## Quality control standards for high-throughput RNA in situ hybridization data generation

Consistent data quality and internal reproducibility are critical concerns for high-throughput histological tissue analysis systems. Maintenance of quality standards across longitudinal and lateral axes are demonstrated in the following sets of replicate experiments described here in **Section I**: Evaluation of run controls, and, **Section II**: Reproducibility of ISH data across ISH runs and independent riboprobe syntheses. The data generated for these validation experiments is quantitated and presented in graphical formats at the end of each section.

### I. Evaluation of run controls for the ABA automated ISH platform

The automated process of in situ hybridization (ISH) described in ABA Process Overview and Data Production Process includes standard controls on every run as a quality control procedure, and as a means of comparing experimental variability over time.

Every Tecan-automated ISH run contains a positive control slide (incubated with *Drd1a* riboprobe, **Appendix 1**) and a negative control (incubated in hybridization buffer without riboprobe). After each ISH run, the control slides are used to qualitatively score the ISH run as 'Pass' or 'Fail,' to determine whether data from the ISH run will progress into the data analysis pipeline.

To obtain a qualitative assessment of run-to-run variability in ISH data, the following longitudinal analysis was performed on control slides sampled from ISH runs executed over the course of six months. Paired sets of control mouse brain sections from the slides used in this analysis are shown in **Figure 1**. Positive and negative control slides were scanned, stitched and white-balanced according to standard procedures, and subsequent images were analyzed.

Based upon the observed gene expression pattern of *Drd1a*, subregions of the positive control section were selected for densitometric analysis across reciprocal negative and positive control sections, as well as across multiple experimental runs (**Figure 2**). Three regions of interest (ROIs) per section were chosen to compare relative levels of expression, such that areas of potentially high (**Figure 2**; Boxes **a**, **a'**), moderate (**Figure 2**; Boxes **b**, **b'**), and low (**Figure 2**; Boxes **c**, **c'**) expression were analyzed. Matched ROIs were manually selected from each paired set of positive and negative controls, as shown on the right side of **Figure 2**, and densitometric values were calculated for the ROIs using ImagePro software.

The mean values of pixel density contained within each bounding box for corresponding regions in positive and negative control sections are shown in **Table 1**. Normalized values of this data are also shown in **Table 2**, demonstrating statistically significant, consistent gene expression profiles across positive and negative control sections, as measured by signal intensity. **Figure 3** presents this data graphically, where hatched bars denote values calculated from ROIs in potentially high-expressing regions (**a**, **a'**), lightly-shaded bars denote ROIs from moderately-expressing regions (**b**, **b'**) and solid bars show ROIs derived from low- or non-expressing regions (**c**, **c'**).

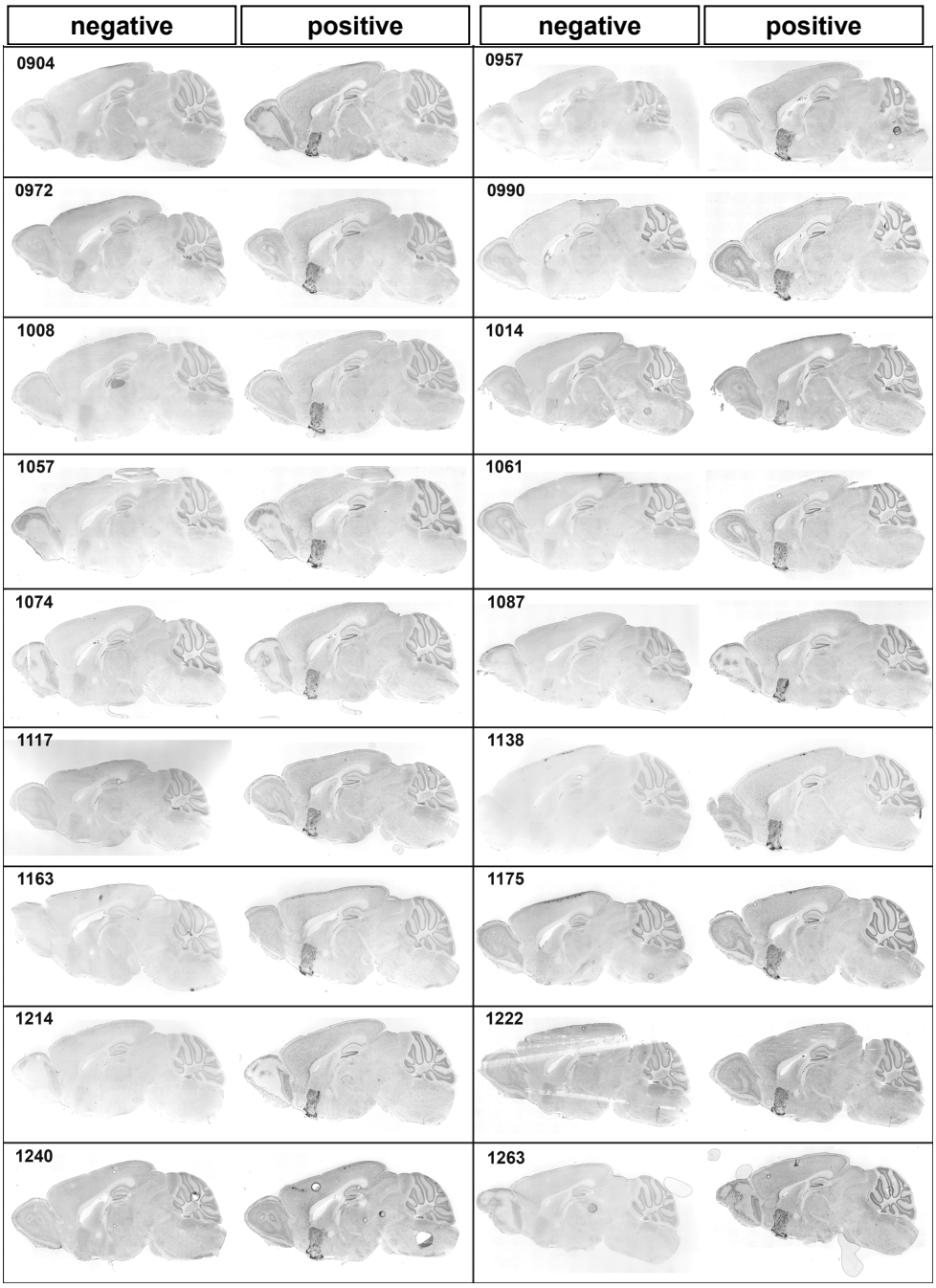


Figure 1 - *Paired sections from control slides from 18 representative ISH experimental runs, collected over a six month period of time. Run identification codes are indicated on the upper left of each pair of controls. Paired sections are shown for negative control sections (first and third columns) and positive control sections hybridized with Drd1a riboprobe (second and fourth columns). Images were scanned at 10X resolution according to standard procedures, then stitched and white balanced using ImagePro software.*

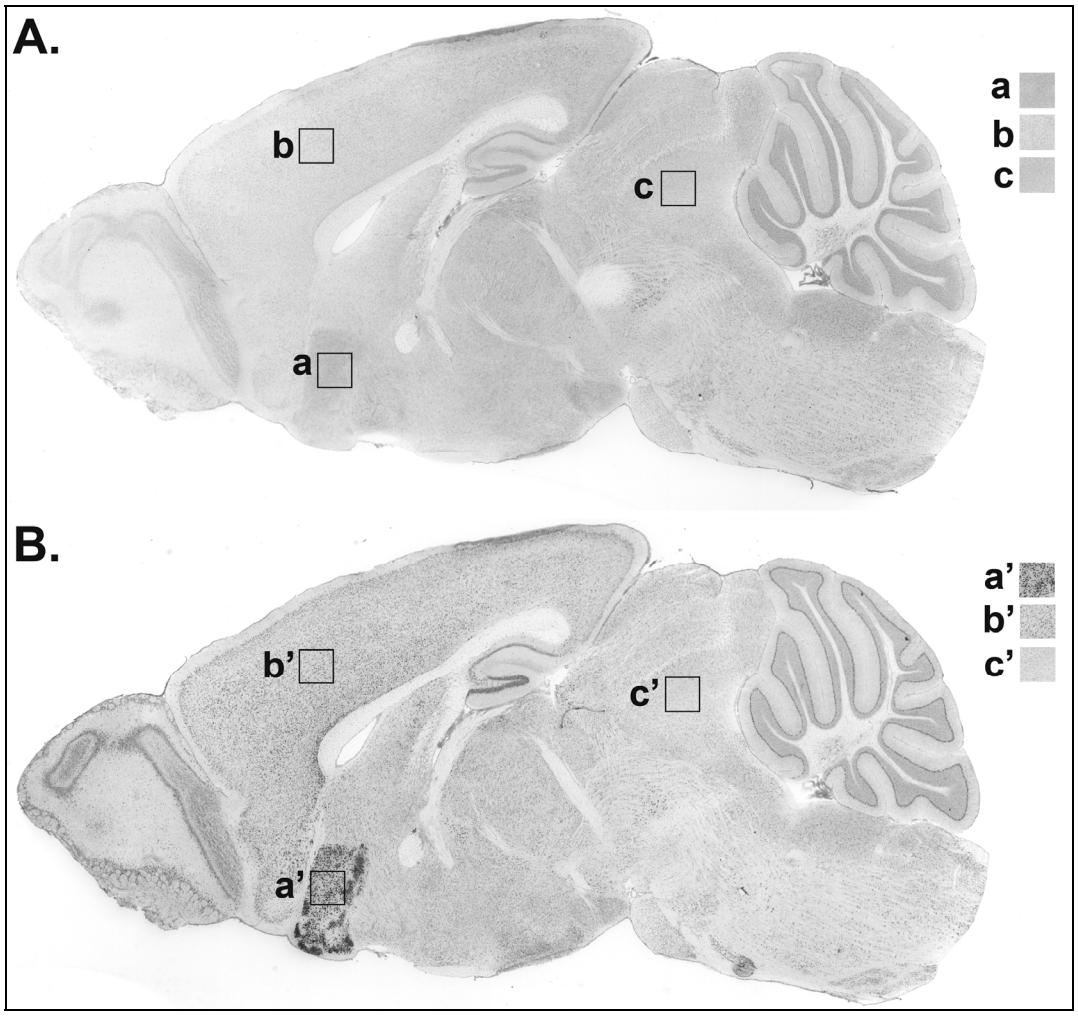


*Figure 2* **-** *Representative tissue sections from negative and positive control ISH slides (from Run 0904). Sagittal mouse brain cryosections were collected and processed according to standard procedures. The section shown in Panel* ***A*** *was one of four sections on the negative control slide, which was incubated in hybridization buffer without riboprobe. Panel* ***B*** *shows a paired section run simultaneously, but hybridized with 300 ng/ml Drd1a antisense riboprobe. Images were scanned at 10X magnification, stitched and white-balanced using ImagePro software. Matched regions of interest (ROIs) were manually selected and quantitated by densitometric analysis using corresponding 500x500 pixel bounding boxes on the negative control section (****a****,* ***b****,* ***c****) and positive control section (****a'****,* ***b'****,* ***c'****).*

| Run Date | ISH Run ID | a | a' | b | b' | c | c' |
|---|---|---|---|---|---|---|---|
| **09-29-05** | **0904** | 194 | 155 | 210 | 198 | 209 | 216 |
| **10-25-05** | **0957** | 218 | 170 | 222 | 199 | 222 | 211 |
| **11-01-05** | **0972** | 192 | 160 | 202 | 207 | 207 | 203 |
| **11-09-05** | **0990** | 215 | 165 | 222 | 207 | 217 | 220 |
| **11-17-05** | **1008** | 201 | 163 | 216 | 210 | 215 | 209 |
| **11-21-05** | **1014** | 201 | 178 | 205 | 197 | 200 | 196 |
| **12-14-05** | **1057** | 218 | 161 | 214 | 206 | 221 | 218 |
| **12-20-05** | **1061** | 216 | 163 | 213 | 204 | 214 | 209 |
| **01-04-06** | **1074** | 209 | 175 | 222 | 209 | 210 | 209 |
| **01-11-06** | **1087** | 217 | 153 | 210 | 200 | 214 | 205 |
| **01-25-06** | **1117** | 200 | 167 | 199 | 198 | 198 | 208 |
| **01-30-06** | **1138** | 223 | 170 | 218 | 210 | 222 | 224 |
| **02-07-06** | **1163** | 217 | 176 | 220 | 203 | 215 | 218 |
| **02-15-06** | **1175** | 199 | 160 | 202 | 194 | 207 | 209 |
| **03-08-06** | **1214** | 221 | 164 | 217 | 200 | 219 | 216 |
| **03-09-06** | **1222** | 195 | 151 | 210 | 184 | 201 | 197 |
| **03-22-06** | **1240** | 195 | 158 | 201 | 189 | 204 | 201 |
| **03-30-06** | **1263** | 205 | 143 | 214 | 183 | 223 | 199 |

Table 1 - *Mean densitometric intensity values derived from ROIs selected from negative (**a**, **b**, **c**) and positive (**a'**, **b'**, **c'**) control sections. Data is represented on a scale of 255 (least intense) to 1 (most intense).*

*P values of **a, a'** = 6.19227E-12; **b, b'** = 1.44928E-5; **c, c'** = 0.13355. At a 95% confidence level, only the case (**c**, **c'**) is not significant. This is the expected result as this region corresponds to an area of no visually significant gene expression.*

| ISH Run ID | a | a' | b | b' | c | c' |
|---|---|---|---|---|---|---|
| **0904** | 61 | 100 | 45 | 57 | 46 | 39 |
| **0957** | 37 | 85 | 33 | 56 | 33 | 44 |
| **0972** | 63 | 95 | 53 | 48 | 48 | 52 |
| **0990** | 40 | 90 | 33 | 48 | 38 | 35 |
| **1008** | 54 | 92 | 39 | 45 | 40 | 46 |
| **1014** | 54 | 77 | 50 | 58 | 55 | 59 |
| **1057** | 37 | 94 | 41 | 49 | 34 | 37 |
| **1061** | 39 | 92 | 42 | 51 | 41 | 46 |
| **1074** | 46 | 80 | 33 | 46 | 45 | 46 |
| **1087** | 38 | 102 | 45 | 55 | 41 | 50 |
| **1117** | 55 | 88 | 56 | 57 | 57 | 47 |
| **1138** | 32 | 85 | 37 | 45 | 33 | 31 |
| **1163** | 38 | 79 | 35 | 52 | 40 | 37 |
| **1175** | 56 | 95 | 53 | 61 | 48 | 46 |
| **1214** | 34 | 91 | 38 | 55 | 36 | 39 |
| **1222** | 60 | 104 | 45 | 71 | 54 | 58 |
| **1240** | 60 | 97 | 54 | 66 | 51 | 54 |
| **1263** | 50 | 112 | 41 | 72 | 32 | 56 |

Table 2 - *Densitometric values are normalized to reflect relative intensity of gene expression across negative (**a**, **b**, **c**) and positive (**a'**, **b'**, **c'**) control sections. On an intensity scale of 1 to 255, where absence of signal (white) is equal to 255 and most intense signal (black) equals 1, values have been normalized to reflect relative signal abundance as a measure of 255-[Mean$_{densitometric\ intensity}$].*

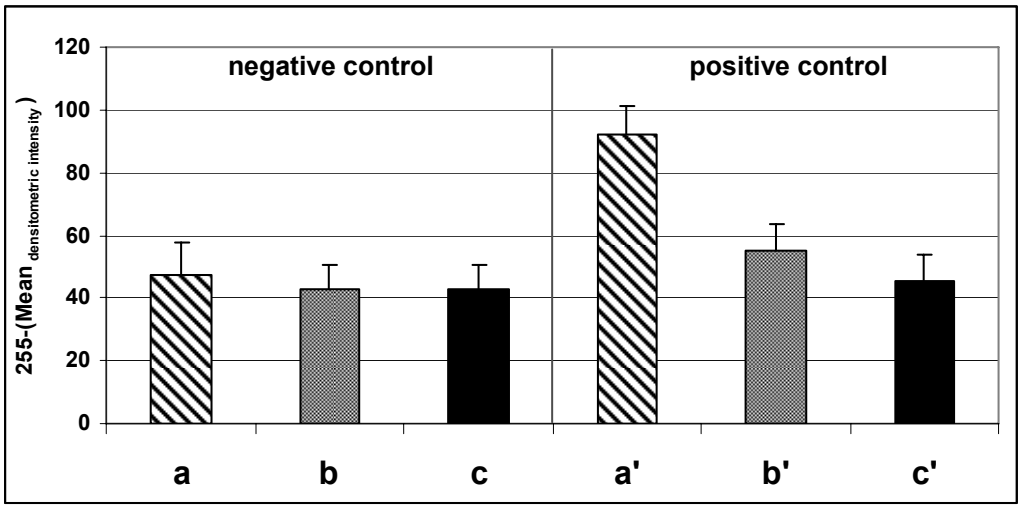


Figure 3 - *Average of the mean densitometric intensity for ROIs from negative and positive control sections. On an intensity scale of 1-255 where absence of signal (white) is equal to 255 and highest signal intensity (black) equals 1, values have been normalized on the Y axis to reflect relative signal abundance as 255-[Mean densitometric intensity]. Intensity scores from negative control slides (**a**, **b**, **c**) are shown on the left, and data from positive control slides (**a'**, **b'**, **c'**) are on the right side of the graph. Hatched bars denote values calculated from ROIs in potentially dense or high intensity regions (**a**, **a'**), lightly shaded bars denote ROIs from moderate intensity regions (**b**, **b'**) and solid bars show ROIs derived from low intensity regions (**c**, **c'**). n=18; error bars denote standard deviation of the mean.*

The run control data collected from the 18 ISH runs shown here is typical for results seen over the course of the ABA project. Although densitometric analysis of selected regions is a simplified means of quantifying gene expression, and does not involve specific segmentation of the putatively expressing cells, it provides data demonstrating platform consistency across multiple ISH runs. The information derived from these controls also serves the purpose of assessing whether data from individual runs is valid for further analysis.

## II. Reproducibility of ISH data across ISH runs and independent riboprobe syntheses

The primary sources of variability on the ABA semi-automated ISH platform are differential hapten incorporation in riboprobes, day to day variability associated with batch reagent preparation, and biological variability across brain samples. To assess the first two of these variables, an experiment was designed to generate replicate data across a series of days, using riboprobes generated in parallel. This design minimizes biological variability to the extent possible and involves processing on consecutive serial sections from the same brains from day to day to maximize comparability over time (**Table 3**). Sample riboprobes were selected for comparison across independent syntheses by *in vitro* translation (IVT) based on demonstrated gene expression profiles that were cell-type and/or highly regional. The specific genes assayed include *Calb1*, *Calb2*, *Cst3* (an astrocyte marker), *Dkk3*, *Gad1* (a pan-GABAergic interneuron marker), *Man1a*, *Plp1* (an oligodendrocyte marker), *Pvalb* and *Nov*. Processing these genes on full sagittal series through a single brain hemisphere allows not only visual comparison, but quantitative analysis using informatics methods described in detail in the Supplemental Document Informatics Data Processing.

| *ISH Run* | brain 1 | | brain 2 | |
|---|---|---|---|---|
| **Day 1** | **IVT-1** *Series 1* | **IVT-2** *Series 5* | **IVT-3** *Series 1* | **IVT-4** *Series 5* |
| **Day 2** | **IVT-1** *Series 2* | **IVT-2** *Series 6* | **IVT-3** *Series 2* | **IVT-4** *Series 6* |
| **Day 3** | **IVT-1** *Series 3* | **IVT-2** *Series 7* | **IVT-3** *Series 3* | **IVT-4** *Series 7* |
| **Day 4** | **IVT-1** *Series 4* | **IVT-2** *Series 8* | **IVT-3** *Series 4* | **IVT-4** *Series 8* |

Table 3 - *Experimental design to assess variability across independent probe syntheses. Probes for each of 9 genes were independently generated by in vitro translation (IVT) 4 times (IVT-1, IVT-2, IVT-3, IVT-4). For each gene, each independently synthesized probe was hybridized on series brain sections, consisting of 25µm-thick, 200µm-spaced sections through a single hemisphere, from the same brain over a span of 4 days (Series 1-4). Since there are only 8 series per brain, 2 probes are used on sections from Brain 1, and the other 2 probes are used on Brain 2.*

Low magnification (10X) images of ISH data at selected mid-sagittal planes of section across experimental conditions for each gene are shown in **Figures 4-12**. Images were collected according to standard procedures, as described in Data Production Process. Consistency across the entire data set for each gene is evident at this scale. Some minor overall color variation is visible that correlates most strongly with the day to day replicates, which is likely to be a result of reagent preparation variability and NBT/BCIP precipitation.

To quantitate experimental variability, precise densitometric values were calculated for images collected according to standard procedures, and the data were subjected to statistical analysis. The results of these evaluations are shown in **Figures 13-17**, using values for ISH signal intensity (light-to-dark gradation in color) and density (amount of color coverage across a given area).

The data presented in the following figures demonstrates that the standardized ABA platforms used to generate ISH data result in high quality, internally-consistent results. At the magnification level shown here, signal intensity and ratios of signal-to-noise are comparable across multiple probe syntheses and different brain samples. Data quality is also consistent when evaluated at higher magnification (data not shown). The plots shown in **Figure 17** illustrate slightly greater day-to-day variability than probe-to-probe variation, indicating that Tecan ISH platform reagent preparation introduces greater error into data quality than that produced in IVT probe synthesis. Some sectioning artifacts are visible (such as bubbles in tissue in **Figure 4**, Panels 7, 8); however, the signal intensity and quality over multiple sections is consistent and uniform for each probe tested.

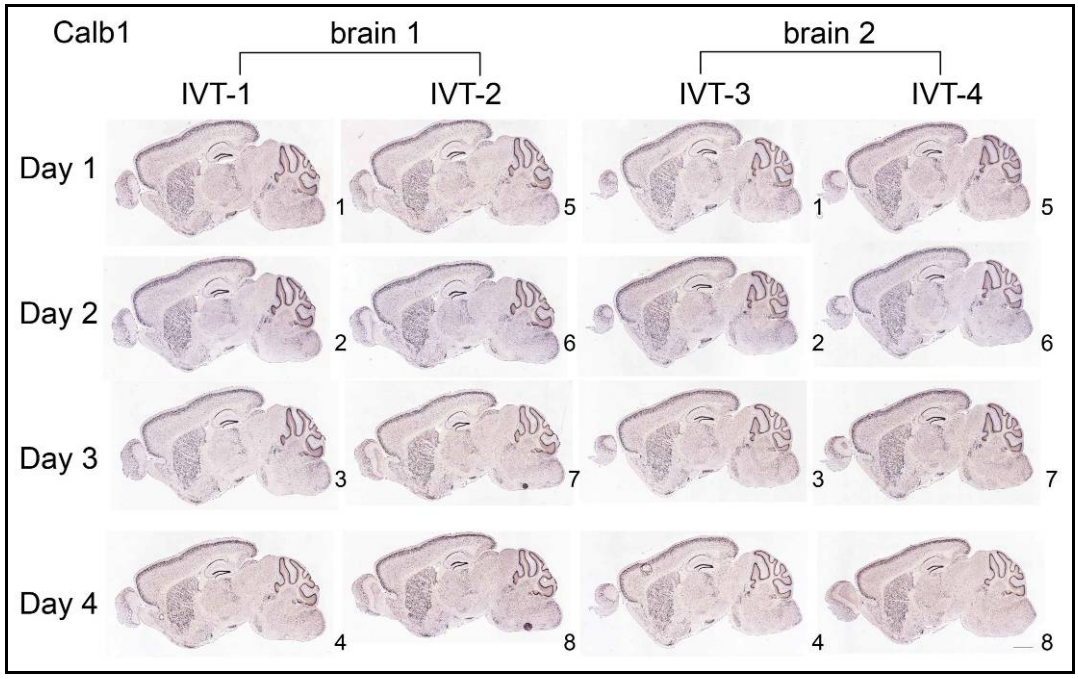


Figure 4 - *IVT and ISH run replicates for Calb1. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

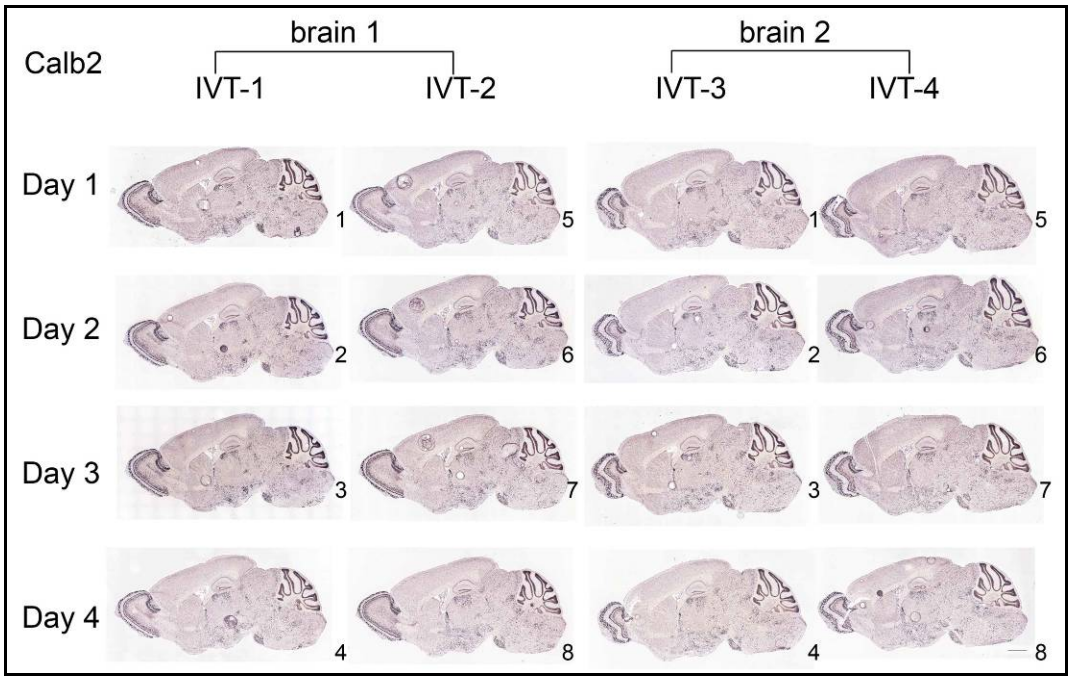


Figure 5 - *IVT and ISH run replicates for Calb2. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

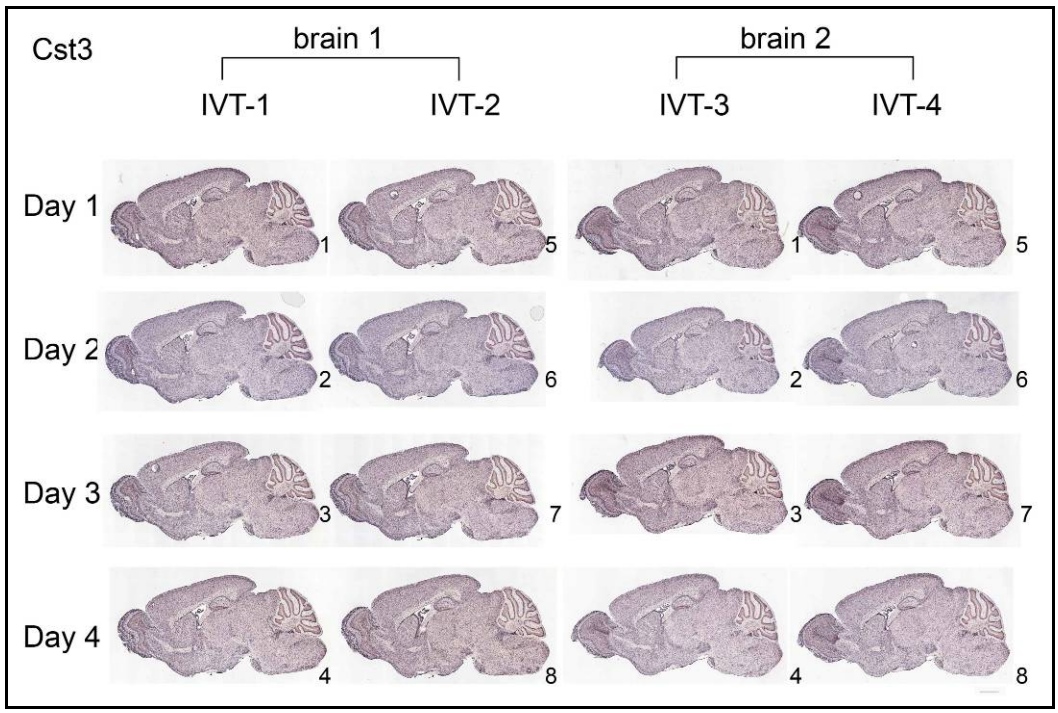


Figure 6 - *IVT and ISH run replicates for Cst3. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

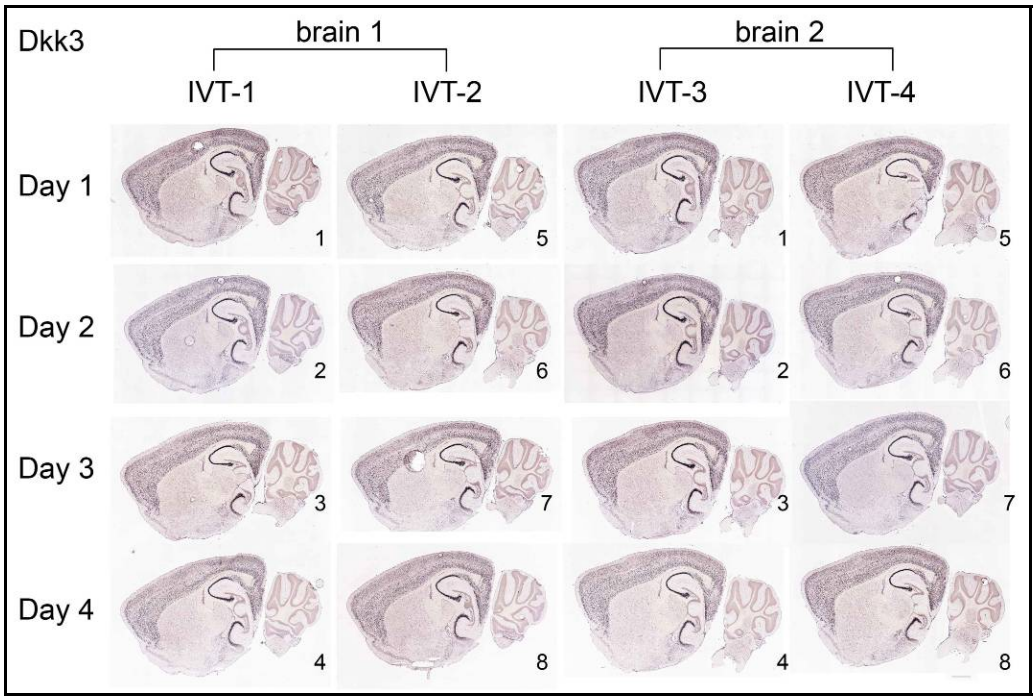


Figure 7 - *IVT and ISH run replicates for Dkk3. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

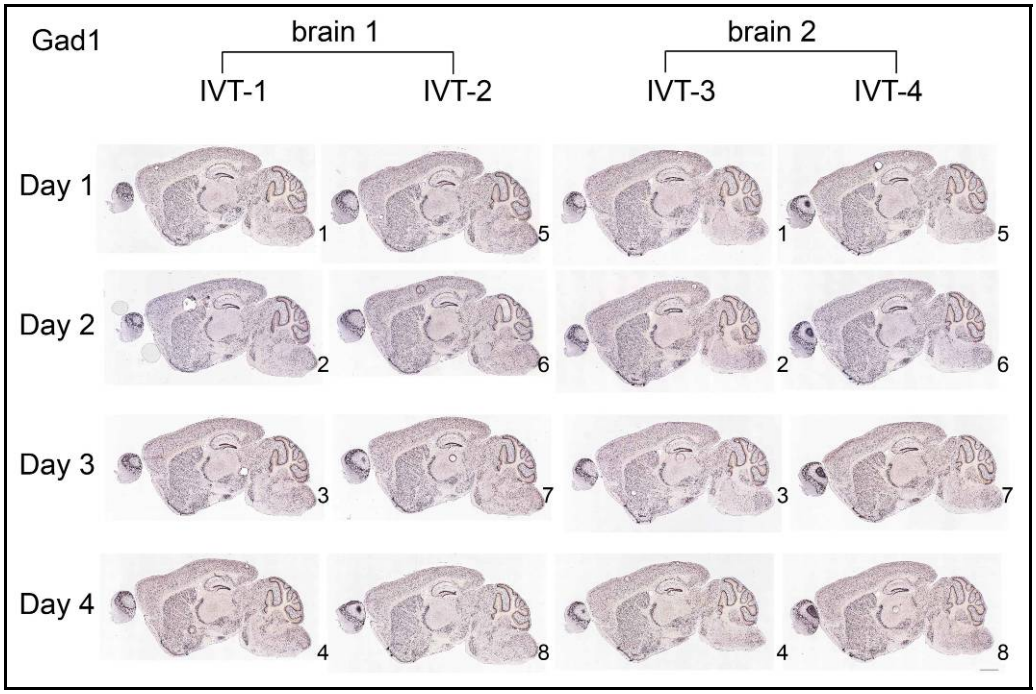


Figure 8 - *IVT and ISH run replicates for Gad1. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

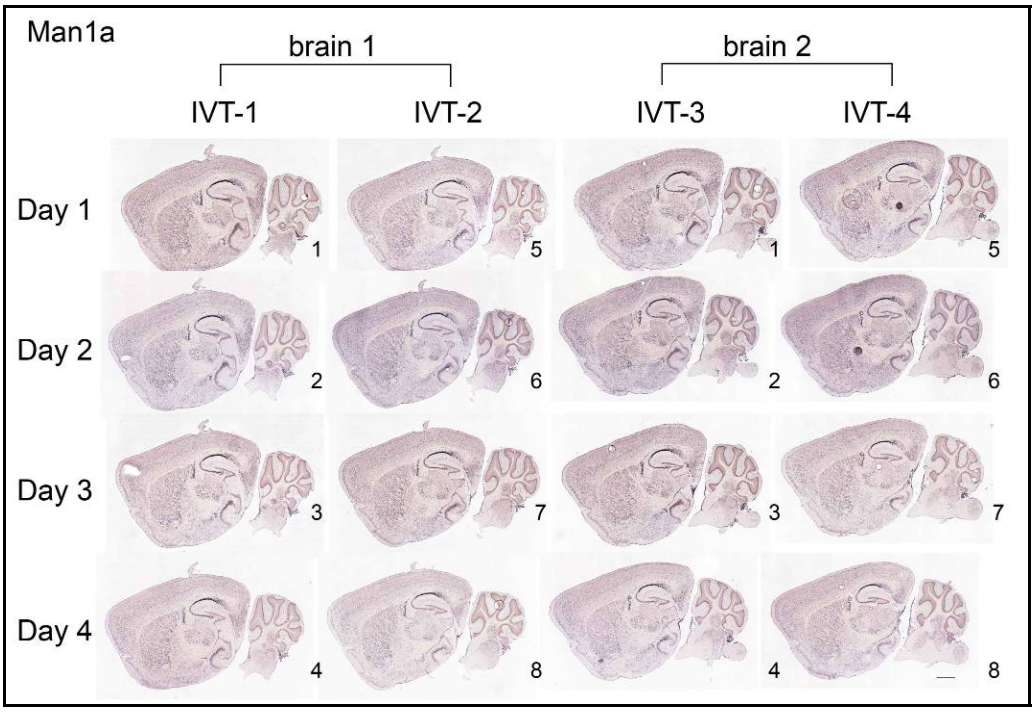


Figure 9 - *IVT and ISH run replicates for Man1a. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

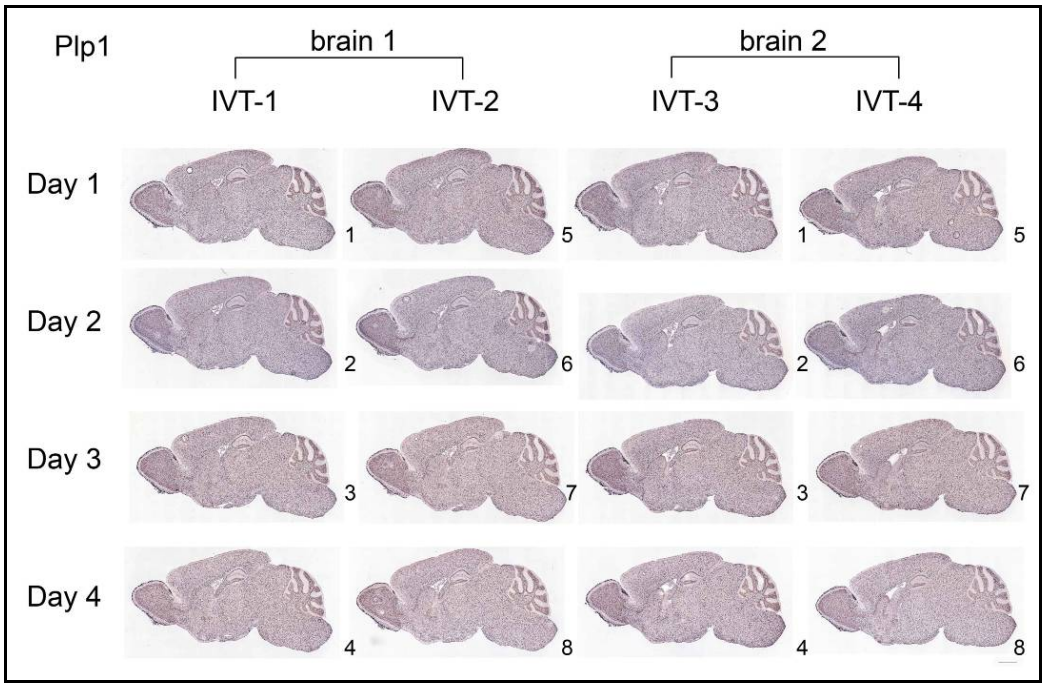


Figure 10 - *IVT and ISH run replicates for Plp1. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

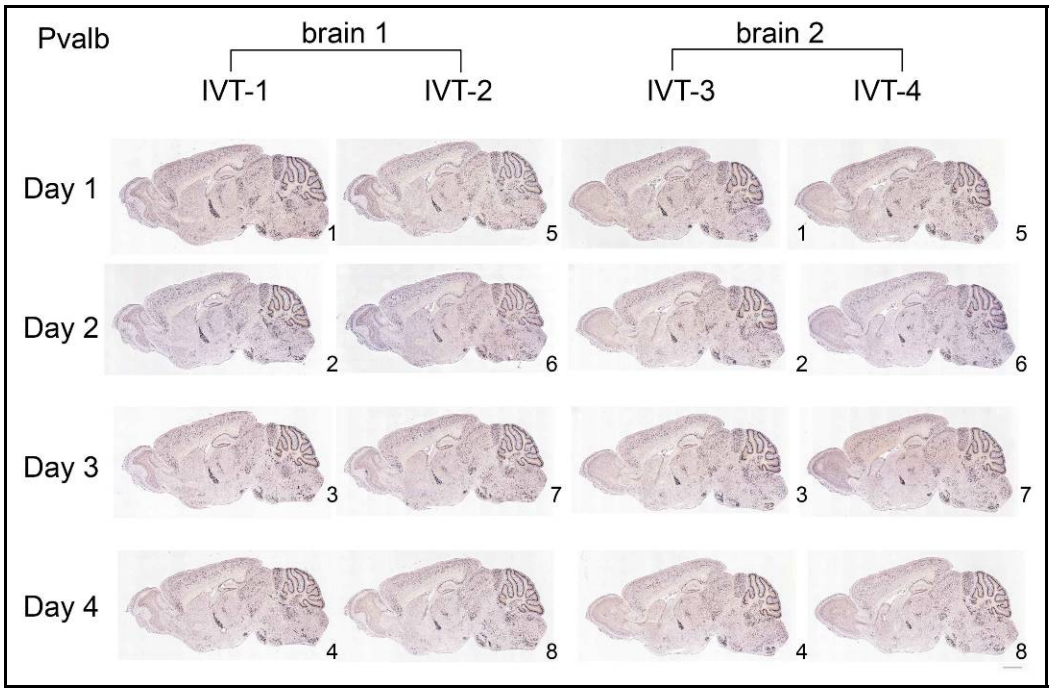


Figure 11 - *IVT and ISH run replicates for Pvalb. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

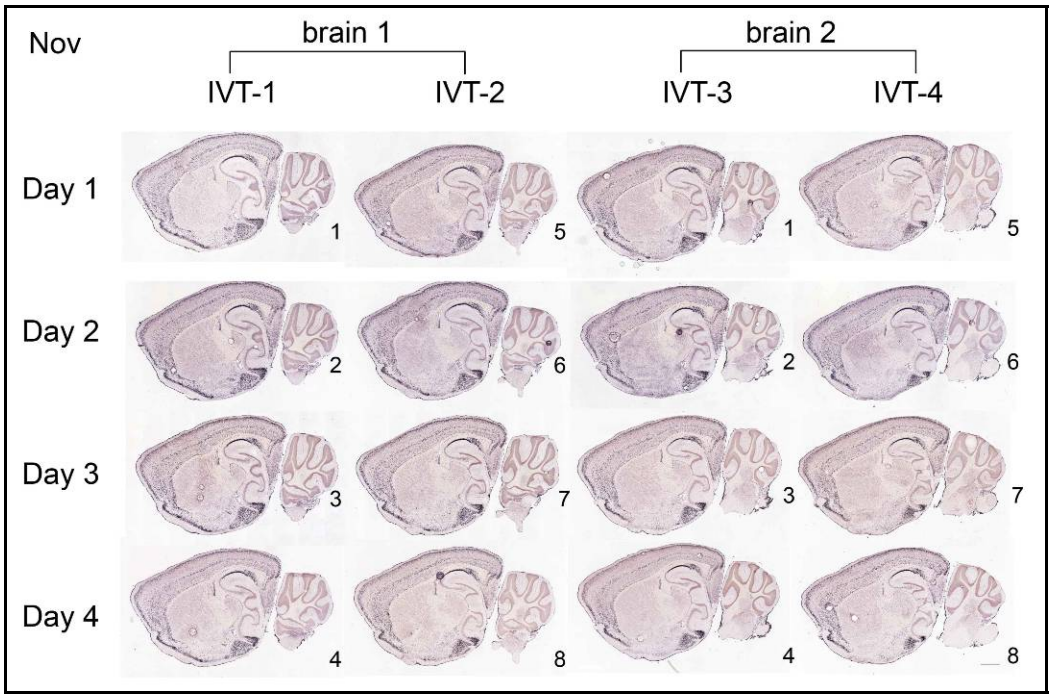


Figure 12. - *IVT and ISH run replicates for Nov. Four independent riboprobe syntheses (IVT-1 through IVT-4) were used as probes on four different days (Day 1 through Day 4). Sections displayed are serial sections (1-8) at a representative mid-sagittal plane of section for each of the 16 conditions. Scale bar: 200 μm.*

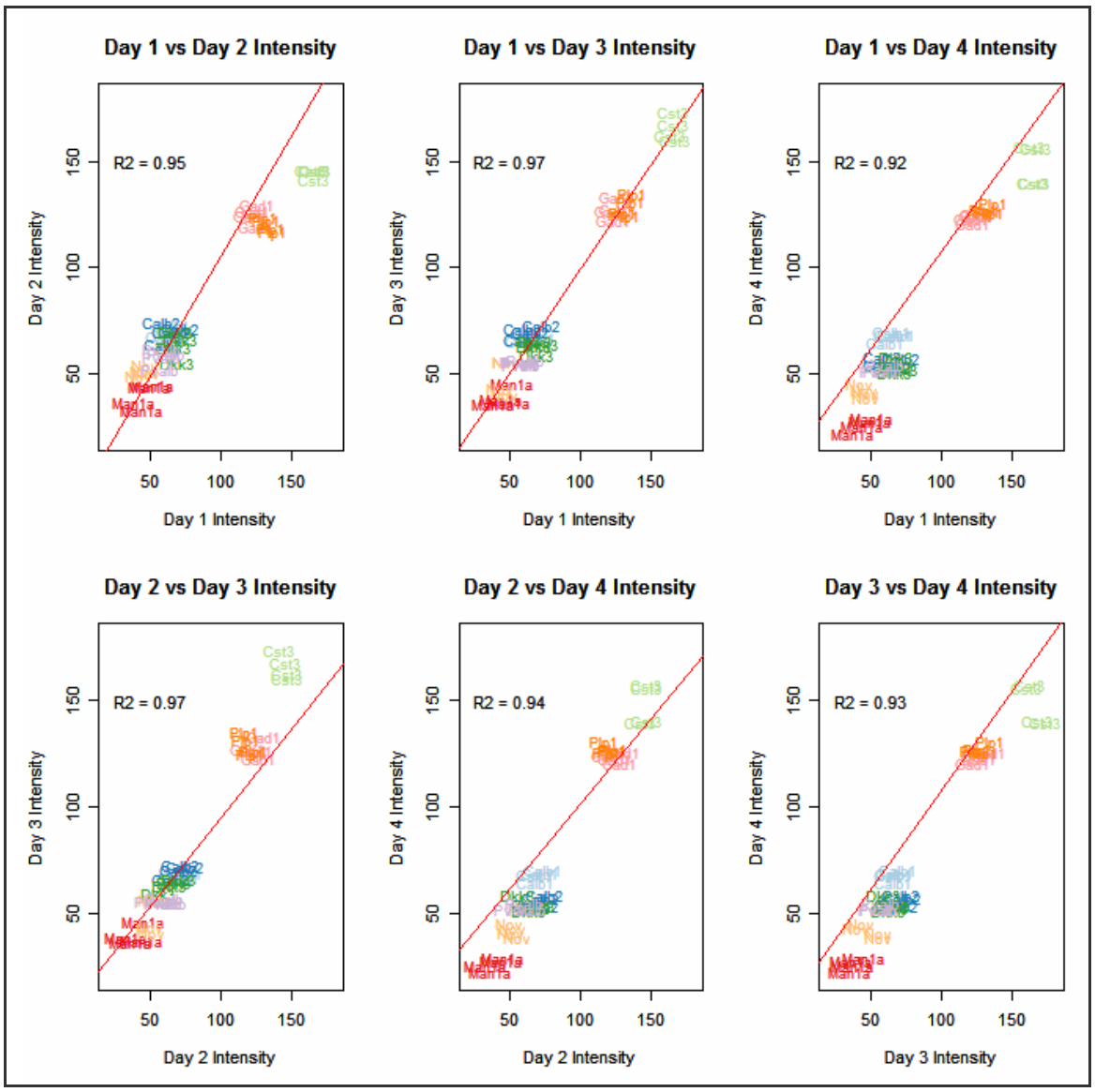


Figure 13 - *Intensity of gene expression as measured by ISH signal strength, compared across days. Intensity is defined as the product of average pixel intensity times the fractional area of expressing cells in the brain for the given gene compared to a model (with complete coverage). See* Informatics Data Processing *for detailed definitions of intensity (displayed on the X and Y axes above) and density (Figure 14). In each plot, four independent probes for each gene run on a given day are compared to each other by day. Ideal measured values would be equivalent from day to day with linear regression $R^2 = 1$. Clustering of the labeled probes for each gene is shown with colored gene labels. The average $R^2$ value across all day pairs is 0.94.*

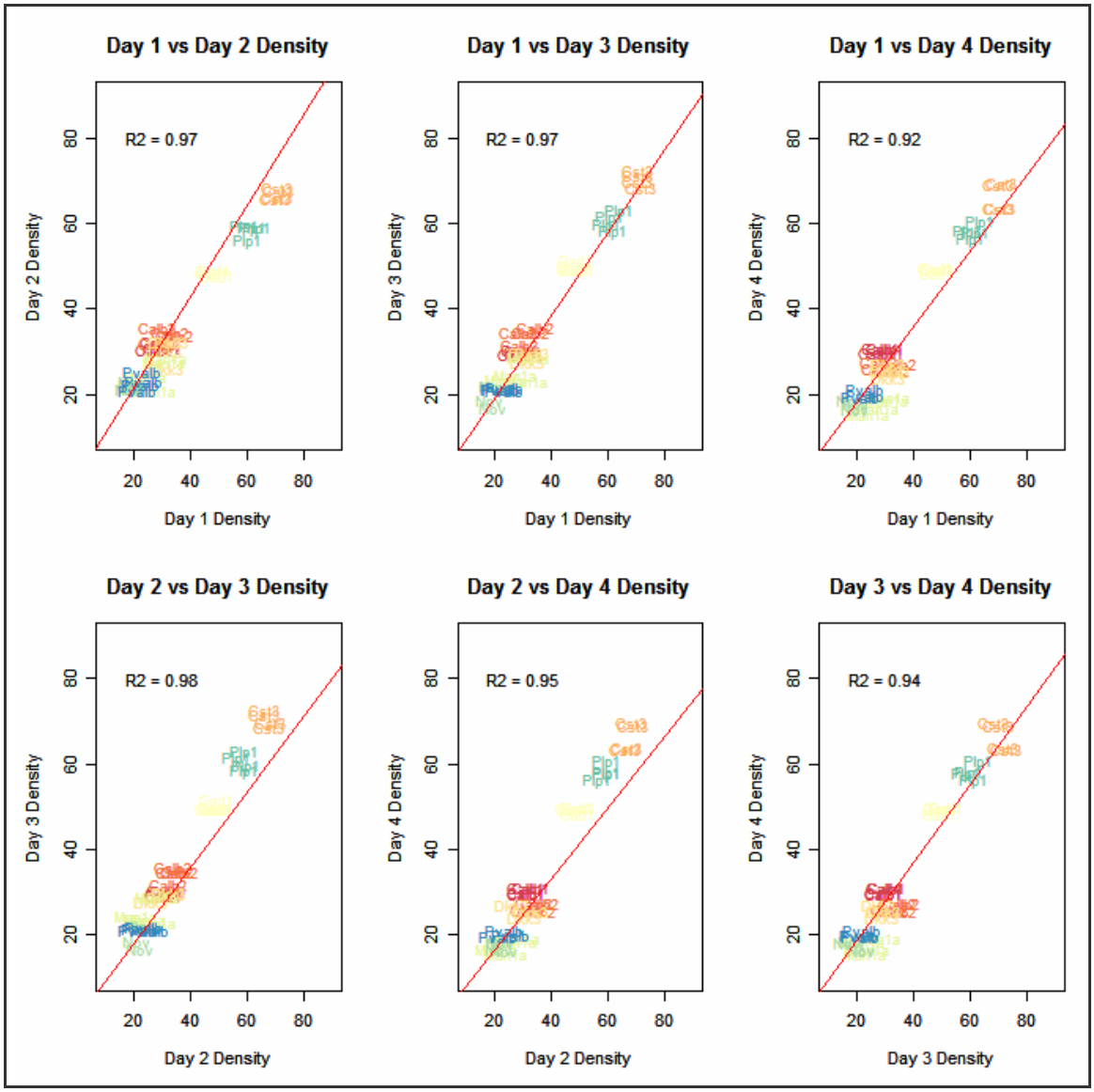


Figure 14 - *Density of gene expression as measured by ISH signal strength, compared across days. Similar inter day variation as for intensity measurements shown in Figure 13, but defined as the ratio of the number of expressing cells in the brain divided by the number of expressing cells in a model defined by a class of widely or ubiquitously expressing genes. The average $R^2$ value across day pairs is 0.95.*

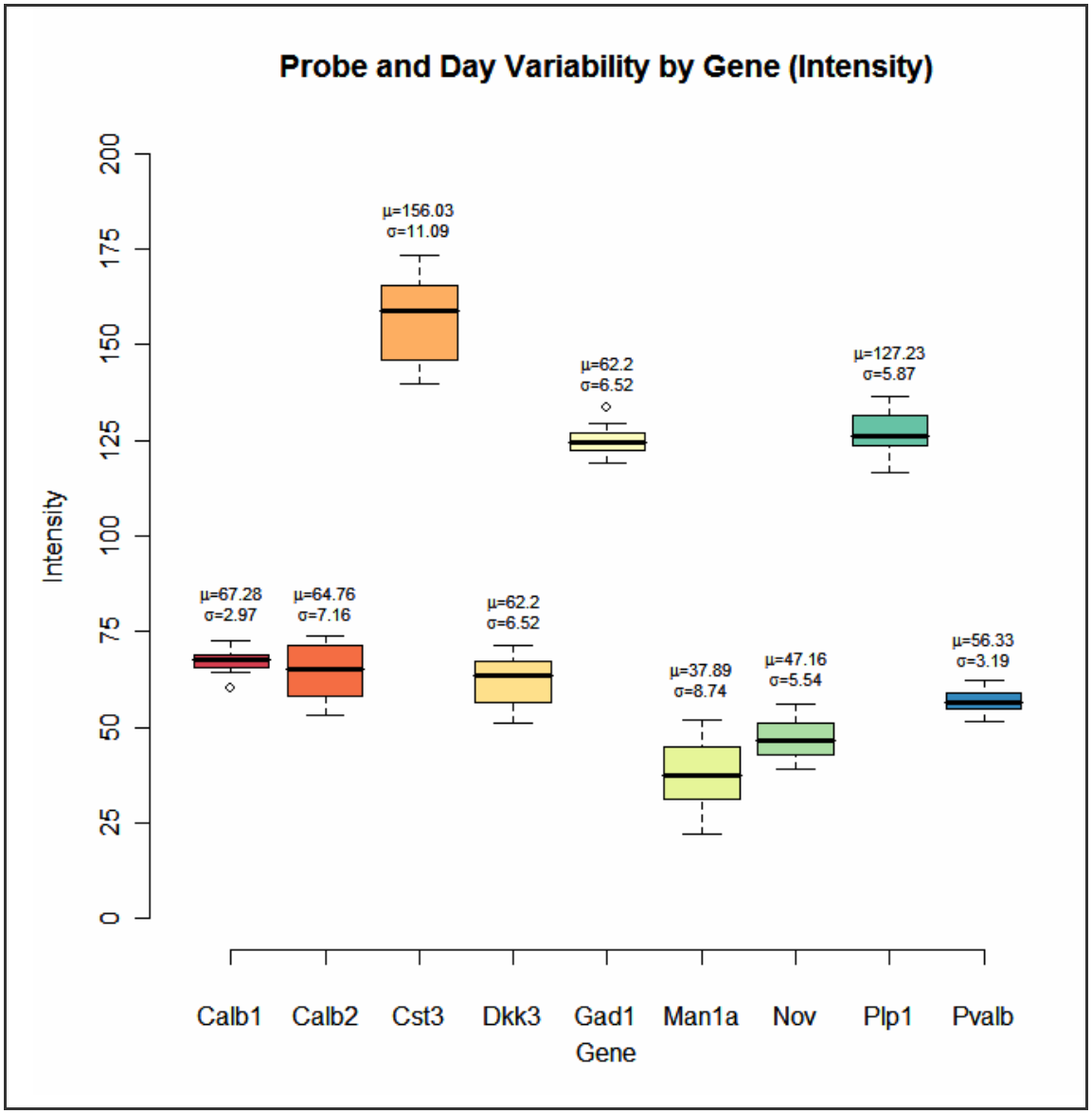


Figure 15 - *A distribution plot of the intensity of gene expression values of 4-day by 4-probe measurements for each of 9 genes is displayed. See* Informatics Data Processing *for detailed definitions of intensity (displayed on the Y axis above) and density (Figure 16). The boxplots show the median and inner quartile range of 16 points. The mean and standard deviation for each set is shown above individual probe boxplots.*

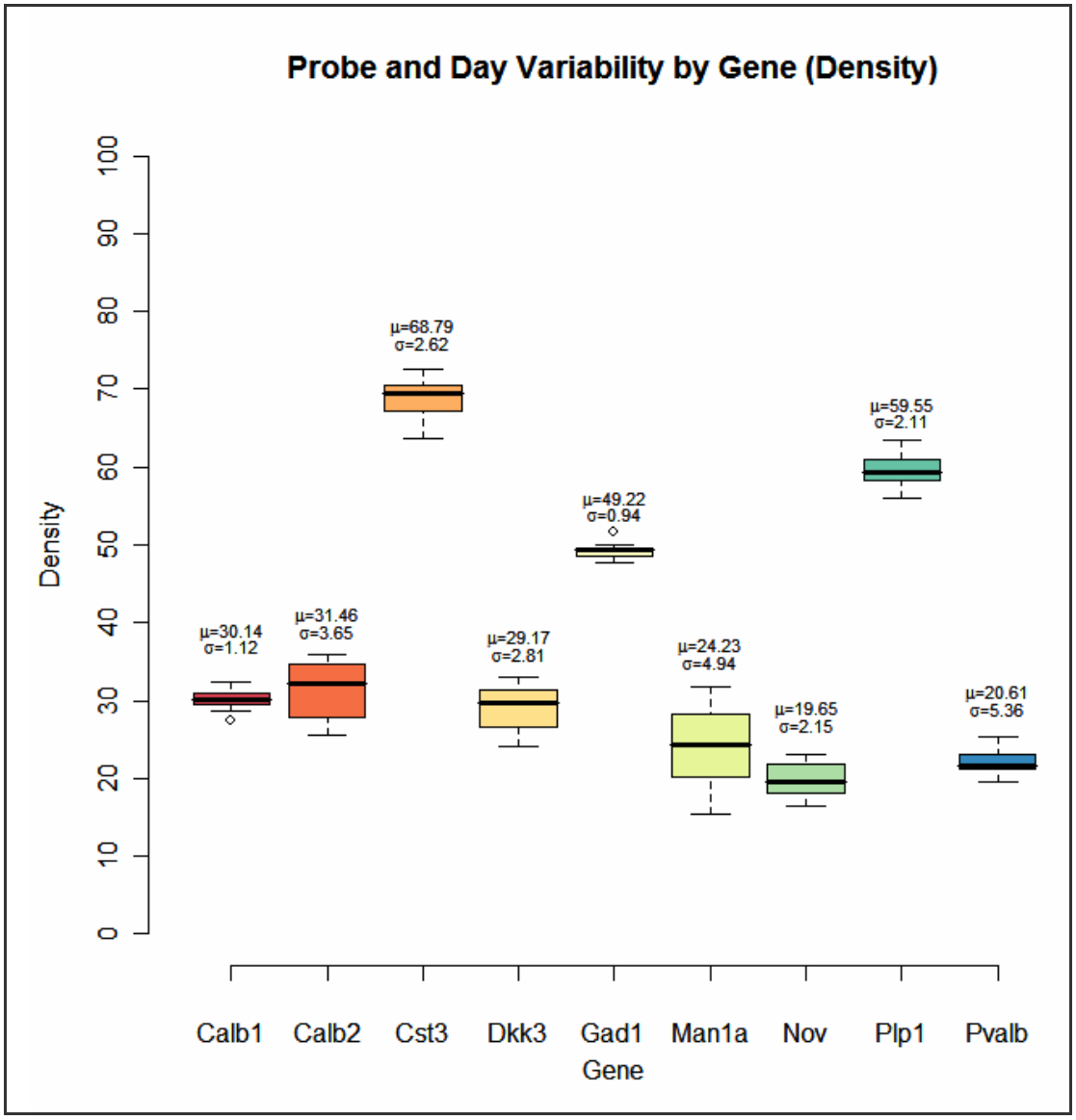


Figure 16 - *A distribution plot of the density of gene expression values of 4-day by 4-probe measurements for each of 9 genes is displayed. The boxplots show the median and inner quartile range of 16 points. The mean and standard deviation for each set is shown above individual probe boxplots.*


Figure 17 - *Coefficient of variation (CV) plots by gene, with day and probe values for both intensity (left panels) and density (right panels) measurements. Each curve in the top panels shows the CV for each of 9 genes using 4 replicates of probes on a fixed day, for intensity and density. The mean CV is shown in the dotted black line.*

*The bottom two panels graph values by day, holding the probes constant. The plots illustrate mildly more day to day variation than probe to probe variation. The average CV for probes across four days for intensity is 5.47%, for density it is 4.88%. The average CV for days across four probes for ISH intensity values is 8.81%, while for density the analogous figure is 7.78%. The reason for the difference is largely due to comparatively low expression values with higher variance in the single gene Man1a.*

## Appendix 1: Positive control riboprobe

Dopamine receptor D1A (*Drd1a*), 1155 nt from Entrez Gene ID: 13488

5'-
aggacagaagctgttccagtcctgaaggttcctctcccaaggaagctccgagaaccctcctgggggcgggggcagca
atctgtaggtgggcaaggtagcaggaggggcgctgcttccctggcacctggcctgggattcctttcccaaatccatcccag
agatttttctgcatgaggaggggaacacttgctctgctggagggactcgttgagtccaggggttttgggagaagtgactct
aaagcaaggtaggtggtcttgatggacttgtccaggggggtgatgggctgagattttgtgtagccaaataggaggtctgcac
agagtattggtgaacgactttgtgtttccctttgctttttaggcatttggagagatgtggcaccaggggcttgaaggagaaatg
catgtattttaggctgtgtctcagaagaagaggcagcatccctgaaaagtgactgagattgaccaggaagaggccccag
actcttagagtatgcttaaaagcctatgcattccttggggaatctagggaccagcggatgccagggaacagcgacctgg
agcactgaacccagaagacaggtggaaagcaagcggctcttcttcctggtatggcttggattgctatggagatgctcctg
atggaacaccattgtgcttttgtccagacagcaactggggctggagaaggggctgggtggtgagtgattgggggaagtct
ggctaagcctggccaagaacgtgagggctaagccaccggaagtgctttccttctggaagatggctcctaacacttctacc
atggatgagactggcctgccagtggagagggacttctcctttcgcatcctcacagcctgttttctgtccctgcttatcctgtcca
ctctcttagggaataccttgtctgtgccgctgtcatcaggtttcgacacctgcggtccaaggtgaccaacttctttgtcatctct
ttagctgtgtcagatctcttggtggctgtcttggtcatgccctggaaagctgtggctgagattgctggcttttggccctttgggtcc
ttttgtaacatttgggtagcctttgacatcatgtgctccacggcatccatccttaacctctgtgtgatcagcgtggacaggtattg
ggctatct
-3'