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High-throughput multiplexed transcript analysis yields enhanced resolution of $5HT_{2C}$ receptor mRNA editing profiles

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Non-standard Abbreviations:

$5HT_{2C}$:	2C-subtype of serotonin receptor
bp:	base pair
HTMTA:	high-throughput multiplexed transcript analysis
mRNA:	messenger RNA
nt:	nucleotide
pre-mRNA:	pre-messenger RNA

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ABSTRACT

RNA editing is a post-transcriptional modification in which adenosine residues are converted to inosine (A-to-I editing). Commonly employed methodologies to quantify RNA editing levels involve either direct sequencing or pyrosequencing of individual cDNA clones. The limitations of these methods lead to a small number of clones characterized in comparison to the number of mRNA molecules in the original sample, thereby producing significant sampling errors and potentially erroneous conclusions. We have developed an improved method for quantifying RNA editing patterns that increases sequence analysis to an average of over 800,000 individual cDNAs per sample, substantially increasing accuracy and sensitivity. Our method is based on the serotonin 2C receptor ($5HT_{2C}$) transcript, an RNA editing substrate where up to five adenosines are modified. Using a high-throughput multiplexed transcript analysis we were able to quantify accurately the expression of twenty $5HT_{2C}$ isoforms, each representing at least 0.25% of total $5HT_{2C}$ transcripts. Furthermore, this approach allowed the detection of previously unobserved changes in $5HT_{2C}$ editing in RNA samples isolated from different inbred mouse strains and dissected brain regions, as well as editing differences in alternatively spliced $5HT_{2C}$ variants. This approach provides a novel and efficient strategy for large-scale analyses of RNA editing and may prove to be a valuable tool for uncovering new information regarding editing patterns in specific disease states and in response to pharmacologic and physiologic perturbation, further elucidating the impact of $5HT_{2C}$ RNA editing on central nervous system function.

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INTRODUCTION

The 2C-subtype of serotonin receptor ($5HT_{2C}$) is a member of the G-protein coupled receptor superfamily and has been implicated in the central regulation of mood, appetite, metabolism, and sleep-wake cycle regulation (Backstrom et al., 1999; Monti and Jantos, 2006; Nonogaki et al., 1998; Schmauss, 2003; Sodhi et al., 2001; Tecott et al., 1995). Transcripts encoding the $5HT_{2C}$ receptor are subject to an RNA processing event in which specific adenosine residues are modified by hydrolytic deamination and converted to inosine moieties (A-to-I editing) (Burns et al., 1997). Inosine is recognized as guanosine by the cellular translation machinery (Higuchi et al., 1993), leading to alterations in the coding potential of the transcript and subsequent alterations in receptor function. $5HT_{2C}$ pre-mRNAs can be edited at up to five sites (sites A, B, E, C, and D) that can alter codons for three critical amino acids in the putative second intracellular loop of the receptor. Combinatorial editing at these five positions can generate up to 32 mRNA isoforms encoding 24 different receptor proteins (Burns et al., 1997; Niswender et al., 1998). RNA editing profiles have been shown to vary significantly between brain regions (Burns et al., 1997; Wang et al., 2000), among inbred mouse strains (Du et al., 2006; Englander et al., 2005; Hackler et al., 2006), in response to pharmacologic treatment (Englander et al., 2005; Gurevich et al., 2002; Sodhi et al., 2005) and in affective disorders (Backstrom et al., 1999; Schmauss, 2003; Sodhi et al., 2001), implicating the modulation of RNA editing levels in the regulation of $5HT_{2C}$ receptor function. The fully-edited (VGV) isoform of the human $5HT_{2C}$ receptor (encoding valine, glycine and valine at amino acid positions 156, 158 and 160, respectively) exhibits reduced constitutive activity and decreased G protein-coupling efficacy when compared to the genomically-encoded (INI) isoform in heterologous expression systems (Berg et al., 2001; Burns et al., 1997; Fitzgerald et al., 1999; Niswender et al., 1999; Wang et al., 2000). As the increased editing of $5HT_{2C}$ RNAs generates receptors less likely to assume a G-protein coupled high-affinity conformation (R*) (Niswender et al., 1999), the observed potency of agonists with increased affinity for this receptor state is disproportionately reduced (Werry et al., 2008) and the specificity of agonist-directed trafficking of receptor stimulus is lost (Berg et al., 2001).

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The most common methodology to quantify site-specific editing has involved reverse-transcription polymerase chain reaction (RT-PCR) amplification of specific RNAs followed by a modified primer-extension analysis (Burns et al., 1997; Rueter et al., 1995); however, this analytical paradigm is less useful for analyses of editing patterns in transcripts with multiple editing sites, such as $5HT_{2C}$ mRNAs. The two most commonly used methods to quantify editing of $5HT_{2C}$ RNA isoforms involve either direct sequencing or pyrosequencing analysis of individual cDNA clones (Iwamoto et al., 2005; Sodhi et al., 2005; Werry et al., 2008). While these methods yield unambiguous results, the accuracy of such methods is directly related to the number of clones sequenced, which can vary widely between studies and has been reported to be as few as 10 clones per sample (Sodhi et al., 2001). The limited number of clones analyzed can produce significant sampling errors that may either obscure or overestimate differences between experimental groups. Recent studies have analyzed $5HT_{2C}$ editing using 454 deep sequencing technology, increasing the number of $5HT_{2C}$ cDNAs sequenced to nearly 800, while simultaneously avoiding the laborious subcloning, bacterial transformation and preparation of individual cDNA clones (Wahlstedt et al., 2009). Despite the increased throughput, from 7 to 16 predicted mRNA isoforms remained undetectable in mouse brain samples between embryonic day 15 and postnatal day 21, possibly missing rare isoforms that could represent important species in minor neuronal subpopulations.

We have developed a High-Throughput Multiplexed Transcript Analysis (HTMTA) based upon the massively parallel, short-read sequencing technology available on the Illumina/Solexa platform (Bentley et al., 2008). This approach allows an improved method for quantifying $5HT_{2C}$ receptor RNA editing patterns with substantially increased accuracy and sensitivity. Using eight barcodes to maintain sample identity, this multiplexed strategy has been employed to analyze RNA editing profiles for $5HT_{2C}$ transcripts from up to 56 RNA samples simultaneously using a single Illumina Genome Analyzer flow cell. This adaptation of new sequencing technology provides a substantial improvement in the quantification of isoform-specific $5HT_{2C}$ mRNA expression compared to previously used methods and should provide a powerful strategy for

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identifying previously undetected variations in RNA editing patterns in specific physiologic or disease states and in response to pharmacologic manipulation.

MATERIALS AND METHODS

Mice

Inbred male mice of different strains were purchased from The Jackson Laboratories (Bar Harbor, ME; C57BL/6, DBA/2J, BALB/cJ) or Taconic (Germantown, NY; 129S6). Mice were maintained on a 12-h light, 12-h dark cycle in a humidity- and temperature-controlled environment with *ad libitum* access to water and standard laboratory chow. All animal studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

RNA Isolation

Animals were sacrificed at eight weeks of age and brain tissue was either flash-frozen (whole brain samples) or dissected into specific brain regions (Glowinski and Iversen, 1966) and homogenized immediately. All tissue was homogenized in Tri-Reagent (Ambion, Austin, TX); total RNA was isolated according to the manufacturer's instructions and quantified by spectrophotometery at A_{260} (Nanodrop; Thermo Scientific, Wilmington, DE).

HTMTA Primer Design

Each antisense oligonucleotide primer was designed to contain a 24 nucleotide (nt) region complementary to exon 6 (72-95nt downstream from the D site) of the $5HT_{2C}$ gene preceded by a distinct 5nt barcode sequence, a 37nt region for "read 2" sequencing primer hybridization, and a 24nt adapter sequence that can anneal to the complementary oligonucleotide bound to the Illumina flowcell surface (Bentley et al., 2008). Specifically, the 8 barcodes consist of palindromic 5nt sequences that allow the identity of a sample to be determined unambiguously with up to 2 errors in the barcode sequence. The sense oligonucleotide primer contains 30nt complementary to exon 5 (13-42nt upstream from the A site) and a 29nt adapter sequence that can anneal to a second complementary oligonucleotide

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bound to the flowcell surface (Bentley et al., 2008) (Figure 1A). The "read 1" sequencing primer corresponds to the 30nt region of $5HT_{2C}$ complimentarity in the sense primer. The adapter sequences and "read 2" sequencing primer sequences were modeled for use in paired-end sequencing with the Illumina Genome Analyzer (Illumina Inc., San Diego, CA) as described previously (Bentley et al., 2008). Primer sequences are detailed in Supplementary Table S1.

Sequence Analysis

Pyrosequencing analyses of $5HT_{2C}$ receptor RNA editing profiles were performed as described previously (Sodhi et al., 2005). For HTMTA, first strand cDNA was synthesized in a 10μl reaction from 1μg of RNA using avian myoblastosis virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions in a reaction containing 0.75μg of a specific bar-coded antisense primer. Parallel control reactions lacking reverse transcriptase were performed for all samples. The total reverse-transcription volume was included in a 25μl PCR amplification reaction with Phusion DNA polymerase (Finnzymes, Woburn, MA) according to the manufacturer's recommendations using 0.75μg of the HTMTA sense primer. All reactions were amplified for 28 cycles unless otherwise specified. Amplified products were separated on a 2% agarose gel and purified from excised gel slices using the Wizard SV Gel and PCR Purification Kit (Promega, Madison, WI). PCR amplicons corresponding to $5HT_{2C}$ RNA 2 (249bp) were isolated for all experiments except for comparisons of editing in alternatively spliced $5HT_{2C}$ mRNAs. For these studies, PCR amplicons corresponding to both $5HT_{2C}$ RNA 2 and RNA 3 (334bp) were isolated in a single excised gel slice. The concentration of gel-purified fragments was measured by spectrophotometry (A_{260}) and ~20ng of each individual sample was pooled with seven other products containing unique barcodes and subjected to paired-end sequencing with the Illumina Genome Analyzer as described (Bentley et al., 2008) using sequencing primers detailed in Supplementary Table S1. Sequencing data were filtered as indicated in Figure 1B to eliminate sequences derived from either RNA 3 or human transcripts where appropriate. Read 2 sequences were used to distinguish both barcode and $5HT_{2C}$ splice

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form identities, while sequences obtained from read 1 were used to determine the editing profile of individual cDNA clones.

Statistical Analysis

Statistical differences between the editing profiles of individual edited isoforms were determined using a Poisson linear model. This model included the number of reads for each isoform as the outcome variable, with the main independent variable being Group (e.g. brain region 1 vs. brain region 2). To allow sample comparisons, the logarithm of total isoform count was calculated for each sample and included in the model as an offset variable so that statistical significance of the variable Group would indicate whether the average proportion (observed count/total count) of the isoform is significantly different for the two samples. In addition, an over-dispersion parameter was included in the model to account for greater variability in the dataset than those accounted by assuming the Poisson model. The editing level at each individual site (A-E) was analyzed similarly, except for replacing individual isoform count with the count at each site as the outcome variable.

To estimate the number of samples needed to detect an isoform with abundance level p with probability t, we assumed a binomial model and used the formula Pr (an editing pattern occurs for at least 1 sample among n samples) = t = 1- Pr (an editing pattern does not occur for all n samples) = $1-(1-p)^n$.

RESULTS

Validation of HTMTA analyses for $5HT_{2C}$ mRNA editing profiles

To develop a high-throughput method for quantifying $5HT_{2C}$ receptor RNA editing patterns, we adapted the Illumina Genome Analyzer whole-transcriptome sequencing technology (Bentley et al., 2008) to report sequence data from an average of 828,354 (±93,282) individual RT-PCR amplicons derived solely from $5HT_{2C}$ receptor transcripts (Figure 1A). To decrease the total cost of analysis, we included 5 nucleotide barcode sequences in the anti-sense primers, allowing multiplexing of sequencing reactions derived from eight separate RNA

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samples while maintaining sample identity. HTMTA data from a representative flow cell lane yielded 9,564,125 raw sequence reads and we used a bidirectional sequencing approach that allowed us to distinguish and eliminate any RNA 1- or RNA 3-derived $5HT_{2C}$ sequences from read 2 (Supplemental Table S2). The data were further filtered to isolate 7,438,012 reads with identifiable barcodes precisely matching the mouse $5HT_{2C}$ RNA 2 reference sequence (77.8% recovery rate; Figure 1B).

Since extensive RT-PCR amplification can selectively bias template-to-product ratios in multi-template PCR (Kanagawa, 2003), we determined whether the number of amplification cycles could affect the observed editing profile by performing control studies in which identical PCR reactions were amplified for 19, 22, 25, 28, 31, or 34 cycles prior to cluster generation. Sequence analysis revealed that editing profiles did not change significantly between 19 and 34 cycles (*data not shown*). All subsequent PCR amplification was performed for 28 cycles, as this was the lowest cycle number for which a robust PCR product could routinely be observed for gel-purification (*data not shown*).

To determine if the increased sensitivity provided by HTMTA results in the false discovery of contaminating DNA molecules or RNA misidentification due to polymerase errors, a non-edited human $5HT_{2C}$ cDNA clone (INI) was used as a control for detection of other potential edited species within the sample. Results from this analysis revealed the identification of non-INI-encoding species representing 0.25% of total $5HT_{2C}$ expression, indicating that HTMTA is accurate within 0.1% when analyzing the level of editing at each individual site (A-E) and within 0.25% when determining the relative expression of individual isoforms (Supplemental Figure S1). Furthermore, repeated analysis of a single RNA sample isolated from mouse whole brain (C57BL/6J) revealed a low level of intra-sample variability for editing at each site (n = 3; mean percentage of editing at each site ± SEM; A = 79.48 ± 0.34; B = 68.16 ± 0.35; E = 4.01 ± 0.09; C = 23.91 ± 0.34; D = 62.98 ± 0.15).

We compared $5HT_{2C}$ RNA editing profiles determined by HTMTA with pyrosequencing analysis (Iwamoto et al., 2005; Sodhi et al., 2005) of 426 total cDNA clones generated from five separate C57BL/6 whole brain mouse RNA samples. While each technique detected similar

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levels of site-specific editing upon analyses of the same RNA samples (Figure 2; $p > 0.05$), HTMTA revealed substantially decreased inter-animal variability compared to pyrosequencing, suggesting that the variance detected by pyrosequencing among these inbred mice was largely due to insufficient sampling (Figure 2 and Table 1). Of the 32 possible mRNA isoforms, HTMTA detected 20 isoforms at frequencies >0.25% of total $5HT_{2C}$ transcripts (the error threshold for these experiments; supplemental Figure S1). RNAs encoding the ISI, VSI and VGI receptor isoforms were readily observed, though they were absent from pyrosequencing analyses, and RNAs encoding the VDI and VDV isoforms were present at levels 3-fold greater than determined by pyrosequencing.

Differential editing of alternatively spliced $5HT_{2C}$ transcripts.

Alternative splicing of $5HT_{2C}$ pre-mRNAs can generate three distinct mRNA species from the use of different 5'-splice sites located in exon 5 and intron 5. Use of the proximal and distal 5'-splice sites results in the generation of mRNAs encoding the truncated non-functional receptors $5HT_{2C}$-tr (Canton et al., 1996) and $5HT_{2C}$-COOHΔ (Wang et al., 2000), respectively, whereas use of the intermediate splice junction generates RNA 2 which encodes the full-length, functional $5HT_{2C}$ receptor (Figure 1A). $5HT_{2C}$R-tr transcripts (RNA 1) lack a 95nt region encoding the second intracellular loop and the fourth transmembrane domain of the receptor which encompasses the five editing sites. $5HT_{2C}$-COOHΔ mRNAs (RNA 3) include an additional 90nt region of intron 5 containing a premature, in-frame stop codon (Wang et al., 2000). During the initial characterization of RNA 3, standard sequence analysis of 21 cDNA clones revealed that this mRNA was edited to completion at all five sites (Wang et al., 2000). Such increased editing was hypothesized to result from an increased time interval for A-to-I conversion provided by the maintenance of an essential duplex structure in mature RNA 3 mRNAs (Wang et al., 2000). During the preparation of the C57BL/6 whole brain samples for HTMTA analysis, RT-PCR amplicons derived from RNA 2 and RNA 3 were co-excised and purified to reexamine the RNA editing profiles between these two alternatively spliced $5HT_{2C}$ mRNA species. Analyses of these transcripts revealed significant increases in editing at the A, B, E, and C sites in RNA 3

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transcripts compared to RNA 2 (Figure 3), corresponding to a significant increase in three highly-edited isoforms [ABEC (+1.36%, $p<0.01$), ABCD (+22.42%; $p<0.001$), and ABECD (+4.15%; $p<0.000001$)], confirming that editing of RNA 3 is increased significantly compared to RNA 2.

$5HT_{2C}$ RNA editing in commonly-used inbred mouse strains

Previous studies have reported inconsistent variations in $5HT_{2C}$ RNA editing profiles between inbred mouse strains (Du et al., 2006; Englander et al., 2005; Hackler et al., 2006). To reassess $5HT_{2C}$ RNA editing patterns in these strains, HTMTA sequencing was performed using whole brain samples isolated from 129S6, DBA/2J, and BALB/cJ mice and compared to results obtained from C57BL/6J samples (Figure 4; Supplemental Table S3). The C57BL/6J, DBA/2J and BALB/cJ strains showed remarkable similarities in $5HT_{2C}$ editing profiles with no significant differences in editing levels observed at any of the five sites (Figure 4) and only a small decrease in expression of the AD isoform detected in BALB/cJ when compared to C57BL/6J samples (Supplemental Table S3). Analysis of $5HT_{2C}$ RNA editing patterns in 129S6 mice however, provided a significantly different profile when to compared to C57BL/6 animals; editing at the A and B sites was significantly reduced (-9.75% and -16.4%, respectively) while editing at the E and D sites was significantly increased (+1.86% and +4.52%, respectively) (Figure 4). Similar differences were observed when 129S6 mice were compared with both the DBA/2J and BALB/cJ strains (*data not shown*). Quantitative analysis of specific $5HT_{2C}$ RNA species revealed that the expression of seven isoforms was significantly decreased and 18 isoforms was significantly increased in 129S6 mice when compared to the C57BL/6J strain (Supplemental Table S3) and similar changes were observed when comparing 129S6 mice to both the DBA/2J and the BALB/cJ strains (Supplemental Table S3 and *data not shown*).

$5HT_{2C}$ RNA editing in dissected brain regions

The expression pattern for $5HT_{2C}$ receptors in the central nervous system is suggestive of specific roles in normal physiology and also, when dysregulated, in the development of cer-

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tain disease states such as obesity, anxiety, epilepsy, sleep disorders and motor dysfunction (Werry et al., 2008; Wright et al., 1995). Previous studies using conventional sequence analyses of cDNAs isolated from dissected rat, mouse and human brains identified the region-specific expression of as many as 12 major $5HT_{2C}$ receptor isoforms (Burns et al., 1997; Niswender et al., 1999; Werry et al., 2008) that may allow the differential modulation of region-specific responses to ambient or stimulated levels of neurotransmitter. Decreased editing of $5HT_{2C}$ mRNAs in the choroid plexus represents the most consistent difference observed previously (Burns et al., 1997; Wang et al., 2000). Since a failure to identify significant changes in isoform distribution between other dissected brain regions may have been limited by a reduced statistical power resulting from the relatively small number of sequences used for these analyses, we sought to evaluate $5HT_{2C}$ mRNA editing in six distinct brain regions isolated from C57BL/6J animals. Significant increases in site-specific RNA editing were identified in the olfactory bulb, frontal cortex and striatum when compared to data obtained from whole brain RNA (Figure 5A) and further analysis of $5HT_{2C}$ mRNA profiles from these dissected regions revealed numerous changes (both increases and decreases) in the relative expression of specific mRNA isoforms (Figure 5B). Though fewer differences were observed in the striatum when compared to whole brain RNA editing profiles, expression of five isoforms was significantly decreased and expression of four isoforms was significantly increased in the striatum (Figure 5B and Supplemental Table S4). Conversely, less $5HT_{2C}$ RNA editing was observed in the hippocampus, with significant reductions in editing at the A, B, and C sites (Figure 5A) that were reflected as subsequent changes in the encoded $5HT_{2C}$ receptor profile (Figure 5B).

RNA samples isolated from hypothalamus revealed significantly increased levels of editing at the A and B site sites when compared to whole brain RNA and a reduction in editing at the E, C, and D sites (Figure 5A). Consistent with previous results (Burns et al., 1997); Werry et al., 2008; Pandey et al., 2006; Wang et al., 2000), characterization of choroid plexus RNA revealed the largest differences in $5HT_{2C}$ RNA editing. Though editing was significantly decreased at the A, B, and C sites when compared to whole brain RNA, there was a small, but significant increase in the level of editing at the D site (Figure 5). These alterations in site-specif-

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ic editing were reflected as a change in the overall profile of $5HT_{2C}$ mRNA expression in the choroid plexus where the relative level of all but two (BEC and BECD) of the 32 mRNA isoforms were affected (Figure 5B and Supplemental Table S4).

DISCUSSION

Here we show that quantitative analysis of $5HT_{2C}$ RNA editing profiles using a high-throughput strategy based upon the Illumina sequencing platform (Bentley et al., 2008) represents a significant improvement over previously developed low-throughput sequencing technologies. This approach is rapid, efficient and cost-effective compared to currently existing paradigms involving the sequencing or pyrosequencing of isolated cDNA clones and is easily adaptable to any RNA editing substrate, providing a reduction in both inter- and intra-sample variability (Figure 2 and *data not shown*). By generating an average of over 800,000 sequence reads per individual RNA sample, this approach provides both increased sensitivity for the detection of rare $5HT_{2C}$ isoforms and increased statistical power compared to conventional sequencing or pyrosequencing methodologies (Burns et al., 1997; Iwamoto et al., 2005; Sodhi et al., 2005; Wang et al., 2000; Werry et al., 2008). To estimate the minimum number of individual cDNA sequences necessary to detect a specific $5HT_{2C}$ isoform, we employed a binomial model to determine that a minimum of 2994 clones must be sequenced to simply detect a rare isoform (0.1% abundance) with 95% probability (Table 2). This suggests that the ability of previous sequencing technologies to accurately quantify rare isoforms or demonstrate small changes in editing profiles between different RNA samples may have been limited.

HTMTA primers were developed initially to amplify all three possible spliced forms of $5HT_{2C}$ mRNA simultaneously (Canton et al., 1996; Wang et al., 2000) (Figure 1A), thereby allowing quantification of both RNA editing and alternative splicing patterns. However, the relative expression levels for RNA 1 and RNA 2 varied significantly between identical RT-PCR reactions and with amplification cycle number, preventing a reliable measurement of splicing ratios from RT-PCR amplicons (*data not shown*). Since the five editing sites are not contained within RNA 1 due to the use of an upstream 5'-splice junction (Canton et al., 1996), only the

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editing profiles of RNA 2 and RNA 3 can be compared (Figure 3). Comparisons of editing patterns between these alternatively spliced $5HT_{2C}$ mRNAs revealed significant increases in the editing of RNA 3, most likely resulting from the retention of a 90nt region within intron 5 that is essential for A-to-I conversion to allow continued editing of RNA 3 after splicing has been completed. Despite these editing differences however, the potential consequences of increased editing for RNA 3 mRNAs have yet to be elucidated (Wang et al., 2000).

Previous comparisons of $5HT_{2C}$ RNA editing profiles in different mouse strains have yielded conflicting results, although these studies did not feature overlapping experiments that could be compared directly. Such studies reported either no changes in whole brain RNA editing between the C57BL/6, DBA/2J, and 129S1 strains (Du et al., 2006), decreased editing in the amygdala of both DBA/2J and BALB/cJ mice when compared to C57BL/6 mice (Hackler et al., 2006), or substantial decreases in editing in the forebrain neocortex for BALB/cJ (but not 129S6) when compared to C57BL/6 animals (Englander et al., 2005). In the current study, we found significant alterations in whole brain RNA editing in 129S6 mice when compared to the C57BL/6, DBA/2J, or BALB/cJ strains (Figure 4 and Supplementary Table S3). Observed changes in 129S6 whole brain $5HT_{2C}$ RNA editing appeared comparable to previously published results, though the present method revealed numerous statistically significant differences that were not identified previously (Du et al., 2006). Binomial probability analysis (Table 2) suggests that the strain-specific differences observed previously in dissected brain regions (Englander et al., 2005; Hackler et al., 2006) may require further analysis due the small number of cDNA clones analyzed and a lack of adequate statistical power.

HTMTA analysis of $5HT_{2C}$ RNA editing profiles in dissected mouse brain regions supported previous results (Burns et al., 1997; Niswender et al., 1999; Wang et al., 2000; Werry et al., 2008), yet also identified additional differences in region-specific RNA editing patterns. When compared to whole brain samples, editing levels were increased in the olfactory bulb, frontal cortex, and striatum, decreased in the hippocampus, or showed mixed changes in the hypothalamus and choroid plexus (Figure 5 and Supplementary Table S4). Subtle variations in editing profiles between brain regions could reflect small neuronal subpopulations or specific

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pathways whose editing patterns deviate substantially from the surrounding tissue. While it has not yet been determined whether small changes in editing profiles produce detectable changes in the function of cells expressing $5HT_{2C}$ receptors, recent studies have described genetically-modified mice expressing only the fully-edited (VGV) isoform of the receptor that exhibit phenotypic alterations including severe reductions in fat mass, hyperphagia and increased energy expenditure (Kawahara et al., 2008), demonstrating the importance of normal patterns of $5HT_{2C}$ RNA editing *in vivo*.

HTMTA represents a new adaptation of the massively parallel, short-read sequencing technology available on the Illumina/Solexa Genome Analyzer to accurately determine the RNA editing profile of multiple biological samples simultaneously. This assay is easily adaptable to any RNA editing substrate, providing a facile strategy to quantify editing of numerous ADAR substrates. The improved sensitivity and statistical power provided by this methodology increases the ability to accurately quantify variations in $5HT_{2C}$ RNA editing that may be important in affective disorders or in response to pharmacologic manipulation.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Summary of the HTMTA sequencing strategy. **A**. The structure and alternative splicing of $5HT_{2C}$ pre-mRNA in the exon 5-6 region is presented (*top*). The location of RT-PCR primers are indicated (black arrows). Adapter sequences for paired-end sequencing on the Illumina Genome Analyzer (dark blue) were incorporated into each primer. An annealing region for a sequencing primer (light blue) and one of eight distinct barcode sequence (yellow) were incorporated into the antisense primer. The sequence of the non-edited RNA, beginning immediately after the primer sequence, is specified below the pre-mRNA RT-PCR diagram. The positions of the five edited adenosines are indicated in bold (sites A, B, E, C and D) and the amino acids encoded by the non-edited (upper) and fully edited transcripts (lower) are indicated below their respective codons. The position of the 36nt sequence obtained from read 1 is indicated between the dashed lines on both the RT-PCR and pre-mRNA diagrams. Two sequencing primers (read 1 and read 2, orange arrows) were used for paired-end sequence analysis of RT-PCR amplicons generated from $5HT_{2C}$ mRNA (*bottom*). Each of eight unique barcode sequences incorporated into the RT-PCR amplicon for sample identification is provided in the yellow inset. **B**. Flow chart of data sorting criteria. Total sequence reads obtained per RNA sample from HTMTA were culled to an appropriate data set by meeting a series of inclusion criteria including: 1) barcode sequences are identifiable; 2) RNA 2 or RNA 3 alternatively spliced isoforms are identifiable; 3) nucleotides at the editing site are either A or G; and 4) the sequence of read 1 precisely matches the mouse $5HT_{2C}$ RNA 2 reference sequence (Supplemental Table S2). The representative example is derived from analysis of eight distinct barcoded samples. The number of reads that fail to meet each inclusion criterion is indicated to the right, followed by the percentage of the total reads that these excluded sequences represent. The number of sequence reads meeting each criterion is indicated below the inclusion description in each rectangle.

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Figure 2. Comparison of RNA editing patterns using HTMTA and pyrosequencing methodologies. A. Quantitative analysis of site-specific $5HT_{2C}$ editing in RNA isolated from five, independent whole brain mouse samples as determined by HTMTA. **B**. Quantitative analysis of $5HT_{2C}$ editing using the same RNA samples as above by pyrosequencing. The mean percentage of site-specific editing is listed below each site (± SEM).

Figure 3. Quantitative analysis of $5HT_{2C}$ RNA editing for alternatively spliced RNA 2 and RNA 3. HTMTA determination of site-specific editing is presented for RNA 2 and RNA 3 (mean ± SEM, n=5, $*p<0.05$).

Figure 4. Analysis of $5HT_{2C}$ mRNA profiles in inbred mouse strains. HTMTA of site-specific editing patterns from whole brain RNA isolated from the C57BL/6J, 129S6, DBA/2J, and BALB/cJ inbred mouse strains (mean ± SEM, n=5 per strain, $* p<0.0001$).

Figure 5. Analysis of brain region-specific $5HT_{2C}$ RNA editing profiles in C57BL/6J mice. $5HT_{2C}$ editing profiles were determined by HTMTA from RNA isolated from whole brain (WB) and dissected brain regions including cortex (Ctx), striatum (Str), hippocampus (Hip), hypothalamus (Hyp), olfactory bulb (OB) and choroid plexus (CP) (mean ± SEM; n≥4, $*p<0.05$, $**p<0.01$, $***p<0.001$). **A**. Site-specific editing patterns from whole brain and dissected brain regions; the percentage of editing at each site is indicated. **B**. Quantitative analysis of the major $5HT_{2C}$ RNA (R) and encoded protein (P) isoforms resulting from RNA editing are represented as percentage of total sequence reads (NE, non-edited).

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Table 1. Comparison of $5HT_{2C}$ mRNA editing profiles from C57BL/6J mouse whole brain samples using HTMTA and pyrosequencing methods.

Protein isoform	RNA isoform	HTMTA			Pyrosequencing		
		Total Reads	% of total	SEM	Total Reads	% of total	SEM
INI	Non-edited	244,399	7.692	0.153	32	7.530	1.343
VNI	A	192,775	6.080	0.180	19	4.558	1.413
	AB	346,950	10.975	0.358	48	11.356	1.245
MNI	B	16,798	0.529	0.013	1	0.244	0.244
IDI	E	9,593	0.303	0.009	2	0.494	0.494
ISI	C	35,986	1.133	0.036	0	0.000	0.000
INV	D	215,924	6.817	0.202	23	5.442	1.098
VDI	AE	6,946	0.218	0.008	0	0.000	0.000
	ABE	16,856	0.529	0.022	1	0.241	0.241
VSI	AC	27,964	0.882	0.016	0	0.000	0.000
	ABC	171,158	5.408	0.113	20	4.692	0.992
VNV	AD	126,821	3.981	0.100	19	4.407	0.852
	ABD	1,113,379	35.326	0.426	186	43.263	4.120
MDI	BE	213	0.007	0.001	0	0.000	0.000
MSI	BC	1,804	0.057	0.004	0	0.000	0.000
MNV	BD	28,591	0.901	0.021	4	0.940	0.238
IGI	EC	2,795	0.088	0.003	0	0.000	0.000
IDV	ED	9,221	0.290	0.008	1	0.208	0.208
ISV	CD	51,051	1.607	0.053	6	1.458	0.598
VGI	AEC	7,525	0.237	0.004	0	0.000	0.000
	ABEC	8,637	0.273	0.010	0	0.000	0.000
VDV	AED	5,888	0.186	0.009	0	0.000	0.000
	ABED	38,767	1.225	0.029	2	0.455	0.280
VSV	ACD	28,514	0.896	0.025	3	0.717	0.477
	ABCD	425,393	13.447	0.199	55	13.178	3.402
MGI	BEC	56	0.002	0.001	0	0.000	0.000
MDV	BED	555	0.018	0.003	2	0.485	0.297
MSV	BCD	4,439	0.141	0.007	1	0.247	0.247
IGV	ECD	1,984	0.063	0.004	0	0.000	0.000
VGV	AECD	5,116	0.160	0.004	0	0.000	0.000
	ABECD	16,865	0.531	0.010	1	0.241	0.241
MGV	BECD	72	0.002	0.000	0	0.000	0.000

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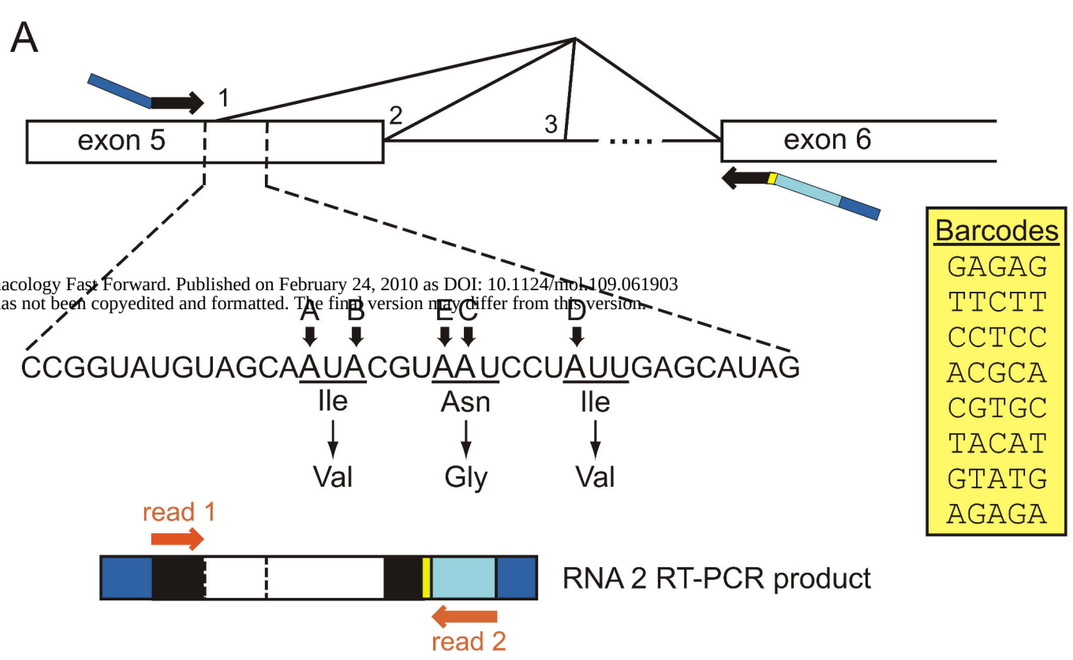
Table 2. Estimated sample sizes to detect a low abundant isoform with high probability (confidence) using the HTMTA method.

Isoform Abundance	Probability (confidence) of detecting isoform					
	99%	95%	90%	80%	50%	10%
10%	44	28	22	15	7	1
1%	458	298	229	160	69	10
0.10%	4,603	2,994	2,301	1,609	693	105
0.01%	46,049	29,956	23,025	16,094	6,931	1,054
0.001%	460,515	299,572	230,257	160,943	69,314	10,536

Figure 1.



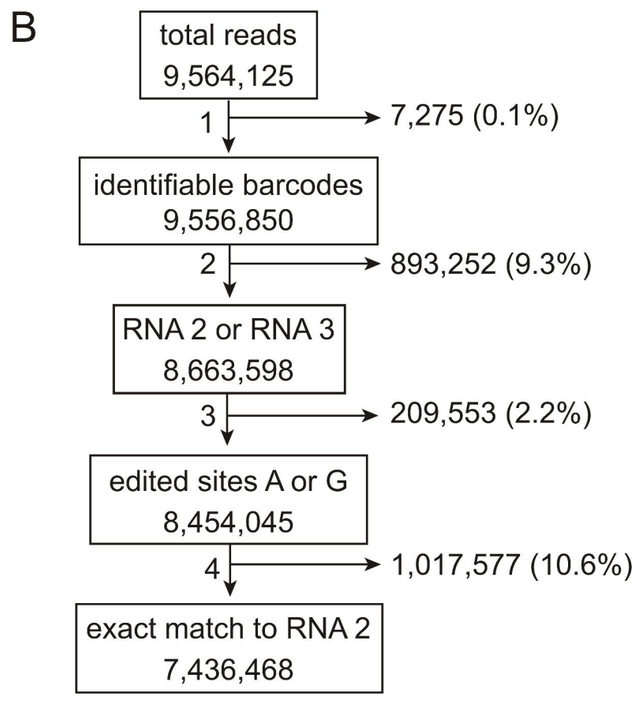
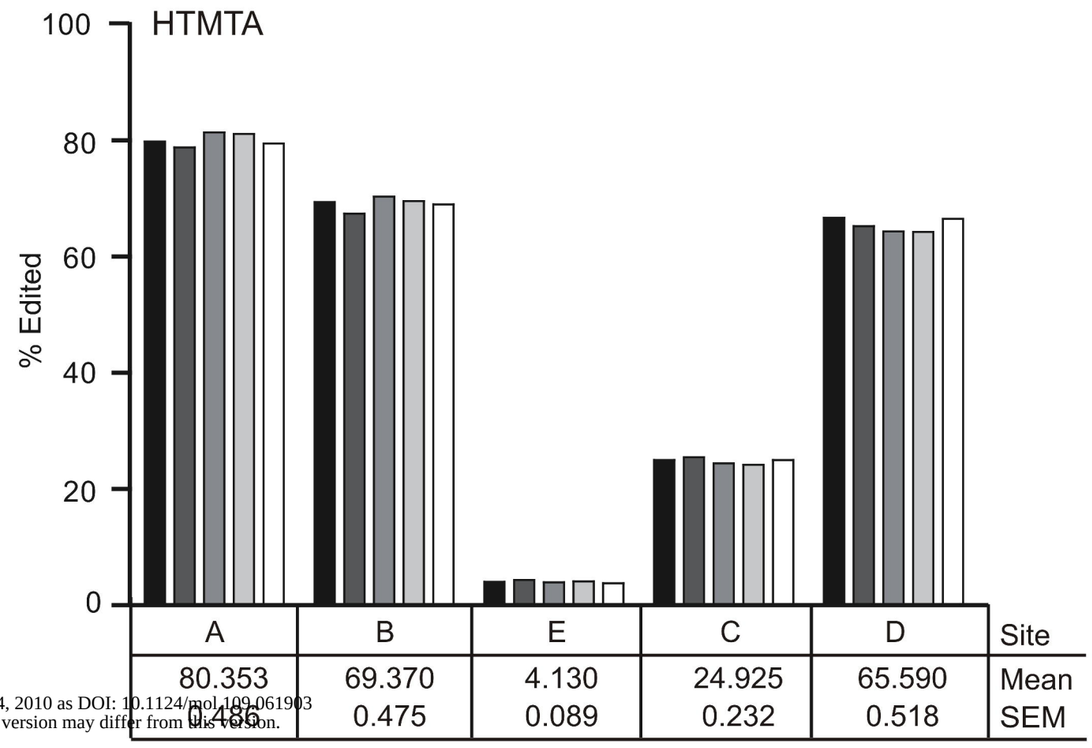
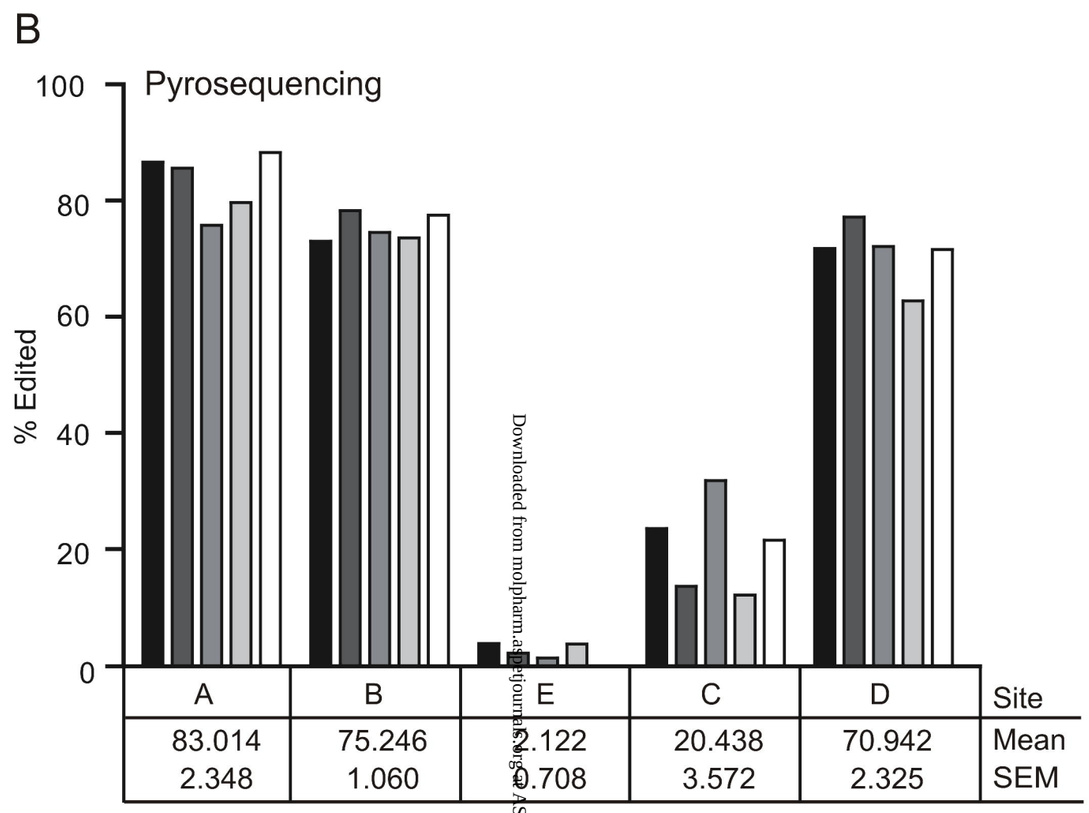


Figure 2.





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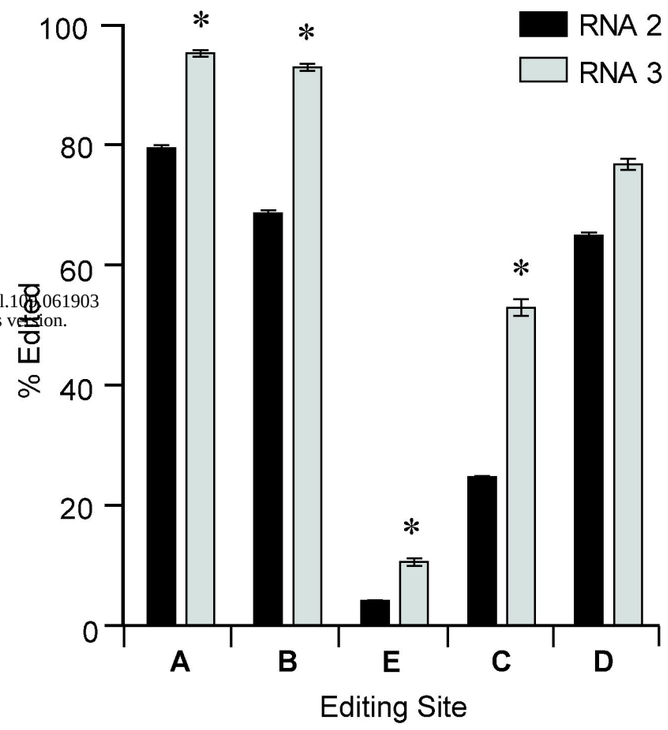

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Figure 4.

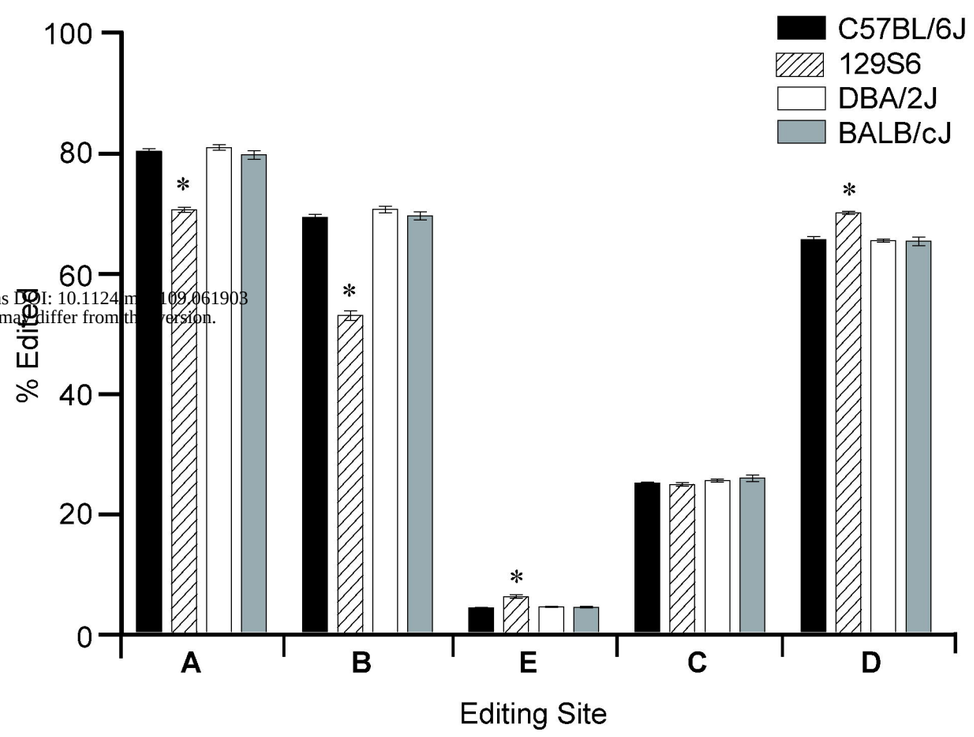

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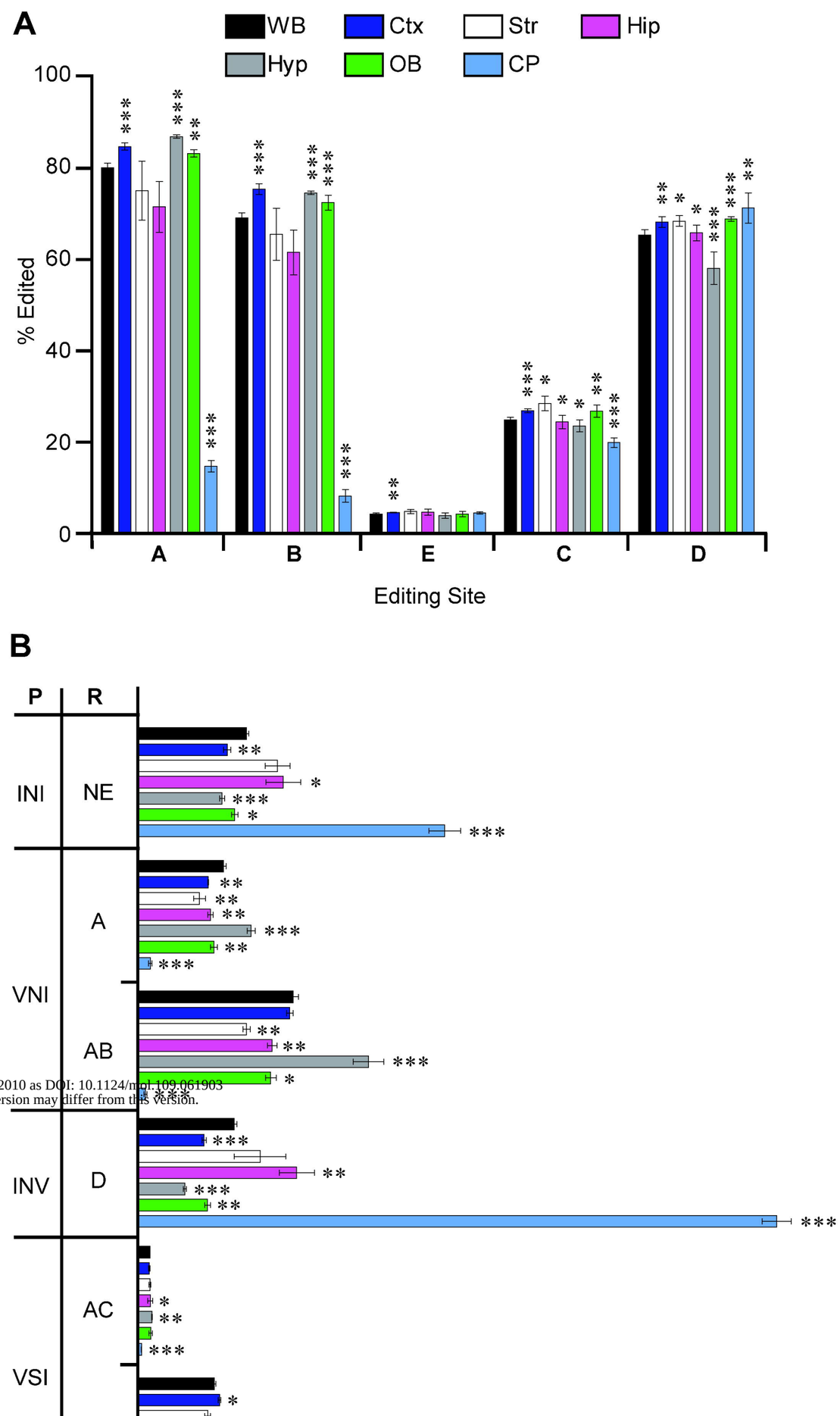
Figure 5.



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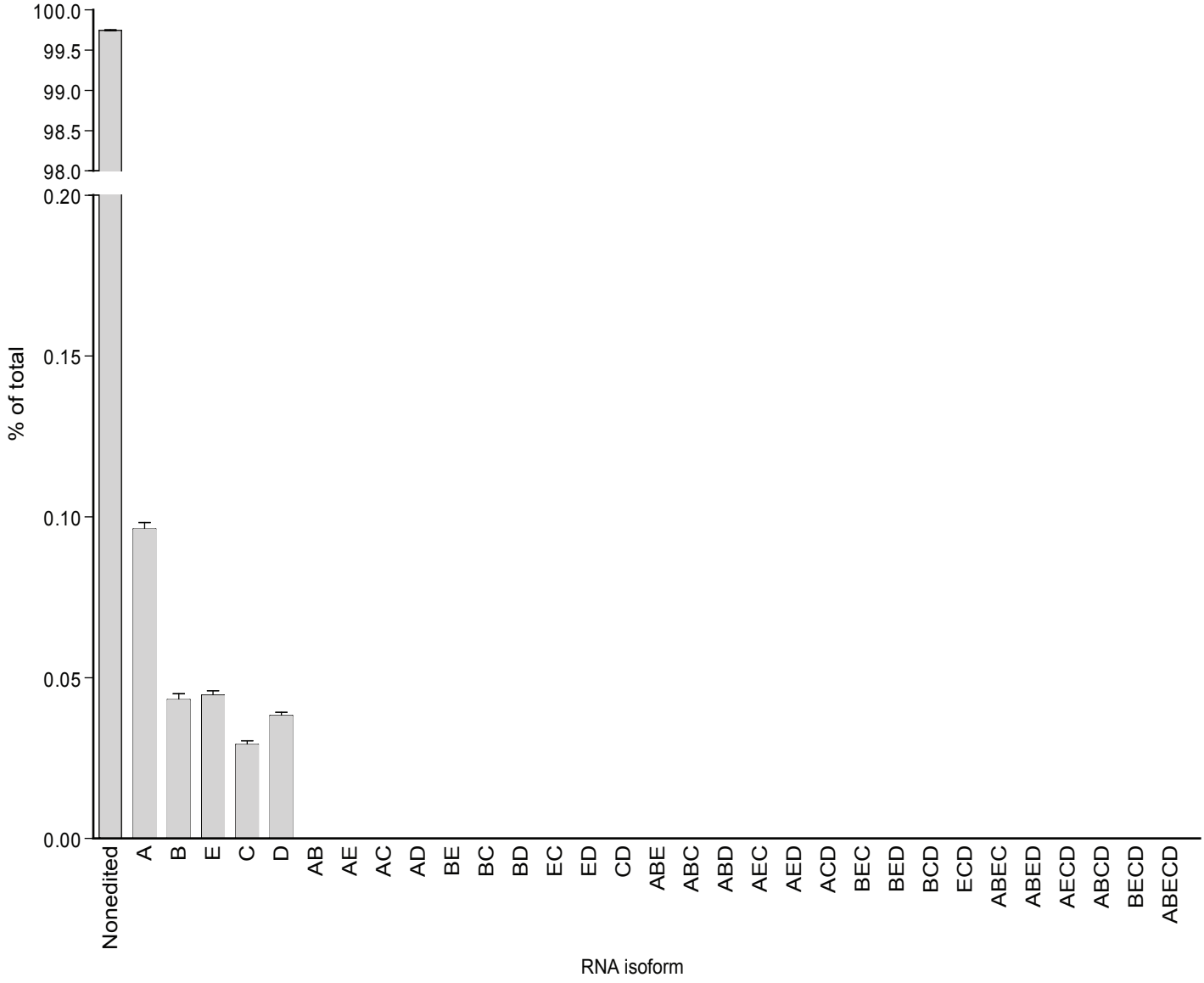

Figure S1. Quantification of false discovery. A non-edited human 5HT$_{2C}$ cDNA clone was analyzed by HTMTA to determine the level of editing isoforms resulting from A-to-G errors (n=5, mean ± SEM)