B-actin does not show the characteristics of a reference protein in human cortex.

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Abbreviations: BA = Brodmann's area, BD = bipolar disorders, DI = duration of illness, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, qPCR = quantitative Polymerase Chain Reaction, MDD = major depressive disorders, PMI = post-mortem interval, RT = room temperature (RT), TTBS = Tween Tris buffered saline.

Keywords: β-actin, human cortex, loading control, reference protein, Western blotting

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Abstract

Levels of a reference protein must be the same as a proportion of total protein in all tissues and, in the study of human diseases, cannot vary with factors such as age, gender or disease pathophysiology. It is increasingly apparent that there may be few, if any, proteins that display the characteristics of a reference protein within the human central nervous system (CNS). To begin to challenge this hypothesis, we used Western blotting to compare variance in levels of the "gold standard" reference protein, β-actin, in Brodmann's area 9 from 194 subjects to variance of total

transferred protein measured as intensity of Ponceau S staining. The coefficient of variance of sum intensity measurements for β-actin levels across all donors was 47% compared to 24% and 27% for the sum intensity of Ponceau S staining measured using two different detection techniques. These data strongly suggest that the level of β-actin, proportional to total protein, is not constant in human cortex which raises further doubt about the use of reference proteins to normalise data in human CNS studies. Considering our data, we suggest an alternative approach to presenting data from Western blotting of human CNS.

1 Introduction

With the widespread use of quantitative Polymerase Chain Reaction (qPCR) to measure levels of mRNA in the human central nervous system (CNS) from subjects with psychiatric disorders, it has become standard practice to normalise expression levels of genes of interest against up to 3 reference genes [1]. The notion of normalising qPCR data using reference genes was based on the posit that such genes were ubiquitously expressed at the same level across all tissues [2]. In addition, the level of expression of a reference gene could not change with variables such as gender, age and drug treatments and could not be affected by disease pathophysiologies. The logic of normalising results to reference genes was that this would remove experimental variabilities such as inefficiency of extracting RNA from tissue and differences in levels of gene expression between CNS regions [3]. However, like others [4-7], we have suggested that it is very difficult to find genes expressed in the human CNS that fulfil the rigorous criteria required of reference genes [3]. This is probably because the human CNS is made up of multiple cell types, present in different combinations across different CNS regions. In this regards, the ongoing use of reference genes to

normalise qPCR data is of concern because genes such as β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are still frequently used as reference genes for gene expression studies despite evidence to show they fail many of the criteria used to define a reference gene [3, 8, 9].

The practice of normalising data to a reference protein, also known as a loading control, is now a common practice in Western blotting [10]. Different to the definition of reference genes, a reference protein must be present at a constant proportion of the total protein in a tissue [11]. In addition, similar to the criteria for reference genes, reference proteins must not be affected by variables such as disease pathophysiology, gender or drug treatments [11]. The validity of the concept of a reference protein has become particularly important as it has been stated that "To obtain any reliable information about the expression levels of proteins on western blots, it is essential that an appropriate loading control is used. This is an absolute requirement for publication-quality work." (http://www.abcam.com/primary-antibodies/loading-controls---why-bother). This proposition has been put forward despite findings at the level of mRNA [3] and protein [10, 12-18] that the notion of a reference protein is untenable when studying complex tissue such as human CNS. To address this issue, we measured levels of the commonly used reference protein, β-actin, in Brodmann's area (BA) 9 from 194 subjects who had, or had not, suffered from a psychiatric disorder. BA 9 was chosen for this study because it is a region within the dorsolateral prefrontal cortex which is recognised to be affected by the pathophysiology of disorders such as schizophrenia [19] and mood disorders [20], which makes it an appropriate region to test the requirement that levels of a reference protein in such a region should not change with disease pathophysiology. We also addressed the proposal that the total level of transferred protein, as measured using Ponceau S staining [21], could be used to normalise data from Western blotting.

2 Materials and Methods

2.1 Human Tissue Collection and Processing

Human CNS tissue was collected postmortem with approval from the Ethics Committee of the Victorian Institute of Forensic Medicine and the research was conducted in accordance with all relevant guidelines and procedures. All tissue was collected from cases coming to the Victorian Institute of Forensic Medicine after gaining written consent from the nearest next-of-kin. All cadavers were refrigerated within 5 hours of being found. The CNS was removed at autopsy and the left hemisphere rapidly processed and frozen to -70^{o}C using a standardised procedure [22] by the same individual in a way designed to minimise autolytic effects [23]. The pH of the brain tissue was measured as described previously [24] as this is a good measure of overall tissue preservation [25].

All tissue was provided by the Victorian Brain Bank at the Florey Institute for Neuroscience and Mental Health. The tissue for these studies was taken from BA 9, which is bounded by the lateral surface of the frontal lobe from an area that includes the middle frontal gyrus superior to the inferior frontal sulcus.

2.2 Case History Review

Demographic, clinical, pharmacological and CNS collection data were collected during a case history review conducted using the Diagnostic Instrument for Brain Studies (DIBS) [26]. Where appropriate, psychiatric disorders were diagnosed as described previously according to Diagnostic and Statistical Manual of Mental Disorders (DSM) IV criteria [27]. From the data collected using the DIBS, duration

of illness (DI) was calculated as the time from first contact with a health service professional because of issues with mental health to death and post-mortem interval (PMI) was calculated as the time from death to autopsy. Where death was not witnessed, tissue was only collected from subjects who had been seen alive within 5 hours of the body being found, in these instances the PMI was taken as the midpoint between the person being found and being last seen alive.

2.3 Tissue Processing

To minimise inter-operator and equipment-based variability in this study all protein analyses and Western blotting were carried out by a single operator. In addition, all Western blotting was carried out using the same power pack, each gel was run in the same position in a single electrophoresis tank and protein transfer was achieved using the same Trans-blot cell, hence only one gel was run each day. Transfer buffer was made fresh each morning and stored in the cold room until needed. Single production lots of primary and secondary antibody were used for all assays to eliminate the effect of batch to batch variation.

For each subject, 100 mg of BA 9 was homogenized using a Teflon-glass homogenizer into 1 ml homogenization buffer (100 mM Tris, 1% SDS, 1 mM Na_3VO_4) until a homogenous homogenate was formed (10-20 strokes), the protein concentration was determined using a modified Lowry Method (Bio-Rad DC) protein assay, and the homogenate was stored at -80°C until required. Immediately prior to polyacrylamide gel electrophoresis and following dilution, the total protein concentration in each sample was again determined using the Bio-Rad DC protein assay.

2.4 Quality Control Case

To assess intra- and inter-gel variation, a homogenate from 1 subject, the Quality Control Case (QCC), was prepared from BA 9 exactly as for all other cases. Aliquots of this homogenate were stored at – 80°C until required. Before any cases were analysed one sample of QCC was thawed, loaded onto 12 lanes of a Mini-gel and subjected to the Western blot protocol described below. The sum intensity of Ponceau S staining in each lane were measured using the UVP Biospectrum® Imaging System with VisionWorks® Image Acquisition and Analysis Software to give an estimate of intra-gel variation. The sum intensity of the chemiluminescence band for β-actin was measured in each lane as described below. From these measurements an estimate of intra-gel variation could be obtained. A sample of QCC was subsequently thawed and run in two lanes of every subsequent mini-gel and these data were used to gain an estimate of the overall inter-gel variation for the study.

2.5 Western Blot Analyses

To determine levels of β-actin in each subject, on the day of analysis homogenate aliquots of five randomly selected subjects were thawed and diluted in deionised water to give a final protein concentration of 2.0 µg/µl which was confirmed using the BioRad DC protein assay. The homogenate was then diluted into an equal volume of 2 × sample buffer (0.5 M Tris– HCl pH 6.8, containing 20% glycerol, 10% SDS, 10% 2-β- mercaptoethanol and 0.05% bromophenol blue) to give 1.0 µg/µl protein and denatured for 5 min at 95°C. After denaturation, 20 µl (20 µg protein) from each sample was loaded, in duplicate, onto a 4% stacking gel along with Broad Range Molecular Weight Standards

(BioRad) and 0.25 µg/µl bovine serum albumin as a molecular weight check. The samples were resolved using a 15% SDS-PAGE gel at 150 V in a BioRad Mini-PROTEAN Tetra-Cell until the sample buffer had migrated to 1 cm from the bottom of the gel. Each gel was used to resolve proteins in homogenates from 5 cases and 1 QCC in duplicate; therefore, for this study 39 gels were run to process all the cases studied. Once resolved, the proteins on each gel were transferred onto BioRad nitrocellulose membrane, using an overnight transfer in Towbin Transfer Buffer (25 mM Tris pH 8.3 containing 192 mM glycine and 20% methanol) in a Bio-Rad Mini Trans-blot® electrophoretic transfer cell (40 mA constant). Foam pads, blotting paper, nitrocellulose membrane and gel were equilibrated in cold transfer buffer for 20 minutes before the transfer was commenced. A Mini Trans-Blot system was used, with platinum wire electrodes. No magnetic stir-bar was used during transfer.

The transferred proteins on each membrane were visualised by staining with 0.2% Ponceau S Red in 3% trichloroacetic acid for 5 min, then washed thrice for 5 min with deionised water. The sum intensity of staining of the transferred proteins for each subject was imaged using both an Epson scanner (Figure 1A) and a UVP Biospectrum® Imaging System (Figure 1B) with associated VisionWorks® Image Acquisition and Analysis Software. The image obtained from the Epson scanner was converted to grayscale and analysed using the "Plot Lanes" function on ImageJ® (U. S. National Institutes of Health, Bethesda, Maryland, USA) to give the sum intensity of protein staining, expressed as sum intensity × 10^{-1}, along each gel lane. For the UVP Biospectrum, a white light image was taken with an aperture of 3.5 and each lane was defined using the "1D Analysis" function. Following a straight-line background correction, the sum intensity of Ponceau S staining was

measured within “defined columns” that encompass each gel lane prior to being expressed as the sum intensity × 10^{-5}.

Once the Ponceau S stain images were obtained, each membrane was placed in blocking buffer (Tween Tris buffered saline (TTBS): 100 mM Tris pH 7.5, 0.9% sodium chloride and 0.1% Tween 20 containing 5% blotting grade non-fat milk) for 1 hour at room temperature (RT) with gentle agitation. The nitrocellulose membrane was then exposed to anti-Actin antibody (MAB1501, Merck, NJ, USA) (diluted 1:200,000) in blocking buffer for 1 hour at RT with gentle agitation and subsequently washed 3 × 5 min in TTBS. The nitrocellulose membrane was then exposed to a 1:5000 dilution of polyclonal goat anti-mouse IgG: horse radish peroxidase conjugated antibody (P0447, DAKO, Denmark) in blocking buffer for 1 hour at RT with gentle agitation, followed by 3x 5 min washes in TTBS. The membrane was incubated with Amersham ECL™ at RT for 5 min and the sum intensity of the chemiluminescence band was measured at 45 sec exposure on the UVP Biospectrum® Imaging System with VisionWorks® Image Acquisition and Analysis Software (Figure 1C) and expressed as sum intensity × 10^{-2}.

2.6 Western Blot Analyses: Normalisation to Reference protein

To further assess the impact of using β-actin as a reference protein we also expressed the levels of Ponceau S staining of the homogenate measured using two methodologies from each donor normalised to the signal for β-actin.

2.7 Statistical Analyses

All analyses were completed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). Independence of frequency of gender and suicide completion between groups were tested for using the χ^2 test whilst variation in experimental data was identified using unpaired t-tests. Variance in demographic, CNS collection, disorder history or experimental data with diagnoses was identified using One-way ANOVA followed by a Tukey's multiple comparisons test to identify potential sources of variance. Relationships between experiment data and numeric demographic and CNS collection data were interrogated using Pearson's correlation analysis.

3 Results

In this study, the intensity of Ponceau S staining and levels of β-actin were measured in homogenates prepared from BA 9 from 194 subjects made up of 136 males and 58 females. These subjects had a mean age of 49 yr, a mean brain weight of 1377 g, a mean PMI of 42 hr and a mean CNS pH of 6.32 (Table 1). Tissue was collected from 70 subjects with no history of a psychiatric or neurological disorder (Unaffected), 75 subjects with schizophrenia, 24 subjects with major depressive disorders (MDD), 17 subjects with bipolar disorders (BD) and 8 suicide completers with no identifiable psychiatric or neurological illness.

When the subjects within this study were separated according to diagnoses, there were no significant differences in age, PMI, or brain weight between cohorts (Table 1). By contrast, there were significant differences in gender ratio, CNS pH and suicide completion between the cohorts.

The rates of suicide completion remained significantly different between subjects with different psychiatric diagnoses (χ^2 = 11.5, d.f. = 2, p = 0.003) but these groups did not differ in DI (Table 1).

3.1 Western blotting: β-actin

For the QCC, the intra-gel sum intensity of signal for β-actin ranged from 1317 to 1522 with a coefficient of variation of 3.9%. The QCC signal for β-actin across all other gels (inter-gel) ranged from 646 to 1914 with a coefficient of variation of 29.2 %.

The sum intensity of signal for β-actin across case samples ranged from 212 to 4461 with a coefficient of variation of 47% (Figure 1B). The sum intensity of β-actin did not vary significantly with gender, suicide completion (whether or not subjects had a psychiatric disorder) or diagnoses (Table 2). The signal intensity from β-actin did not strongly correlate with age (r^2 = 0.02, p = 0.08), CNS pH (r^2 = 0.02, p = 0.04), PMI (r^2 = 0.002, p = 0.57), CNS weight (r^2 < 0.0001, p = 0.99) or DI (r^2 = 0.006, p = 0.44).

3.2 Ponceau S Staining Analyses: Epson scanner

For the QCC, the intra-gel sum intensity of signal for Ponceau S staining measured using the Epson scanner ranged from 5730 to 5830 with a coefficient of variation of 3.2%. The QCC inter-gel signal for Ponceau S staining measured using the Epson scanner across all other gels ranged from 3120 to 7885 with a coefficient of variation of 23.4 %.

The sum intensity of Ponceau S staining for all cases in this study measured on the Epson scanner ranged from 2382 to 10025 with a coefficient of variation of 24% (Figure 1B). The sum intensity of Ponceau S staining did not vary significantly with gender, suicide completion (whether or not subjects had a psychiatric disorder) or diagnoses (Table 2). The sum intensity of Ponceau S staining did not strongly correlate with age (r^2 = 0.04, p = 0.008), CNS pH (r^2 = 0.002, p = 0.57), PMI (r^2 = 0.001, p = 0.59), CNS weight (r^2 < 0.006, p = 0.33) or DI (r^2 = 0.001, p = 0.73).

3.3 Ponceau S Staining Analyses: UVP Biospectrum®

For the QCC, the sum intensity of signal for Ponceau S staining measured using the UVP Biospectrum® ranged from 1846 to 3144 with a coefficient of variation of 14.7%. The QCC inter-gel signal for Ponceau S staining measured using the Epson scanner across all other gels ranged from 934 to 4618 with a coefficient of variation of 27.4 %.

The sum intensity of Ponceau S staining for all cases in this study measured using the UVP Biospectrum® ranged from 1171 to 4727 with a coefficient of variation of 27% (Figure 1D). The sum intensity of Ponceau S staining did not vary significantly with gender, suicide completion (whether or not subjects had a psychiatric disorder) or diagnoses (Table 2). The Ponceau S staining sum intensity did not strongly correlate with age (r^2 < 0.0001, p = 0.99), CNS pH (r^2 = 0.002, p = 0.58), PMI (r^2 < 0.0001, p = 0.98), CNS weight (r^2 = 0.001, p = 0.64) or DI (r^2 = 0.001, p = 0.74).

3.4 *Western Blot Analyses: Normalisation to Reference protein*

When the intensity of Ponceau S staining measured using the UVP Biospectrum® or the Epson scanner for each case was normalised to β-actin the coefficients of variation was 67.8 % and 66.8 % respectively (Figure 1E). The levels of Ponceau S staining normalised to β-actin did not vary significantly with gender, suicide completion or diagnoses (Supplementary Table 1). The levels of Ponceau S staining normalised to β-actin did not strongly correlate with age (UVP: r^2 = 0.001, p = 0.63; Epson: r^2 = 0.004, p = 0.41), CNS pH (UVP: r^2 = 0.010, p = 0.16; Epson: r^2 = 0.020, p = 0.04), PMI (UVP: r^2 =0.0008, p = 0.69; Epson: r^2 = 0.0007, p = 0.72), CNS weight (UVP: r^2 =0.006, p = 0.30; Epson: r^2 = 0.004, p = 0.37) or DI (UVP: r^2 =0.02, p = 0.16; Epson: r^2 = 0.001, p = 0.72).

3.5 Methodological correlations

There were no strong correlations between the sum intensity of Ponceau S staining measured using the Epson scanner and the UVP Biospectrum® (r^2 = 0.009; p = 0.20). There were no strong correlations between the sum intensity of Ponceau S staining measured using the Epson scanner or the UVP Biospectrum® and the sum intensity signal for β-actin (r^2 = 0.08; p < 0.0001 and r^2 = 0.003; p = 0.45, respectively).

4 Discussion

The quality control data from this large study using human cortical tissue shows that Western blotting should still be regarded as a semi-quantative approach to measuring levels of a protein in a biological sample [10]. However, the objective of this study was to determine if measuring levels of

β-actin in human cortical tissue gave data that was consistent with the suggestion that the protein can be used as a reference protein to reduce overall variability when measuring proteins in human CNS [28]. Our data from BA 9 from 194 subjects, some of which had a psychiatric disorder, does display some of the characteristics of a reference protein in that levels of β-actin did not vary with gender, age, PMI, CNS pH, CNS weight, diagnoses, DI or suicide completion. However, these findings may be CNS specific as it has been reported that in human muscle levels of β-actin decrease with age [29].

One critical characteristic of a reference or loading protein is that it should show no variation as a proportion of total protein between individual cases [11]. In this study we used a quality control homogenate (the QCC) to gain an estimate of the intra- and inter-gel variation for Ponceau S staining and the intensity of the β-actin signalling. The inter-gel variation for the measurement of Ponceau S staining measure two detection systems and β-actin were between 23 and 28%. Critically, whilst the variation of Ponceau S staining across the 194 subjects we studied was ~ 25%, the coefficient of variation of levels of β-actin was 47%. These data show the variance in sum intensity of Ponceau S staining across all samples was no higher than that of the variance of the QCC, suggesting little additional subject to subject variation. By contrast, the variation of levels of β-actin across all subjects was ~ 17% higher than that for the QCC, suggesting individual variances between donors. Our data therefore suggests that levels of β-actin, as a proportion of total protein, is not constant in every individual and β-actin fails a core criterion for a reference protein, which is to be present in all tissues from all individuals at a constant proportion of total tissue protein [11].

To further model the impact of normalising results to levels of β-actin in human cortex, we normalised levels of Ponceau S staining for each case to β-actin and showed the coefficient of variation for these data increased to > 60%. These data perhaps best support the hypothesis that the use of β-actin, and perhaps other reference or loading proteins, introduce rather than reduce variance into a Western blot study.

Our finding that β-actin does not constitute a constant proportion of total tissue protein in human CNS is similar to a finding using the human derived cell line MDA-MB-231 cells where levels of β-actin measured using Western blotting did not reflect the starting total protein concentration [13]. Moreover, data in the mouse shows levels of β-actin vary between tissue [10]. These data all argue that β-actin, and possibly other proteins such as GAPDH, should not be used as reference proteins unless they are shown to have all the characteristics of such proteins in the tissue or cell to be studied.

In considering alternative approaches to the use of reference proteins, others have suggested that using carefully measured total protein loaded onto a gel [10], total protein measured in a gel using stain free technologies [29] or the intensity of a Ponceau S staining of transferred proteins [21] as alternative normalisation measurements. Our data suggests normalising to Ponceau S staining would introduce less variability than normalising to β-actin but our QCC data suggest that the variability introduced by using transferred protein levels would still be significant.

Given current controversy around the use of reference proteins it is important to acknowledge that measuring proteins using Western blotting is semi-quantitative, not quantitative [30]. In addition, for a difference in levels of proteins between diagnoses using the non-transformed Western blot data to be artefactual, there would need to be some form of systematic effect during tissue processing, electrophoresis, blotting or protein detection that would give a bias towards one diagnostic cohort. To minimise such effects, practical solutions controlling for variation in methodology may offer a better solution than the use of data normalisation. For example, whilst the individual(s) completing the analyses should remain blind to diagnoses, coded samples could be provided in a way that ensured tissue from each disorder was present in each batch of tissue to be processed, if batch processing was necessary. Similarly, samples from each diagnostic group should be present on each gel if multiple gels are to undergo electrophoresis and blotting onto membranes. Using a similar approach to that taken in many quantative assays [31], a "quality control" sample could be run on every gel and the data on the repeated measures of levels of the protein of interest in that sample can be used to calculate inter-blot variation. This would add value because, if the difference between diagnostic groups is greater than the inter-blot variation of the quality control, this would add confidence to concluding that differences between diagnoses was not simply due to methodological-related variation.

It is important to recognise that the use of Western blotting remains a powerful tool for the study of protein levels in human CNS tissue. Given the problems in normalising such data using reference proteins or protein staining, it may be most appropriate to present non-derived Western blot data when comparing protein levels between those with a disorder and controls. If such data is presented alongside appropriate quality control data, it would be possible to determine if changes in

proteins with variables such as diagnoses were greater than the methodological variance. In addition to an analysis of non-derived Western blot data, it would be valuable to show that total protein in stain free gels [29] or integrated measures of Ponceau S staining [21] did not vary with diagnoses. Alternatively, other more stable measures of protein levels, such as staining with Sypro®-Ruby [32], Amido Black [32] or post staining with Coomassie R-250 [33] may be more useful as they provide more stable read-outs when measuring protein loading of gels. Perhaps most compelling would be to show levels of a protein known not to be affected by the pathophysiology of the disorder of interest did not vary with diagnoses would be a good planned negative control where changed protein levels are reported. A bonus in using raw data would be that such analyses would negate all the well-documented problems in the statistical analyses of data expressed as a ratio [34-36]. Finally, proteomic technologies are being used to try and identify proteins that fulfil all the criteria of a reference of loading protein [37]. Whilst such approaches may discover potential reference or loading proteins it will be critical that such proteins have all the characteristics of such a protein to avoid introducing variance into Western blotting above that of the methodology itself.

5 References

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Conflict of interest statement:

The authors have declared no conflict of interest.

Table 1: Demographic, disorder and CNS collection related data (mean ± SEM) for donors and tissue used in this study

Population	n	Age (yr)	Gender (M/F)	pH	PMI (hr)	Brain Weight (gm)	Suicide Y/N	DI (yr)
All	194	49 ± 1.2	136 / 58	6.32 ± 0.02	42 ± 1.0	1377 ± 12	69 / 125	
Unaffected	70	49 ± 1.8	56 / 14	6.32 ± 0.02	42 ± 1.7	1395 ± 18	0 / 70	
Sz	75	47 ± 2.1	50 / 25	6.25 ± 0.03	44 ± 1.6	1385 ± 20	33 / 42	18 ± 1.7
MDD	24	53 ± 3.8	12 / 12	6.46 ± 0.06	43 ± 3.3	1312 ± 31	20 / 4	10 ± 2.2
BD	17	53 ± 4	10 / 7	6.31 ± 0.05	36 ± 3.6	1311 ± 52	8 / 9	18 ± 3.8
Suicide	8	44 ± 5	8 / 0	6.44	36	1455 ± 63	8 / 0	

				± 0.08	± 4.7			
	F or χ^2	0.91	12.8	3.74	1.98	2.07	73.2	0.21
	d.f.	4,189	4	4,180	4,188	4,168	4	2,98
	p	0.46	0.01	0.01	0.10	0.09	< 0.0001	0.80

Table 2: Sum intensities of Ponceau staining (mean ± SEM) measured using an Epsom Scanner or a UVP Biospectrum® and sum β -Actin measured using Western blotting in BA 9 postmortem tissue, with comparisons between (A) gender (male: M; female: F), suicide completion (All Suicide Completion), suicide completion in subjects with a diagnosable psychiatric disorder (Suicide Completion with Diagnosis) (Y: yes; N: no) (B) subjects with no psychiatric disorders and subjects with schizophrenia (Sz), Major Depressive Disorder (MDD) and Bipolar Disorders (BD) or suicide completion without a psychiatric diagnosis (Suicide Completers)

Variable		**β -Actin*10^{-2}**	**Epson Scanner*10^{-1}**	**UV Spec*10^{-5}**
A				
Gender	M	1655 ± 70	5607 ± 111	2709 ± 65
	F	1642 ± 88	5638 ± 195	2759 ± 91
	p	0.91	0.88	0.67

All Suicide Completion	Y	1593 ± 66	5422 ± 115	2732 ± 68
	N	1757 ± 98	5969 ± 170	2709 ± 84
	p	0.16	0.007	0.84
Suicide Completion	Y	1561 ± 81	5952 ± 175	2783 ± 111
with Diagnosis	N	1754 ± 102	5514 ± 160	2700 ± 92
	p	0.15	0.07	0.57
B				
Diagnoses				
Unaffected		1618 ± 100	5359 ± 163	2692 ± 86
Sz		1618 ± 84	5793 ± 148	2707 ± 85
MDD		1808 ± 152	5817 ± 312	2832 ± 143
BD		1654 ± 150	5427 ± 249	2751 ± 244
Suicide Completers		1774 ± 368	6088 ± 634	2780 ± 171
	F	0.36	1.47	0.19
	d.f.	4,189	4,189	4,189
	p	0.83	0.21	0.94

Figure Legend:

Figure 1: Typical images optioned of Ponceau S staining of proteins transferred to a nitrocellulose gels obtained using an Epson scanner (A) and the UVP Biospectrum® (B), as well as a Western blot showing β-actin in human Brodmann's area 9 (C). D: The coefficients of variation from the measurement of Ponceau S staining measured using an Epson scanner or the UVP Biospectrum® analyser and β-actin in Brodmann's area 9 obtained from 194 individuals, some of whom had a psychiatric disorder. E: The coefficients of variation for Ponceau S staining imaged with an Epson scanner or the UVP Biospectrum normalised to levels of β-actin.