanne Wehner (University of Colorado) - to routinely meet and recommend practices to better define preclinical research with HD mouse models. They advised that CHDI develop standardized and rigorous testing criteria and identify an organization that could implement such methodologies.

PsychoGenics, Inc. was selected by CHDI as the appropriate contract research organization, a research collaboration that endures to this day. Subsequently, CHDI decided it was critical to establish a centralized repository to breed, maintain, and distribute mouse models to HD researchers that they fund, a concept consistent with practices recommended by the advisory group to maintain quality control over the health and genetic purity of each of the models and to distribute them on request to research labs around the world. The Jackson Laboratory is that centralized mouse model repository.

The Jackson Laboratory's mission is to discover precise genomic solutions for disease and empower the global biomedical community in our shared quest to improve human health. We are pleased to work jointly with colleagues at CHDI and PsychoGenics Inc. to present this guide in a series of specialized field guides to assist the research community.

This field guide describes many of the established and emerging models and their known advantages, disadvantages, peculiarities and pitfalls, and propose standardized best practices that will allow direct comparison of results across laboratories. In particular, we want to emphasize the importance of understanding how different models reflect disease phenotypes and mechanisms; the 'best model' to use will depend greatly on the question being addressed. Our ultimate goal is to improve the translation of results to the clinic and accelerate the pace of HD therapeutic research.

We intend this field guide to be a living document that is amended periodically, so we encourage input and feedback from the HD research community - please contact CHDI at HDmice@chdifoundation.org or The Jackson Laboratory at micetech@jax.org

Important information on obtaining HD mice

- Requests for HD mouse models or any JAX® Mice should be directed to The Jackson Laboratory at 1-207-288-5845 or orderquest@jax.org
- For technical questions contact The Jackson Laboratory at micetech@jax.org
- For further details, advice, or discussion of any of the HD mouse models listed in this manual contact CHDI at HDmice@chdifoundation.org
- For detailed information on testing in HD mouse models, procedures and services contact PsychoGenics, Inc. at NDG@psychogenics.com or see www.psychogenics.com

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Introduction

Field Guide

HD is a fatal neurodegenerative disorder characterized by movement and psychiatric disturbances as well as cognitive impairment (Bates and Jones, 2002). The prevalence of HD is 3 to 12 cases per 100,000 people, with the most frequent age of onset in the thirties or forties (Evans *et al.*, 2013; Pringsheim *et al.*, 2012). Patients develop involuntary movements known as chorea, which progresses over time. Patients and caregivers are most profoundly affected, however, by cognitive impairment, behavioral abnormalities and personality changes. Life expectancy is generally 15 to 20 years after symptoms begin.

HD is an autosomally dominant, inherited disease that is caused by an unstable trinucleotide repeat expansion in the *huntingtin* (*HTT*) gene. Normal *HTT* alleles contain from 6 to 35 glutamine-encoding CAG repeats, while patients with HD in almost all cases carry alleles with more than 39 repeats, which translate as an expanded polyglutamine domain in the huntingtin (*HTT*) protein. Alleles with 36 to 39 CAG repeats are associated with reduced penetrance (Rubinsztein *et al.*, 1996). The age at onset of motor symptoms inversely correlates with CAG repeat number — that is, the higher the repeat number, the earlier the onset of motor dysfunction. In individuals who carry *HTT* alleles with 55 or more CAG repeats, disease symptoms may become apparent before 20 years of age, even in early childhood.

Since the discovery of the *HTT* gene in 1993 (The Huntington's Disease Collaborative Research Group, 1993), numerous HD mouse models have been genetically engineered (Figiel *et al.*, 2012; Heng *et al.*, 2008; Pouladi *et al.*, 2013). These models have contributed significantly to our understanding of HD pathogenesis and offer tremendous potential to evaluate new therapeutics (Crook and Housman, 2011; Gil and Rego, 2009; Li *et al.*, 2005; Switonski *et al.*, 2012). The models differ in the methods by which they were engineered, their CAG repeat numbers, genetic backgrounds, and disease onset, severity and presentation. They also vary in their sensitivity to environmental factors such as health status and enrichment (Carter *et al.*, 2000; Hockly *et al.*, 2002; van Dellen *et al.*, 2000; Wood *et al.*, 2010). Although comprehensive direct comparisons of all of the models under identically controlled experimental conditions have not been conducted, the HD research community as a whole has gained an enormous appreciation for the variables that may affect study design and experimental outcomes.

Part I. Overview of selected genetically-modified mouse models of Huntington's disease

There are a variety of mouse models available for the study of HD that develop behavioral and motor deficits relevant to the human disease. For the purpose of this manual we will focus exclusively on the genetically-modified mouse models. For information on chemically-induced lesion models, such as those induced with 3-nitropropionic acid or quinolonic acid, please see the excellent review of these chemical models in Ramaswamy *et al.*, 2007 (Ramaswamy *et al.*, 2007).

The genetic models can be grouped into three broad categories according to how they were engineered. The first two categories aim to study evident/overt phenotypic endpoints; **N-terminal transgenic** animals carry the 5' portion of the human *HTT* gene, which contains the CAG repeats, whereas **full-length transgenic** models carry the full-length *HTT* sequence and express full-length HTT protein containing expanded polyglutamine repeats. The third category are **knock-in models** in which the HD mutation is replicated by directly engineering CAG repeats of varying length into the mouse *huntingtin* (*Htt*) genomic locus. Models within each of these three broad categories differ in their CAG repeat numbers (and stability of the stretch), the size and species of origin (mouse or human) of the huntingtin protein, the promoters that drive expression of the HTT proteins, and their background strain. As a consequence of how and for what purpose each was engineered, each model exhibits a somewhat different characteristic phenotype.

In the context of model building, it is worth noting the DNA sequence of the stretch of DNA in exon 1 that encodes the polyglutamine. In humans, the polyglutamine is encoded by a pure stretch of CAG codons that abuts CAACAG codons that also encode glutamine residues. By convention it is the length of the pure CAG tract that defines whether the allele is considered to be in the normal (6-26 repeats) or high-end normal (27-35) range of the distribution. In the disease range CAG lengths include incompletely penetrant (36-39), adult-onset (40-50), or juvenile (~55+) alleles for HD.

We emphasize the polyglutamine-encoding sequence of the human *HTT* gene in HD as some of the mouse models described below do not specifically mimic the (CAG)_nCAACAG DNA sequence and this is especially important to note when considering certain mechanisms relevant to HD pathophysiology.

Furthermore, immediately 3' to the (CAG)_nCAACAG tract in the human *HTT* gene, a polyproline rich stretch of amino acids is encoded in exon 1 that has been shown to be an important structural feature of both the *HTT* mRNA and resulting protein. Differences exist at both the nucleic acid and protein sequence between mouse and human versions of this polyproline stretch. In the HD models described below, some models utilize human while others utilize mouse polyproline encoding sequences in exon 1. The importance of the polyproline differences between human *HTT* and mouse *Htt* exon 1 is still under investigation (Zheng and Diamond, 2012).

I.1. N-terminal transgenic models

The N-terminal transgenic lines, also known as partial fragment lines, were among the first HD mouse models generated. They carry a small portion of the 5' end of the human *HTT* gene, including exon 1 that contains the CAG repeat region. The N-terminal transgenic lines generally have an accelerated phenotype relative to other genetically engineered lines (Menalled *et al.*, 2009a) and develop progressive neurological abnormalities, including loss of coordination, tremors, hypokinesia, abnormal gait, neuropathology and premature death. The N-terminal transgenic lines demonstrated that an N-terminal mutant HTT fragment was sufficient to elicit HD-like neurological phenotypes in the mouse. The R6/1 and R6/2 lines, which express mutant human exon 1 HTT, were the first transgenic lines produced (Mangiarini *et al.*, 1996) and the R6/2 mouse has been the most extensively studied and utilized mouse model of HD to date. Another N-terminal transgenic mouse is the N171-82Q line (Schilling *et al.*, 1999) expressing a 171 amino acid mutant HTT fragment under the regulation of the mouse prion promoter, which directs expression primarily in the brain. These and other N-terminal transgenics are available from The Jackson Laboratory (JAX) and/or CHDI Foundation (Table 1). The table lists each strain's common and full name, CAG repeat number, background strain, CHDI and/or JAX stock number, and genetic characteristics in brief.

Because all of these N-terminal containing transgenic mice were engineered via pronuclear injection, each transgene is integrated in a random fashion at a unique site in the mouse genome. Moreover, some may carry multiple concatemers of the transgene at the integration site. Recently, the insertion site and sequence of the R6/2 transgene was determined using high-throughput sequencing (Chiang *et al.*, 2012). The results show that the injected transgene underwent rearrangement upon insertion into mouse chromosome 4 between positions 96409585 and 96414930. In the allele, a fully intact transgene copy is flanked by 2 rearranged sequences that do not contain the full exon 1 encoding DNA. These data are consistent with the restriction map that was constructed of this genomic region (Mangiarini *et al.*, 1996) and indicate that the R6/2 transgene functions as a single copy integrant. Additionally, a segment of Gram-positive bacterial sequence (likely originating from cloning vector contamination) is inserted just upstream of the *HTT* promoter that drives the expression of the intact copy. Insertion of the R6/2 transgene resulted in a 5.4 kb deletion of mouse chromosomal DNA near the integration site, a demonstration that transgenic insertions can be very disruptive to the resident mouse sequences. Restriction mapping (Mangiarini *et al.*, 1996) as well as CAG repeat instability experiments (Gonitel *et al.*, 2008; Mangiarini *et al.*, 1997) indicate that the R6/1 line also contains a single copy integrant, but high-throughput sequencing results were inconsistent with these and other extensive data sets, leaving the question of copy number integration in the R6/1 line unresolved. Capture sequence methodologies like that reported by Chiang *et al.* (2012) should be employed to reveal the transgene structure, sequence and potential disruptive effects on native mouse chromosomal DNA. In HD, to date this has only been done to our knowledge for the R6/1 and R6/2 transgenic mouse lines but should be extended to other transgenic mouse lines to fully define the structure and sequence of the transgenes as they exist *in situ*.

These data emphasize the need to fully analyze transgene DNA and RNA sequences from transgenic animals. Expression levels of mRNA and protein from the integrated transgenic DNA are dependent both on the site of integration and on the number

of intact transgene copies that are inserted at the locus. Founder lines, therefore, are typically screened for high expression levels, and lines with the most rapid onset and severity of disease are typically selected for analysis. Please note, however, that the CAG repeat sequences, which are of human origin: (CAG)_nCAACAG, carried in these transgenes are subject to instability of the length of the tracts both in germ line (see Figures 1 and 2) and somatic cells (Figure 3). Therefore, care must be taken to evaluate the CAG repeat length inherited in each transgenic animal chosen when maintaining colonies and generating cohorts for studies. This is readily accomplished by polymerase chain reaction (PCR) using genomic DNA harvested from a tail biopsy of each mouse, and measuring the PCR product size via capillary electrophoresis (see Part II, below). Failure to do so can result in generational drift in the CAG repeat number (Figures 1 and 2). Indeed, this phenomenon has been a significant issue in the R6/2 mice, where the initial report defined a mean CAG repeat size of approximately 150 (Mangiarini *et al.*, 1996). Through generational drift and selective breeding, either intentionally or unintentionally, many sublines of R6/2 mice now exist with CAG repeat sizes ranging from 43 to more than 600 (Cowin *et al.*, 2011; Cummings *et al.*, 2012; Dragatsis *et al.*, 2009; Menalled *et al.*, 2009a; Morton *et al.*, 2009; Jenny Morton, personal communication). As discussed above, the phenotype of the R6/2 varies greatly as a function of CAG repeat size. Interestingly, however, the relationship is not linear in R6/2, nor does a large CAG repeat number necessarily lead to an earlier onset and more severe phenotype. An inverse relationship of CAG size to onset of HD motor signs, electrophysiological dysfunction in striatal neurons and nuclear inclusion formation applies fairly well in R6/2 mice carrying 50 to 160 CAG repeats (Cummings *et al.*, 2012). When the CAG repeat size approaches or exceeds 200, however, the onset of HD motor symptoms and pathology tends to be delayed as repeat size increases (Dragatsis *et al.*, 2009; Morton *et al.*, 2009). The reason for this inverted U-shaped, curvilinear relationship between CAG size and onset of behavioral abnormalities in R6/2 mice is not clear, but there is some evidence suggesting that expression of *HTT* mRNA may decrease as a function of CAG repeat size above a certain threshold (Dragatsis *et al.*, 2009; CHDI, unpublished data). Decreased *HTT* mRNA and protein expression from highly CAG-expanded *HTT* could account for, at least in part, a delay in disease onset. Additionally, it has been postulated that highly expanded, mutant *HTT* may have limited access to the nucleus, which may result in a different neuropathology similar to that observed in R6/2 mice with reduced CAG repeat number (Dragatsis *et al.*, 2009). Therefore, knowing how many CAG repeats HD transgenic mice carry, and monitoring the CAG repeat lengths in breeding and experimental animals, is absolutely critical to reduce experimental variation.



Table 1. HD mouse strains maintained at and available from The Jackson Laboratory colonies

Common Name	Repeat Length	Allele Type	Genetic Background	Strain Name/Standardized Nomenclature	CHDI Number	Stock Number	Gene Characteristics	Comments/References
R6/1	116	Tg fragment	C57BL/6J	Tg(HDexon1)61Gpb/J	CHDI-81001009	006471	Htt promoter, exon 1 of human Htt	www.jax.org/jaxmice/strain/006471; strain name at CHDI's colony: B6.Cg-Tg(HDexon1)61Gpb/115JChdi; [Bayram-Weston et al., 2012b; Brooks et al., 2012e; Hodges et al., 2008]
R6/1	116	Tg fragment	BALB/cByJ	Tg(HDexon1)61Gpb/J		007578	Htt promoter, exon 1 of human Htt	www.jax.org/jaxmice/strain/007578; [Bayram-Weston et al., 2012b; Brooks et al., 2012e; Hodges et al., 2008]
R6/1	116	Tg fragment	B6CBA mixed	Tg(HDexon1)61Gpb/1J		002809	Htt promoter, exon 1 of human Htt	www.jax.org/jaxmice/strain/002809; [Bayram-Weston et al., 2012b; Brooks et al., 2012e; Hodges et al., 2008]
R6/2	128	Tg fragment	B6CBA mixed	Tg(HDexon1)62Gpb/3J	CHDI-81001000	006494	Htt promoter, exon 1 of human Htt	www.jax.org/jaxmice/strain/006494; strain name at CHDI's colony: B6CBA-Tg(HDexon1)62Gpb/125JChdi; [Menalled et al., 2009a]
R6/2	160	Tg fragment	B6CBA mixed	Tg(HDexon1)62Gpb/1J		002810	Htt promoter, exon 1 of human Htt	www.jax.org/jaxmice/strain/002810; [Carter et al., 1999; Cummings et al., 2012; Mangiarini et al., 1996]
R6/2	116	Tg fragment	C57BL/6J	Tg(HDexon1)62Gpb/110JChdi	CHDI-81001001	370203	Htt promoter, exon 1 of human Htt	[Cummings et al., 2012; Mangiarini et al., 1996]
R6/2	116	Tg fragment	DBA/2J	Tg(HDexon1)62Gpb/110JChdi	CHDI-81001002	370558	Htt promoter, exon 1 of human Htt	[Cummings et al., 2012; Mangiarini et al., 1996]
R6/2	168	Tg fragment	C57BL/6J	Tg(HDexon1)62Gpb/160JChdi	CHDI-81001003	370239	Htt promoter, exon 1 of human Htt	[Cummings et al., 2012; Mangiarini et al., 1996]
R6/2	168	Tg fragment	DBA/2J	Tg(HDexon1)62Gpb/160JChdi	CHDI-81001004	908764	Htt promoter, exon 1 of human Htt	[Cummings et al., 2012; Mangiarini et al., 1996]
R6/2	251	Tg fragment	C57BL/6J	Tg(HDexon1)62Gpb/240JChdi	CHDI-81001005	371097	Htt promoter, exon 1 of human Htt	[Dragatsis et al., 2009; Morton et al., 2009]
R6/2	251	Tg fragment	DBA/2J	Tg(HDexon1)62Gpb/240JChdi	CHDI-81001006	908765	Htt promoter, exon 1 of human Htt	[Dragatsis et al., 2009; Morton et al., 2009]
R6/2	293	Tg fragment	C57BL/6J	Tg(HDexon1)62Gpb/280JChdi	CHDI-81001007		Htt promoter, exon 1 of human Htt	[Dragatsis et al., 2009; Morton et al., 2009]
R6/2	293	Tg fragment	DBA/2J	Tg(HDexon1)62Gpb/280JChdi	CHDI-81001008	908766	Htt promoter, exon 1 of human Htt	[Dragatsis et al., 2009; Morton et al., 2009]
Tg100	100	Tg fragment	B6SJL mixed	Tg(HD)63Aron/J		004360	rat NSE promoter, first 3kb of human Htt cDNA	www.jax.org/jaxmice/strain/004360; [Laloret et al., 2001]
Htt-1600-37	160	Tg fragment	FVB/NJ	Tg(GFAP-Htt*160Q)37X/J		012630	N-terminal (208aa); GFAP promoter, human Htt, 160Q repeat	www.jax.org/jaxmice/strain/012630; [Bradford et al., 2009]
N171-82Q	82	Tg fragment	B6C3H mixed	Tg(HD82Gin)81Dbo/J		003627	Prnp promoter, 171aa human Htt	www.jax.org/jaxmice/strain/003627; [Schilling et al., 1999]
HD_N171(TT); HQ-021	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002021	370308	CMV early enhancer/chicken beta actin promoter/171aa human Htt	Unpublished. N171 Htt transgene inserted at mouse Gt(ROSA)26Sor (rosa 26) locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
HD_A463(TT); HQ-020	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002020	370339	CMV early enhancer/chicken beta actin promoter/171aa human Htt	Unpublished. A463 Htt transgene inserted at mouse rosa 26 locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
HD_DS536(TT); HQ-019	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002019	370346	CMV early enhancer/chicken beta actin promoter/171aa human Htt	Unpublished. S536 Htt transgene inserted at mouse 26 locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
HD_DS52(TT); HQ-017	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002018	370313	CMV early enhancer/chicken beta actin promoter/171aa human Htt	Unpublished. D552 Htt transgene inserted at mouse rosa 26 locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
HD_DS86(TT); HQ-016	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002017	370332	CMV early enhancer/chicken beta actin promoter/171aa human Htt	Unpublished. D586 Htt transgene inserted at mouse rosa 26 locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
HD_1000aa(TT); HQ-022	145 [mixed CAA-CAG repeats]	Targeted Tg (rosa26)	C57BL/6	Gt(ROSA)26Sor ^{tm2BHTTThre} /JChdi	CHDI-81002022	370323	CMV early enhancer/chicken beta actin promoter/1000 aa human Htt	Unpublished. N1000 Htt transgene inserted at mouse rosa 26 locus. Part of a mouse series expressing different Htt length fragments from rosa 26 to allow for comparable patterns and levels of Htt expression.
YAC72	72	Tg full-length	FVB/NJ	Tg(YAC72)2511Hay/J		003640	Full length, human Htt	www.jax.org/jaxmice/strain/003640; [Hodgson et al., 1999]
YAC128	100, 126 [mixed CAA-CAG repeats]	Tg full-length	C57BL/6	Tg(YAC128)53Hay/JChdi	CHDI-81001013	370231	Full-length; human Htt	[Bayram-Weston et al., 2012c; Brooks et al., 2012a; Slow et al., 2003; Van Raamsdonk et al., 2007; Pouladi et al., 2012]
YAC128	100, 126 [mixed CAA-CAG repeats]	Tg full-length	FVB/NJ	Tg(YAC128)53Hay/J		004938	Full-length; human Htt	www.jax.org/jaxmice/strain/004938; [Pouladi et al., 2012; Slow et al., 2003]
BAC HD	97 [mixed CAA-CAG repeats]	Tg full-length	FVB/NJ	Tg(HTT*97Q)IXwy/J	CHDI-81001010	017487	Full-length, floxed exon 1; human Htt	www.jax.org/jaxmice/strain/017487; strain name at CHDI's colony: FVB-Tg(HTT*97Q)IXwy/97JChdi; common name at JAX: BACHD-L; [Gray et al., 2008]
BAC HD	97 [mixed CAA-CAG repeats]	Tg full-length	C57BL/6	Tg(HTT*97Q)IXwy/JChdi	CHDI-81001011	370192	Full-length, floxed exon 1; human Htt	[Gray et al., 2008; Menalled et al., 2009a]
BAC HD	97 [mixed CAA-CAG repeats]	Tg full-length	FVB/NJ	Tg(HTT*97Q)IXwy/J		008197	Full-length, floxed exon 1; human Htt	www.jax.org/jaxmice/strain/008197; [Gray et al., 2008; Menalled et al., 2009a]
BAC HD-SA	97 [mixed CAA-CAG repeats]	Tg full-length	FVB/NJ	Tg(HTT*1)IXwy/J	CHDI-81001021	017485	Full-length, human Htt Ala-13, 16-Ser, BAC	www.jax.org/jaxmice/strain/017485; [Gu et al., 2009]
BAC HD-SD	97 [mixed CAA-CAG repeats]	Tg full-length	FVB/NJ	Tg(HTT*1)BXwy/J	CHDI-81001022	370874	Full-length; human Htt Asp-13, 16-Ser, BAC	[Gu et al., 2009]
CAG 140 KI	146	Knock-in	CBA/J	Htt ^{tm1Mc} /140JChdi	CHDI-81003001	370235	Endogenous murine Htt gene, chimeric human/mouse exon 1	[Menalled et al., 2003]

CAG 140 KI	146	Knock-in	C57BL/6J	<i>Ht^{tm1Mc}/140JChdi</i>	CHDI-81003002	370232	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	(Menalled <i>et al.</i> , 2003)
z0175 KI	198	Knock-in	C57BL/6J	<i>Ht^{tm1Mc}/190JChdi</i>	CHDI-81003003	370437	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	(Menalled <i>et al.</i> , 2012b)
HdhQ20	20	Knock-in	C57BL/6J	<i>Ht^{tm2Men}/20JChdi</i>	CHDI-81003005	370504	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Generated with a pure CAG tract followed by CAACAG similar to the human <i>HTT</i> gene (Wheeler <i>et al.</i> , 1999; White <i>et al.</i> , 1997)
HdhQ50	50	Knock-in	C57BL/6J	<i>Ht^{tm2Men}/J</i>	CHDI-81003016	003454	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Generated with a pure CAG tract followed by CAACAG similar to the human <i>HTT</i> gene www.jax.org/jaxmice/strain/003454; strain name at CHDI's colony: B6.129- <i>Ht^{tm2Men}/50JChdi</i> ; (Wheeler <i>et al.</i> , 1999; White <i>et al.</i> , 1997)
HdhQ80	86	Knock-in	C57BL/6J	<i>Ht^{tm11Pp}/80JChdi</i>	CHDI-81003007	370506	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Generated with a pure CAG tract followed by CAACAG similar to the human <i>HTT</i> gene
HdhQ92	98	Knock-in	C57BL/6J	<i>Ht^{tm2Men}/J</i>	CHDI-81003008	003597	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Generated with a pure CAG tract followed by CAACAG similar to the human <i>HTT</i> gene www.jax.org/jaxmice/strain/003598; strain name at CHDI's colony: B6.129- <i>Ht^{tm2Men}/92JChdi</i> ; (Bayeram-Weston <i>et al.</i> , 2012a; Trueman <i>et al.</i> , 2008; Wheeler <i>et al.</i> , 1999; White <i>et al.</i> , 1997)
HdhQ111	116	Knock-in	C57BL/6J	<i>Ht^{tm2Men}/111J</i>	CHDI-81003000	003598	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Generated with a pure CAG tract followed by CAACAG similar to the human <i>HTT</i> gene (Lloret <i>et al.</i> , 2006; Wheeler <i>et al.</i> , 1999)
HdhQ50	50	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/50J</i>		016521	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/http/strain/016521; (Sathasivam <i>et al.</i> , 2013)
HdhQ100	100	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/100J</i>		016522	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/016522; (Sathasivam <i>et al.</i> , 2013)
HdhQ150	150	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/150J</i>		004595	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/004595; (Heng <i>et al.</i> , 2007; Lin <i>et al.</i> , 2001)
HdhQ200	200	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/200J</i>		016523	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/016523; (Heng <i>et al.</i> , 2010a)
HdhQ250	250	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/250J</i>		016524	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/016524
HdhQ315	315	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/315J</i>		016525	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/016525
HdhQ365	365	Knock-in	C57BL/6J	<i>Ht^{tm2Chd1}/365J</i>		021193	Endogenous; murine <i>Htt</i> , expanded CAG	www.jax.org/jaxmice/strain/021193
Hd(HD_1000aa) KI	145 (mixed CAA-CAG repeats)	Knock-in	C57BL/6J	<i>Ht^{tm1HTT}/JChdi</i>	CHDI-81003009	370266	CAG promoter/1000 aa human <i>Htt</i> replaces Endogenous murine <i>Htt</i> gene	Unpublished. Knock-in mouse where N1000 (1450) expression from a strong CAG promoter replaces endogenous normal <i>Htt</i> mouse allele expression.
Hdh CAG 45 KI	45	Knock-in	C57BL/6NJ	<i>Ht^{tm1Men}/45JChdi</i>	CHDI-81003010	370606	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with pure CAG45. Corresponding mouse <i>Htt</i> (CAG CAA CAG CAA AAn); n = 9. For addressing somatic instability and RNA toxicity biology.
Hdh (CAGCAA)9 KI	45 (mixed CAA-CAG repeats)	Knock-in	C57BL/6NJ	<i>Ht^{tm2Men}/45JChdi</i>	CHDI-81003011	370612	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with mixed <i>Htt</i> (CAG CAA CAG CAA CAA AAn); n = 9. Corresponding mouse <i>Htt</i> CAG45. For addressing somatic instability and RNA toxicity biology.
Hdh CAG80 KI	80	Knock-in	C57BL/6NJ	<i>Ht^{tm2Men}/80JChdi</i>	CHDI-81003012	370610	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with pure <i>Htt</i> CAG 80. Corresponding mouse <i>Htt</i> (CAG CAA CAG CAA AAn); n = 16. For addressing somatic instability and RNA toxicity biology.
Hdh (CAGCAA)16 KI	80 (mixed CAA-CAG repeats)	Knock-in	C57BL/6NJ	<i>Ht^{tm2Men}/80JChdi</i>	CHDI-81003013	370604	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with mixed <i>Htt</i> (CAG CAA CAG CAA CAA AAn); n = 16. Corresponding mouse <i>Htt</i> CAG80. For addressing somatic instability and RNA toxicity biology.
Hdh CAG105 KI	105	Knock-in	C57BL/6NJ	<i>Ht^{tm2Men}/105JChdi</i>	CHDI-81003014	370615	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with pure <i>Hdh</i> CAG 105. Corresponding mouse <i>Hdh</i> (CAG CAA CAG CAA CAA AAn); n = 21. For addressing somatic instability and RNA toxicity biology.
Hdh (CAGCAA)21 KI	105 (mixed CAA-CAG repeats)	Knock-in	C57BL/6NJ	<i>Ht^{tm2Men}/105JChdi</i>	CHDI-81003015	370618	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Unpublished; Knock-in mouse with mixed <i>Htt</i> (CAG CAA CAG CAA CAA AAn); n = 21. Corresponding mouse <i>Htt</i> CAG105. For addressing somatic instability and RNA toxicity biology.
Hdh^{ms45}	N/A	Knock-out	C57BL/6	<i>Ht^{tm1Men}/J</i>		002688	Knock-out exons 4 & 5; murine <i>Htt</i>	www.jax.org/jaxmice/strain/002688; (Duyao <i>et al.</i> , 1995)
Hdh RNAiTT	N/A	Knock-down	C57BL/6NTac	<i>Gf(ROSA)Z6</i> <i>Sor^{sh3H1}/Tad</i> - <i>Hdh</i> - <i>Htt</i> <i>Men</i> /TacChdi	CHDI-81002001		Conditional <i>Htt</i> Knock-down	Unpublished. <i>Htt</i> shRNA under inducible regulation cassette at mouse <i>rosa 26</i> locus.
Hdh RNAiTT	N/A	Knock-down	C57BL/6J	<i>Gf(ROSA)Z6</i> <i>Sor^{sh3H1}/Tad</i> - <i>Hdh</i> - <i>Htt</i> <i>Men</i> /JChdi	CHDI-81002002		Conditional <i>Htt</i> Knock-down	<i>Htt</i> shRNA under inducible regulation cassette at mouse <i>rosa 26</i> locus. (Menalled <i>et al.</i> , 2009b)
Hdh RNAiTT	N/A	Knock-down	C57BL/6NTac	<i>Gf(ROSA)Z6</i> <i>Sor^{sh3H1}/Tad</i> - <i>Hdh</i> - <i>Htt</i> <i>Men</i> /TacChdi	CHDI-81002023		Conditional <i>Htt</i> Knock-down	Unpublished. <i>Htt</i> shRNA under inducible regulation cassette at mouse <i>rosa 26</i> locus.

• Requests for mouse strains from CHDI Foundation should be directed to The Jackson Laboratory at 1-207-288-5845 or orderquest@jax.org.

• Animals from lines with a *Stock Number* starting with "0" are part of the JAX collection and can also be found at The Jackson Laboratory (www.jax.org/jaxmice/query). These animals are housed under JAX standard husbandry conditions; more information about these mice can be found at www.jax.org.

• Animals from lines with a *CHDI number* are part of the CHDI repository that are bred and housed at The Jackson Laboratory. *CAG* repeat lengths represent the average length found in mice from the CHDI colony. These lines are housed under specific conditions (see more below). For any background information about mice in the CHDI collection, contact CHDI Foundation (HDmice@chdifoundation.org).

• HD lines carrying (CAA-CAG) repeats do not present germ line instability.

• The strain names follow MGI guidelines for the nomenclature of mouse strains (www.informatics.jax.org/mgihome/nomen/).

• References cited may describe mouse models in a background strain different from the one presented in this table.

• Abbreviations: Tg: transgenic, N/A: not applicable

I.2. Full-length transgenic models

HD models in which the full-length *HTT* transgene is carried in either a yeast or a bacterial artificial chromosome (YAC and BAC, respectively) are also available (Hodgson *et al.*, 1999; Slow *et al.*, 2003; Gray *et al.*, 2008); see Table 1. Unlike the shorter N-terminal fragment models, these larger transgenic constructs have a tendency to integrate into the genome at a single genomic locus and typically in low copy numbers (i.e., 1-3 transgene copies). Also, the large size of the YAC and BAC constructs typically insulates the *HTT* transgenes from the influences of cis-DNA sequences in the mouse genome that can negatively or positively affect expression levels. As a result, *HTT* mRNA and protein expression levels in YAC- and BAC- derived full-length transgenic models tend to correlate well with the number of transgene copies that are inserted. The well-studied YAC128 transgenic mouse, for example, expresses multiple copies of full-length human *HTT* with 100 and 126 glutamine repeats, encoded by a tract that is composed primarily of CAG codons but also contains 9 interspersed CAA codons (that also encode glutamine) (Pouladi *et al.*, 2012), at roughly the same level (~75%) as the endogenous mouse *Htt* (Slow *et al.*, 2003). In contrast, the BAC HD mouse model, which expresses full-length human *HTT* containing 97 codons of a mixed CAG/CAA repeat tract, expresses human *HTT* at levels 1.5 to 2 times higher than the endogenous mouse *Htt* (Gray *et al.*, 2008), a function of higher numbers of transgene copies inserted.

Unlike the N-terminal transgenic animals, which develop disease phenotypes early and have shortened life spans, the full-length transgenic models develop disease phenotypes gradually over many months and show relatively normal survival (Gray *et al.*, 2008; Menalled *et al.*, 2009a; Van Raamsdonk *et al.*, 2005b). Although the neurodegenerative phenotype of the full-length transgenic models is slower to develop than the N-terminal transgenic models, the full-length transgenic models have higher construct validity because the full human *HTT* gene is expressed within the context of its endogenous genomic regulatory elements. These full-length models may offer certain advantages over the N-terminal fragment transgenics, particularly when testing experimental therapies targeted directly at the human *HTT* gene or protein. Curiously, in both the YAC128 and BAC HD full-length transgenic mouse models, weight gain is observed (Gray *et al.*, 2008; Van Raamsdonk *et al.*, 2007) (see Figure 4). The phenomenon seems to be related to the full-length *HTT* gene dosage and may be mediated through mouse *Igf1* expression or other, as yet uncharacterized, molecules that regulate food intake and metabolism (Pouladi *et al.*, 2010). The unusual weight gain in the YAC128 and BAC HD mouse models can actually confound motor endpoint evaluation since body weight, in addition to mutant huntingtin, has been reported to influence activity levels (Gray *et al.*, 2008; Kudwa *et al.*, 2013; Menalled *et al.*, 2009a). Additionally, these models may not be well suited for studies evaluating the effect of mutant *HTT* on the metabolic changes and weight loss typically seen in HD patients. Unexpected phenotypes like this, which can complicate and confound experimental results, can occur in any transgenic model system in which a human transgene is expressed in a murine host.

With regards to germ line and somatic instability in the YAC128 and BAC HD mouse models, some important considerations should be noted. First, in the YAC transgenics the polyglutamine stretch in exon 1 is encoded by a CAG tract that is interspersed with 9 CAA codons (Pouladi *et al.*, 2012). These interspersed CAA codons appear to be enough to stabilize the tract to be resistant to germ line instability as the YAC128 mouse CAG-CAA tract length does not drift over generations. Furthermore, it has been recently shown that somatic instability is minimal in the YAC128 mice (unpublished observations; Pouladi and Wheeler). Similarly, in BAC HD mice, the polyglutamine tract is encoded by an ordered sequence comprised of both CAG and CAA

codons. Mixing CAG and CAA codons has been shown to confer resistance of the tract to expansions and contractions (Gray *et al.*, 2008). Germ line and somatic instability is not seen over generational time in BAC HD mice. In contrast, the N-terminal *HTT* fragment transgenic models described above, whose tracts are comprised of (CAG)_nCAACAG sequence, exhibit both germ line and somatic instability in individual mice and over generational time (Menalled *et al.*, 2009a; Slow *et al.*, 2003). The DNA sequence encoding the polyglutamine tract has two important implications that should be considered when using the BAC HD and YAC128 mice in HD research. First, routine measurement of CAG-CAA tract size in each BAC HD and YAC128 animal used for breeding and/or experimentation is not necessary as the tract sizes are highly stable in the germ line (Gray *et al.*, 2008; Slow *et al.*, 2003). Second, while this stability is convenient in terms of maintaining and breeding these mice for experimentation, questions remain regarding the result of such CAG-CAA sequences on HD pathophysiology in the mouse, especially in the context of the potential role of RNA structure and toxicity (Banez-Coronel *et al.*, 2012) and the importance of somatic instability (Kennedy and Shelbourne, 2000; Swami *et al.*, 2009) in eliciting such pathophysiology. Essentially, the BAC HD mice and to some extent the YAC128 mice do not exactly model the HD mutation at the DNA sequence level and care should be taken in using these models where RNA structure and somatic instability mechanisms are under investigation. That being said, the BAC HD and YAC128 mice present with features of HD pathophysiology, suggesting that any influence of a mixed CAG-CAA repeat is likely only partial at most. Notably, a recent head-to-head comparison of the YAC128 and BAC HD models showed some critical differences in transcriptional dysregulation and *HTT* aggregate formation, despite being comparable in overall general health and motor-related behavioral phenotypes (Pouladi *et al.*, 2012). It is possible that these differences arise due to the nucleotide sequence differences of the polyglutamine tract, namely the relative contributions of CAA codons present in exon 1 in each of these transgenic mouse models.

I.3. Knock-in models

Unlike the N-terminal and full-length HD transgenic models, knock-in (KI) HD models are generated by homologous recombination techniques using mouse embryonic stem cells; a specified number of CAG repeats are introduced directly into the mouse *Htt* gene. One obvious advantage to these models is the expression fidelity that results from the CAG repeats being carried in the context of the mouse *Htt* gene; the variability in tissue distribution and expression levels observed in microinjection-based HD transgenic models (due to transgene copy number variation and position effects resulting from random transgene integration) is eliminated. Like HD patients, these HD knock-in mice also are heterozygous for one wild-type *Htt* allele and one CAG-expanded allele, an important consideration since the contributions of the remaining wild-type *Htt* gene to disease in HD patients remain controversial (Aziz *et al.*, 2009; Cattaneo *et al.*, 2005; Djousse *et al.*, 2003; Farrer *et al.*, 1993; Lee *et al.*, 2012; Wexler *et al.*, 1987). A key differentiation among the HD knock-in mouse models reported to date, however, is whether the expanded CAG tract is inserted into an otherwise unaltered mouse *Htt* exon 1 or into a humanized exon 1 sequence. In the former case, the knock-in lines express a mutant form of mouse *Htt* (Lin *et al.*, 2001; Sathasivam *et al.*, 2013), but in the latter case the knock-in lines express a mutant *Htt* with a chimeric mouse-human *HTT* (Levine *et al.*, 1999; Menalled *et al.*, 2003; Shelbourne *et al.*, 1999; Wheeler *et al.*, 1999). Therefore, if preclinical strategies require the presence of a human *HTT* gene and protein sequence then the knock-in series of mice should not be used.

In addition to the well-controlled *Htt* gene dosage (1 normal and 1 mutant allele) and commensurate expression levels, a powerful advantage of the HD knock-in mouse models is the ability to generate allelic series of knock-in mice that differ only in their CAG repeat size in exon 1. These series, which are available on congenic C57BL/6J inbred backgrounds (see Table 1), allow for experimental studies designed specifically to investigate the influence of CAG size on the desired outcome measures.

Numerous HD knock-in mouse models have been described. For a comprehensive review of them, please see Menalled, 2005. For the purposes of this manual, we will summarize three major subcategories of the HD knock-in mouse models and their use in HD research.

The first group is an allelic series of knock-in mice generated and described by Wheeler and MacDonald at Massachusetts General Hospital (MGH) (Wheeler *et al.*, 1999). These knock-in mice have a human exon 1 sequence engineered with CAG repeats of 18, 48, 78, 90 and 109 targeting one allele of mouse *Htt*. Each tract is followed by CAACAG (CAA also encodes glutamine as present in the human *HTT* gene). The targeting construct also contained 268 bp of human intron 1 that replaced 124 bp of mouse intron 1 3' to the consensus 5' splice donor sequence. In addition, a loxP and vector sequence has been retained 5' to exon 1, which is a remnant from a floxed neomycin-resistance gene cassette that was used for selection in ES cells. The most well-described mouse in this knock-in series is the *HdhQ111* mouse (111 glutamines encoded by a (CAG)₁₀₉CAACAG sequence (Wheeler *et al.*, 2000)). The MGH allelic knock-in series exists on multiple strain genetic backgrounds (Lloret *et al.*, 2006), but the C57BL/6J background has been studied most extensively (see Table 1).

The second group is an allelic series of knock-in mice generated and created by Lin and Detloff at the University of Alabama, Birmingham (UAB) (Lin *et al.*, 2001). These knock-in mice have expanded (CAG)_nCAACAG tracts where N is 50, 100, 150, 200, 250, 315 and 365 in the mouse *Htt* exon 1 sequence (Heng *et al.*, 2010b; Lin *et al.*, 2001; Sathasivam *et al.*, 2013). The 150 CAG-repeat line, denoted *HdhQ150*, is the most well studied of this series. Unlike the MGH series, these mice contain no human sequence; 100% of the mutant *Htt* expressed in these mice is derived from the mouse *Htt* gene. In generating these mice the mouse *Htt* exon 1 sequence was replaced with an HPRT gene selectable marker (positive selection) and then exchanged with a CAG-expanded mouse *Htt* exon 1 sequence (negative selection). This strategy leaves no vector or loxP DNA sequence footprint in the targeted *Htt* alleles, an advantage from the standpoint of retaining the highest degree of genetic purity in these knock-in mouse models. A key difference from the MGH allelic series is that the *Htt* exon 1 sequence 3' to the CAG tract in the Detloff KI mice encodes a mouse polyproline stretch (vs. human polyproline stretch in MGH KI mice); recent evidence suggests that the specific polyproline sequence can influence *Htt* protein aggregation and neuropathological phenotypes (Zheng *et al.*, 2012). Further studies are underway that should shed light on the relative importance of these sequence differences between the MGH and Detloff knock-in mouse series. As with the MGH allelic series, the Detloff series has been bred onto a congenic C57BL/6J genetic background, which has allowed direct head-to-head comparison studies of all of these mice without the confounding complications of differing genetic backgrounds (see Table 1).

The third group generated knock-in mice using a strategy distinct from that taken by the MGH and UAB groups. These mice, developed by Zeitlin, are heterozygous for a *Htt* allele with an expanded CAG repeat size of 71, 94 and 140 (Levine *et al.*, 1999; Menalled *et al.*, 2003). The 71 and 94 repeat size lines carry an arginine codon in position 42 of the

polyglutamine tract. This mutation occurred during the propagation of the plasmid constructs and it has been shown that this arginine does not interfere with the ability of the mutant protein to form aggregates (Levine *et al.*, 1999). However, because of this mutation, these mice have generally not been well studied. The 140 repeat size knock-in mouse (CAG140) has been well characterized and retains a pure CAG tract, (CAG)_nCAACAG, encoding polyglutamine (no arginine codon) (Hickey *et al.*, 2008; Menalled *et al.*, 2003). Additionally, the targeting strategy taken by Zeitlin was to humanize the exon 1 amino acid sequence including the polyproline stretch. So, both the MGH and Zeitlin knock-in mice encode human exon 1 sequence in the context of a full length mouse *Htt* locus while the Detloff knock-in mice are entirely mouse *Htt* sequence. The specific gene-targeting strategy employed by Zeitlin, however, left an intact neomycin gene selection cassette behind in the 5' flank of the targeted mouse *Htt* gene. The impact of this gene cassette on HTT expression levels has not been well documented, but existing data suggest that it is minimal (unpublished observations). The modified mouse *Htt* locus also carries 10 bp of human intron 1 while 94 bp of mouse intron 1 has been deleted. Germ line expansions of the CAG tract in the CAG140 mouse led to the identification of a mouse that has a CAG repeat size of 175 at PsychoGenics. This mouse was selectively bred to create a new subline, named zQ175 (Heikkinen *et al.*, 2012; Menalled *et al.*, 2012b). Subsequently, the CAG repeat number in the zQ175 expanded further, and a line was selectively stabilized at a CAG repeat of approximately 190. This line, still confusingly named zQ175, is identical to the CAG140 knock-in mouse except for CAG tract size. Like the MGH and Detloff mice, these mice are available on congenic C57BL/6J backgrounds (see Table 1).

It should be noted that all of the HD knock-in mice that carry pure CAG expansions in exon 1 are subject to germ line and somatic CAG repeat instability (Hunter *et al.*, 2005; Kennedy and Shelbourne, 2000; Lee *et al.*, 2011). In fact, this phenomenon was used by the Detloff group to generate sublines of their knock-in mice that have differing CAG repeat numbers.

CHDI has now commissioned the production of another series of HD knock-in mice that are matched in their polyglutamine length (45, 80, and 105) but differ specifically in the DNA sequence encoding polyglutamine. One series of mice uses a pure CAG expansion comprised of (CAG)_nCAACAG while the other uses a mixed CAG-CAA repeat expansion, specifically (CAG CAA CAG CAA CAA)_n where n = 9, 16 or 21 (see Table 1). These mice are not yet characterized but should be useful models to specifically explore the effect of interrupted CAG tracts and the importance of pure CAG tracts on somatic instability and RNA structure mechanisms in HD pathophysiology.

In general, all of these knock-in models present milder initial behavioral abnormalities than transgenic lines. At later ages, however, they typically develop more robust behavioral abnormalities and huntingtin aggregate pathology (Brooks *et al.*, 2012b; Brooks *et al.*, 2012c; Brooks *et al.*, 2006; Heng *et al.*, 2010b; Hickey *et al.*, 2008; Lin *et al.*, 2001; Menalled *et al.*, 2012b). Most of the knock-in lines have normal life

Huntington's Disease Resources — www.jax.org/jaxmice/neurobiology/huntingtons

Access the world's largest collection of models of Huntington's disease.

spans, although the homozygous zQ175 and *Hdh*Q150 mice reach end-stage disease at around 22-23.5 month of age and have shortened life spans (Menalled *et al.*, 2012b; Woodman *et al.*, 2007). Most HD investigators have focused on using high Q length (92 or greater) knock-in mice for preclinical studies as lower Q length mice do not appear to demonstrate robust measurable phenotypes, at least based on reports to date. Furthermore, most reports using knock-in mouse models have focused on homozygotes as the phenotypes studied are generally more robust compared to heterozygous knock-in mice. The choice of a knock-in HD mouse model therefore requires careful consideration of two variables: the parental CAG repeat size and the zygosity of the mutant allele. Each of these affects the robustness of the outcome measure being sought and the age when deficits are measurable. However, each of these variables has been the subject of debate in the context of attempts to best mimic HD pathophysiology in the mouse. First, while, knock-in alleles provide apparent high level construct validity in that the endogenous *Htt* gene is being modified to express expanded CAG repeats, the size of the repeats most studied (e.g., 111, 150, 175, 200) are well above the normal range for adult-onset HD and exceed typical sizes even for juvenile-onset HD. This concern can be at least partially mitigated by investigations using an allelic series of knock-in mouse models expressing mutant *Htt* with a range of CAG sizes. We suggest that, when possible, it is best to investigate the mechanism in question as a function of CAG repeat size, utilizing knock-in mice containing fewer CAG repeats after initial observations are made in higher repeat mice. Of course this is dependent on whether the phenotype of interest can be measured in these lower repeat mice. Second, it has been argued that heterozygous knock-ins, as stated above, better genocopy the majority of human HD patients, who typically carry a dominant, single *HTT* allele with expanded CAG repeats. Although homozygous mice typically do present with more robust phenotypes, particularly with respect to behavior, further comprehensive investigations have also uncovered phenotypes in heterozygous knock-in mice (Heikkinen *et al.*, 2012; Heng *et al.*, 2007; Lin *et al.*, 2001; Menalled *et al.*, 2012b; Rising *et al.*, 2011). The key question is whether homozygous and heterozygous knock-in mouse models are equally valid in modeling HD. The current evidence in humans suggests that homozygous knock-in mice are a reasonable model of human disease. Patients that are homozygous for the mutant *HTT* allele have been studied and do not show any obvious clinical differences in age of motor onset from heterozygous patients (Lee *et al.*, 2012; Myers *et al.*, 1989; Wexler *et al.*, 1987). However, no human data exist from patients carrying homozygous mutations with CAG repeats sizes in the ranges that are being used in the knock-in mouse models (92+), continuing to raise some concerns. We recommend that investigators consider studying their mechanism of interest as a function of gene dosage using wild-type, heterozygous and homozygous knock-in mice so as to be in a position to best understand the role of zygosity in the outcomes measured in these disease models.

Part II. Best practices for managing HD mouse colonies

II.1. Monitoring CAG repeats

Recommendations: Select breeders by CAG repeat length, and control for maternal influence.

Somatic and germ line instability is a phenomenon associated with HD and many other repeat expansion diseases. As the normal, wild-type repeat grows beyond a threshold length, the size of successive expansions and the likelihood of another unstable event increases in the subsequent generation. The larger expansions in CAG repeats are seen with paternal transmission of the mutation (Duyao *et al.*, 1993; Trottier *et al.*, 1994).

The instability of the CAG repeat with respect to the patterning of paternal and maternal inheritance is less well documented in mice. As mentioned above, however, many of the N-terminal transgenic and knock-in models demonstrate both germ line and somatic instability (Gonitel *et al.*, 2008; Kennedy and Shelbourne, 2000; Lee *et al.*, 2011; Mangiarini *et al.*, 1997; Wheeler *et al.*, 1999), meaning that continuous monitoring of animal models containing CAG repeats is essential for preserving the phenotypic characteristics of the strain caused by the repeat expansion. Similar to human observations, increases of CAG repeat lengths in progeny has been observed in colonies commonly maintained through the male line (Figure 1). Most of the lines carrying over 150 CAG repeats arose spontaneously from animals carrying shorter CAG repeat expansions as a result of the instability of the CAG repeat transmission. Contractions of the CAG repeat length can also occur in HD mouse models; in the R6/2 CAG 120 line, repeat contractions were observed when the CAG repeat length of mutant females used during breeding (via ovarian-transplanted wild-type females) was not monitored (Figure 2).

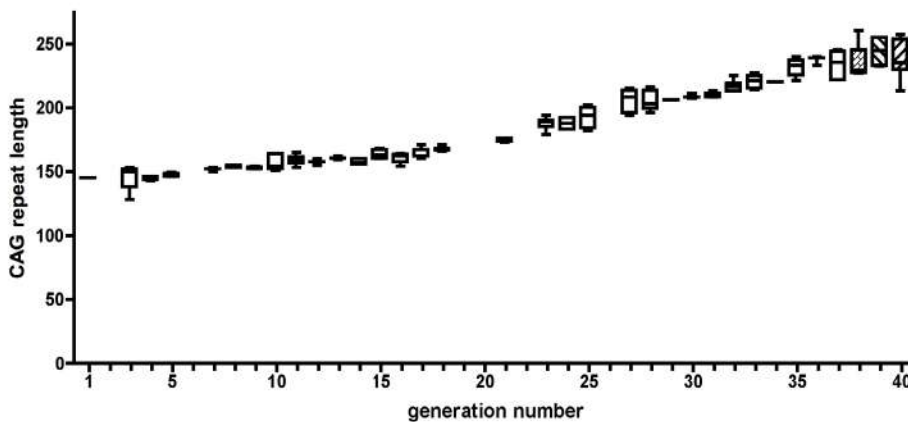


Figure 1: CAG repeat length of the progeny obtained from male R6/2 animals. Unpublished data generously provided by A.J. Morton.

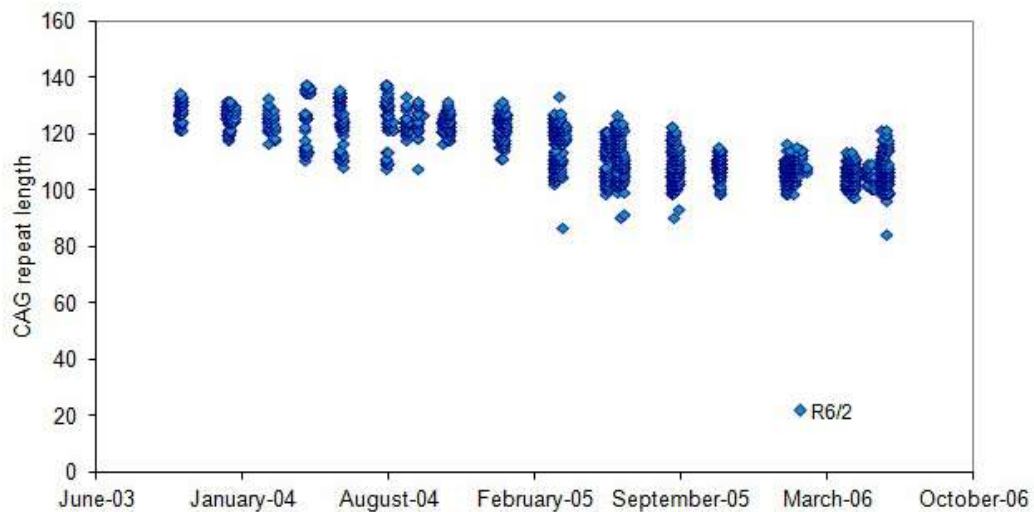


Figure 2: CAG repeat length of the progeny obtained from ovarian-transplanted females when CAG repeats of breeder animals was not monitored.

It is important to note that CAG instability is not only evident in the mouse germ line, but also in somatic cells. It is therefore expected that the mean CAG size present in brain subregions and some peripheral tissues will change with age (Figure 3). Investigators should consider measuring mean CAG repeat size of *HTT* in target tissues and ages of interest for their specific experiments. These data may give a more accurate spatial and temporal depiction of mutation size length in experimental paradigms.

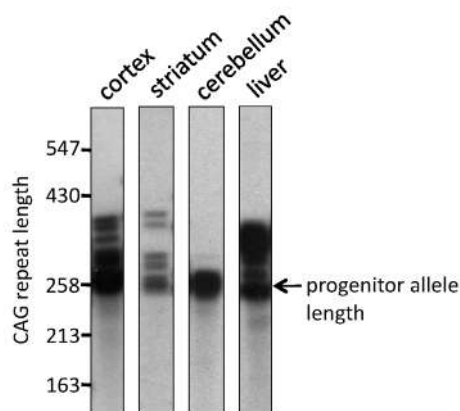


Figure 3: Small pool PCR amplification of CAG repeat from HTT exon 1 R6/2 animals. Unpublished data generously provided by E. Larson, D. Monckton and A.J. Morton.

At JAX, HD mouse models containing unstable trinucleotide expansions are closely monitored using a PCR-based assay in which a fluorescently-labeled product is subsequently analyzed by capillary electrophoresis using the 3730 DNA Analyzer made by Applied Biosystems (Carlsbad, CA). The following sections outline the various assays employed to assess trinucleotide repeat size in HD mouse models and provide historical data regarding CAG repeat size stability (or instability) in each strain.

a. N-terminal transgenics and YAC models

The R6/2, R6/1, and YAC128 HD models can all be genotyped and assessed for CAG repeat size using the following primer set: 5'-ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3' (6-FAM labeled) and 5'-GGC GGC TGA GGA AGC TGA GGA G-3'. As in the assay above, the fluorescently labeled PCR product is subsequently sized on the ABI 3730 analyzer. The product size produced from each strain depends on the number of CAG repeats that it carries. Each PCR product has approximately 86 base pairs of sequence flanking the CAG repeat sequence. Table 2 outlines the product sizes observed and the incidence of expansion and contraction observed in the colonies of the strains maintained at JAX. In addition to this assay, commercial vendors, such as Laragen Inc. (Culver City, CA), offer fee-for-service genotyping to determine CAG repeat size. (Note: Due to the volume of mice produced in its R6/2 colony, JAX routinely outsources genotyping to Laragen, whose assay uses primers that produce a product with ~122bp flanking sequence around the CAG repeat region.)

Table 2. Expected CAG repeat sizes for R6/2, R6/1 and YAC128 lines

Name	Product Size	Repeat Size	Mode of Inheritance	Sex	Repeat Range Observed (Ave +/- Std Dev)	% Incidence of Contraction	% Incidence of Expansion	N
R6/2 (120 CAG)	483	120	maternal	F	119.63 +/- 1.69	0	20.0	15
				M	119.34 +/- 2.16	10.29	19.11	68
			paternal	F	119.97 +/- 1.53	7.69	7.69	52
				M	120.09 +/- 1.71	10.41	8.33	48
R6/2 (160 CAG)	598	160	maternal	F	159.02 +/- 5.00	3.0	4.0	95
				M	159.73 +/- 2.45	7.0	12.0	76
			paternal	F	159.09 +/- 11.03	4.0	3.0	68
				M	161.44 +/- 2.12	12.0	11.0	65
R6/1	430	115	maternal	F	117.47 +/- 4.13	1.8	21.8	27
				M	117.75 +/- 3.79	2.4	14.4	20
			paternal	F	116.85 +/- 3.65	5.0	32.0	41
				M	118.46 +/- 3.36	9.0	16.0	81
YAC128	364/430	93/116	maternal	F	92.6 +/- 0.45 - 116.8 +/- 0.35	0	0	11
				M	92.5 +/- 0.56 - 116.8 +/- 0.52	0	0	15
			paternal	F	92.5 +/- 0.26 - 116.6 +/- 0.39	0	0	19
				M	92.5 +/- 0.52 - 116.6 +/- 0.47	0	0	32

Table 2 presents the CAG repeat size and related PCR product size when monitoring the R6/2, R6/1, and YAC128 lines. The incidence of expansion/contraction, expressed as a percentage of the number of mice assayed, is provided. For both R6/2 lines, breeding through the female requires the use of ovarian-transplanted, hemizygous females crossed with non-carrier males. Expansion or contraction was defined as CAG values falling outside +/- standard deviation of the mean.

The YAC128 line has historically demonstrated 2 bands using this assay, each with a remarkably stable CAG repeat size of either 93 or 116.

b. Knock-in models

We have chosen the Detloff series to serve as examples for monitoring CAG repeats in knock-in mice (see Table 2). Each of these strains employs the same assay to detect the CAG repeat. A fluorescein-tagged (FAM-labeled) forward primer 5'-CCC ATT CAT TGC CTT GCT G-3' is paired with reverse primer 5'-GCG GCT GAG GGG GTT GA-3'. Product sizes will vary with each strain according to the number of CAG repeats in each allele. PCR amplification generates a product with 198 base pairs of flanking sequence on either side of the repeat expansion. In wild-type mice, the product size is approximately 217 base pairs. In animals harboring a CAG repeat expansion, both the wild-type product and the mutant expansion product are amplified in this reaction. Our criteria for determining the acceptable repeat range is set by a +/- 5 CAG repeat error. Animals falling above or below this range are sacrificed; they are not used for colony maintenance or distributed to the scientific community. Table 3 presents CAG repeat instability in the Detloff knock-in allelic series.

Table 3. CAG repeat size, product size, and incidence of contraction/expansion

Name	Product Size	Repeat Size	Mode of Inheritance	Sex	Repeat Range Observed (Ave +/- Std Dev)	% Incidence of Contraction	% Incidence of Expansion	N
HdhQ150	655	150	maternal	F	150.79 +/- 3.55	22.0	11.0	9
				M	148.27 +/- 4.33	10.0	20.0	10
			paternal	F	154.62 +/- 13.64	16.0	16.0	32
				M	157.27 +/- 10.72	23.0	23.0	56
HdhQ50	350	50	paternal	F	50.4 +/- 0.7	0.0	0.0	17
				M	50.7 +/- 0.7	0.0	0.0	17
HdhQ100	498	100	paternal	F	103.0 +/- 3.0	14.3	14.3	7
				M	102.8 +/- 2.7	10.7	0.0	28
HdhQ200	800	200	maternal	F	217.7 +/- 6.27	10.0	10.0	20
				M	213.26 +/- 10.38	16.7	13.3	30
			paternal	F	218.7 +/- 6.31	9.1	18.2	11
				M	214.6 +/- 7.8	11.1	5.5	18
HdhQ250	950	250	paternal	F	263.3 +/- 7.9	7.1	7.1	14
				M	263.9 +/- 6.5	6.2	9.3	32
HdhQ315	1095	315	paternal	F	291.4 +/- 5.1	20.0	20	10
				M	293.8 +/- 3.9	21.4	21.4	14

Table 3 presents the repeat size and expected PCR product size for some of the knock-in mice created by Dr. Peter Detloff and colleagues. The incidence of expansion or contraction, expressed as a percentage of the number of mice assayed, is also provided when breeding using either male (M) or female (F) HET animals. Expansion or contraction was defined as CAG values falling outside +/- 1 standard deviation of the mean.

II.2. Breeding and husbandry of experimental animals

Recommendations: Select breeders by CAG repeat length, and control for maternal influence.

Some of the HD mouse lines present important breeding challenges. R6/2 females, for example, are generally sterile, with the exception of those with very low (<90) or very high (>300) CAG repeats, and consequently, many R6/2 colonies are maintained through the male line exclusively (Mangiarini *et al.*, 1996). However, transgenic male R6/2 mice have a short fertility window and, similar to what is observed in humans, the CAG repeat expands with paternal transmission (Kremer *et al.*, 1995; Mangiarini *et al.*, 1997). Fortunately, there is a viable alternative mating scheme in which wild-type females transplanted with ovaries from transgenic R6/2 females are bred to non-carrier (wild-type) males; this mating scheme is the preferred method for generating cohorts of experimental animals. As mentioned above, the CAG repeat length of the donor female and the offspring need to be monitored to avoid reductions in CAG repeat length in the colony. Ovarian-transplanted females have a breeding efficiency of around 30% - 50%. Ovarian transfers are routinely performed for the R6/2 mice at The Jackson Laboratory. Breeding challenges are also observed in the R6/1 line, in which male transgenic mice lose their fertility between 3-4 months of age (G. Bates, personal communication, Weller *et al.*, 2003).

No reported breeding deficits have been observed in the other HD transgenic mouse lines. Typically, transgenic male carriers are paired with non-carrier females, a breeding scheme that has multiple advantages: first, transgenic males can be bred to multiple females simultaneously—trios of 2 females and 1 male are advantageous—which allows breeding using a smaller number of mice. Further, males can be bred with new pairs of females in consecutive weeks to further maximize their productivity. Finally, breeding through the male germ line eliminates concerns regarding potential unknown maternal-infant interactions, since wild-type females are used.

Mice that are typically bred in large production colonies at JAX are bred in either trios or pairs, and males and females are co-housed while litters are born and throughout weaning to maximize breeding. Mice are weaned at 3 weeks +/- 3 days of age and no pre-wean discarding is performed to control for litter sizes. Littermates are often weaned together in holding cages along with mice from other litters. These practices are optimal for a large-scale production facility. At PsychoGenics, however, breeding mice for the purpose of conducting preclinical trials, especially those involving behavioral studies, have employed different considerations. Since preclinical trials often require a large number of mice born within a narrow time frame, PsychoGenics only includes mice born within 3 days of one another in each experimental cohort. This could be important for the R6/2 lines, where phenotype changes significantly from week to week. Experimental animals are selected from litters having 4 to 8 pups as another effort to control and standardize potential maternal effects. Culling of larger litters, if necessary, is done on postnatal day 3 in a way that allows for a balanced-sex litter.

For husbandry, moderate enrichment should be used in the breeding and holding cages (Hockly *et al.*, 2002). Additionally, mice should be group housed, preferably in mixed genotypes (gene-positive and littermate controls), unless it is not warranted under the specific experimental approach. This is currently not the practice for large-scale mouse production colonies at The Jackson Laboratory, where weaned mice are co-housed by sex and genotype with shaving bedding used as the source of enrichment. We recommend that upon receiving production scale HD mice from JAX, investigators acclimate the mice to their vivarium and adapt the husbandry conditions to those recommended in this manual as closely as possible (see Table 4). However, we recommend that male mice not co-housed at wean not be introduced to new males as this can prompt aggression and fighting. If regrouping of animals is necessary they should be closely monitored and separated in the event of fighting. Even then, some lines should not be regrouped given their aggressive nature (e.g., FVB).

As emphasized throughout this document, screening of CAG repeats in all breeders as well as in experimental animals is critical for genetic and phenotypic quality assurance.



Typical moderately enriched husbandry recommended for HD mouse models

II.3. Strain background

Recommendation: Use animals on a congenic strain or F1 hybrid genetic background in the preclinical testing battery.

Many HD mouse models are now available on different congenic strain backgrounds (see Table 1), allowing for the selection of mouse model and background strain combinations that are most suitable for the particular HD phenotype under evaluation. For example, if a behavioral testing battery includes cognitive tests that require the animals to use visual cues, BAC HD and YAC128 lines on the C57BL/6J background may be preferred over the same lines on the FVB/N background since these mice are homozygous for *Pde6b^{rd1}*, which causes retinal degeneration and blindness from an early age (Farley *et al.*, 2011). In addition, the relatively high aggression levels in FVB/N mice, especially males, cause problems during experiments that require long-term group-housing. Likewise, the C57BL/6J congenic background has its own limitations; for example, these mice develop age-related hearing loss and become deaf to certain frequencies as they age.

At the 2002 Hereditary Disease Foundation Workshop, panelists recommended using F1 hybrid animals for experiments, but maintaining the breeding lines in congenic backgrounds. Using F1 hybrid mice reduces the impact of homozygous recessive alleles that contribute to undesirable characteristics in each of the congenic parental lines. As long as the two congenic backgrounds are not homozygous for the same recessive alleles, the F1 hybrid mice produced from crossing them will be heterozygous for these loci and will not develop the confounding phenotypes.

Numerous preclinical studies have been conducted using R6/2 mice on the mixed B6CBA genetic background. A B6CBA mixed background is not the same as that of an F1 hybrid. F1 hybrids are the first-generation offspring from crossing two inbred or congenic strains of different genetic backgrounds—for example, the offspring of crossing C57BL/6J females with CBA/J males. When crossing hemizygotes on B6CBA mixed background with B6CBAF1/J, which is commonly done in with the R6/2 mice, any given allele may be homozygous for the C57BL/6 alleles, homozygous for the CBA allele or heterozygous. This can be a problem if the C57BL/6 or CBA strain used carries deleterious alleles. For example, while retinal phenotypes are not an issue for the CBA/Ca strain, the CBA/J strain carries the *Pde6b^{rd1}* mutation. Indeed, in our experience, approximately 30% of R6/2 offspring generated from hemizygote X B6CBAF1/J crosses are homozygous for *Pde6b^{rd1}*, which is homozygous in the CBA/J parental strain and profoundly affects animals motor and cognitive performance (Menalled *et al.*, 2012a). The effects of the *Pde6b^{rd1}* mutation can be overcome either by backcrossing the mutant B6CBA animals either to C57BL/6J wild-type mice or

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to B6CBA/CaJ F1 hybrid mice (JAX Stock Number 001201), which are the F1 hybrid offspring from crossing C57BL/6J females with males from the CBA substrain, CBA/CaGnLeJ, that does not carry the *Pde6b^{rd1}* mutation.

We also found that R6/2 mice on mixed B6CBA background are susceptible to seizures. Interestingly, seizures were not observed when the CBA/CaJ substrain was used to generate transgenic animals by crossing hemizygous on a C57BL/6J congenic background to CBA/CaJ mice. While it is not clear if the lack of seizures observed in these animals is due the CBA/CaJ substrain itself or by virtue of working in an F1 experimental animal, this breeding scheme has been demonstrated to eliminate seizures in the R6/2 experimental mice.

Multiple R6/2 transgenic lines are available on congenic C57BL/6J backgrounds (see Table 1). To minimize genetic variability in preclinical trials, these mice can be used “as is” and maintained by backcrossing to C57BL/6J to produce cohorts of hemizygous and wild-type littermates. Alternatively, they can be crossed to inbred CBA/J, CBA/CaJ or CBA/CaGnLeJ mice, for instance, to produce true F1 hybrids that may show less phenotypic variability than mice produced from crossing R6/2 hemizygotes with B6CBAF1/J hybrid partners (Menalled *et al.*, 2012a).

Regardless of which mating scheme and strain backgrounds are chosen, the background is likely to affect the HD phenotypes that the mice manifest (Lloret *et al.*, 2006; Menalled *et al.*, 2009a; Van Raamsdonk *et al.*, 2007). For this reason, it is absolutely critical to include a thorough description of the genetic background and the mating schemes used to propagate them, when reporting results in preclinical testing studies.

It is noteworthy that the availability of HD mouse models on congenic backgrounds enables the search for genetic modifiers of the HD phenotype. As we have described above, the crossing of congenic HD mice (R6/2 mice on a congenic C57BL/6 background, for example) to an unrelated inbred strain, such as DBA/2J, creates an F1 hybrid strain whose phenotypes can be compared with the parental congenic strain. Further backcrosses to DBA/2J or intercrosses between the F1 mice to produce F2s can be used to begin to map, with quantitative trait locus (QTL) analysis, the locations of genes that may contribute to any phenotypic differences observed between the F1 and the parental C57BL/6 congenic strain. Additionally, information regarding potential modifying genes can be uncovered by backcrossing congenic HD transgenic and knock-in lines to BxD recombinant inbred (RI) panels. Because each member of an RI panel carries a unique complement of alleles from each parental strain, and the regions of the genome contributed by each parent are mapped, regions of the genome that contain candidate modifying alleles often can be identified readily for further analysis (Cowin *et al.*, 2012).



Part III. Preclinical research with HD mouse models: study design considerations

Selection of mouse model and design of a battery for preclinical testing

As mentioned in Part I and indicated in Table 1, HD mouse models vary with respect to the specific aspects of the human disease that they recapitulate. Each model is valuable but has limitations, and the model you select for use in preclinical testing phases will depend on the molecular targets of the intervention and the outcome domain(s) under investigation. The reasons why positive results from preclinical trials fail to translate successfully to clinical trials may have more to do with methodological deficits than to problems inherent with the mouse models themselves. This assertion is supported by the inability of several groups to reproduce the beneficial effects reported for some therapies, even when using the same mouse models. (Chen *et al.*, 2000; Ferrante *et al.*, 2002; Menalled *et al.*, 2010; Smith *et al.*, 2003; Stack *et al.*, 2006). Positive findings should be replicated both within laboratories and across laboratories prior to the HD research community accepting that a positive preclinical result is accurate. Further, expert panels, NINDS (Landis *et al.*, 2012), and CHDI Foundation have recently recommended that positive therapeutic efficacy be demonstrated in at least two different animal models of HD prior to considering clinical testing. Therefore, standardization of preclinical testing methods is critical in order to generate reliable and reproducible results (Hockly *et al.*, 2003). In addition, it is crucial when reporting results of preclinical trials to include detailed descriptions of mouse origin, husbandry conditions, CAG repeat length, and the experimental protocols followed, as well as how the data are collected, analyzed and reported. We hope that the recommendations provided below will improve the comparisons of results between laboratories, and aid in uncovering therapies that may be more translatable into successful trials (Table 4). These recommendations are in alignment with the NINDS and CHDI Foundation visions, and with the continuing efforts of the HD research community to execute meaningful preclinical trials (Bates and Hockly, 2003; Hockly *et al.*, 2003; Landis *et al.*, 2012).



Table 4. Standards for preclinical testing allowing robust and comparable inter-laboratory results¹

Animals and husbandry	
Genetic background	<ul style="list-style-type: none"> • Report strain and vendor. • When possible, use congenic lines and/or F1 background lines.
Sex	<ul style="list-style-type: none"> • Include both females and males².
Sample size	<ul style="list-style-type: none"> • Justify by power analysis for the measure(s) of interest.
Environmental enrichment and husbandry	<ul style="list-style-type: none"> • Utilize moderate enrichment conditions by including nesting material, shredded paper, plastic bones, and play tunnels. • Keep experimental animals group-housed unless prevented by severe male aggression or by testing protocols (e.g. food scheduling). Report incidence of separation of animals into single-housing cages (due to fighting, etc) during each trial. • Within each line, house animals in balanced, mixed-genotype groups. Tailor husbandry to line needs (e.g., provide wet feed to R6/2, minimize noise to prevent audiogenic seizures if these occur in a given colony, etc.).
Breeding scheme	<ul style="list-style-type: none"> • Genotypes to be studied should be generated in the same litter to ensure comparable uterine environment and maternal influences.
Experimental design	
Animals' assignment	<ul style="list-style-type: none"> • Assign animals in a (semi-) randomized fashion such that: <ul style="list-style-type: none"> ◦ Animals from each litter are distributed across the different treatment groups. Animals from litters with less than 3 pups should not be included. ◦ CAG repeat lengths are comparable in all mutant mice across the different experimental groups. ◦ If both females and males are included, sex is balanced across experimental groups • If baseline behavioral data are collected before treatment is initiated, distribute animals across the treatment groups in a balanced way to allow comparable baseline behavioral performance and body weight across the groups. • Report the allocation method followed.
Blinding	<ul style="list-style-type: none"> • Experimenters should not be aware of genotype and treatment when administering the treatment, testing the mice, or analyzing the data.
Readouts included in the battery	<ul style="list-style-type: none"> • Select key readouts from the phenotype that are relevant to the clinic and are meaningful to the treatment under evaluation. • If survival is included as an endpoint, include a clear description of how it is assessed (e.g., surrogate markers vs. actual death).
Data analysis	<ul style="list-style-type: none"> • Define <i>a priori</i>, what constitutes an outlier and what the disposition of the outlier will be in the experiment. • Define in advance events that will warrant removing an animal from a treatment group.
Statistical analysis	<ul style="list-style-type: none"> • Justify statistical tests for each readout evaluated prior to the start of the experiments (please see section III.12 for details).
Evaluation of data validity	<ul style="list-style-type: none"> • In the absence of positive controls, compare the performance of wild-type and mutant vehicle-treated groups with groups run previously to confirm that animals performed as expected.
Histology	<ul style="list-style-type: none"> • Utilize unbiased stereological methods when quantifying features (i.e.: inclusion bodies, neurons, etc) (Glaser <i>et al.</i>, 2012).
Replication of effects	<ul style="list-style-type: none"> • Independent replication. Include in the manuscript precise and comprehensive descriptions of the animals, husbandry conditions, protocols used, as well as the lighting conditions and phase of the diurnal cycle utilized for all the behavioral endpoints. Report how the data are analyzed and reported to facilitate the replication by independent research groups. • It is recommended that potential therapies be tested in a second mouse model either by the same or different research group.
Additional outcome measures	<ul style="list-style-type: none"> • Consider other measures that can be included: gene profiling, proteomics, histopathology (cell number, volumetric analysis, neuron morphology, synapse loss), MRI, MRS etc.

¹ The standards presented in this table adhere to and expand the core set of standards proposed by NINDS (Landis *et al.*, 2012).

² Sex differences have been observed in numerous endpoints of interest (i.e.: survival, motor deficits (Dorner *et al.*, 2007; Menalled *et al.*, 2012b; Hickey *et al.*, 2005), immobility time in forced-swimming test (Renoir *et al.*, 2012), social behaviors (Pietropaolo *et al.*, 2011), response to environmental enrichment (Ransome and Hannan, 2013; Wood *et al.*, 2010; Zajac *et al.*, 2010). In addition, sex differences were also observed in pharmacokinetic, neuropathology, etc. (Menalled *et al.*, 2010). Not surprisingly, responses to therapeutic interventions were different between sexes (Du *et al.*, 2012; Ma *et al.*, 2007; Reiner *et al.*, 2012; Renoir *et al.*, 2012; Wood *et al.*, 2010; Wood *et al.*, 2011). Therefore, preclinical studies should always include both sexes, and sex should be included as a factor in the data analysis.

III.1. Litters

Recommendation: Wean experimental animals at the same age into cages containing animals from multiple litters, segregated by genotype.

Animals for the experimental groups should be weaned at the same age (optimally ± 1 day), and mice from each litter should be distributed across the different experimental groups. If more than one animal per litter must be allocated in one experimental arm, the animals should be of different genotypes. No more than two animals from one litter should be allocated into one experimental group. These recommendations will reduce effects of litter origin on the experimental results (Hockly *et al.*, 2003). Experimental cohorts with mice from multiple litters should be established at weaning because aggressive behavior at older ages is likely to prevent group-housing. (Mice group-housed after weaning, especially males, are more intolerant of other mice and often fight aggressively.)

III.2. Environmental conditions

Recommendations: Group-house experimental animals by mixed genotypes, with moderate enrichment. Provide lines known to present with limited mobility and dehydration issues (e.g., R6/2) with lowered water bottle spouts and mashed wet food supplementation.

The beneficial effects of environmental enrichment in rodent cognitive and motor performance are well known (van Praag *et al.*, 2000). In the HD field, numerous studies using R6/2 and R6/1 also have shown that offering even minimally-enriched living conditions (e.g., providing group-housed animals with a play tunnel) improves the animals' performance and promotes the survival of the mutant mice (Carter *et al.*, 2000; Hockly *et al.*, 2002; van Dellen *et al.*, 2000; van Dellen *et al.*, 2008). Since the goal is to find robust therapeutic agents, we recommend maintaining the animals under group-housed conditions with a moderate level of enrichment (e.g., play tunnel, plastic bone and shredded paper) for preclinical testing purposes. Exceptions for the enrichment practices must be judged on an individual basis. For instance, single-housed mice may be needed for experiments aimed at measuring circadian activity or testing protocols requiring food scheduling. Also, mice coming from production colonies (i.e. JAX) should be acclimated and adapted to the specific conditions at institutional vivaria.

For some lines, like R6/2, where motor deterioration can result in poor nourishment (Carter *et al.*, 2000), additional enrichment provisions are recommended. For these mice, providing water bottles with lowered water spouts and mashed wet food supplementation ensures continued access to water and food as the disease conditions worsen. Mashed wet food should be added to the cages daily. It can be prepared by mixing crushed diet with water to form a slurry, or purchased from commercial rodent diet vendors, such as Bio-Serv (www.bio-serv.com) or ClearH2O (www.clearh2o.com). In addition to providing food and water, supplementary feeding provides entrainment of circadian behaviors (Maywood *et al.*, 2010). HD mouse lines that do not show profound motor deterioration and significant weight loss may not need these additional measures to remain healthy.

The facilities where the animals are housed must follow the guide for the care and use of laboratory animals (National Research Council, 2011) to ensure that the mice are maintained under suitable temperature, humidity, and air-quality conditions, that they are provided standardized chow and water; and that their exposure to pathogens is limited. At JAX, animals are kept using a 14-hour light/10-hour dark cycle. In most research facilities, a cycle of 12 light/12 dark is commonly used. Therefore, animals imported from JAX need to be allowed to acclimate to the light-cycle condition before testing starts. For certain experiments or paradigms, different light-cycle conditions may be required (e.g.; circadian experiments). The description of the lighting conditions used should be included when reporting data. Finally, it is important to maintain low noise levels during both testing and non-testing conditions; R6/2 mice in particular are prone to audiogenic seizures on some genetic backgrounds, such as mixed C57BL/6J x CBA/J (Cepeda-Prado *et al.*, 2012; Cummings *et al.*, 2009), and extra care should be taken when handling them during routine maintenance and testing to minimize this phenomenon's incidence.

III.3. Sex

Recommendation: Include equal numbers of female and male mice in each experimental arm.

Sex-specific differences in HD mouse model phenotypes have been reported; for example:

- In the R6/2 (CAG 120) N-terminal transgenic model, analysis of more than 200 transgenic mice revealed that male hemizygotes have a shorter life span than females (Mean survival \pm S.E.M.: males, 116.50 ± 2.02 days; females, 127.00 ± 2.73 days; log-rank test, $p < 0.005$, unpublished observations).
- In the BAC HD full-length transgenic model, motor deficits detected in open-field behavioral assays were more severe and robust in mutant females than in mutant males (Menalled *et al.*, 2012b).
- In the zQ175 knock-in mouse, female but not male homozygotes demonstrated hypoactivity in open field tests during the light phase of the diurnal cycle (Menalled *et al.*, 2012b).

These examples highlight the importance of using both female and male mice in preclinical drug testing studies. Using sex-balanced groups may require significant extra effort, especially for R6/2 lines that must be maintained by breeding sub-fertile transgenic males or ovarian-transplanted, hemizygous females. Exceptions to this recommended practice should be made only under specific experimental circumstances.

III.4. CAG repeat

Recommendation: When using lines with unstable CAG repeat length, quantify the CAG repeat number in all the experimental animals; all animals in all experimental groups should carry comparable CAG repeat sizes.

As discussed previously, the CAG repeat is unstable in various N-terminal transgenic mouse lines and in some knock-in mouse lines so it must be quantified in every experimental animal before it is assigned to a test group. Also, the CAG repeat of the animals in each treatment arm should be comparable. CAG repeat sizing of HD mice should be done using high-resolution methods as described above. Assays based on agarose gel electrophoresis typically do not provide sufficient resolution to accurately measure CAG repeat numbers. If labs do not have access to the appropriate equipment for determining CAG repeat length, CAG repeats can be evaluated on a fee-for-service basis by Laragen Inc. (www.laragen.com).

As discussed in Part II, BAC HD and YAC128 mouse models typically have a stable polyglutamine tract (Gray *et al.*, 2008), so routine monitoring of CAG tract size in every animal is not mandatory.

III.5. Sample size

Recommendation: Use power analysis to determine the sample size needed for the different readouts to ensure meaningful statistical results.

Since preclinical testing is costly, time consuming, and labor intensive, it is important not only to conduct testing rigorously but also to use an appropriate sample size to detect the significance of any effects observed in the readouts of interest. Running experiments with a smaller sample size than the one calculated by statistical power carries a greater risk for obtaining a false negative result. To calculate the sample size, power analysis should be conducted on data collected previously for each of the primary endpoint measures.

III.6. Animals' assignment to experimental arms and blinding

Recommendation: Make sure experimenters are blind to animals' genotype and treatments.

As mentioned previously, animals from each litter should be assigned to different experimental groups to avoid litter-of-origin effects. Also, the different experimental groups should be matched in terms of CAG repeat size, sex, and number of animals per cage. Further, if baseline measures are taken before treatment is started (body weight or behavioral performance, for example), the distribution of the animals across the experimental groups should be adjusted to allow the different arms to be matched by the features measured. Experimenters collecting and evaluating data should be blind to the treatment that was administered to each group and to the animals' genotype, assuming that data from both mutant and wild-type mice are to be evaluated.

III.7. Behavioral batteries

There is no single, universally-valid testing battery to evaluate behavior in HD mouse models. Many different tests have been performed by different laboratories in different HD models. It is beyond the scope of this field guide to review all of the tests and phenotypes that have been observed. Rather, we choose to highlight the major domains worth pursuing in HD mice and to point to specific precautions that we are aware of for each. Behavioral batteries should be organized as best as possible around the domains expected to be modified by the treatment. Age of testing depends on deficits detected in the domain of interest in the specific line. When reporting data, include a detailed description of the protocol followed as well as the lighting conditions and phase of the diurnal cycle in which tests were performed.

Below are recommendations on the most commonly used behavioral endpoints:

- a) **Open-field locomotion.** Open-field tests evaluating spontaneous locomotor activity, exploration, and rearing activity are among the most valuable tests to include in a behavioral battery. Open-field tests can report on overall motor activity, an important phenotype in HD mouse models. As well as evaluating the effect of a therapeutic approach on the motor activity deficits of the HD model under investigation, the open-field test may also unmask undesired side effects (for example, sedation) associated with a dosage or compound. Along these lines, including open-field tests in studies to investigate dose selection may reveal dosing thresholds under which any undesired locomotor side effects are no longer observed.

Available automatic open-field systems (square, circular, or rectangular arenas) use photocell beams, video tracking, or electromagnetic detector technologies to detect and track animals' activity. Testing provides information regarding the activity of the animals in a novel environment (in the first five minutes of a session) and also allows observation of the animals' behavioral profile as the environment becomes more familiar (in a 30- to 60-minute test). Testing can be scheduled during either the dark or the light phase of the diurnal cycle. Rodents are more active during the dark phase of the diurnal cycle; therefore, it is not surprising that discrimination between genotypes improves during testing in the dark phase, under dim red lights (Hossain *et al.*, 2004; Menalled *et al.*, 2012b). This is of particular importance when working with knock-in HD models, in which testing in the dark phase helps unveil more robust motor deficits (Hickey *et al.*, 2008; Menalled *et al.*, 2012b; Menalled *et al.*, 2003). To avoid the inconvenience of testing at night, colonies can be maintained under reverse light cycles. Some lines, such as R6/2, present deficits that are so profound and severe that there is enough statistical power to allow testing during the light phase of the diurnal cycle. For studies in which evaluation is performed during light cycle, standard and uniform lighting conditions should be used and properly reported in such studies.

Regardless of diurnal cycle considerations, the open-field chambers must be cleaned in between the testing of animals and at the end of the testing day to prevent the odors of urine and feces from previous mice from influencing the behaviors of the next mouse.

Our analyses of the BAC HD line suggest that the decreased locomotor activity observed in the mutant mice during open-field tests is influenced by their increased body weight relative to controls (Kudwa *et al.*, 2013; Menalled *et al.*, 2009a). Our data suggest that activity results in the open-field should be interpreted with caution in mouse models where body weight increases due to transgene overexpression (YAC128 and BAC HD mice).

b) Automated home-cage analysis. Automated home-cage analysis systems allow the testing of animals under non-stressed conditions, thereby eliminating any subjectivity or bias. Food and water are provided in the system, permitting the testing to go over multiple days. This allows examination of behaviors during both the light and dark phases of the diurnal cycle. As mentioned above, because mice are nocturnal animals, home-cage activity typically is higher during the dark period; therefore, it is the best period during which to detect deficits and treatment-mediated amelioration in lines with only modest abnormalities (e.g., knock-in lines). Home-cage systems also allow the quantifying of animal activity during periods of peak activity, namely when room lights go off and when they go on, without experimenters' interference or the influences of a novel environment, as occurs when mice are transferred to the unfamiliar open-field system. The systems available allow the evaluation of single-housed animals in home cages (HomeCageScan) (Pallier and Morton, 2009; Rose *et al.*, 2009), or in home-cages with running wheels, or with telemetry receivers with metabolic readouts (PhenoMaster), as well as group-housed animals (IntelliCage and PhenoCube®). PhenoCube® is a high-throughput platform that assesses circadian, exploratory activity, and social and motor behavior exhibited by group-housed mice. The PhenoCube® system is built on IntelliCage units, significantly modified in order to apply computer vision to allow the tracking of individual animals at all times and to determine the type of behavior they display. We have used the PhenoCube® to show circadian deficits in BAC HD and young R6/2 mice (Balci *et al.*, 2013; Oakeshott *et al.*, 2011a).

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- Cryorecovery services
- Sponsored strain distribution

- c) **Grip strength.** In patients with HD, grip strength and grip force coordination are impaired and continue to decline with disease progression (Kouider *et al.*, 2010; Quinn *et al.*, 2001; Reilmann *et al.*, 2001). In many HD mouse models, deficits in grip strength have been detected (Menalled *et al.*, 2009a). Grip strength can be indirectly assessed by measuring the latency for a mouse to fall while hanging from wire or from a mesh/cage lid that the investigator turns upside down. Mice are held about 20 cm from the bench top. Wild-type mice often are able to hold for a few minutes, but a limit is usually set at 60 seconds. In addition, systems are available that are able to determine actual muscle strength by measuring the force required to pull the mouse away from a triangular bar or a metal grip. These systems provide reliable and robust grip strength assessments and, therefore, are recommended for preclinical testing. Usually, testing requires multiple trials at each time point (3 to 5 consecutive measures). Using an experienced experimenter can reduce variability.



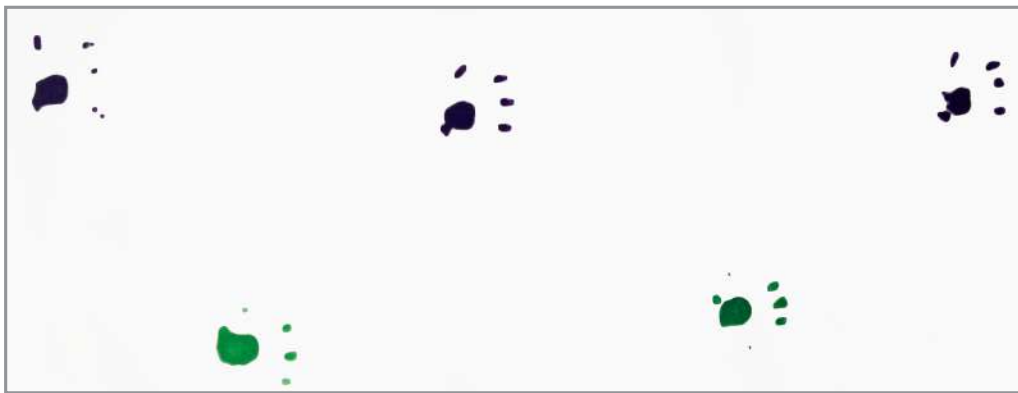
Muscle weakness in HD mouse models can be measured by grip strength meter

- d) **Rotarod.** Rotarod testing can be done using accelerating or constant-speed protocols. Accelerating protocols are recommended with HD mouse models because they typically reveal deficits at younger ages compared with constant-speed protocols (Pallier *et al.*, 2009). In a well-developed accelerating protocol, all mice have to fall from the rods during testing, providing data that permit parametric statistical analysis (Hockly *et al.*, 2003). In addition, the rods in a properly calibrated apparatus must be smooth enough to prevent animals from clinging to them as a strategy to avoid performing at challenging speeds, but at the same time, must provide enough traction to prevent animals from slipping off the rods while running. Rotarod equipment is available from multiple vendors, and some modifications of the rods may be needed (Hockly *et al.*, 2003).

In each testing day the recommended testing protocol has a training trial at a constant speed of 4 RPM for 5 minutes, followed by a resting period of at least 1 hour, followed by 3 consecutive accelerating 5-minute trials with the speed changing from 0 to 40 RPM during each trial. An inter-trial interval of at least 30 minutes is recommended. The latency to fall from the rod during the testing trials is measured and averaged per mouse per day. Testing during the dark or light phase of the diurnal cycle has not been shown to affect the performance of zQ175 animals in this test (Menalled *et al.*, 2012b). Ages and frequency of testing need to be tailored to the mouse model of interest. R6/2 lines, for example, have an early onset of deficits with a faster progression than both

full-length transgenic or knock-in animals, and as a result they require testing at shorter intervals. Furthermore, we found that in R6/2 (CAG 110) B6CBA mice that are susceptible to seizures, testing at ages older than 10 weeks induced fatal seizures in a subset of animals when the CBA/J strain is used to generate the genetic background of B6CBA. Another important consideration when including rotarod in a behavioral testing battery is body weight. Body weight can affect rotarod performance and it has been demonstrated that the rotarod deficits detected in the BAC HD mutant animals are partially due to their increased body weight (Gray *et al.*, 2008; Menalled *et al.*, 2009a). The effect of treatment on body weight should be assessed when using rotarod performance as an endpoint measure during the evaluation of potential therapies in HD mice.

- e) **Gait analysis.** Gait disturbances are characteristic of HD patients, and gait abnormalities have also been observed in many HD mouse models (e.g., R6/2, BAC HD, YAC128, HdhQ111, HdhQ150, zQ175, and CAG 140 KI; Carter *et al.*, 1999; Heng *et al.*, 2007; Lin *et al.*, 2001; Menalled *et al.*, 2009a; Menalled *et al.*, 2003). In those studies footprint analysis was used to measure gait; animals' forelimbs and hindlimbs were painted with nontoxic paint, they were placed at the beginning of a long, narrow alley and as the animals walked their footprints were recorded on the paper placed on the alley floor - stride length, variability, width and/or overlap were then measured. Today, several automatic systems are available to assess gait, under either forced walking (Digigait, GaitScan, TreadScan) or free walking conditions (CatWalk, GaitScan, Runway, NeuroCube®). Most, if not all, of these systems can record data similar to that obtained in a traditional footprint test and can analyze also gait dynamics. Deficits in the BAC HD as well as CAG 140 and zQ175 KI mouse lines have been observed using the CatWalk system (Abada *et al.*, 2013) and the NeuroCube® system, respectively (in preparation).



Gait disturbances are common in HD mouse models

- f) **Beam tests.** Balance beam tests assess fine motor deficits in HD mouse models. Deficits in beam tests have been observed in several mouse lines, including R6/2, R6/1, N171-82Q, YAC128, zQ175, and HdhQ150 knock-in mice (Brooks *et al.*, 2012a; Brooks *et al.*, 2012b; Brooks *et al.*, 2012e; Loh *et al.*, 2013; Southwell *et al.*, 2009). A ledged, tapered balance beam (modified from the original version created by Tim Schallert (Schallert, 2006)) allows the measuring of an animal's latency to turn, latency to transverse, and number of foot slips. The ledge provides a place for the animal to step when a foot slips and prevents the development of compensatory mechanisms that might alter the motor performance of the animals. In comparison with rotarod, beam tests may be less sensitive at measuring motor coordination; deficits often are not detected in beam tests until after deficits in rotarod have appeared. Nevertheless, once detected,

beam deficits have been observed to worsen with time (Brooks *et al.*, 2012a; Brooks *et al.*, 2012b; Brooks *et al.*, 2012e; Loh *et al.*, 2013).

- g) Cognitive testing.** Including relevant cognitive testing protocols in preclinical testing has become a top priority for HD investigators recently because cognitive deficits appear early during disease progression and constitute a heavy burden for HD patients and their families. Much progress has been made in detecting relevant deficits in various domains, including temporal information processing, response acquisition and inhibition, reversal learning, and set-shifting tasks (Brooks *et al.*, 2012d; Brooks *et al.*, 2012f; Morton *et al.*, 2006; Oakeshott *et al.*, 2011b; Trueman *et al.*, 2012a; Trueman *et al.*, 2012b). When developing tests or assessing cognitive deficits in HD mouse models, researchers should keep in mind the particular limitations of the model being used and understand how these limitations may confound cognitive testing results. Understanding a model's limitations will help in selecting the appropriate experimental protocols to use with a particular line. For more specific information about cognitive testing in HD mice please contact PsychoGenics at NDG@psychogenics.com.

Below are some of the limitations associated with specific HD mouse models or with specific tests that should be considered when choosing cognitive testing protocols.

- i. A variety of water maze tasks have been designed to test cognitive function in rodents; some examples include the two-choice swim tank test, T maze test, and Morris water maze test. In these tests, animals are trained to reach a submerged escape platform. Although many HD models present significant motor deficits, most animals can still swim at the ages used for testing. Because swimming speed may be reduced in HD mutants, however, it is important that conclusions regarding performance be based on choice endpoint measures and not solely on the latency to reach the platform.
- ii. In water maze tasks, the temperature of the water must be controlled throughout the experiment. Mice need to be dried and placed on a heat pad after testing to avoid hypothermia. Investigators working with R6/2 mice should know that these mice demonstrate metabolic deficits that prevent them from controlling body temperature as efficiently as their wild-type littermates. Therefore, due to the effect of water temperature, these animals' cognitive performances may differ from those of controls, even in the absence of any real cognitive deficits. The existing literature suggests, however, that only severe hypothermia significantly effects cognitive performance in the mice (Panakhova *et al.*, 1984; Santucci and Riccio, 1986).
- iii. Visual acuity is needed in many cognitive assays (e.g., touchscreen assay, Morris water maze). As mentioned in Part II, approximately 30% of the R6/2 (CAG 120) mice maintained on the C57BL/6J x CBA/J mixed background are homozygous for the *Pde6b^{rd1}* mutation, which causes retinal degeneration and blindness and affects motor and cognitive performance (Menalled *et al.*, 2012a). Suggestions for overcoming this phenotype via careful breeder selection are described above. Other HD mouse lines, including the BAC HD and YAC128 lines, are congenic on the FVB/N genetic background, which also carries *Pde6b^{rd1}* and suffers from retinal degeneration. Fortunately, congenic strains on the C57BL/6 genetic background, which does not carry the *rd1* allele, exist for both of these transgenics, and the use of the latter strains should be considered when testing these models in vision-based protocols.

- iv. In assays that require training mice to go toward a light, (e.g., two-choice swim tank test), investigators should consider that differences in anxiety levels between mutant and wild-type controls may confound the animals' performances and the conclusions drawn from them. Standard practice in experimental psychology is to always counterbalance across subjects for all stimuli used for training and to avoid pairs (or sets) of stimuli that animals do not find equally preferable. Therefore, using mutants and controls that show differences in anxiety behavior may produce uninterpretable results when using the light as a cue in cognitive tests.
 - v. Tone is used in cognitive assays, such as in fear conditioning, and it is contraindicated when examining R6/2 mice if the particular colony is susceptible to audiogenic seizures. With this model, only context fear conditioning should be assessed.
 - vi. A major symptom in neurodegenerative disorders is apathy, which equates with what is termed "incentive motivation" in experimental psychology. Motivation is an important domain within cognition because it energizes and increases the chances that a response will happen. It is important, therefore, to consider motivation as an underlying process when testing for other cognitive domains such as executive function (Oakeshott *et al.*, 2012).
- h) Affective phenotype analysis.** Patients with HD exhibit affective symptoms. Numerous research groups have reported emotional/affective abnormalities in various HD mouse models using forced swim, tail suspension, elevated plus or zero maze, etc. (Abada *et al.*, 2013; File *et al.*, 1998; Naver *et al.*, 2003; Orvoen *et al.*, 2012; Pang *et al.*, 2009; Pouladi *et al.*, 2009; Renoir *et al.*, 2011). Currently there is no consensus in the field regarding which of these assays should be used in preclinical testing since the interpretation of results from some of these tests is not straightforward.

III.8. Body weight

Recommendation: Include body weight in every preclinical battery.

Body weight is an important metric and should be measured at least weekly during the evaluation of new therapeutic interventions for HD. Because HD patients often lose weight as the disease progresses, body weight, itself, can be used as an endpoint. Indeed, many models (e.g., R6/2, R6/1, N171-82Q, zQ175 KI, HdhQ111, and CAG 140 KI) recapitulate the reduced body weight characteristic of human HD. Knock-in models present mainly a failure to gain weight, the onset of which varies from model to model (Figure 4). In the fragment models, a failure to gain weight is followed by a weight-loss phase (Figure 4). Therefore, therapies that halt, delay, or ameliorate the characteristic weight loss in such HD mouse models could be promising. Weight loss, however, might also be an undesirable side effect of a specific treatment and needs to be reported when observed. As mentioned above, most of the full-length transgenic HD mouse models, such as BAC HD and YAC128, present significantly increased body weight (Gray *et al.*, 2008; Slow *et al.*, 2003), which has been found to negatively impact rotarod performance and locomotor activity assessments (body weight curves of the BAC HD line are shown in Figure 4). Consequently, the effects of treatments on the performance of BAC HD and YAC128 lines in these tests need to be considered in the context of their effects on body weight.



Weight gain is observed in both the YAC128 and BAC full length transgenic animals. This image of the in YAC128 transgenic mouse on a C57BL/6J background compared to a wild-type control mouse illustrates the difference in size of these transgenic animals.

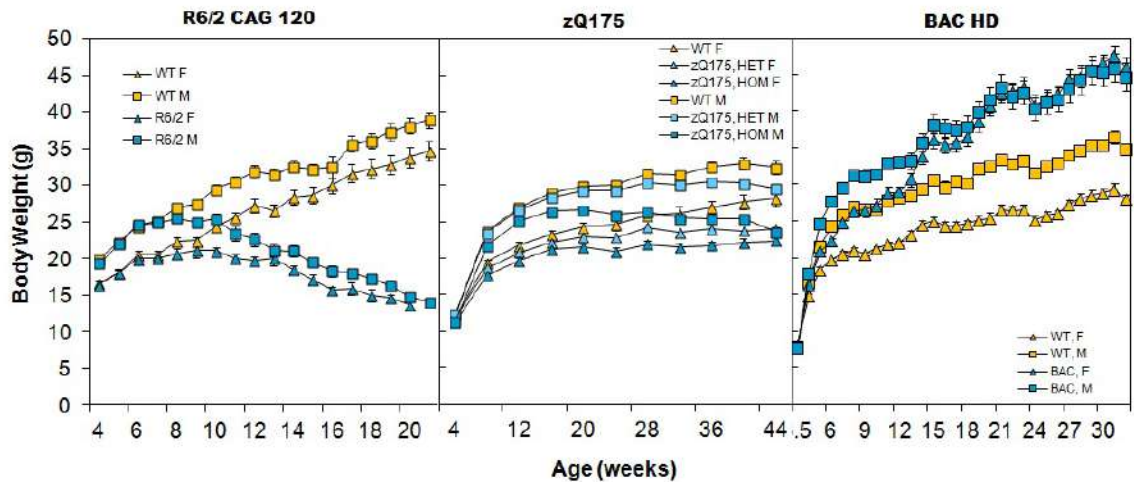


Figure 4: Body weight curves for the R6/2 CAG 120, zQ175 and BAC HD mice (Menalled et al., 2009a; Menalled et al., 2012b).

III.9. Survival

Recommendation: When death can't be used as an endpoint in survival analysis, caution is needed to select a surrogate marker for survival that predicts death reliably.

A compound's ability to improve survival is a valid outcome to measure in those mouse models presenting with premature death. The criteria used to assess survival, however, can vary greatly from one study to the next. Criteria that have been used include the loss of the animals' responsiveness to tactile stimulation, reduced responses to the opening of the cage, decreased body weight or body temperature, longer latencies in the righting reflex, and outright death (lack of heartbeat). When choosing a surrogate marker for survival it is important to consider its ability to reliably predict death. Surrogate markers, such as the lack of response to gentle prodding, lower body temperature, and reduced responses to the opening of the cage may not predict death reliably. Naturally, outright death as the endpoint when survival is included in the trial is most optimal. Death as an outcome, however, often is not acceptable in many institutional vivaria, particular if it is preceded by a prolonged period of moribundity. Animal care and use protocols often require that moribund animals be euthanized immediately, which can affect survival endpoint data. Under such circumstances, surrogate markers have to be employed, and care must be taken to apply them as consistently as possible and to thoroughly describe them when publishing results. In these cases where surrogate measures are employed, we recommend that the data are published as "endstage disease" rather than "survival".

III.10. Molecular and histological outcomes

In addition to behavioral analyses, molecular and histopathology outcomes should be considered in preclinical studies with HD mouse models. Because the specific parameters measured are highly dependent on the nature of the preclinical study, we will not focus on the specific merits of any particular measurements. Instead, we simply summarize the molecular and histological measurements that are most commonly used in assessing treatment efficacy in HD mouse models, as well as some important considerations to their use.

- a. Soluble and aggregated huntingtin (HTT) protein levels.** Measuring mutant and normal soluble HTT protein levels in the HD mouse models, especially in experiments evaluating the preclinical efficacy of candidate therapeutic compounds, is very important. Any effect of a compound on reducing mutant HTT expression levels could potentially effect the animal's disease status or subsequent disease progression. Such measurements have not been reported often in the literature on preclinical studies with HD mice, perhaps due to inherent difficulties in quantitatively assessing soluble or aggregated mutant and normal HTT protein levels from mouse tissue. Recently, however, multiple HTT protein assay systems that overcome these difficulties have been developed. These systems include a TR-FRET assay (Baldo *et al.*, 2012) and a Meso Scale Discovery assay to measure soluble HTT protein levels (in preparation). HD investigators interested in these assays should contact CHDI. Several assay platforms are also now available to quantify aggregated mutant HTT protein in tissues, including TR-FRET (Baldo *et al.*, 2012), Seprion assay (Sathasivam *et al.*, 2010), and AGERA (Weiss *et al.*, 2007) assays.
- b. Gene expression analyses.** Expression profiles of genes known to be dysregulated in HD mouse models, including *Darpp32*, *Drd2*, *Cnr1*, *Pde10a* in the striatum and *Bdnf* promoter transcripts in the cortex, can be run as molecular markers of disease. QPCR methods to assess expression have been well described for these and other genes known to be dysregulated in the HD mouse models (Benn *et al.*, 2008; Cha *et al.*, 1999; Cha *et al.*, 1998). In addition, measuring *Htt* mRNA levels using qPCR or alternative methods, such as branched DNA assay platforms is recommended to evaluate therapeutic intervention on *Htt* transcription levels (Kordasiewicz *et al.*, 2012). Global gene expression analyses, such as Affymetrix microarrays or RNAseq, can also be conducted to examine the effect of a therapeutic intervention on gene networks (Borovecki *et al.*, 2005; Kuhn *et al.*, 2007) .
- c. Histopathology: brain regional volume and neuronal cell counts.** Histopathology analyses routinely used to evaluate therapeutic outcomes in HD mouse models include staining with antibodies such as EM48 or S830 (Bayram-Weston *et al.*, 2012a; Bayram-Weston *et al.*, 2012b; Bayram-Weston *et al.*, 2012c; Bayram-Weston *et al.*, 2012d; Moffitt *et al.*, 2009; Van Raamsdonk *et al.*, 2005a), which reveal intranuclear and extranuclear aggregates/inclusion bodies in different brain regions (Figure 5, Table A1). The histological methods for aggregate staining are generally not useful as quantitative measures when compared to the TR-FRET or Seprion assays mentioned above. The distribution of aggregates throughout the mouse brain is extremely complex. However, immunohistochemistry is useful for visualizing any gross changes to aggregate distribution.

Measurement of brain regional volumes (e.g., striatal, cortical, hippocampal and whole brain volumes) using MRI or histological methods has been well described for HD models and invariably show regional decreases as a function of gene dosage and CAG repeat length (Aggarwal *et al.*, 2012; Carroll *et al.*, 2011; Cheng *et al.*, 2011; Sawiak *et al.*, 2009). Further, some investigators have measured neuronal cell counts and have documented decreases in HD mouse models, most notably in R6/2, N171-82Q, zQ175 and YAC128 mice (Heikkinen *et al.*, 2012; Hickey *et al.*, 2008; McBride *et al.*, 2006; Slow *et al.*, 2003; Stack *et al.*, 2005). Such measures are best done using unbiased, stereoscopic methods to accurately count cells in brain regions, thereby allowing the analysis of therapeutic interventions on these measures (see Heikkinen *et al.*, 2012).

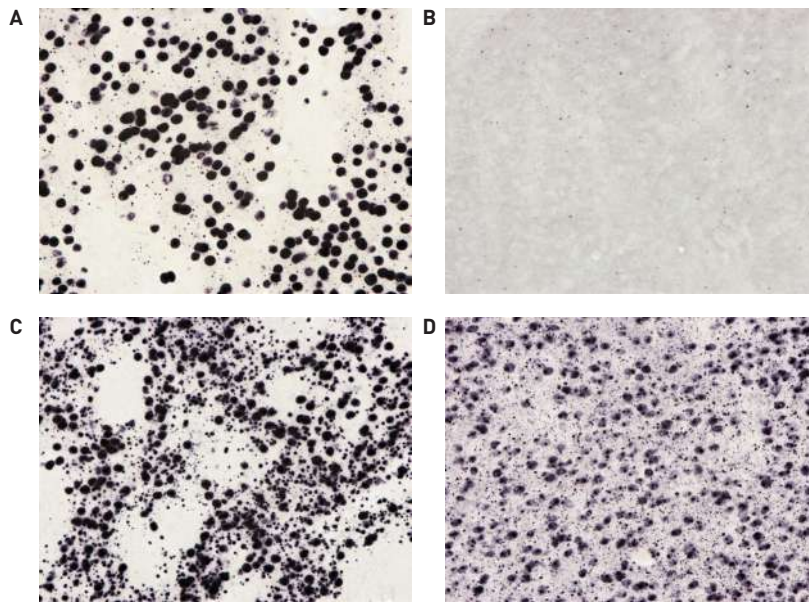


Figure 5: Representative huntingtin inclusion body staining (antibody S830) in the striatum from **A.** 12 month YAC128, **B.** 12 month BAC HD, **C.** 12 month zQ175 (heterozygous) and **D.** 10 week R6/2 mice. Images generously provided by A. Osmand, University of Tennessee, [unpublished data].

III.11. Pharmacokinetics, pharmacodynamics and dosage selection

Studies describing significant effects of drugs in ameliorating HD-related phenotypes in HD mouse models are common in the literature. Too frequently, however, such studies have not been reproducible when repeated by other groups. This is not surprising since between animal dosing and demonstrable efficacy there are a number of processes that need to be verified to ensure that the efficacious observations are true consequences of the drug and its mechanism of action. For drug studies done in HD mice, dosage formulation, selection, and route of administration on the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics of the compound of interest should be fully considered.

- a. Dose and vehicle formulation.** Ideally, dosing should be done with the drug in solution to achieve maximal drug absorption and reproducibility of absorption with multiple dosing. If a solution cannot be achieved, then a vehicle with excipients that maximize the amount of drug in solution and that can produce a formulation with an overall very fine suspension should be selected. CHDI has compiled a list of vehicles (with excipients) that are well tolerated after multiple dosing in genetically-modified HD animals, (see Table A2 in the Appendix). Drugs should also be tested at multiple dosage levels.

- b. Selection of dosing route in the mouse.** The dose route in PK/PD studies should match the dose route intended in the efficacy studies. In general, oral gavage, subcutaneous or intraperitoneal injection is preferred due to the ease of administration. For compounds targeted to the brain that do not cross the blood-brain barrier (e.g., antibodies, antisense nucleotides, etc.), direct administration into the brain (intracerebral ventricular, intraparenchymal, etc.) should be considered.
- c. PK/PD correlation.** For most central targets it is necessary that the drug distribute into the brain parenchyma. After dose administration, measuring the drug concentration in the blood and brain is recommended (PK). In addition, if possible, PD effect(s) (i.e., the resulting action of a compound to receptor binding, signal transduction, etc.) directly related to the concentration of the drug at the target (after factoring the drug potency and off-rate contributions, among others) should be measured to determine whether drug has reached its intended target.

The frequency of dosing for the PK/PD study will depend on the target under consideration and the characteristics of the drug. If the PD effect can be observed following a single (acute) dose, then a single dose at multiple concentrations is recommended. The dose levels should be selected on the basis of the concentration that the compound needs to reach in the brain to obtain a log or semi-log PD effect between 0 and 100% of the response. If the target needs to be engaged over a certain time period to elicit a PD effect, or for several days to elicit an efficacious outcome, then the elimination half-life of the drug needs to be considered in determining the frequency of dosing needed to maintain the drug concentrations above the levels. Further recommendations for pharmacokinetic, pharmacodynamic and dosage selection criterion can be provided by CHDI (larry.park@chdifoundation.org)

III.12. Statistical analyses of the data

A large number of preclinical results cannot be replicated, and a likely cause is the use of erroneous statistical methods (Brunner *et al.*, 2012). Studies on mouse models should be set up just as clinical studies, and the methods described below set the stage for such a strategy.

- The primary endpoint measure that will be the focus of the study should be declared before the start of the study. Any alternative endpoint, manipulation or data transformation should be considered, and described, as exploratory.
- The statistical test to be used and follow-up tests, such as post-hocs, and levels of significance, should also be declared before the study starts.
- Exploratory statistics (e.g., evaluating data with multiple statistical tests) should be used only to predict results and choose future experiments. They should not be used to determine whether a particular result is significant.
- Analysis of variance should be used whenever more than one factor is analyzed, and interaction among factors should be followed up by the appropriate post-hoc; for example, in a mixed (within and between) two-factor ANOVA, a significant interaction should be followed with a simple main effect analysis, rather than with a simple t-test that would use the wrong error term.

- When repeated measures are used, the appropriate within subjects factor should be used to properly account for inter- and intrasubject variability and to account for expected carry-over effects. Use of independent tests at each age analyzed, for example, increases the experiment-wide alpha and, thus, the chance of finding a false-positive is higher than the conventionally desired five percent.
- Although ANOVA is well-suited for these purposes, other analyses are available, namely latent growth and hierarchical linear models. These models have promise for the handling of some experimental designs, although each one has its own advantages and caveats.
- It is unfortunately common to consider that a treatment is significant when the difference between a control wild-type group (either treated or untreated) and a treated model group is not significant. This practice is wrong and contrary to the basics of scientific practice, particularly statistical analysis. Failing to find a difference is not evidence, in any way or form, in favor of any hypothesis. One can only say that the experiment *failed to reject* the hypothesis that the two groups being compared are different. The only way one could conclude that a treatment is significant is by showing a significant difference between the mutant control and mutant treated groups.
- The choice between parametric versus non-parametric tests should be considered carefully. Choice data and latency data can be analyzed with parametric methods if they are relatively normal (i.e., neither clustered at an extreme nor very skewed). Neurological assessment data resulting in yes/no or other categorical results should be analyzed with non-parametric methods, such as chi-square analysis. Survival data can be assessed with Kaplan-Meier analysis, using the Mantel-Cox log-rank statistic.
- Missing data should be treated with care. If possible, software that allows missing cells should be used (SAS). Replacing data with the average of the group is a possible, but less desirable, option. Replacing data with an arbitrary value (such as a maximum time for latency data in the case the behavior was not observed in the test time) is possible, but forces the use of non-parametric tests, as the underlying assumption of an interval or ratio scale is no longer valid.
- The sample size should be based on power analysis, which should be based on previous data collected in similar circumstances (same model, same test).
- Power analysis should be done on the main effects and the interactions of the ANOVA to be used, not just on genotypic effects. For example, if a longitudinal study hopes to find slower progression in mutants treated with a drug (a difference in slopes), then the expected result would be a treatment type x treatment time effect in the mutant subject, and, therefore, the study should ensure sufficient power for the interaction, not for the main effect.

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Table A1. HD strain phenotypes

Name	Repeat Length	Allele Type	Genetic Background	Strain Name	CHDI Stock	JAX Stock	Gene Characteristics	Motor Symptom	Rotarod	Brain Atrophy	Clasping	Inclusions/aggregates	Body Weight	Cognition	Survival	Phenotype References
R6/2	120	Tg fragment	B6CBA mixed	B6CBA-Tg(HDexon1)629pb/125JChdi	CHDI-81001000	006494	Htt promoter; Exon 1; human <i>HTT</i>	Hypoaactivity from 6 wks	↓ From 4-6 wks	↓ From 6 wks	From 6 wks	From 4 wks	↓ From 8 wks	From 9 wks	Median: 16.1 wks	Menalled et al., 2009
R6/2	110	Tg fragment	C57BL/6J	B6.Cg-Tg(HDexon1)629pb/110JChdi	CHDI-81001001		Htt promoter; Exon 1; human <i>HTT</i>	Hypoaactivity from 6 wks, ↓ rearing from 4 wks	↓ From 4 wks	↓ From 6 wks	From 6 wks	From 4 wks	↓ From 12 wks	From 9 wks	Median: ~20 wks	Unpublished observations
R6/2	240	Tg fragment	C57BL/6J	B6.Cg-Tg(HDexon1)629pb/240JChdi	CHDI-81001005		Htt promoter; Exon 1; human <i>HTT</i>	Hypoaactivity from 8 wks	↓ From 8 wks	↓ From 12 wks	From 11 wks	From 6 wks	↓ From 9 wks M, 11 wks F	From 11 wks	Median: ~24 wks	Unpublished observations
YAC128	120	Tg full-length	C57BL/6	B6.FVB-Tg(YAC128)53Hay/97JChdi	CHDI-81001013			Hypoaactivity from 8 to 32 wks	↓ From 4 wks	Not examined	Not detected	Not examined	↑ From 16 wks in males, ↓ from 8 wks in females	Not examined	Similar to WT leexamined up to 1 y)	Menalled et al., 2009
YAC128	120	Tg full-length	FVB/NJ	FVB-Tg(YAC128)53Hay/J		004938	Full-length; human <i>HTT</i>	Hypoaactivity from 32 wks (dark phase)	↓ From 4 wks	Not examined	Not detected	Not examined	↑ From 32 wks in males, ↓ from 48 wks in females	Not examined	Similar to WT leexamined up to 1 y)	Menalled et al., 2009
BAC HD	97-(mixed)	Tg full-length	FVB/NJ	FVB-Tg(HTT*97Q) LXwy/97JChdi	CHDI-81001010	017487	Full-length; floxed exon 1; human <i>HTT</i>	Hypoaactivity from 28 wks	↓ From 4 wks	↓ From 16 mo	Not detected	Not examined	↑ From 12-16 wks	Not examined	Similar to WT	Menalled et al., 2009
BAC HD	97-(mixed)	Tg full-length	C57BL/6	B6.FVB-Tg(HTT*97Q) LXwy/97JChdi	CHDI-81001011		Full-length; floxed exon 1; human <i>HTT</i>	Hyperactivity 4 wks F, Hypoaactivity from 8 wks—mainly in females	↓ From 4 wks	↓ From 16 mo	Not detected	Not examined	↑ From 4 wks	No deficits in nose poking [74 wks]	Similar to WT leexamined up to 1 y)	Menalled et al., 2009; Oakshott et al., 2011
CAG140 KI	125-140	Knock-in	C57BL/6J	B6.129S1-Htt<tm1Mfc>/140JChdi	CHDI-81003002		Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	No deficits detected	Not detected	Not examined	Not detected	Not examined	↓ From 26 wks	No deficits up to 39 wks	Similar to WT leexamined up to 1 y)	Unpublished observations
zQ175 KI	190	Knock-in	C57BL/6J	B6.129S1-Htt<tm1Mfc>/190JChdi	CHDI-81003003		Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Hypoaactivity from 8 wks (dark phase)	↓ From 30 wks	↓ From 12 mo (Br); 8 mo (Str)	Not detected	From 2-4 months	↓ From 8 wks	Deficits detected at 14 wks	Median: 90 wks	Menalled et al., 2012
Hdh020	18-20	Knock-in	C57BL/6J	B6.129(Cg)-Htt<tm2Mem>/20JChdi	CHDI-81003005		Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	No deficits detected	Not tested	Not examined	↔	Not examined	↔	Not tested	↔	Unpublished observations
Hdh080	75-85	Knock-in	C57BL/6J	B6.129(Cg)-Htt<tm1.1Pfs>/80JChdi	CHDI-81003007		Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	No deficits detected	Not tested	Not examined	↔	Not examined	↔	Not tested	↔	Unpublished observations
Hdh092	85-95	Knock-in	C57BL/6J	B6.129(Cg)-Htt<tm4Mem>/92JChdi	CHDI-81003008	003597	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	No deficits detected	Not tested	Not examined	↔	Not examined	↔	Not tested	↔	Unpublished observations
HdhQ111	120	Knock-in	C57BL/6J	B6.129(Cg)-Htt<tm3Mem>/111JChdi	CHDI-81003000	003598	Endogenous murine <i>Htt</i> gene, chimeric human/mouse exon 1	Hypoaactivity from 37 wks	↓ From 24 wks F	Not examined	Not detected	Not examined	↓ From 45 wks	No deficits up to 61 wks	Similar to WT leexamined up to 1 y)	Unpublished observations

Data presented for the CHDI-81001001 and CHDI-81001005 correspond to animals examined under a B6.CBA/CaJF1 background strain.

HOM vs. WT comparisons are presented in the KI lines.

Appendix

Table A2. Vehicles used in drug testing studies in R6/2 mice that did not show adverse effects¹

Vehicle	Route of administration
100% water	IP, PO
Saline (0.90% w/v of NaCl)	SC, IP, PO
Phosphate buffered saline pH 7.4 (commercially available; in general containing NaCl [8.01 g/L], KCl [0.20 g/L], Na ₂ HPO ₄ • 2 H ₂ O [1.78 g/L], KH ₂ PO ₄ [0.27 g/L])	SC, PO
0.1M Sodium phosphate buffer	PO
0.3% Tween-80 in saline	PO
10% HPβCD (hydroxypropyl-β-cyclodextrin) in water	PO, SC
0.5% Tween-80 in water	PO
0.5% Methylcellulose in water	PO
0.5% Carboxymethylcellulose (CMC), 1 % Lutrol F68 in water	IP, PO
100% 50mM citrate pH 4.5	PO
1% Lutrol in 50mM citrate pH 4.5	IP, PO
0.5% Carboxymethylcellulose (CMC), 1 % Lutrol F68 in 50 mM citrate pH 4.5	IP, SC, PO
20% Polyethylene glycol (PEG 400), 80% 50 mM citrate pH 4.5	PO, SC
0.9% NaCl, 0.5% Sodium Carboxymethylcellulose, 0.5% benzyl alcohol, 0.4% Tween-80 in water	PO
2% n-Methyl pyrrolidone (NMP), 18% propylene glycol, 10% Solutol HS15, 70% (1% meglumine in water)	SC
2% n-Methyl pyrrolidone (NMP), 18% polyethylene glycol (PEG 400), 10% solutol HS15, 70% (1% Lutrol F68 in water)	SC
30% Solutol HS15 in PBS	SC
10% Solutol HS15 in saline	SC
5% Phospholipon water dispersion	IP
5% Gum Arabic in water	PO

¹ Unpublished observations. Vehicles and excipients have not been tested in a systematic manner. Thus, absence of a vehicle on this list should not be associated with unacceptability. In general, pH should be kept between 4.5 and 8 and HPβCD as an excipient should not exceed 10%. Aqueous-based vehicles are preferred; R6/2 mice will not tolerate viscous formulations. IP: intraperitoneal, SC: subcutaneous, PO: per oral.

Acknowledgments

This work was funded by CHDI Foundation.

The authors wish to thank Charles Miller, Ph.D. (The Jackson Laboratory) for project management and review, Maria Beconi, Ph.D. (Retrophin, Inc.) for her contributions, and the following reviewers for their helpful discussion and insightful comments:

Gillian Bates, Ph.D. (King's College London)

Jeff Carroll, Ph.D. (Western Washington University)

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The authors wish to thank the Senior Scientific Advisors to CHDI, Ethan Signer, Ph.D and Allan Tobin, Ph.D. for their insight and guidance.

The authors also wish to thank CHDI *in vivo* advisory panel, namely Dr. Gillian Bates, Kings College London, Dr. Michael Fanselow, UCLA, Dr. Dan Goldowitz, University of British Columbia, Dr. Holly Moore, Columbia University, Dr. Jenny Morton, University of Cambridge and Dr. Jeanne Wehner, University of Colorado, for their valuable advice in developing guidelines for working with HD mouse models.

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