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Methodological considerations for gene expression profiling of human brain

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Abstract

Gene expression profiles of postmortem brain tissue represent important resources for understanding neuropsychiatric illnesses. The impact(s) of quality covariables on the analysis and results of gene expression studies are important questions. This paper addressed critical variables which might affect gene expression in two brain regions. Four broad groups of quality indicators in gene expression profiling studies (clinical, tissue, RNA, and microarray quality) were identified. These quality control indicators were significantly correlated, however one quality variable did not account for the total variance in microarray gene expression. The data showed that agonal factors and low pH correlated with decreased integrity of extracted RNA in two brain regions. These three parameters also modulated the significance of alterations in mitochondrial-related genes. The average *F*-ratio summaries across all transcripts showed that RNA degradation from the AffyRNAdeg program accounted for higher variation than all other quality factors. Taken together, these findings confirmed prior studies, which indicated that quality parameters including RNA integrity, agonal factors, and pH are related to differences in gene expression profiles in postmortem brain. Individual candidate genes can be evaluated with these quality parameters in post hoc analysis to help strengthen the relevance to psychiatric disorders. We find that clinical, tissue, RNA, and microarray quality are all useful variables for collection and consideration in study design, analysis, and interpretation of gene expression results in human postmortem studies.

Keywords: Microarray; Gene expression; Postmortem brain tissue; pH; RNA quality; Agonal factors

1. Introduction

It has been a challenge to locate precise candidate genes for complex psychiatric disorders using methods that were successful for simple Mendelian disorders. Complex psychiatric disorders are not caused by one gene, but rather by multiple genes (Mitchell et al., 1993; Craddock and Jones, 1999; Shastry, 2005). Complex disorders have been difficult to map for reasons of disease heterogeneity, misclassification and environmental influences. An accurate, yet comprehensive gene expression profile of brain tissue may result in better understanding of the genotype and phenotype relationships (Nestler et al., 2002; Bunney et al., 2003; Mirnics and Pevsner, 2004; Altar et al., 2005; Erraji-Benchekroun et al., 2005; Newton et al., 2005).

One highly used technique of gene expression profiling in psychiatric disorders has been microarray studies that use postmortem brain tissue (Barrett et al., 2001; Luo and Geschwind, 2001; Mirnics and Pevsner, 2004) followed by quantitative real time PCR to confirm candidate genes (Jurata et al., 2004; Mimmack et al., 2004). Microarray is a high-throughput method used to screen thousands of genes for alterations in expression between groups. The resulting data has suggested novel pathways linked to psychiatric disorders (Bunney et al., 2003; Hosack et al., 2003; Mootha et al., 2003a,b).

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The reliability and reproducibility of microarray results (Auer et al., 2003; Buesa et al., 2004; Shergill et al., 2004) must be constantly evaluated (Konradi et al., 2004; Ryan et al., 2004) and is an important question. Previously reports have shown that standard factors such as age, postmortem interval, and medical and family history from proxy respondents increased data reliability (Deep-Soboslay et al., 2005). It has been reported that the most critical aspect of postmortem research is the integrity of the sample (Mirnics and Pevsner, 2004; Tomita et al., 2004) and the pH (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006; Lipska et al., 2006). It has been suggested that samples used for gene expression studies must be of the highest quality (or matched quality) to represent underlying molecular pathophysiology (Dumur et al., 2004; Mirnics and Pevsner, 2004) and most investigators will attempt to avoid samples with highly degraded RNA. However, inherent in many comorbid psychiatric disorders (e.g. drug overdose and suicide), potential subjects are likely to have varying degrees of quality, and thus elimination of subjects with less than ideal quality would severely restrict research avenues in psychiatric disorders. Therefore, many studies match samples based on several quality parameters. Human postmortem brain tissue profiling has been challenging for several reasons beyond these quality issues (Mirnics et al., 2001, 2006). Polygenic, epigenetic, and environmental factors affect gene profiling (Mirnics et al., 2001). A presumed narrow range of gene expression in brain tissue due to homeostatic mechanisms restricts the fold-change of differential gene expression observed in microarray analyses of psychiatric disorders (Mirnics and Pevsner, 2004). Another complication reported was the dynamic range of gene expression of the transcriptome. For example, in the hippocampus there was about a 2000-fold difference between highly expressed genes compared to rare transcripts (Evans et al., 2002). Furthermore, RNA transcription was significantly regulated in the opposite direction to protein (Greenbaum et al., 2003), possibly due to mRNA stability, mRNA turn-over, mRNA steady-state transcription differences, and translation differences. Additionally, pharmacological treatments affected the transcriptome and since most patients with severe psychiatric disorders received medication, while controls do not receive psychiatric drugs, this further complicated interpretation of gene expression results.

In an effort to obtain well-characterized samples, which can be utilized for data analysis, investigators have begun to assess quality parameters thought to be important in postmortem brain tissue studies. One parameter often examined is the clinical quality. One aspect of clinical quality is to obtain correct retrospective psychiatric diagnosis (Deep-Soboslay et al., 2005). Medical records and next-of-kin interviews were complementary methods for confirmation of the diagnosis of cases and also useful to account for the lack of a psychiatric history in the controls (Brent et al., 1993; Kelly, 1996; Isometsa, 2001; Deep-Soboslay et al., 2005). Another facet of clinical quality is the agonal state of the patient. There is no consensus method for assessing agonal state (Hardy et al., 1985; Tomita et al., 2004) and thus, the precise effects on microarray quality have yet to be decided (Johnston et al., 1998; Buesa et al., 2004; Iwamoto et al., 2005). pH might be a more objective measure of clinical and tissue quality (Johnston et al., 1998) because pH was inversely correlated to the agonal state (i.e. the sum of the number of agonal factors as described in Tomita et al., 2004) and the duration of agonal state (measured in minute, hour, day). In order to assess this correlation correctly of pH and gene expression profiling, the data should be approached with caution because not all mRNA levels were affected by pH (Barton et al., 1993; Preece and Cairns, 2003; Buesa et al., 2004). For example, about 28% of mitochondrial-related transcripts were affected by pH (Vawter et al., 2006). Many researchers have used pH measurements as a substitute for clinical assessments of agonal state and duration (Johnston et al., 1998; Preece and Cairns, 2003; Buesa et al., 2004; Mirnics et al., 2004). Previous analyses showed that agonal factors were not perfect predictors of microarray and tissue quality and thus, other methods are being developed to assess microarray and sample quality (Li et al., 2005).

Differences in agonal state are clearly associated with differences in both pH and RNA quality (Harrison et al., 1991; Hynd et al., 2003; Tomita et al., 2004). Acidosis in human postmortem brain tissue can be caused by agonal factors such as coma, hypoxia, pyrexia, seizures, dehydration, hypoglycemia, multiple organ failure, head injury, and ingestion of neurotoxic substances, which can affect RNA integrity (Hardy et al., 1985; Harrison et al., 1991, 1995; Barton et al., 1993; Morrison-Bogorad and Pardue, 1995; Hynd et al., 2003). Along with agonal factors per se, rapidity of death played a role in the outcome of the tissue quality (Harrison et al., 1991; Hynd et al., 2003; Tomita et al., 2004). The influence of agonal factors on alterations of neurochemicals in human brain was initially demonstrated by researchers that measured the level of the inhibitory neurotransmitter (GABA) and the biosynthetic enzyme level of glutamic acid decarboxylase (GAD) in schizophrenia and Huntington's chorea (Bird et al., 1977; Spokes, 1979; Spokes and Iversen, 1979; Spokes et al., 1980). GABA was decreased in control brains due to hypoxia and long-term illness, but was even more reduced in Huntington's cases (Bird et al., 1977; Spokes, 1979; Spokes and Iversen, 1979; Spokes et al., 1980). Hypoxia was a key complication of major agonal events (Buesa et al., 2004) and it affected gene expression in human postmortem brain (Burke et al., 1991). Hypoxia was reported to 'cause' a reduction in pH (Hardy et al., 1985; Kingsbury et al., 1995; Corbett et al., 1996) possibly by increasing tissue lactate (Hardy et al., 1985; Yates et al., 1990). A decreased pH was also associated with an increased mitochondrial DNA copy number and an increased number of mitochondrial DNA transcripts in postmortem human brain (Vawter et al., 2006). However, lower pH was correlated with long agonal duration, thereby leading to speculation that mtDNA copy number and mtDNA transcription is influenced by pH as well as events occurring in the agonal phase. These results suggest that pH is a useful monitor for agonal events. However, it cannot be stated that lower pH will decrease RNA quality due to an effect such as acid hydrolysis within the pH range commonly observed in postmortem brain. Others have reported that a lower pH was associated with compromised RNA integrity (Harrison et al., 1995). Thus, pH and RNA integrity are strongly correlated

measures but it cannot be assumed that this represents a 'cause and effect' relationship.

Another factor used to assess postmortem samples is tissue quality. Different parameters have been used to assess tissue quality, notably brain pH, gross neuropathological examination, postmortem interval (PMI), and freezer time. Brain pH, as discussed above, has been shown to be related to agonal state and RNA integrity (Harrison et al., 1995; Kingsbury et al., 1995; Li et al., 2004; Tomita et al., 2004). Postmortem human brain tissue was evaluated using housekeeping gene expression by reverse transcription real-time PCR. The results showed pH was significantly correlated with the gene expression score for four housekeeping genes (Miller et al., 2004). Furthermore, when hippocampal gene expression profiles were examined in schizophrenia, pH was found to contribute to a variation in expression that was greater than any other factor evaluated (Mexal et al., 2006). Additionally, it was reported that pH affected the gene expression of mitochondrial-related genes (Li et al., 2004; Iwamoto et al., 2005; Vawter et al., 2006) and if the effects of pH were not controlled in a post hoc analysis, the gene expression profile results would be a reflection of pH (Li et al., 2004; Mexal et al., 2006; Vawter et al., 2006). It appears that not all mitochondrial-related transcripts are affected to the same degree by pH, however large pH effects were seen in non-mitochondrial-related gene expression (Vawter et al., 2006).

As a surrogate measure of tissue quality, pH, was reported to be stable in brain tissue after death and during freezer storage (Buesa et al., 2004). Different regions of the brain may be used for pH measurements, as stable pH measurements in 10 brain regions for three subjects were shown (Johnston et al., 1998). Cortex has been suggested to be a usable surrogate tissue for pH measurements (Mexal et al., 2006). The range and average pH measurement of postmortem brain collections varies between brain banks. Part of this variability may be due to the method of pH measurement (Preece and Cairns, 2003; Miller et al., 2004; Middleton et al., 2005; Torrey et al., 2005). Although, a more likely explanation is that this variation is due to the observation that agonal factors are variably related to pH, as discussed above.

Human studies have not shown a clear relationship between PMI and RNA quality (Harrison et al., 1995; Cummings et al., 2001; Catts et al., 2005). On the other hand, animal studies, which are more carefully controlled, have shown that an increase in the length of the PMI decreased total RNA amounts (Taylor et al., 1986). A recent study of murine samples reported that increased PMI was associated with decreased pH and decreased RNA integrity measured by 28S/18S ratio (Catts et al., 2005). The acceptable maximum PMI for human studies was reported as 36–48 h (Johnson et al., 1986; Barton et al., 1993; Soverchia et al., 2005). Furthermore, brain refrigeration following death will ultimately slow autolysis and maintain pH homeostasis (Buesa et al., 2004).

Tissue freezer storage time following autopsy was shown to cause degradation of the poly(A) tail region of RNA (Johnston et al., 1998). The loss of the poly(A) tail was thought to result in loss of the rest of the message (Bernstein and Ross, 1989), as this caused an exonuclease involved in RNA degradation to assemble (Ford et al., 1997; Yang et al., 2003; Wilusz and Wilusz, 2004). Another concern, was that loss of the poly(A) tail can impede the priming of the oligo-dT in the cDNA synthesis step of the microarray protocol. Furthermore, while the RNases involved in degradation may not be active at low temperatures, when a sample is subjected to freeze–thaw cycles it may result in degradation (Johnston et al., 1998).

A third aspect of sample assessment for gene expression profiling is the RNA quality, which is critical for subsequent microarray analysis (Dumur et al., 2004). Various criteria have been used to evaluate the integrity of RNA (Buesa et al., 2004; Dumur et al., 2004; Miller et al., 2004). The total RNA ratio, as determined by the Agilent Bioanalyzer, measures the fraction of the area in the 18S and 28S regions. These areas are compared to the total area under the curve and the result is the ratio of large molecules to small molecules. This reading is not sufficient to serve as a universal integrity number. It is now suggested that this reading is better when accompanied with the RNAA integrity number (RIN) algorithm. The RIN algorithm does not use the ratio of the ribosomal bands to determine integrity rather; it uses the entire electrophoretic trace. This tool allows for a robust and reliable prediction of RNA integrity (Schroeder et al., 2006). Another reported advantage of the Agilent Bioanalyzer was reliability and efficiency compared to standard agarose gel (Grissom et al., 2005).

Additionally, cRNA synthesized by in vitro transcription can be assessed as a measure of RNA quality by observing the median length on the Agilent Bioanalyzer (Dumur et al., 2004; Ryan et al., 2004) and by spectrophotometer to gauge the A_{260}/A_{280} absorbance measurements. The synthesis of high quality cDNA and cRNA were associated with the quality of the initial total RNA (Dumur et al., 2004; Carter et al., 2005).

The stability of human postmortem brain tissue mRNA for use in microarray analyses and real time PCR was shown to be an essential prerequisite for further downstream molecular analysis (Bahn et al., 2001; Lipska et al., 2006). For example, when RNA was manually degraded, it was shown that 75% of the differential gene expression was actually due to RNA integrity differences between the samples (Auer et al., 2003). Furthermore, the gene expression patterns showed that RNA degradation led to both up and down regulated genes (Auer et al., 2003; Lee et al., 2005). This was demonstrated by examining degraded total RNA samples at different time points and comparing the results to non-degraded RNA. At each time point, there were a significant number of genes that showed increased expression in the degraded samples when compared to the non-degraded RNA samples. One explanation for this is that RNA fragmentation may have caused a more efficient synthesis of cDNA. However, the RNases active during the freeze–thaw cycles are unpredictable and consequently lead to varying degrees of degradation (Grissom et al., 2005).

RNA degradation can be complex due to the structure of RNA (Hollams et al., 2002) and the sequence of the 3′-untranslated region (UTR) (Berger et al., 2005). The 3′-UTR sequence may have altered the stability of some RNA transcripts (Berger

et al., 2005) that harbor the AU-rich elements (AREs) and iron-responsive elements (IREs), both of which play a role in destabilizing some RNAs (Hollams et al., 2002). mRNA degradation occurred from the 3′ end, the 5′ end, or from internal positions (Buesa et al., 2004). The loss of the 5′ cap led to 5′ → 3′ decay and loss of the poly(A) tail led to 3′ → 5′ decay by exonuclease activity (Buesa et al., 2004; Fritz et al., 2004; Wilusz and Wilusz, 2004), but the predominant degradation pathway in mammals was not determined (Yang et al., 2003; Wilusz and Wilusz, 2004). It has been observed that low degradation caused a reduction in transcript length, but did not reduce the total amount of transcripts (Ryan et al., 2004). However, when genes were organized by functional classes the variation of decay rates between classes of mRNAs was significantly different (Yang et al., 2003).

Affymetrix gene expression probes on the U133 series of chips were designed toward the 3′ end and also further toward the 5′ end of several housekeeping genes (GAPDH and ACTB). The ratio of 3′/5′ expression was evaluated as a measure of transcript degradation. Although, because the site of degradation is unknown, in theory, it is possible to have a low 3′/5′ ratio (meaning relatively intact RNA) in a slightly degraded sample (Dumur et al., 2004; Ryan et al., 2004). Several *studies* have reported that slight RNA degradation does not have a substantial effect on the number of genes detected in the "Present Call" reading on the Affymetrix arrays (Schoor et al., 2003; Ryan et al., 2004; Lee et al., 2005). These studies examined only a small subset of the probesets on the arrays due to the restrictions in the design of each experiment (Schoor et al., 2003; Lee et al., 2005) and furthermore, it was shown that for each transcript the exact mechanism of RNA degradation was unclear (Ryan et al., 2004).

An often-overlooked aspect of discussions of degradation of mRNA is that frequently a spurious increased expression was found using microarray technology (Bahn et al., 2001; Auer et al., 2003; Schoor et al., 2003; Buesa et al., 2004; Ryan et al., 2004; Lee et al., 2005). RNA degradation was induced by an in vitro perturbation experiment and a list of 31 genes was found to be significantly different due to RNA degradation alone (Auer et al., 2003). Our group recently published a list of genes found to be affected by pH in three brain regions (Vawter et al., 2006). The complete Excel table can be downloaded here: http://pritzkerneuropsych.org/data/archive/File022206.aspx. We considered agonal-pH sensitive genes in a control group analysis only, and found 570 genes that were dysregulated across two or more brain regions (DLPFC, ACC, or CB) meeting a fold-change criteria of ±1.25 and in the top 5% ranked differential gene expression values. This data suggested labile transcripts in postmortem tissue may be used advantageously to indicate degradation and/or an imbalance due to agonal-pH factors. Implementing a protocol to qualitatively assess RNA integrity significantly improved the quality of microarray data (Carter et al., 2005).

The final quality parameter in gene expression profiling is the microarray quality. Affymetrix MAS 5.1 software determines whether each transcript was reliably detected using a percent present call (%PC) and a scaling factor (SF), which adjusted the average signal intensity to a preset constant. The %PC and SF obtained from the microarray results were used as gross indicators of RNA degradation or abundance (Ryan et al., 2004; Lee et al., 2005). When RNA was experimentally degraded the %PC was 40%, and with intact RNA the %PC was 54% (Lee et al., 2005). Thus, chips with lower present calls in a sample set must be treated with caution during analysis if the differences are significant this could be due to true biological differences or quality covariables. Prior reports have shown that differences in mRNA quality produced significant changes in microarray %PC and SF (Ryan et al., 2004; Lee et al., 2005).

Agonal factors, pH, and RNA integrity were each related to the post hoc microarray measure called the average correlation index (ACI), which was a chip quality indicator (Tomita et al., 2004). Another method for examining microarray similarity involved hierarchical clustering of samples and gene expression results (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006). Hierarchical clustering of samples by pH was independently replicated in a set of 105 DLPFC (Iwamoto et al., 2005) and a set of 24 human hippocampal samples (Mexal et al., 2006). Not surprisingly, the composition of groups based upon pH and agonal factor states was recommended as criteria for matching samples in human postmortem studies of single gene and protein expression, for examples see (Hardy et al., 1985; Harrison et al., 1991; Kingsbury et al., 1995; Johnston et al., 1998; Preece and Cairns, 2003).

Postmortem brain tissue is a limited resource and a major effort has been put forth to obtain well-characterized subjects. By evaluating the above quality factors in this paper the results will aid in future study design, analysis, and result interpretation. The present study was undertaken to address four broad quality indicators for evaluating the quality of postmortem samples for gene expression profiling.

2. Methods

2.1. Quality control indicators

Quality control indicators were analyzed for 98 anterior cingulate (ACC) and 91 matched cerebellum (CB) samples with microarray profiling results using U133A and U133P Affymetrix chips. Included in the 98 ACC samples were bipolar disorder ($n = 16$), schizophrenia ($n = 19$), control ($n = 42$), and major depression ($n = 18$) samples. However, diagnostic groupings were not used to assess quality. *Clinical quality* was assessed by agonal risk and agonal duration that yielded agonal factor scores (AFS). *RNA quality* was determined based on the 28S/18S rRNA readings and the RNA integrity number (RIN) both obtained from the Agilent Bioanalyzer. The 3′/5′ glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin (ACTB) housekeeping gene ratios were from the Affymetrix Microarray Suite 5.1 (MAS 5.1) report. Standard denaturing agarose gels were also run for RNA quality, and the AffyRNAdeg software program to compute RNA degradation (see Section 2.4 below). RNA degradation was evaluated using four quality indicators in two brain regions. *Tissue quality* was assessed by pH measurement, post mortem interval (PMI) and freezer time vari-

ables. *Microarray chip quality* was evaluated using the percent present call, scaling factor, average correlation index (ACI) of the chip (Tomita et al., 2004), and by gene clustering after array processing (Type 1/Type 2) (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006).

2.2. Agonal factor score (AFS)

We calculated the AFS based on data collected for each subject, which included the patient's physical health, medication use, psychopathology, substance abuse and details of death. This information was obtained from the medical examiner's conclusions, coroner's investigation, medical records and family interviews. Agonal risk and agonal duration scores were summed to give the final AFS for each subject as described in a prior study (Tomita et al., 2004).

2.3. pH measurements

Brain pH measurements were taken using a 50–100 mg piece of frozen cerebellar cortical slice. The frozen tissue was mixed with 1.0 mm glass beads (BioSpec Products, Bartlesville, OK) and distilled deionized water to form a 10% (w/v) solution. This solution was homogenized by shaking with a Bead-Beater (Biospec Products) for 60 s at 4 °C. The homogenate was then centrifuged at 5000 rpm for 2 min at 4 °C and then equilibrated to room temperature for 10 s and the pH was measured. The pH meter (Corning, Cypress, CA) was calibrated with three standard buffer solutions (pH 4, 7, and 10). The pH was measured in a second laboratory by the same the technique on the same samples and the results were highly concordant between the laboratories ($r = 0.97$, $n = 10$). This was also repeated in a third laboratory ($r = 0.99$, $n = 7$). To use a tissue for a single point calibration of our pH measurement technique, we subjected postmortem non-human primate brain cerebella to the same measurement pH method and found the average pH was 7.24 ± 0.15 ($n = 6$). This non-human primate experiment had an absence of agonal factors and had a short PMI, which may explain why a higher average pH was found, compared to postmortem human brain collections.

2.4. RNA quality measurements

(1) Total RNA was extracted from ACC ($n = 98$) and CB ($n = 91$) and evaluated on Agilent Technologies 2100 BioAnalyzer (Palo Alto, CA) to obtain the 28S/18S ratio.
(2) The RNA integrity number (RIN) was determined using Agilent Technologies 2100 Expert Software. The RIN is a software tool used to aid in the estimation of RNA integrity and to compare RNA integrity across samples (Imbeaud et al., 2005). This tool reads the entire electropherogram rather that just the 28S/18S ribosomal bands. The RIN reflected the presence and absence of degradation products; where higher RNA degradation was assigned a lower RIN value.
(3) A measure of RNA integrity was acquired via the microarray gene chip analysis based on the 3′/5′ ratio of signal intensities of the probe sets for the housekeeping genes glyceraldehyde phosphate dehydrogenase (GAPDH) and β-actin (ACTB) derived from Affymetrix MAS 5.1 signal intensity.
(4) A post hoc microarray measure of RNA degradation was proposed which utilized the R program function AffyRNAdeg (R program, Function to assess RNA degradation in Affymetrix GeneChip data) (Cope, 2005). This measurement was based on the fact that Affymetrix arrays have individual probes tiled in a 3′ to 5′ direction along the transcript, therefore an algorithm was created to measure the decay of transcript abundance, i.e. the signal decline within each gene on the Affymetrix GeneChip. The calculations were executed based on the assumption that primer transcription starts at the 3′ end and therefore, probe intensities should be lower at the 5′ end of a probeset compared to the 3′ end if RNA is degraded. This program calculates the average probe intensity based on location in the probeset and produces a plot of the means for each chip by probe. The slope of this graph was used as a measure of the severity of degradation (Gautier et al., 2004).
(5) Total RNA samples were run on a denaturing agarose gel according to a protocol from Ambion (Austin, TX). The agarose gel was quantitated on the BioRad ChemiDoc System and the 28S/18S ratio was determined. The Agilent 28S/18S readings were compared with the results of the same set of samples run on the agarose gel by a paired *t*-test.

2.5. Microarray chip quality

In the current sample, differential gene expression was determined using GC content robust multi-array average (gcRMA) (Wu et al., 2003). We applied a Unigene 4 custom chip definition file (Dai et al., 2005), which is available at: http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download_v4.asp. RMA was used to process the cel files for signal intensity and the signal intensity was used for gene clustering (Type 1/Type 2) after array processing (Li et al., 2004; Iwamoto et al., 2005; Mexal et al., 2006). The percent present call, the scaling factor and the average correlation index (ACI) of the chip were determined using MAS 5 generated values (Tomita et al., 2004). Pathway enrichment of differential gene expression was examined by gene set enrichment analysis (GSEA) (Mootha et al., 2003a,b).

The relationship of the quality parameters to the total variance was estimated by two methods. The receiver operator characteristic plots of sensitivity and specificity were used to determine the inter-relationships of the quality covariables in determining microarray outcome. This was determined quantitatively by measuring the area under the ROC curve and comparing different quality variables. However, this provided an assessment related to microarray outcomes, so a direct approach was to look at the total variation accounted for by each quality covariable across all genes in ANCOVA analyses. The *F*-ratios for each covariate was averaged across all probesets on the Affymetrix U133A platform.

3. Results

3.1. Clinical quality

The current sample consisted of anterior cingulate ACC ($n = 98$) and matched cerebellum CB ($n = 91$) samples, i.e. 91 subjects had data for two brain regions (Table 1). Cases were included in the present data in which both medical records and next-of-kin interviews were obtained (Table 1). The controls were also ascertained with the same method so a similar level of rigor was applied to the controls. We did not assess case–control differences but assessed all samples together to minimize analyses, and to maintain power with a large number of subjects. The subjects were diagnosed as bipolar disorder (16%), major depression (18%), schizophrenia (20%) or controls (46%).

We established two groups of subjects based upon agonal factor scores (AFS = 0 versus AFS > 0) and compared these groups for differences in tissue, RNA, and microarray quality. The three categories of quality indicators (tissue pH, RNA quality, and microarray quality) were significantly different between AFS = 0 and AFS > 0 samples for both brain regions (Fig. 1; Table 2).

An over-representation analysis revealed that the mitochondrial pathway of gene expression was affected by AFS. Analysis of ACC showed that mitochondrial enzymes were significantly over-represented when comparing AFS = 0 and AFS > 0 (Table 3). Agonal duration and pH were significantly correlated ($r = -0.43$, $p < 0.0001$). These results ($n = 98$) agreed with our previously published data which included 40 subjects (Vawter et al., 2006).

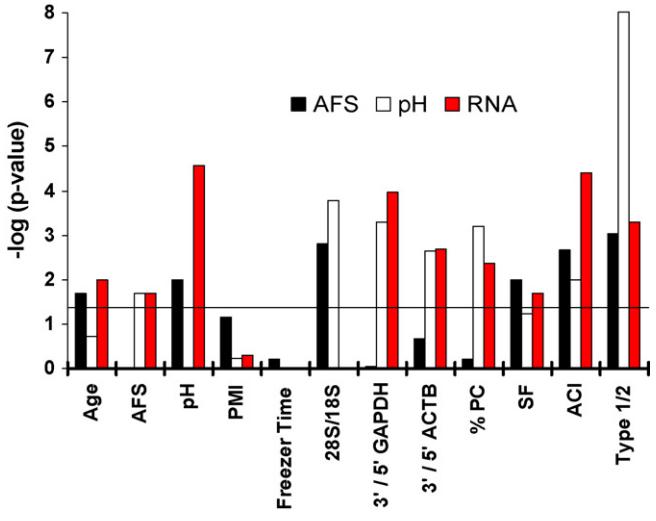

Fig. 1. The significance of comparing groups separated by differences in high vs. low RNA quality, clinical quality, and tissue quality is shown for multiple variables commonly used in controlling gene expression profiling experiments and in particular microarray results (%PC, SF, ACI, and Type 1/2). The data is from Table 2 for the anterior cingulate cortex and shows on the *y*-axis the significance of *t*-test (*p*-value transformed by −log 10) and the *x*-axis shows the individual variables. The abbreviations are same as shown in Table 1. The legend shows three different groups (AFS, pH, and RNA).

Table 1
Summary of four quality control measures for brain samples from anterior cingulate ($n = 98$) and cerebellum ($n = 91$)

Region	Clinical		Tissue			RNA			Microarray chip			
	Age	AFS 0/AFS > 0[a]	pH	PMI (hours)	Freeze time (years)	28S/18S ratio	3′/5′ GAPDH[b]	3′/5′ ACTB[c]	RIN[d]	% PC[e]	SF[f]	ACI[g]	Type 1/2[h]
Anterior cingulate cortex (ACC) ($N = 98$)	51.98 (13.40)	75/23	6.82 (0.24)	24.14 (8.63)	2.96 (3.34)	1.80 (0.37)	1.68 (0.70)	2.93 (1.80)	5.95 (1.35)	43.02 (5.22)	1.42 (1.57)	0.94 (0.06)	71/27
Cerebellum ($N = 91$)	51.64 (13.20)	75/16	6.83 (0.24)	23.72 (8.41)	3.15 (3.40)	1.89 (0.46)	1.70 (0.77)	3.55 (3.03)	6.58 (1.66)	43.94 (5.48)	2.57 (4.16)	0.96 (0.05)	64/27

The categories of the four quality control indicators are displayed for each brain region. There were 91 common.
[a] AFS = agonal factor score, number refers to the number of subjects with AFS = 0/AFS > 0.
[b] GAPDH = glyceraldehyde phosphate dehydrogenase.
[c] ACTB = β-actin.
[d] RIN = RNA integrity number.
[e] %PC = percent present call.
[f] SF = scaling factor.
[g] ACI = average correlation index.
[h] Type 1/2 = hierarchical clustering group membership which is defined by cluster membership.

Table 2
Quality control assessment

Brain	Criteria	Clinical quality		Tissue quality			RNA quality				Microarray chip quality			
		Age	AFS* 0/≥1	pH	PMI (hours)	Freezer time (years)	28S/18S	3′/5′ GAPDH	3′/5′ ACTB	RIN	% PC	SF	ACI	Type 1/2*
ACC	AFS = 0 (n = 75)	50.49 (13.87)	N/A	6.87 (0.20)	25.04 (8.48)	2.85 (3.08)	1.86 (0.36)	1.68 (0.72)	3.02 (1.97)	6.05 (1.08)	43.19 (4.87)	1.06 (0.93)	0.95 (0.04)	61/14
	AFS ≥ 1 (n = 23)	56.83 (10.57)	N/A	6.69 (0.31)	21.22 (8.67)	3.33 (4.15)	1.60 (0.31)	1.66 (0.64)	2.62 (1.06)	5.73 (1.81)	42.45 (6.33)	2.58 (2.48)	0.90 (0.08)	10/13
	p-Value	**0.02**	N/A	**0.01**	0.07	0.61	**0.001**	0.90	0.21	0.44	0.61	**0.01**	**0.002**	**0.0009**
CB	AFS = 0 (n = 75)	50.49 (13.87)	N/A	6.87 (0.20)	24.27 (8.28)	2.85 (3.08)	1.96 (0.45)	1.62 (0.77)	3.62 (3.28)	6.58 (1.66)	45.18 (3.89)	1.37 (2.10)	0.97 (0.04)	61/14
	AFS ≥ 1 (n = 16)	57.06 (7.65)	N/A	6.64 (0.33)	21.16 (8.80)	4.53 (4.48)	1.56 (0.39)	1.97 (0.71)	3.24 (1.35)	4.99 (1.64)	38.09 (7.83)	7.01 (5.95)	0.90 (0.07)	3/13
	p-Value	**0.01**	N/A	**0.02**	0.21	0.17	**0.00125**	0.09	0.45	**0.0001**	**0.0025**	**0.0017**	**0.01**	**<0.000005**
ACC	pH ≥ 6.6 (n = 81)	51.23 (13.67)	67/14	N/A	23.89 (8.22)	2.97 (3.26)	1.75 (0.37)	1.52 (0.53)	2.55 (1.40)	6.09 (1.25)	44.13 (4.26)	1.19 (1.20)	0.96 (0.03)	69/12
	pH < 6.6 (n = 17)	55.53 (11.71)	9/8	N/A	25.35 (10.60)	2.96 (3.82)	1.49 (0.41)	2.46 (0.88)	4.70 (2.40)	5.34 (1.56)	37.71 (6.20)	2.46 (2.54)	0.91 (0.06)	2/15
	p-Value	0.19	**0.02**	N/A	0.60	1.00	**0.00017**	**0.0005**	**0.0022**	0.12	**0.0006**	0.06	**0.01**	**<0.000005**
CB	pH ≥ 6.6 (n = 74)	50.76 (13.44)	66/8	N/A	23.81 (8.42)	3.19 (3.32)	1.92 (0.43)	1.58 (0.72)	3.34 (3.06)	6.73 (1.66)	44.81 (4.65)	2.03 (3.41)	0.97 (0.03)	62/12
	pH < 6.6 (n = 17)	55.53 (11.71)	9/8	N/A	23.37 (8.64)	2.96 (3.82)	1.73 (0.56)	2.11 (0.84)	4.49 (2.81)	6.03 (2.13)	40.14 (7.18)	3.80 (4.92)	0.91 (0.09)	2/15
	p-Value	0.15	**0.002**	N/A	0.85	0.82	0.21	**0.03**	0.15	0.27	**0.02**	0.17	**0.02**	**<0.000005**
ACC	28S/18S ≥ 1.5 (n = 73)	49.84 (13.62)	64/14	6.86 (0.22)	23.99 (7.92)	2.83 (3.31)	N/A	1.55 (0.57)	2.73 (1.78)	6.08 (1.35)	43.57 (5.10)	1.29 (1.53)	0.96 (0.03)	60/13
	28S/18S < 1.5 (n = 25)	58.24 (10.67)	11/9	6.72 (0.27)	24.59 (10.62)	3.35 (3.48)	N/A	2.07 (0.89)	3.48 (1.78)	5.56 (1.45)	41.42 (5.35)	1.76 (1.69)	0.93 (0.05)	11/14
	p-Value	**0.01**	0.02	**0.00002**	0.51	0.93	N/A	**0.0001**	**0.002**	0.11	**0.004**	**0.02**	**0.00003**	**0.0005**
CB	28S/18S ≥ 1.5 (n = 76)	51.33 (13.50)	66/10	6.86 (0.24)	24.17 (8.10)	3.03 (3.28)	N/A	1.50 (0.54)	3.06 (2.53)	6.91 (1.45)	45.00 (4.55)	1.80 (2.89)	0.97 (0.03)	60/16
	28S/18S < 1.5 (n = 15)	53.27 (11.84)	9/6	6.63 (0.19)	21.49 (9.87)	3.72 (4.04)	N/A	2.58 (1.10)	6.07 (4.06)	4.49 (1.44)	38.53 (6.69)	5.20 (6.02)	0.91 (0.08)	4/11
	p-Value	0.58	**0.02**	**0.0002**	0.34	0.55	N/A	**0.002**	**0.01**	**0.0006**	**0.002**	**0.05**	**0.02**	**0.0001**

Each quality control measure is based on the criteria listed below for both anterior cingulate (ACC) and cerebellum (CB). The averages (±standard deviation) are shown for each criterion and significant differences between the groups were determined by t-test. The resulting significant p-values are shown in bold. The abbreviations are the same as used in Table 1. N/A indicates that due to *a priori* selection of quality by AFS, pH, and RNA these produced highly significant differences therefore the p-value was not shown.

* p-Values for AFS and Type 1/2 were calculated using Fisher's exact test.

Table 3
Differential expression of genes in the mitochondrial pathway

Brain region	Pathway search	Pathway	Subjects with AFS = 0 compared to subjects with AFS > 0	Subjects with pH ≥ 6.6 compared to subjects with pH < 6.6	Subjects with 28S/18S ≥ 1.5 compared to subjects with 28S/18S < 1.5
ACC	KEGG	Oxidative phosphorylation	4.85E−02	NS*	1.00E−02
	Gene ontology	Mitochondria	NS	4.30E−02	1.30E−02
CB	KEGG	Oxidative phosphorylation	4.00E−03	2.70E−02	NS
	Gene ontology	Mitochondria	4.30E−02	6.50E−03	NS

Differential gene expression was determined in two brain regions and used for ranking in an over-representation analysis (GSEA gene set enrichment analysis, Mootha et al., 2003a,b). The over-representation analysis used subject permutation to correct *p*-values for multiple test comparisons.
* NS: not significant.

3.2. Tissue quality for gene expression profiling

Some brain banks have collected brains ∼10–30 years older than the current sample (Breese et al., 1997; Hakak et al., 2001; Konradi et al., 2004). Because of the high average age of these cases, investigators routinely utilized microscopic neuropathological examinations to rule out age-related neurodegeneration in cases and controls. In the present study, subjects were relatively young at the time of death (Table 1) and were excluded if they showed clinical signs of dementia prior to death. Therefore a neuroanatomist performed gross neuropathological examinations of all subjects after the brain had been sliced into 1 cm coronal slabs to rule out hemorrhage, gross infarcts, and lesions. Any subjects with gross neuropathology were excluded.

The pH and AFS measures were significantly correlated ($r = -0.44$, $p < 0.01$, d.f. = 89) therefore the residual variation between pH and AFS must be accounted for by other unmeasured variables. We found that subjects with zero agonal factors, which included only short agonal duration of minutes and no prolonged illnesses had a range of pH from 6.3 to 7.26 (Fig. 2). The average pH was 6.83 ($n = 98$) and the median was 6.87 (Fig. 2).

In order to assess the statistical relationships of pH on RNA integrity and microarray quality, the subjects were arbitrarily divided into high pH (≥6.6) and low pH (<6.6). In a statistical sense, pH significantly affected measures of RNA quality and microarray quality for both brain regions by *t*-test comparisons of the high and low pH groups (Fig. 1; Table 2).

The PMI was not statistically associated with pH or RNA integrity (Fig. 1; Table 2). This was not surprising because the average PMI was 24.1 ± 8.6 h and during the collection process any sample with a PMI over 48 h would not be collected. Furthermore, a majority of the postmortem cases received from the coroner's office were placed in the refrigerator within hours after death pending autopsy. The average ±standard deviation of the postmortem time to refrigeration was 6.5 ± 4.2 h. The average freezer time ±standard deviation for the 98 ACC samples was 2.9 ± 3.3 years and there were no significant relationships between freezer time and RNA quality (Tables 2 and 4). However, an in vitro perturbation of RNA to assess one freeze–thaw cycle that could occur during prolonged freezer storage conditions of small RNA aliquots modestly increased RNA degradation (next section).

The mitochondrial gene pathways were significantly different between the groups with high pH (≥6.6) and low pH (<6.6) using an over-representation analysis of GO terms for cellular, biological, and molecular components (Vawter et al., 2006). The mitochondrial pathway was significantly over-represented between the high pH and low pH groups for the KEGG analysis and the GO mitochondrion cellular component term (Table 3). These results were similar to the comparisons of the AFS groups (see Section 3.1 above), as pH and the AFS (sum of the number of agonal factors) were significantly correlated ($r = -0.44$, $p < 0.01$, Fig. 2).

Table 4
The top 5% of dysregulated genes in ACC comparing different groups based on AFS, pH and 28S/18S were compared to genes listed in Auer et al. (2003) that were found to be dysregulated due to RNA degradation

Gene symbol	Auer et al. up/down	AFS = 0 vs. AFS = +1	pH ≥ 6.6 vs. pH < 6.6	28S/18S ≥ 1.5 vs. 28S/18S < 1.5
GNAS	Down		X	X
CHD1	Down	X		
GDI2	Down		X	
ATP5A1	Down		X	X
YWHAZ	Down		X	X
PCBP1	Down		X	X
TEGT	Down	X		
B2M	Down	X		X
NME1	Up		X	X
ATP5B	Up		X	X

There were 31 genes listed in Auer et al. (2003) changed by RNA degradation. Comparisons of the top 5% of dysregulated genes between our ACC samples revealed 194 genes in common between AFS and pH, 202 genes in common between AFS and 28S/18S and 417 genes in common between pH and 28S/18S. There were 146 genes in common between the three groups.

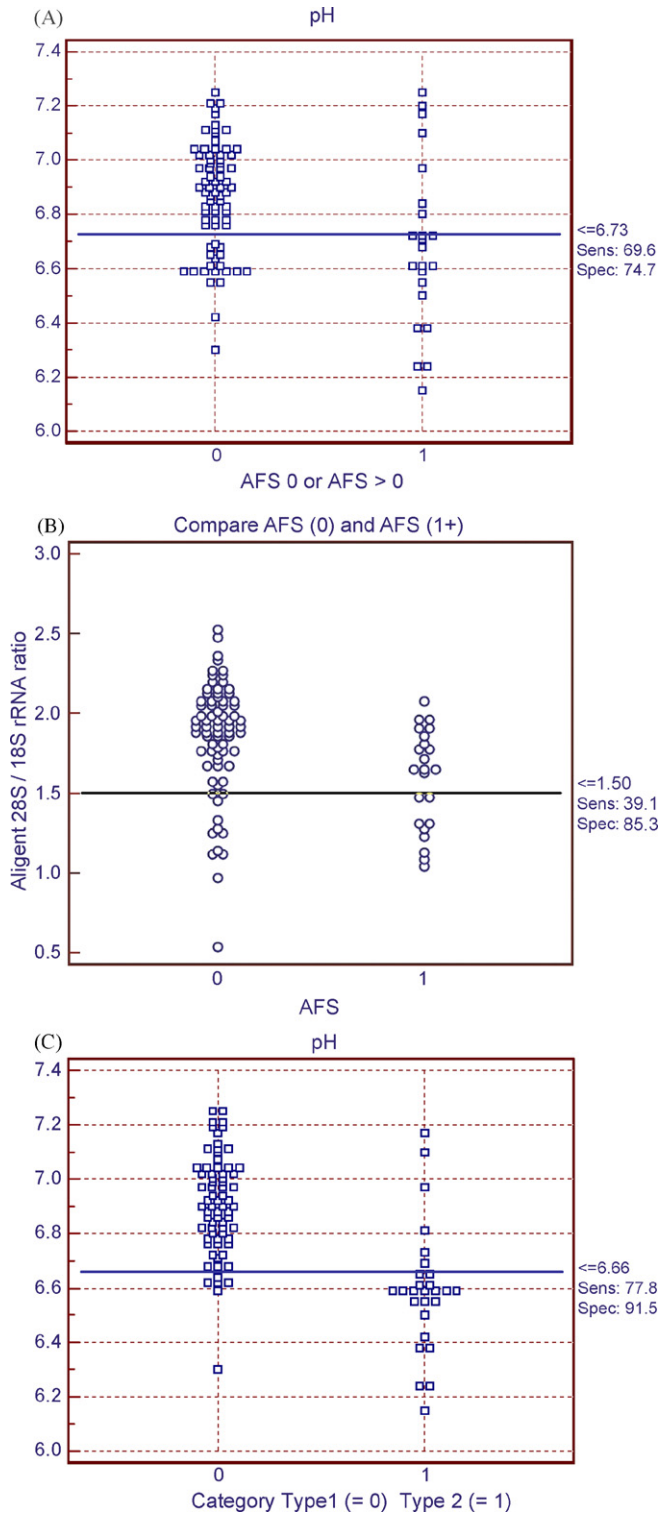

Fig. 2. (A) Sensitivity of pH and (B) RNA to AFS 0 and AFS > 0. The majority of 98 cases show a pH above an arbitrary cut point of 6.73 and RNA quality above a cut-point of 1.4, however there are outliers within AFS = 0 and AFS 1 categories. The combined use of three quality measures of pH, RNA, and AFS results in less differences in gene expression as a result of agonal-pH effects (Vawter et al., 2006). (C) Type '1' and '2' microarray outcome strongly depends on pH (Li et al., 2004). (B) Histogram of RNA quality (28S/18S) by AFS. (C) Histogram of pH by Type '1' and Type '2' samples. The type '1' and '2' refer to groups formed in hierarchical clustering that were associated with pH differences between clusters.

3.3. RNA quality

We induced RNA degradation in vitro by freeze–thawing RNA extracts from brain to simulate what might be observed during prolonged freezer storage, as aliquots in small volumes may freeze–thaw on occasion especially when moving samples in and out of the freezer. RNA samples that were fresh frozen, thawed once at room temperature and then subjected to an additional freeze–thaw cycle showed no differences in the integrity of total RNA determined by quantification of 28S/18S ribosomal RNA ratios on the Agilent 2100 Bioanalyzer. However, the Agilent RNA integrity number (RIN) showed significantly more degradation in the twice thawed samples compared to the thawed once samples ($p = 0.0018$). These results support the ability of the RIN algorithm to robustly detect mild degradation differences between samples compared to the 28S/18S ratio which did not detect any difference.

Total RNA 28S/18S ratios from a set of samples were measured on both the Agilent Bioanalyzer and on a denaturing agarose gel. The paired *t*-test between the methods was significant ($p = 0.03$) and the Agilent 28S/18S readings were consistently higher than the agarose gel 28S/18S ratio, as previously observed (Boris Sokolov, personal communication). Thus, setting a low threshold for Agilent quality translated to a lower quality measurement by conventional formaldehyde gel electrophoresis.

To measure the effect of RNA degradation on other quality parameters, the samples were divided into high (≥ 1.5) and low (<1.5) 28S/18S total RNA based upon Agilent measures. The median length of cRNA was significantly decreased in the low 28S/18S group ($p = 0.03$) compared to the high 28S/18S group confirming that shorter transcripts, as shown by cRNA length, are indeed made from degraded total RNA. Additionally, the samples with pH < 6.6 displayed a shorter size of the cRNA in vitro transcript compared to the higher pH group >6.6 ($p = 0.006$). A poor quality total RNA was associated with shorter cRNA transcripts.

We found significant correlations between the 28S/18S ratio and 3′/5′ GAPDH ratio ($r = -0.64$, $p < 0.01$, d.f. = 89) and between the 28S/18S and 3′/5′ ACTB ($r = -0.48$, $p < 0.01$, d.f. = 89). As expected, the negative correlations indicated higher 3′/5′ signal ratios, which was associated with poorer starting RNA quality. GAPDH 3′/5′ ratio presents a view of short transcript degradation while the ACTB 3′/5′ ratio gives a view of longer transcript degradation in postmortem brain. Hence, this was the reason that samples often showed a large disparity between the two ratios.

We employed an arbitrary criterion for 28S/18S Agilent measure (≥ 1.5) which produced a significant difference in the four quality measures in ACC and in CB (Fig. 2; Table 2). There was a technical problem in measuring the RIN in the ACC, so not all samples were measured. The technical issue might have biased our measures towards a lower RIN value for ACC compared to the CB samples leading to this regional variability. However, the 28S/18S measure was very robust and the mitochondrial pathway was significantly different between the 28S/18S groups in both the KEGG pathway and GO terms for cellular component

in ACC. The significance was also observed in CB for the KEGG pathway (Table 3).

Another question we addressed was if a consistent set of labile genes in degraded samples existed which could be used to survey the degradation and adjust for degradation effects using the labile gene set. The top 5% of genes dysregulated by the AFS, pH, and 28S/18S comparisons were matched to the Auer et al. (2003) gene list. There were 9 genes in common between the 31 genes found by Auer et al. (2003) and the present study indicating that certain labile transcripts are dysregulated when the postmortem brain sample was degraded (Table 4). Furthermore, in the present study there were 146 altered genes in common between all three group comparisons of AFS, pH, and 28S/18S.

3.4. Microarray chip quality

The results presented thus far, have largely focused on the impacts and inter-relatedness of clinical, tissue, and RNA quality. We have also examined pathway analysis for mitochondrial-related transcripts, and will later address the issue of variability of the quality factors across the entire transcriptome. It can be summarized that using only one quality indicator did not account for the total picture of sample variability and selection because each quality indicator was partially and not perfectly correlated (see Table 5 for correlation matrix of quality indicators; Fig. 2).

After the samples were completely processed the data from the microarray chip provided a set of indicators to measure overall chip quality and derivative measures of RNA degradation. The Affymetrix MAS 5.1 software was used to determine whether each transcript was reliably detected using a percent present call (%PC) and a scaling factor (SF), which adjusted the average signal intensity to a preset constant. Microarray chip quality was evaluated in the present study at cut points for the other three quality measures (AFS = 0, pH ≥ 6.6 and 28S/18S ≥ 1.5). Two microarray quality indicators, %PC and SF, were significantly different in both ACC and CB for RNA quality and for pH (tissue quality) group comparisons (Fig. 2, Table 2). Thus, differences in mRNA quality were related to significant changes in microarray %PC and SF.

ACI was significantly different between all three groups (AFS, pH, and 28S/18S shown in Fig. 2 and Table 2) for both brain regions. Employing these same cut points for AFS, pH and 28S/18S revealed significant differences (p-values ranged from 9.6E−09 to 5.2E−04) between the 'Type 1' and 'Type 2' groups for the current samples (Table 2; Fig. 2). The ROC plots and data table showed similar findings that pH and RNA quality were equally related to 'Type 1' and 'Type 2' membership (illustrated in Figs. 2 and 3; Table 6).

We tested more global gene expression parameters in two further methods. First, the AffyRNAdeg function modeled the extent of RNA degradation and thus could possibly control for this effect across samples at the probe level. The slope generated by AffyRNAdeg, which putatively measured the severity of degradation, was found to be significantly correlated with %PC ($r = -0.36$, $p = 0.0005$), 3′/5′ GAPDH ($r = 0.47$, $p < 0.0001$),

Table 5
Correlations of quality control measures. Each quality control measure is organized by the four categories (clinical, tissue, RNA, microarray) and then correlated for cerebellum data

	Clinical quality		Tissue quality			RNA quality				Microarray quality			
	Age	AFS	pH	PMI	Freezer time	28S/18S	3′/5′ GAPDH	3′/5′ ACTB	RIN	cRNA (~nts)	PC	SF	ACI	Type 1/2
Age	1.00													
AFS	0.19	1.00												
pH	−0.20	**−0.44***	1.00											
PMI	−0.05	−0.13	0.12	1.00										
Freezer time	−0.19	0.17	0.00	**−0.44**	1.00									
28S/18S	−0.04	−0.25	**0.33**	0.11	−0.18	1.00								
3′/5′ GAPDH	0.08	0.21	**−0.35**	0.13	−0.01	**−0.64**	1.00							
3′/5′ ACTB	−0.02	−0.02	−0.20	0.21	−0.18	**−0.48**	**0.86**	1.00						
RIN	−0.02	**−0.52**	**0.31**	0.25	**−0.37**	**0.68**	**−0.63**	**−0.37**	1.00					
cRNA (~nts)	−0.15	−0.21	0.22	−0.19	**0.34**	0.14	**−0.42**	**−0.34**	**0.28**	1.00				
PC	−0.04	**−0.46**	**0.38**	0.17	**−0.40**	**0.53**	**−0.47**	−0.17	**0.63**	0.21	1.00			
SF	0.01	**0.52**	−0.25	**−0.34**	**0.45**	**−0.41**	**0.27**	0.02	**−0.70**	−0.22	**−0.82**	1.00		
ACI	−0.06	**−0.53**	**0.46**	−0.03	−0.10	**0.58**	**−0.70**	**−0.49**	**0.63**	**0.39**	**0.65**	**−0.41**	1.00	
Type 1/2	0.17	**0.49**	**−0.61**	0.16	0.01	**−0.42**	**0.47**	0.24	**−0.34**	**−0.33**	**−0.48**	**0.30**	**−0.59**	1.00

The significant correlations are in bold ($p < 0.01$). Abbreviations are the same as in Table 1.
* Bold correlations were significant ($r > |0.267|$, $p < 0.01$, d.f. = 89).

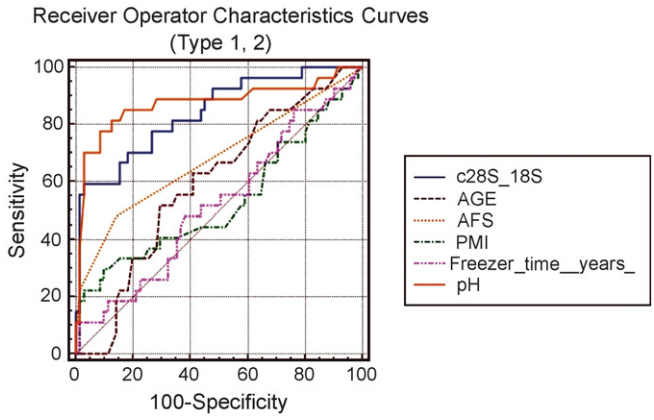

Fig. 3. The diagnostic performance of test variables, or the ability of a variable to discriminate microarray outcome (Type '1' vs. Type '2') is evaluated using receiver operating characteristic (ROC) curve analysis. The ROC curves are shown for six quality variables that relate differentially to microarray outcome. The area under each curve shows a relationship to overall prediction of Type '1' and '2' microarray outcome. The comparison of ROC curves for tissue quality (pH, freezer time, PMI), clinical quality (AFS, age), or RNA quality (28S/18S) shows that tissue quality pH measure is strongly related to Type 1 and Type 2 microarray outcome (Li et al., 2004). Table 6 shows the ROC values for each variable.

3′/5′ ACTB ($r = 0.76$, $p < 0.0001$), and cRNA length ($r = -0.35$, $p = 0.0007$). The AffyRNAdeg slope variable was used as a covariate in PLMfit (Bolstad, 2004) and this reduced the residual variation between duplicate RNA samples run on chips at different laboratories (data not shown).

The second method of testing global gene expression relationships to the four groups of quality covariables was to enter each variable as either a main effect if categorical such as AFS or 'Type 1, 2' or as a covariate if continuous. The *F*-ratios from the ANCOVA for all transcripts were summarized by average *F*-ratio for each variable. Of interest, the slope from the AffyRNAdeg showed the highest average *F*-ratio compared to all other covariates (Fig. 4). The rank of AffyRNAdeg persisted regardless of the type of data normalization used (quantile or grand median centering) or the effect of entering or removing other covariates.

Table 6
The comparison of receiver operator characteristic (ROC) curves for microarray Type 1 or Type 2 outcome (Li et al., 2004)

Quality variable	Area under ROC (maximum area = 1)	Standard error	95% CI
pH	0.874	0.035	0.792–0.933
28S/18S RNA ratio	0.841	0.040	0.754–0.907
AFS*	0.683	0.063	0.581–0.773
Age*	0.597	0.066	0.493–0.694
Freezer time*	0.523	0.066	0.420–0.625
PMI*	0.532	0.066	0.429–0.634

Tissue quality (pH) and RNA quality (28S/18S) strongly associated with accurate detection of Type 1 and Type 2 microarray outcome. The ROC plot for these variables is shown in Fig. 3.

* The area under the ROC column was significantly increased for RNA quality and pH compared to all other variables except the pH.

Table 7
Example of a strategy that used all three sample cut-points and eliminated the poorest quality samples and chips

Brain	Criteria	Clinical quality		Tissue quality			RNA quality				Microarray Chip quality			
		Age	AFS 0/≥1	pH	PMI (hours)	Freezer time (years)	28S/18S	3′/5′ GAPDH	3′/5′ ACTB	RIN**	%PC	SF	ACI	Type 1/2***
ACC	Include ($n = 60$)	49.43 (13.76)	N/A	6.93 (0.16)	22.96 (7.79)	3.35 (3.36)	1.98 (0.22)	1.46 (0.37)	2.49 (1.42)	5.94 (0.93)	44.00 (4.23)	1.07 (1.03)	0.97 (0.02)	56/4
	Exclude ($n = 38$)	56.26 (12.05)	N/A	6.66 (0.26)	25.52 (9.19)	2.51 (3.41)	1.51 (0.36)	2.03 (0.93)	3.60 (2.13)	5.67 (1.58)	41.47 (6.25)	1.97 (2.08)	0.92 (0.05)	12/24
	p-value	**0.01**	N/A	N/A	0.16	0.24	N/A	**0.00074**	**0.0006**	0.35	**0.03**	**0.02**	**<0.000005**	**<0.000005**
CB	Include ($n = 59$)	50.20 (13.81)	N/A	6.94 (0.16)	23.44 (7.99)	3.11 (3.24)	2.06 (0.34)	1.42 (0.48)	3.02 (2.74)	7.11 (1.31)	45.96 (3.08)	1.66 (3.10)	0.98 (0.02)	57/2
	Exclude ($n = 32$)	54.31 (11.75)	N/A	6.62 (0.25)	24.25 (9.26)	3.22 (3.73)	1.57 (0.50)	2.16 (0.97)	4.54 (3.33)	5.72 (1.81)	40.20 (6.86)	4.24 (5.28)	0.92 (0.07)	7/25
	p-value	0.14	N/A	N/A	0.68	0.89	N/A	**0.0002**	**0.03**	**0.002**	**0.00006**	**0.02**	**0.0005**	**<0.000005**

The criteria applied for inclusion were: AFS = 0 AND pH ≥ 6.6 AND 28S/18S ≥ 1.5. The samples that passed all three measures were placed into the 'included' group and the remaining were placed in the group 'excluded.' The two groups were compared by *t*-test separately in two brain regions. The significant *p*-values are in bold. The abbreviations are the same as Table 1. The microarray chip quality was improved in the included compared to the excluded samples. N/A: The *a priori* selection of quality by AFS, pH, and RNA produced highly significant differences in these three measures; therefore the *p*-value was not shown.

** RIN calculation for ACC only included part of the sample due to technical difficulties in the Agilent run.

*** Type 1/2 *p*-values were calculated by Fisher's exact test.

Table 8
An example of applying four quality parameters to the results of anterior cingulate for 98 subjects

	Clinical cut-off (AFS = 0)	RNA cut-off (28S/18S ≥ 1.5)	Tissue cut-off (pH ≥ 6.6)	Chip type cut-off (Type '1')
Number of subjects above cut-off	75	64	60	57
Number of subjects below cut-off	23	14	12	9

The methodology shows that 58% of the initial subject pool will pass all four parameters. Other methods to adjust for the effects of these quality and other postmortem variables on gene expression can be tested in post hoc analysis such as ANCOVA.

3.5. Quality control parameters application in anterior cingulate cortex

We presented evidence that each of the four quality control indicators (clinical, tissue, RNA and microarray quality) were significantly correlated to one another (Table 5), but were not perfectly correlated. A multiple covariable analysis of all transcripts showed that a post hoc measure of putative RNA degradation (AffyRNAdeg) accounted for a large proportion of variance in transcript expression.

All three quality selection parameters were used simultaneously in the current data set using cut points of AFS = 0, pH ≥ 6.6, and 28S/18S ≥ 1.5 as threshold criteria for sample selection as described. The "included" set (AFS = 0, pH ≥ 6.6 and 28S/18S ≥ 1.5) and the "excluded" (AFS ≥ 1, pH < 6.6 and 28S/18S < 1.5) set were compared. When the poor quality samples were "excluded" each microarray quality control indicator was significantly improved in the "included" group (Table 7). Notably, the microarray quality indicators (all post hoc) were significantly improved in eight measures performed in two brain regions (Table 7). Additionally, the mitochondrial pathway was significantly over-expressed between the groups. The thresholds appeared to work very well for this data set in two brain regions in selecting high quality microarray data. The "included" set shows that the cut points improved the four microarray chip quality indicators (%PC, SF, ACI, and Type 1/2) (Table 7), although it is noted that other methods for determining outlier samples could be equally applied at this point. Other methods might include principal component analysis to determine outliers based upon the entire gene expression profile, or performing an ANCOVA with multiple covariates.

Some regional variability in the outcome of the quality parameters was noted. As an example, the RIN values for the high quality 'included' groups in cerebellum and ACC were not comparable. This was likely due to lower starting RIN values in the ACC the technical result of the revised Agilent software reading of the electropherograms. However correlations were calculated between ACC and cerebellum regions for both RIN ($r = 0.42$, $p < 0.01$) and SF ($r = 0.85$, $p < 0.0001$), thus indicating that across regions there was agreement of these values within subjects.

When we employed the four quality cut-offs to the present results the number of samples removed at each stage (Table 8) significantly reduced the study from 98 to 57 samples. This reduction in sample size is only one strategy that we have adopted, and may not be practical when working with degraded samples from fixed tissues, or for certain research questions that require use of tissues obtained under less than ideal conditions.

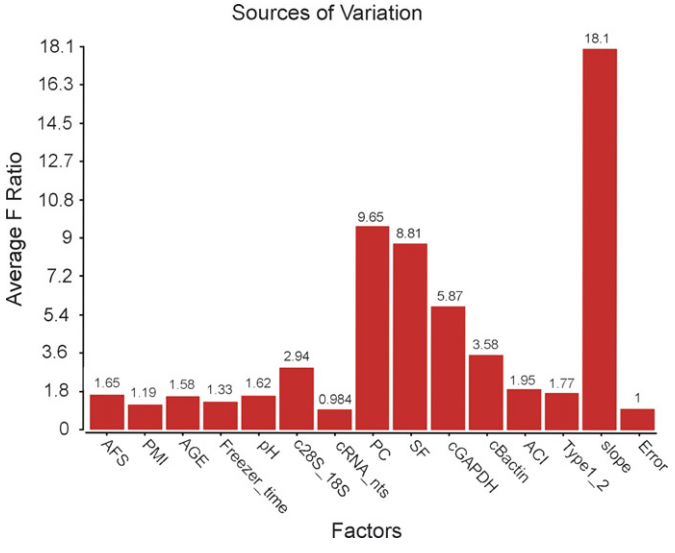

Fig. 4. The sources of variation in an ANCOVA of multiple covariates shows that the RNA degradation accounts for the highest average effect (*F*-ratio) across the entire transcriptome in ACC measured on an Affymetrix U133A chip. The AffyRNAdeg program (Cope, 2005) provides a slope, which is a microarray chip based indicator of the decline in signal across a transcript, thus a putative index of RNA degradation. The abbreviations for each variable are described in Table 1.

4. Conclusions

We systematically evaluated four quality indicators in one study with reference to gene expression results in postmortem brain from two regions. When samples in our study were not balanced well or matched for these quality indicators, the results of pathway analysis identified a brain disorders pathophysiology of mitochondria dysfunction. This pathophysiology is correlated to sample groups that have RNA, clinical, and tissue quality differences. Researchers have implicated mitochondrial-related pathways as a cause in schizophrenia (Prabakaran et al., 2004; Altar et al., 2005) and bipolar disorder studies (Konradi et al., 2004). A slight imbalance in group composition based upon an agonal-pH difference will affect this functional pathway and its related pathways such as apoptosis, proteasome, and chaperone functions (Vawter et al., 2006).

The impact of each quality covariable on gene expression may not be linear across wide ranges. As a first approximation researchers have found it useful to examine the readily accessible measures of clinical, tissue and RNA quality prior to microarray analysis and have used matched pairs of subjects or matched groups on these measures. Across a wide

range there was a potentially large non-linear variation observed with matching strategies. Investigators might adopt an approach of using all samples and making adjustments with regression models, however this would presume that adequate models exist.

RNA quality found to have a large statistical impact on the amount of variation across all transcripts. The R-script AffyRNAdeg (Cope, 2005) can be used post hoc to assess RNA quality to measure the slope across all transcripts in the 3′/5′ direction instead of only using the beta-actin or GAPDH transcripts. Other chip measures such as present calls and scaling factor accounted for a large proportion of variance across transcripts. Most investigators agree that severely degraded samples will not provide useful data and the findings are compromised when total RNA is degraded (Schoor et al., 2003; Auer et al., 2003; Buesa et al., 2004; Tomita et al., 2004; Lipska et al., 2006). However, modest degradation of samples such as observed in postmortem brain collections was addressed in the present paper to attempt to describe different strategies to select samples with minimal RNA degradation and with high clinical and tissue quality. It was shown that by removing samples with agonal factors, low pH and degraded RNA higher quality microarray results, based on post hoc measures, will be observed. Consistent recommendations to use the highest quality RNA for gene expression measurements (Bahn et al., 2001; Auer et al., 2003; Schoor et al., 2003; Buesa et al., 2004; Mexal et al., 2006; Lipska et al., 2006) have led one group of investigators to conclude that "The strongest predictor of gene expression was total RNA quality"(Lipska et al., 2006). Our results are consistent with these reports.

Future microarray knowledge will include how different types of transcripts are affected by postmortem and premortem variables as well as the set of transcripts not affected by these variables. Postmortem brain studies generally utilize one or more of the criteria reviewed for study design and statistical analysis. By adopting only one indicator to accept or reject samples at a certain threshold, an investigator may accept marginal samples. However, in post hoc analysis the impact of these parameters can be determined. We have used multiple criteria to form cohorts of postmortem samples based upon these *a priori* quality parameters of clinical, tissue, and RNA quality indicators and post hoc microarray indicators. We have also used these same parameters as covariables. For variables with a simple linear relationship these approaches are satisfactory, however, we have not tested non-linear models which may address a larger proportion of variance than the simple linear models. After forming a cohort with cut-off criteria this essentially narrows the range that strong effects can operate, so that the potential for case–control effects can emerge. As an example, we have used a set of criteria to reduce the range of strong effects with the following criteria: AFS = 0; the samples must have both medical records and next of kin interview information; the sample pH is minimally in the range of 6.4–6.6; and the RNA integrity measured by Agilent must have a 28S/18S ratio greater than 1.4–1.5 while at the same time taking the RIN value into consideration. We finally use post hoc indicators such as the AffyRNAdeg slope, percent present, scaling factor, ACI, and hierarchical clustering approaches to find outlier chips. These steps taken together will minimize the strong effects of these covariates, but other methods can be utilized to study the same effects and to assess the final quality of the chip.

The evaluation of these four quality categories aids in the study design, characterization and assessment of our samples, analysis, and interpretation of results. Not all transcripts are affected to the same degree by these variables as shown in our ANCOVA results. Thus, meaningful data can be derived if post hoc analysis has ruled out confounding effects on specific transcripts. Post hoc use of these quality covariables will help to either strengthen or weaken a candidate gene depending on the impact of the key variables we describe.

The four quality indicators are broadly related and therefore using a certain combination of these factors improves the quality of the data set, but might never truly separate the low, moderate, and high quality samples completely. By having access to alternative parameters to assess the quality of both the sample and the microarray data, we present an investigator with covariates for study design, selection criteria, or parameters for matching strategies depending on the nature of the study. These quality parameters may assist future investigators for meta-analysis of postmortem brain gene expression studies, such as those that can be conducted with the gene expression arrays deposited at the Gene Expression Omnibus.

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References

Altar CA, Jurata LW, Charles V, Lemire A, Liu P, Bukhman Y, et al. Deficient hippocampal neuron expression of proteasome, ubiquitin, and mitochondrial genes in multiple schizophrenia cohorts. Biol Psychiatry 2005;58(2):85–96.

Auer H, Lyianarachchi S, Newsom D, Klisovic MI, Marcucci G, Kornacker K, et al. Chipping away at the chip bias: RNA degradation in microarray analysis. Nat Genet 2003;35(1):106.

Bahn S, Augood SJ, Ryan M, Standaert DG, Starkey M, Emson PC. Gene expression profiling in the post-mortem human brain—no cause for dismay. J Chem Neuroanat 2001;22(1/2):79–94.

Barrett T, Cheadle C, Wood WB, Teichberg D, Donovan DM, Freed WJ, et al. Assembly and use of a broadly applicable neural cDNA microarray. Restor Neurol Neurosci 2001;18(2/3):127–35.

Barton AJ, Najlerahim PRA, Harrison PJ. Pre- and postmortem influences on brain RNA. J Neurochem 1993;61(1):1–11.

Berger A, Stierkorb E, Nickenig G. The role of the AUUUUA hexamer for the posttranscriptional regulation of the AT1 receptor mRNA stability. Biochem Biophys Res Commun 2005;330(3):805–12.

Bernstein PPS, Ross J. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. Mol Cell Biol 1989;9(2):659–70.

Bird ED, Barnes SE, MacKay J, Iversen AV, Shepherd LLM. Increased brain dopamine and reduced glutamic acid decarboxylase and choline acetyl transferase activity in schizophrenia and related psychoses. Lancet 1977;2(8049):1157–8.

Bolstad Benjamin Milo. Low-level analysis of high-density oligonucleotide array data: background, normalization and summarization. PhD dissertation, University of California, Berkeley, United States–California, 2004.

Breese CR, Marks MJ, Logel J, Adams CE, Sullivan B, Collins AC, et al. Effect of smoking history on [3H]nicotine binding in human postmortem brain. J Pharmacol Exp Ther 1997;282(1):7–13.

Brent DA, Moritz PJ, Allman G, Roth CJ, Schweers C, Balach JL. The validity of diagnoses obtained through the psychological autopsy procedure in adolescent suicide victims: use of family history. Acta Psychiatr Scand 1993;87(2):118–22.

Buesa CMT, Subirada F, Barrachina M, Ferrer I. DNA chip technology in brain banks: confronting a degrading world. J Neuropathol Exp Neurol 2004;63(10):1003–14.

Bunney WE, Vawter BB, Tomita MP, Li H, Evans J, Choudary SJ, et al. Microarray technology: a review of new strategies to discover candidate vulnerability genes in psychiatric disorders. Am J Psychiatry 2003;160(4):657–66.

Burke WJ, Chung OMK, Harmon HD, Miller SK, Berg JPL. Effect of pre- and postmortem variables on specific mRNA levels in human brain. Brain Res Mol Brain Res 1991;11(1):37–41.

Carter DE, Robinson JF, Allister EM, Huff MW, Hegele RA. Quality assessment of microarray experiments. Clin Biochem 2005;38(7):639–42.

Catts VS, Catts SV, Fernandez HR, Taylor JM, Coulson EJ, Lutze-Mann LH. A microarray study of post-mortem mRNA degradation in mouse brain tissue. Brain Res 2005;138(2):164–77.

Cope L. Function to assess RNA degradation in Affymetrix GeneChip data: AffyRNAdeg, 2005, http://www.bioconductor.org.

Corbett RJLA, Sterett R, Tollefsbol G, Garcia D. Effect of hypoxia on glucose-modulated cerebral lactic acidosis, agonal glycolytic rates, and energy utilization. Pediatr Res 1996;39(3):477–86.

Craddock N, Jones I. Genetics of bipolar disorder. J Med Genet 1999;36(8):585–94.

Cummings TJSJ, Yoon LW, Szymanski MH, Hulette CM. Recovery and expression of messenger RNA from postmortem human brain tissue. Mod Pathol 2001;14(11):1157–61.

Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, et al. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res 2005;33(20):e175.

Deep-Soboslay A, Martin AM, Bigelow CE, Herman LB, Hyde MM, Kleinman TMJE. Reliability of psychiatric diagnosis in postmortem research. Biol Psychiatry 2005;57(1):96–101.

Dumur CI, Best NS, Archer AM, Ladd KJ, Mas AC, Wilkinson VR, et al. Evaluation of quality-control criteria for microarray gene expression analysis. Clin Chem 2004;50(11):1994–2002.

Erraji-Benchekroun L, Underwood MD, Arango V, Galfalvy H, Pavlidis P, Smyrniotopoulos P, et al. Molecular aging in human prefrontal cortex is selective and continuous throughout adult life. Biol Psychiatry 2005;57(5):549–58.

Evans SJ, Datson NA, Kabbaj M, Thompson RC, Vreugdenhil E, De Kloet ER, et al. Evaluation of Affymetrix Gene Chip sensitivity in rat hippocampal tissue using SAGE analysis. Serial analysis of gene expression. Eur J Neurosci 2002;16(3):409–13.

Ford LP, Bagga PS, Wilusz J. The poly(A) tail inhibits the assembly of a 3′-to-5′ exonuclease in an in vitro RNA stability system. Mol Cell Biol 1997;17(1):398–406.

Fritz DT, Bergman N, Kilpatrick WJ, Wilusz CJ, Wilusz J. Messenger RNA decay in Mammalian cells: the exonuclease perspective. Cell Biochem Biophys 2004;41(2):265–78.

Gautier L, Cope L, Bolstad BM, Irizarry RA. affy–analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 2004;20(3):307–15.

Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 2003;4(9):117.

Grissom SF, Lobenhofer EK, Tucker CJ. A qualitative assessment of direct-labeled cDNA products prior to microarray analysis. BMC Genom 2005;6(1):36.

Hakak Y, Walker JR, Li C, Wong WH, Davis KL, Buxbaum JD, et al. Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci USA 2001;98(8):4746–51.

Hardy JA, Winblad WP, Gezelius B, Bring C, Eriksson GA. The patients dying after long terminal phase have acidotic brains; implications for biochemical measurements on autopsy tissue. J Neural Transm 1985;61(3/4):253–64.

Harrison PJ, Eastwood HP, Burnet SL, McDonald PW, Pearson BRC. The relative importance of premortem acidosis and postmortem interval for human brain gene expression studies: selective mRNA vulnerability and comparison with their encoded proteins. Neurosci Lett 1995;200(3):151–4.

Harrison PJ, Barton PA, Lowe AJ, Najlerahim SL, Bertolucci A, Bowen PH, et al. Terminal coma affects messenger RNA detection in post mortem human temporal cortex. Brain Res Mol Brain Res 1991;9(1/2):161–4.

Hollams EM, Giles KM, Thomson AM, Leedman PJ. MRNA stability and the control of gene expression: implications for human disease. Neurochem Res 2002;27(10):957–80.

Hosack DA, Dennis Jr G, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. Genome Biol 2003;4(10):R70.

Hynd MR, Lewohl JM, Scott HL, Dodd PR. Biochemical and molecular studies using human autopsy brain tissue. J Neurochem 2003;85(3):543–62.

Imbeaud S, Boulanger GE, Barlet V, Zaborski X, Eveno P, Mueller E, et al. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res 2005;33(6):e56.

Isometsa E. Psychological autopsy studies—a review. Eur Psychiatry 2001;16(7):379–85.

Iwamoto K, Bundo M, Kato T. Altered expression of mitochondria-related genes in postmortem brains of patients with bipolar disorder or schizophrenia, as revealed by large-scale DNA microarray analysis. Hum Mol Genet 2005;14(2):241–53.

Johnson SA, Morgan DG, Finch CE. Extensive postmortem stability of RNA from rat and human brain. J Neurosci Res 1986;16(1):267–80.

Johnston NL, Shore CJ, Torrey AD, Yolken EFRH. Multivariate analysis of RNA levels from postmortem human brains as measured by three different methods of RT-PCR. Stanley Neuropathology Consortium. J Neurosci Methods 1998;77(1):83–92.

Jurata LW, Bukhman YV, Charles V, Capriglione F, Bullard J, Lemire AL, et al. Comparison of microarray-based mRNA profiling technologies for identification of psychiatric disease and drug signatures. J Neurosci Methods 2004;138(1/2):173–88.

Kelly TMMJ. Validity of DSM-III-R diagnosis by psychological autopsy: a comparison with clinician ante-mortem diagnosis. Acta Psychiatr Scand 1996;94(5):337–43.

Kingsbury AE, Nisbet FO, Cairns AP, Bray N, Eve L, Lees DJ, et al. Tissue pH as an indicator of mRNA preservation in human post-mortem brain. Brain Res Mol Brain Res 1995;28(2):311–8.

Konradi C, Eaton M, MacDonald ML, Walsh J, Benes FM, Heckers S. Molecular evidence for mitochondrial dysfunction in bipolar disorder. Arch Gen Psychiatry 2004;61(3):300–8.

Lee J, Hever A, Willhite D, Zlotnik A, Hevezi P. Effects of RNA degradation on gene expression analysis of human postmortem tissues. Faseb J 2005;19(10):1356–8.

Li J, Meng F, Evans SJ, Choudary PV, Tomita H, Vawter MP, et al. Sample matching strategies in gene expression studies of brain tissues. Program No. 923.18. Abstract Viewer/Itinerary Planner. Washington, DC; Society for Neuroscience, online; 2005.

Li JZ, Walsh VM, Tomita DM, Evans H, Choudary SJ, Lopez PV, et al. Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. Hum Mol Genet 2004;13(6):609–16.

Lipska BK, Deep-Soboslay A, Weickert CS, Hyde TM, Martin CE, Herman MM, et al. Critical factors in gene expression in postmortem human brain: focus on studies in schizophrenia. Biol Psychiatry 2006;60(6):650–8.

Luo Z, Geschwind DH. Microarray applications in neuroscience. Neurobiol Dis 2001;8(2):183–93.

Mexal S, Berger R, Adams CE, Ross RG, Freedman R, Leonard S. Brain pH has a significant impact on human postmortem hippocampal gene expression profiles. Brain Res 2006;1106(1):1–11.

Middleton FA, Lewis PL, Levitt DA, Mirnics PK. Altered expression of 14-3-3 genes in the prefrontal cortex of subjects with schizophrenia. Neuropsychopharmacology 2005;30(5):974–83.

Miller CL, Diglisic S, Leister F, Webster M, Yolken RH. Evaluating RNA status for RT-PCR in extracts of postmortem human brain tissue. Biotechniques 2004;36(4):628–33.

Mimmack ML, Brooking J, Bahn S. Quantitative polymerase chain reaction: validation of microarray results from postmortem brain studies. Biol Psychiatry 2004;55(4):337–45.

Mirnics K, Levitt P, Lewis DA. DNA microarray analysis of postmortem brain tissue. Int Rev Neurobiol 2004;60:153–81.

Mirnics K, Lewis MF, Levitt DAP. Analysis of complex brain disorders with gene expression microarrays: schizophrenia as a disease of the synapse. Trends Neurosci 2001;24(8):479–86.

Mirnics K, Pevsner J. Progress in the use of microarray technology to study the neurobiology of disease. Nat Neurosci 2004;7(5):434–9.

Mirnics K, Levitt P, Lewis DA. Critical appraisal of DNA microarrays in psychiatric genomics. Biol Psychiatry 2006;60(2):163–76.

Mitchell P, Mackinnon A, Waters B. The genetics of bipolar disorder. Aust N Z J Psychiatry 1993;27(4):560–80.

Mootha VK, Lepage P, Miller K, Bunkenborg J, Reich M, Hjerrild M, et al. Identification of a gene causing human cytochrome *c* oxidase deficiency by integrative genomics. Proc Natl Acad Sci USA 2003a;100(2):605–10.

Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet 2003b;34(3):267–73.

Morrison-Bogorad M, Pardue ZAS. Heat-shock 70 messenger RNA levels in human brain: correlation with agonal fever. J Neurochem 1995;64(1):235–46.

Nestler EJ, DiLeone BM, Eisch RJ, Gold AJ, Monteggia SJLM. Neurobiology of depression. Neuron 2002;34(1):13–25.

Newton SS, Bennett A, Duman RS. Production of custom microarrays for neuroscience research. Methods 2005;37(3):238–46.

Prabakaran S, Swatton JE, Ryan MM, Huffaker SJ, Huang JT, Griffin JL, et al. Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. Mol Psychiatry 2004;9(7):684–97, 643.

Preece P, Cairns NJ. Quantifying mRNA in postmortem human brain: influence of gender, age at death, postmortem interval, brain pH, agonal state and inter-lobe mRNA variance. Brain Res Mol Brain Res 2003;118(1/2):60–71.

Ryan MM, Huffaker SJ, Webster MJ, Wayland M, Freeman T, Bahn S. Application and optimization of microarray technologies for human postmortem brain studies. Biol Psychiatry 2004;55(4):329–36.

Schoor O, Hennenlotter WT, Corvin J, Stenzl S, Rammensee A, Stevanovic HGS. Moderate degradation does not preclude microarray analysis of small amounts of RNA. Biotechniques 2003;35(6):1192–6, 1198–201.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 2006;7:3.

Shastry BS. Bipolar disorder: an update. Neurochem Int 2005;46(4):273–9.

Shergill IS, Shergill NK, Arya M, Patel HR. Tissue microarrays: a current medical research tool. Curr Med Res Opin 2004;20(5):707–12.

Soverchia L, Ubaldi M, Leonardi-Essmann F, Ciccocioppo R, Hardiman G. Microarrays—the challenge of preparing brain tissue samples. Addict Biol 2005;10(1):5–13.

Spokes EG. GABA in Huntington's chorea. Parkinsonism and schizophrenia. Adv Exp Med Biol 1979;123:461–73.

Spokes EG, Iversen GNLL. Differential effects of agonal status on measurements of GABA and glutamate decarboxylase in human post-mortem brain tissue from control and Huntington's chorea subjects. J Neurochem 1979;33(3):773–8.

Spokes EG, Rossor GN, Iversen MNLL. Distribution of GABA in post-mortem brain tissue from control, psychotic and Huntington's chorea subjects. J Neurol Sci 1980;48(3):303–13.

Taylor GR, Crow CG, Johnson TJ, Fairbairn JA, Perry AF, Perry EKRH. Recovery and measurement of specific RNA species from postmortem brain tissue: a general reduction in Alzheimer's disease detected by molecular hybridization. Exp Mol Pathol 1986;44(1):111–6.

Tomita H, Vawter MP, Walsh DM, Evans SJ, Choudary PV, Li J, et al. Effect of agonal and postmortem factors on gene expression profile: quality control in microarray analyses of postmortem human brain. Biol Psychiatry 2004;55(4):346–52.

Torrey EF, Webster BB, Bartko MJ, Meador-Woodruff JJ, Knable JHMB. Neurochemical markers for schizophrenia, bipolar disorder, and major depression in postmortem brains. Biol Psychiatry 2005;57(3):252–60.

Vawter MP, Tomita H, Meng F, Bolstad B, Li J, Evans S, et al. Mitochondrial-related gene expression changes are sensitive to agonal-pH state: implications for brain disorders. Mol Psychiatry 2006;11(7):663–79.

Wilusz CJ, Wilusz J. Bringing the role of mRNA decay in the control of gene expression into focus. Trends Genet 2004;20(10):491–7.

Wu Z, Irizarry R, Gentleman R, Murillo F, Spencer FA. A model based background adjustment for oligonucleotide expression arrays. Department of Biostatistics Working Papers. Baltimore, MD; Johns Hopkins University; 2003.

Yang E, van Nimwegen E, Zavolan M, Rajewsky N, Schroeder M, Magnasco M, et al. Decay rates of human mRNAs: correlation with functional characteristics and sequence attributes. Genome Res 2003;13(8):1863–72.

Yates CM, Tennant BJ, Gordon MCA. Enzyme activities in relation to pH and lactate in postmortem brain in Alzheimer-type and other dementias. J Neurochem 1990;55(5):1624–30.