Abstract

Miller, Brian Douglas. Rat Peripheral Blood Lymphocytes as a Surrogate for Expression of TCDD inducible genes. (Under the direction of John F. Roberts, Ph.D.)

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is persistent in the environment and the food chain resulting in a chronic exposure to human populations. The effects of this known human carcinogen are evaluated by measuring various TCDD-inducible endpoints. Cytochrome P450 1B1, a recently identified TCDD responsive gene, shows inducibility in both the rat and humans that is similar to Cytochrome P450 1A1, a widely accepted marker for TCDD responsiveness. In order to evaluate a Sprague Dawley rat model to predict human tissue effects with easily accessible human tissue, such as peripheral blood lymphocytes (PBLs), rat PBL and granulocyte CYP1B1 and CYP1A1 expression were compared to expression among several tissues. Two treatment groups were used: an acute group, dosed with a 540 ng/kg single daily dose over a 3-day period followed by a day 4 sacrifice, and a chronic group, dosed biweekly with 7.6 ng/kg/day for 15 weeks. Competitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to show

the different levels of expression of CYP1B1 and CYP1A1 in resting PBLs, granulocytes and various other tissues. CYP1B1 and CYP1A1 were shown to be inducible in liver, adrenal gland, lung, kidney, granulocytes and in PBLs. However, a lower induction for PBLs and granulocytes was observed compared to the highly inducible tissues such as liver and lung. Furthermore, male rats showed a 200-fold induction in CYP1B1 expression in PBLs and 16-fold induction in granulocytes in the chronic group following TCDD exposure compared to a 2 fold induction in PBLs and 2-fold induction in granulocytes for the female rats of the same dosing group. The differences in inducibility between PBLs, granulocytes and tissue may be due to the different levels of TCDD that reach each tissue due to the pharmacokinetics dependent upon that tissue. With the different expression patterns and low copy number of CYP1B1 and CYP1A1 in PBLs and granulocytes in Sprague Dawley rats, their use as surrogates for expression in tissues of interest does not correlate with recent published data for human studies on PBLs exposed to TCDD. Therefore, the comparison needs to be further investigated and the use of PBLs better established as a surrogate for expression.

Rat Peripheral Blood Lymphocytes as a Surrogate for Expression of TCDD Inducible Genes

by

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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science

Physiology

Biography

Brian D Miller was born in Smithfield, North Carolina in 1972. He attended North Carolina State University from 1990-1994. His B.S. in Zoology was awarded in 1994.

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Abbreviations used:

TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Dioxin	2,3,7,8-tetrachlorodibenzo-p-dioxin
CYP1A1	Cytochrome P-4501A1
CYP1B1	Cytochrome P-4501B1
PBL	peripheral blood lymphocytes
RT-PCR	reverse transcription-polymerase chain reaction
IS	internal standard
rcRNA	recombinant RNA

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), is an ubiquitous environmental contaminant with anti-estrogenic effects that originates from contamination of broad leaf herbicides and industrial processes such as paper bleaching. It is classified as both a rodent and a human carcinogen (1), and its persistence in the environment and bioaccumulation in the food chain results in chronic exposure of human populations. Exposure to TCDD in both rodents and humans follows the Aryl hydrocarbon receptor (AhR) agonist mechanism of action of dioxin. TCDD enters the cell and binds with the Ah Receptor causing the release of the proteins p50 and Hsp 90 before translocating to the nucleus. Once in the nucleus a heterodimer is formed between the AhR:Dioxin complex and ARNT (the Ah receptor nuclear translocator protein). This heterodimer then binds to the dioxin response elements in the dioxin response genes, which results in the increased expression of TCDD responsive genes such as Cytochrome P450 1A1 and CYP1B1(2-7). The response and sensitivity observed to TCDD exposure in mammalian species varies with the species and tissue-specific changes in TCDD-responsive genes. (8,9). Analyses comparing the regulation and inducibility of TCDD-responsive genes between rodent models and that of humans may lead to a better understanding of human exposure risk and how that relates to target organ toxicity.

CYP1B1 represents a gene subfamily of Cytochrome P450, a supergene family of heme-thiolate proteins, that is TCDD-inducible to varying extents depending on the tissue in question (10). It is constitutively expressed at high levels in the adrenal gland, an organ not primarily known for detoxification of exogenous substrates, so it may have an endogenous steroid as a substrate. CYP1B1 has been shown to be inducible by TCDD in Sprague Dawley rat liver and kidney in a sex-specific manner (11), and it is responsible for the 4 hydroxylation of 17β-estradiol as well as bioactivation of polycyclic aromatic hydrocarbons and arylamines (12-14). It has even been implicated in benign tumor formation in humans due to its metabolic activity in the formation of catechol estrogen 4-hydroxyestradiol (15,16). This lends credence to the concept that CYP1B1 is an important biomarker for chemical exposure.

CYP1A1 is perhaps the best characterized of the TCDD-inducible genes (17), and its induction is regulated by the same Ah receptor agonist pathway as CYP1B1. CYP1A1 mRNA expression has been closely linked to TCDD exposure showing a detectable affect with as little as 1 ng TCDD/kg body weight (18). The increase in CYP1A1 activity seen with TCDD exposure resulting in liver and skin tumor promotion (19, 20) and multi-site rodent cancers (21, 22) has shown the usefulness of CYP1A1 as a marker for risk assessment.

Human tissues for biomarkers of chemical exposure have been historically difficult to obtain and have been limited to blood, urine, or hair. Obtaining PBLs from human blood and measuring biomarkers of

exposure has had limited yet promising results. For example, Spencer, et al. (23) showed that CYP1B1 expression could be induced by TCDD exposure to PBLs in vitro following mitogen stimulation (23). In human monocytes, CYP1A1 was induced by in the same way as CYP1B1 though CYP1B1 had a lower level of constitutive expression. (23,24). A recent study showing that CYP1B1 may be a major P450 isozyme in human blood monocytes (26), asks if this correlates in the rodent model as well.

Since CYP1B1 is constitutively expressed in uncultured human PBLs but requires mitogen stimulation and dioxin exposure to get a response (27), is this necessarily the case for the Sprague Dawley rat model? The objective of the present study was to quantitatively measure the expression of CYP1B1 in uncultured rat PBLs and granulocytes for an acutely treated group, dosed with 540 ng TCDD/kg body weight over a 3 day period followed by a day 4 sacrifice, and a chronically treated group, dosed biweekly with 7.6 ng TCDD/kg body weight/day for fifteen weeks, to determine if the PBLs or granulocytes were good surrogates for expression of CYP1B1 in other tissues in the Sprague Dawley rat model. The analysis of this dioxin-inducible gene in this rat dose response study may be useful in addressing knowledge gaps in risk assessment for human exposure to dioxin by extrapolation.

Materials and Methods

Experimental Design: Male and female Sprague Dawley Rats (Taconic, Germantown, NY), a benchmark species used for long term TCDD studies by the National Toxicology Program, were obtained and kept three per cage and fed food and water *ad libitum*. Treatment started at 10 weeks of age. Doses were selected to obtain TCDD levels above background but low enough to prevent systemic toxicity and recruitment of binding proteins. Oral gavage of TCDD in corn oil was used to allow for optimal absorption and consistent TCDD levels, similar to that used in physiological pharmacokinetic modeling.

Acute study : Consisted of 10 male and 10 female rats (5 controls and 5 TCDD treated, for each sex) with dosing over 3 consecutive days at 540 ng/kg body weight TCDD and corn oil alone for the 10 total number of controls. All animals were sacrificed at day four.

Chronic study : Consisted of 11 males and 11 females (5 for the controls and 6 for the TCDD treated, for each sex) with dosing done on a biweekly schedule for 15 weeks. Treated animals received 7.66 ng/kg/dose TCDD and corn oil alone for controls.

Necropsy : For both studies, animals were anesthetized with CO_2 and blood was withdrawn into heparinized tubes by cardiac

puncture. Liver, lung, kidney, and adrenal gland samples were snap frozen in liquid nitrogen and then stored at -70^{0}C.

Lymphocyte and Granulocyte Isolation: Peripheral blood lymphocytes and granulocytes were isolated from fresh blood within 3 hours following necropsy using double density Ficoll gradient centrifugation. Blood was diluted 2.5 times with Hank's Balanced Salt Solution and then layered on top of a double density Histopaque (Sigma) step gradient consisting of 25 ml Histopaque 1083 over 25 ml Histopaque 1119. After centrifugation at 700 X g for 30 minutes, both lymphocytes (taken from the interphase between the top plasma layer and the Histopaque 1083) and granulocytes (taken from the interphase between the Histopaque 1083 and the Histopaque 1119) were washed with media [RPMI 1640 (Gibco), 2mM L-glutamine (Gibco) and penicillin (100U/ml)-streptomycin (100ug/ml) (Gibco), and 10% FBS(Hyclone)] and cryopreserved in freezing media [RPMI media as described above except with 20% FBS and 7.5% DMSO (culture grade ATCC)]. Both lymphocytes and granulocytes were frozen in 1-2 ml aliquots at approximately 1 x 10^7 cells/ml using a Cryomed 700A programmed cell freezer, and then stored in liquid nitrogen vapor. The granulocytes were pooled based on sex and treatment.

RNA Isolation: Total RNA was isolated from frozen liver, lung, kidney, and adrenal gland tissue using a double extraction method of guanidine isothiocyanate with DNA removal by acid hydrolysis and

phenol-chloroform-isoamyl alcohol partitioning (TRI Reagent, Molecular Research Center, Inc., Cincinnati, OH). Total RNA was isolated from PBLs and granulocytes after a quick thaw in 37^{0}C water bath followed by a wash in RPMI media (as described above except without FBS) at 200 x g for 10 minutes then a wash, again using HBSS. RNA was kept at -70^{0}C until use.

RNA Gels: Total RNA at a final concentration of 1120 ng/5ul, as determined by UV spectrophotometry at 260nm, was loaded onto RNA formamide denaturing gels, 1.25% SeaKem gold Agarose (Reliant RNA Gel System, FMC Bioproducts, Rockland, ME), to determine the condition of the stored RNA. This analysis was done on a completely random basis and included approximately half of all samples. RNA was run at 75V for 1-1.5hrs and then the gels were stained with ethidium bromide for 30 minutes and destained with distilled water for 30 minutes. The gel was then visualized and the image was captured using still video (Eagle Eye II, Stratagene, La Jolla, CA.)

RT-PCR: Quantitative RT-PCR was performed using a competitive titration assay utilizing a heterologous recombinant internal standard RNA (Fig. 1a and 1b : ref.25,26). A series of dilutions of a specific recombinant RNA internal standard longer than the mRNA target of interest (CYP1B1, CYP1A1 or actin) was used as a competitive template. An internal standard at each titration point,

along with approximately 350ng of total target RNA, as determined by UV spectrophotometry at 260nm, was reverse transcribed in a final volume of 20ul containing a 1 X NEB Buffer [consisting of: 16.6mM $(NH_4)_2SO_4$, 67mM Tris-HCL (pH 8.8), 6.8uM EDTA (pH 8.0), 0.08 mg/ml bovine serum albumin {Calbiochem}, 5 mM 2-mercaptoethanol], 5 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate, 15 units RNase inhibitor, 10 units AMV Reverse Transcriptase, and oligo $(dT)_{15}$. Samples were incubated at 42^0C for 15 minutes followed by inactivation of the reverse transcriptase at 99^0C for 5 minutes. To the completed reverse transcription reaction, a polymerase chain reaction mix was added containing 2.5mM MgCl2, 1X NEB Buffer, 10 pmoles rat forward primer (CYP1A1(ref 25); or CYP1B1 [5'-accgcaacttcagcaacttc-3', or 20bp, 5'-nucleotide 1165]; actin(ref 25)), 10 pmoles rat reverse primer (CYP1A1(ref 25)]; or CYP1B1 [5'-gtgttggcagtggtggcatg-3', 20bp, 3'-nucleotide 1591]; or actin(ref 25)), 1 unit Taq DNA polymerase, and DEPC treated H_2O to a final volume of 40ul. The mixture was added to the completed reverse transcription reaction mixture at ambient temperature. They were then placed in a Perkin Elmer Thermocycler, heated to 94^0C for 4 minutes, then underwent 35 cycles for CYP1A1 and CYP1B1 tissue samples, 40 cycles for CYP1A1 and CYP1B1 lymphocyte and granulocyte samples, and 28 cycles for actin of: 30s at 94^0C, 30s annealing at 60^0C, and 30s at 75^0C; followed by a 4 minute extension at 75^0C. The expected length of the target rat CYP1B1 fragment was 427 bp long.

After RT-PCR, the 309 bp CYP1B1 internal standard product and the CYP1B1 target will differ in length by 118bp. The difference in size between the CYP1A1 target and the CYP1A1 internal standard product was 57bp, and the difference in size of the actin target and the actin internal standard product was 101bp and were consistent with previous work done by Vanden Heuvel, et al (26) All RT and PCR reagents with the exception of the NEB buffer were purchased from Promega.

Internal Standard RNA: The internal standard amplicon consisted of target-specific forward and reverse primers separated by a heterologous spacer molecule derived by amplification using primers specific to the human (GSTM1) gene (Genbank accession # S62935). The amplicon bears a 5' T7 promotor site and a 3' $d(A)_{18}$ tail (27). Using an alignment of the rat and human CYP1B1 cDNAs, two primer sequences were identified that could be used for amplification of both rat and human CYP1B1. The primer pairs span a known intron/exon boundary in human CYP1B1 and the region amplified corresponds to the variable region of the CYP1B1 protein sequence alignment. A glutathione transferase (GSTM1) plasmid template "spacer" fragment and PCR primers of ~60 base pairs were utilized in synthesizing the internal standards. The forward PCR primers contained sequences for the T7 promotor, target mRNA (CYP1B1, CYP1A1, or Actin) and the spacer gene forward primers (GSTM1F2). The reverse PCR primers contained sequences for the spacer gene reverse primer (GSTM1R2),

the target gene (CYP1B1,CYP1A1, or Actin) mRNA reverse primer and a poly $(dT)_{18}$ tail (27). The pooled PCR products from the PCR amplification of the GSTM1 plasmid and the above primers were purified using a Microcentricon 100 then transcribed into RNA using the Ribomax Large Scale Production System and T7 promotor. This was followed by a DNase treatment to remove the GSTM1 DNA template. The RNA was purified utilizing the Tri Reagent system as described previously . Before use, a serial dilution was made of the rcRNA preparation and amplified by PCR with the target gene PCR primers, with and without reverse transcriptase to ensure DNA was not detectable in the rcRNA preparation. This serial dilution series was then used for the internal standard range finding.

The size of the GSTM1 spacer fragment is 269 including the primers. After genomic-PCR, the product will be 346 bp long and after RT-PCR the product will be 309 bp long.

Gel Analysis: Aliquots of the PCR reaction product were analyzed by agarose gel electrophoresis on ethidium bromide stained 2% Metaphor agarose gel (FMC Bioproducts, Rockland, ME) and the image was captured using a Perkin Elmer fluorimager. The density of each band was quantified using the NIH Image Analysis software. Each band was circled and volume/spot was determined. The area and peak height were not used for quantitation due to increased variability of results compared to volume/spot as seen in previous work by Vanden Heuval et al. (26). The log of the ratio of target RNAamplicon to rcRNA amplicon versus the log of the number of

copies of internal standard of RNA in each reaction was graphed. The number of copies of target amplicon was determined by interpolation using a log ratio = 0, which is where the number of molecules of CYP1B1 in 0.350 ug target RNA and that of the internal standard are equivalent. All PCR Reagents were from Promega (Madison, WI). All other reagents, unless otherwise noted, were purchased from Sigma. The primers were made by Bioserve Biotechnologies (Laurel, MD).

Results

The development of an RT-PCR titration assay was used in this study to determine the levels of CYP1B1, CYP1A1 and actin. By taking a 10 fold dilution of an Internal Standard (IS) for a total of 6 reactions, ranging from 10^2 – 10^7 molecules per RT-PCR, a competitive RT-PCR reaction was used to determine expression levels in the RNA isolates. The cDNA derived from these reactions were quantitated and analyzed using image analysis. This initial data from the 6 reactions of 10 fold IS dilutions repeated twice in triplicate were then used to set up 6 more reactions in 2 fold dilutions of IS to further refine the assay for expression levels (n=5). The possibility of genomic DNA contamination in the RNA isolates and IS was ruled out by RT-PCR performed with and without reverse transcriptase. There were no observable products in the reactions where the reverse transcriptase enzyme was omitted.

The constitutive expression of CYP1B1 (table 1; fig.2&3) and CYP1A1 (table 2; fig. 4&5) vary appreciably across tissues in the Sprague Dawley Rat. Using competitive RT-PCR in both the short term and long term treatment groups, constitutive CYP1B1 expression varied from as low as 1.31 copies CYP1B1/350 ng RNA to 6790 copies CYP1B1/350 ng RNA. Constitutive expression of CYP1A1 varied from 246 copies CYP1A1/350 ng RNA to 1,590,000 copies CYP1A1/350 ng RNA. This expression varied based on sex differences as well with female Sprague Dawley rats having significantly higher constitutive expression of CYP1B1 in lymphocytes and CYP1A1 expression in liver than males.

The induced expression of CYP1B1 and CYP1A1, as seen with competitive RT-PCR, continued the variability seen across tissues as with the constitutive expression. The induced expression levels of CYP1B1 varied from as low as 24 copies CYP1B1/350 ng RNA to 42400 copies CYP1B1/350 ng RNA (table 3; fig.6&7). The induced expression of CYP1A1 (table 4; fig.8&9) varied from as low as 1930 copies CYP1A1/350 ng RNA to 17,600,000 copies CYP1A1/350 ng RNA. The fold induction seen in the expression levels were from as low as 1.87 fold to as high as 201.9 fold for CYP1B1 expression (fig.2) and from as low as 1.27 fold to as high as 1441 fold for CYP1A1 expression (fig.3).

By comparing all the results with the actin expression profiles, samples showing wide variation in the number of actin molecules per 350 ng RNA were further evaluated and compared to replicates. This ensured

no technical errors contributed to the variation. The results were further subjected to the student t test and showed coefficients of variance ranging from 6-25%.

Discussion

The primary goal of this study was to determine if rat peripheral blood lymphocytes and granulocytes are a good surrogate indicator for expression of CYP1B1 and CYP1A1 in specific tissues of interest in the Sprague Dawley rat. In addition, would the expression patterns in the rat model correlate with the human population's expression patterns as seen in work by Spencer et al (23)? This study had two major components based on treatment. The acute dosing and chronic dosing were designed using standard Physiologically Based Pharmacokinetic modeling to obtain similar tissue TCDD concentrations with 2 different time periods as well as prevent the possibility of reversible TCDD induced changes (24). Using this method, the acute dosing group should show immediate changes due to TCDD exposure while the chronic dosing group would show the slower adaptive expression changes. Since the dosing regimen and rat population were controlled, the need to account for background exposure and inter-individual variation as with human populations was not necessary.

The expression patterns seen were similar to what has been previously reported in studies by Van Den Heuval et al (25,26) and Walker et al (11). Constitutive expression of CYP1B1 and CYP1A1 were higher in female than male rats for tissues such as liver, lung, kidney, adrenal gland, lymphocytes and granulocytes. The characteristic response to TCDD exposure was also seen with increased expression of both CYP1B1 and CYP1A1 in all tissues measured, albeit at different relative responses. The acute dosing group showed a lower overall response to TCDD exposure than the chronic dosing group, when comparing the overall fold inductions, suggesting that the response to TCDD exposure takes longer to be seen. However, the peripheral blood lymphocytes and granulocytes show a pattern not seen before. The CYP1B1 expression response to TCDD exposure in male rat lymphocytes was almost 200-fold higher over constitutive expression compared to only 2-fold induction for the female rat. This suggests that while the female rat has a higher basal rate of CYP1B1 expression in peripheral blood lymphocytes, the male rat has a higher response to TCDD. A similar response was seen in the chronic study for the granulocytes. The basal rates of CYP1B1 expression were comparable between the female and male rats but the CYP1B1 response to TCDD exposure was seen to be greater in the male rat. CYP1B1 expression in male rats showed a 16-fold increase over constitutive expression compared to the 2-fold increase seen in the female rat. The CYP1B1 expression response seen in the peripheral blood lymphocytes and granulocytes further indicates that

CYP1B1 may be a major P450 isozyme as originally suggested in Baron et al. (28). The expression patterns seen and the low overall copy numbers of CYP1B1 and CYP1A1 in peripheral blood lymphocytes and granulocytes of the Sprague Dawley rat indicate that, unlike the human population, peripheral blood lymphocytes and granulocytes are not a good surrogate of expression patterns for other tissues. Rat peripheral blood lymphocytes and granulocytes are not as easily obtained in high enough numbers due to low volume per animal when compared to the human population. This makes them unsuitable for determining TCDD inducibility in correlation with tissues using conventional RT-PCR due to the high sample requirement for a statistically significant number of data points.

At the time this study was conducted, two new techniques that aid in gene expression/dose response experiments were still in their infancy. To date, real-time RT-PCR and Multiplex RT-PCR have evolved to the point that their potential use in this study's objectives should be evaluated. Real-time RT-PCR, originally developed by Haguchi et al (29) and since improved upon, utilizes fluorescent DNA binding dyes, such as Sybr Green (30), or gene specific probes, such as Taqman, based on the 5' nuclease assay by Holland et al (31), to evaluate the exponential phase of the PCR reaction real time. The incorporation of dye or probe during the annealing step allows for a direct correlation between increasing fluorescent signal and the increasing amount of PCR product. Dye incorporation specificity is

comparable to conventional RT-PCR that utilizes post-PCR staining techniques specific for double stranded DNA (32). Gene specific probes, such as Taqman, utilize 5' fluorescent probes with 3' dye quenchers that are specific to the gene template and sensitive to the amplification kinetics allowing for increased specificity (33). Quantitative real-time RT-PCR has successfully been used in detection of β-lymphocyte monoclonality (34), analysis of immune responses in peripheral blood (35) and for tissue-specific gene expression (36). It is high-throughput and less labor intensive (37) while offering a much wider dynamic range than conventional RT-PCR (38).

Multiplex real-time RT-PCR tries to utilize limited availability of biological sample by amplifying multiple specific targets simultaneously using multiple primer sets (39). In so doing, careful consideration needs to be paid to the choices of chemistries, primer sets and instrumentation to prevent decreased sensitivity and specificity (32). The success of a Multiplex real-time RT-PCR requires that fluorescent probes for each target do not interact (32), PCR master mix concentrations of $MgCl_2$, primers and dNTPs be carefully calculated so as not to inhibit extension (40)(41), and starting material be consistent for each target to optimize extension times and overall amplification (32). The use of Multiplex real-time RT-PCR might enable the quantification of CYP1B1, CYP1A1, AhR, Actin and other housekeeping genes for normalization reducing time and reagent

consumption while generating more data points with the same amount of stating material. The final thing to keep in mind is that current instrumentation may not be capable of detecting the needed number of fluorescent probes and may be the only limiting factor that can't be readily addressed.

In this study, the characteristic response to TCDD exposure was seen with increased expression of both CYP1B1 and CYP1A1 in the liver, lung, kidney, adrenal gland, lymphocyte and granulocyte tissues. The constitutive expression of CYP1B1 and CYP1A1 was higher in female than male rats. When looking at the different treatment groups, the acute dosing group showed a lower overall response to TCDD exposure than the chronic dosing group suggesting that the overall response to TCDD exposure takes longer to be seen. The 1B1 expression response to TCDD exposure in the male rat lymphocytes was almost 200-fold higher over constitutive expression for the chronic study compared to only 2-fold induction for the female rat.

This suggests that while the female rat has a higher basal rate of CYP 1B1 expression in peripheral blood lymphocytes, the male rat has a higher response to TCDD. The expression patterns seen and the low overall copy numbers of CYP 1B1 and 1A1 in peripheral blood lymphocytes and granulocytes of the Sprague Dawley rat indicate that, unlike the human population, peripheral blood lymphocytes and granulocytes are not a good surrogate of expression patterns for other tissues.

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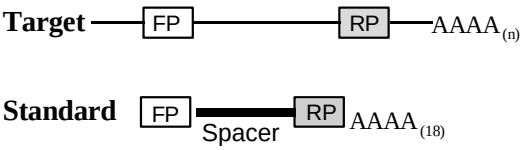
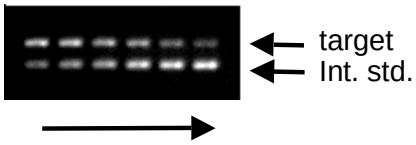
Figure 1a

RNA Quantitation by Competitive RT-PCR

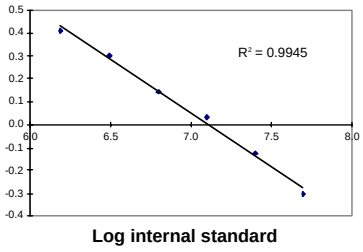


An internal standard consisting of a spacer sequence flanked by target RNA-specific forward (FP) and reverse (RP) primer sites and an (A)18 tail was constructed for each target RNA



Increasing input copies of Internal. standard RNA

350ng total RNA was reverse transcribed in the presence of increasing amounts of a homologous recombinant internal standard RNA. The resulting cDNAs were co-amplified by PCR, separated by agarose gel electrophoresis and stained with ethidium bromide.



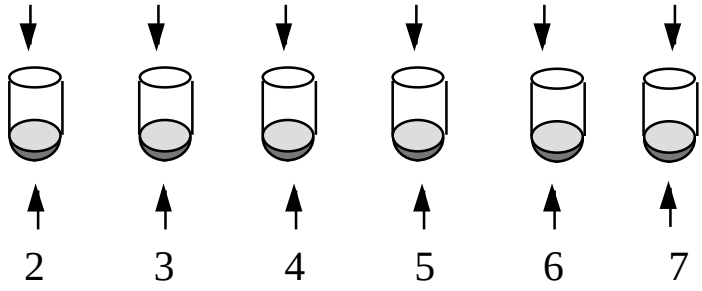
The log ratio of target: internal standard was plotted against the log copies of internal standard RNA. The x-axis intercept, ie where the ratio of target: standard band density is 1 corresponds to the antilog of target copy number.

ie target RNA copies =antilog 7.11 =$1.29Ex10^7$ per 100ng total RNA

Figure 1b

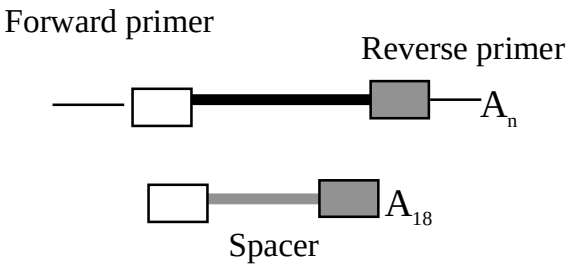
Competitive RT-PCR

Total RNA (350ng)



Internal standard RNA copies x 10^n

Target RNA



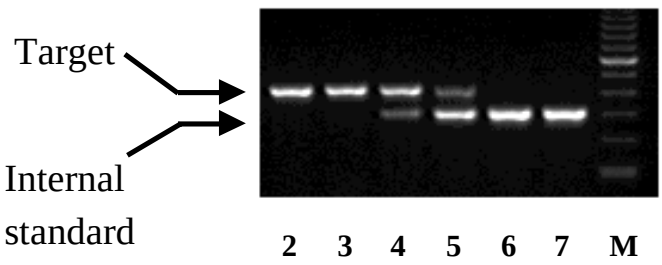
Internal standard RNA

Reverse Transcription

Add PCR Mix + Primers

PCR

Gel Electrophoresis



Quantitate band densities on computerized image

As the internal standard concentration increases, the ratio of PCR product formation of target:internal standard decreases. The concentration of target RNA lies at the crossover point. An exact copy number is determined using a 2x dilution series of internal standard concentrations that cover the crossover point.

Table 1 Control CYP1B1 Expression in 3 Day Study

Sex	Treatment	Tissue	Avg. CYP1B1 molecules	STDEV	Coeff. Var.
Male	3 Day Control	Lung	2.25E+02	1.50E+01	6.66%
Female	3 Day Control	Lung	5.12E+02	4.17E+01	8.14%
Male	3 Day Control	Kidney	6.54E+03	5.66E+01	0.86%
Female	3 Day Control	Kidney	6.79E+03	2.69E+02	3.96%
Male	3 Day Control	Adrenal	4.98E+05	2.06E+04	4.12%
Female	3 Day Control	Adrenal	5.27E+05	2.83E+04	5.37%
Male	3 Day Control	Liver	2.48E+02	6.24E+01	25.16%
Female	3 Day Control	Liver	7.12E+00	4.82E-01	6.77%
Male	3 Day Control	Lymphocytes	1.63E+01	1.06E+00	6.53%
Female	3 Day Control	Lymphocytes	3.60E+01	5.59E+00	15.54%
Male	3 Day Control	Pooled Gran.	2.88E+01	2.83E-01	0.98%
Female	3 Day Control	Pooled Gran.	3.00E+01	1.41E+00	4.71%
Male	Equilibrium Control	Lung	1.74E+02	6.24E+00	3.59%
Female	Equilibrium Control	Lung	1.76E+02	6.11E+00	3.47%
Male	Equilibrium Control	Kidney	6.40E+03	2.12E+02	3.31%
Female	Equilibrium Control	Kidney	6.74E+03	2.62E+02	3.88%
Male	Equilibrium Control	Adrenal	5.28E+05	1.03E+05	19.55%
Female	Equilibrium Control	Adrenal	5.31E+05	2.33E+04	4.40%
Male	Equilibrium Control	Liver	5.93E+00	5.86E-01	9.88%
Female	Equilibrium Control	Liver	7.72E+00	2.25E-01	2.91%
Male	Equilibrium Control	Lymphocytes	1.31E+00	4.95E-01	3.79%
Female	Equilibrium Control	Lymphocytes	3.21E+01	3.29E+00	10.25%
Male	Equilibrium Control	Pooled Gran.	1.84E+01	1.63E+00	8.86%
Female	Equilibrium Control	Pooled Gran.	2.28E+01	1.06E+00	4.66%

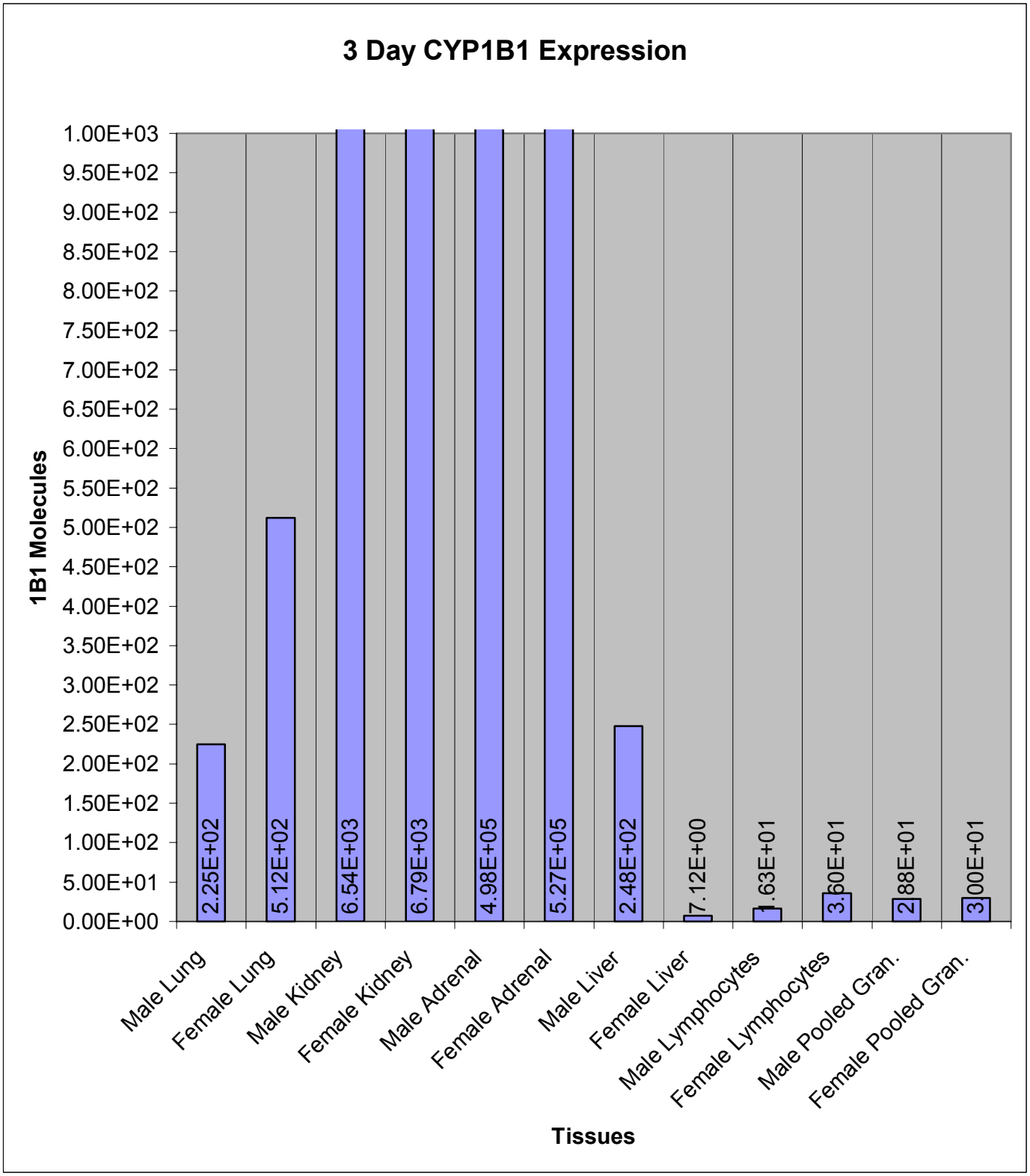
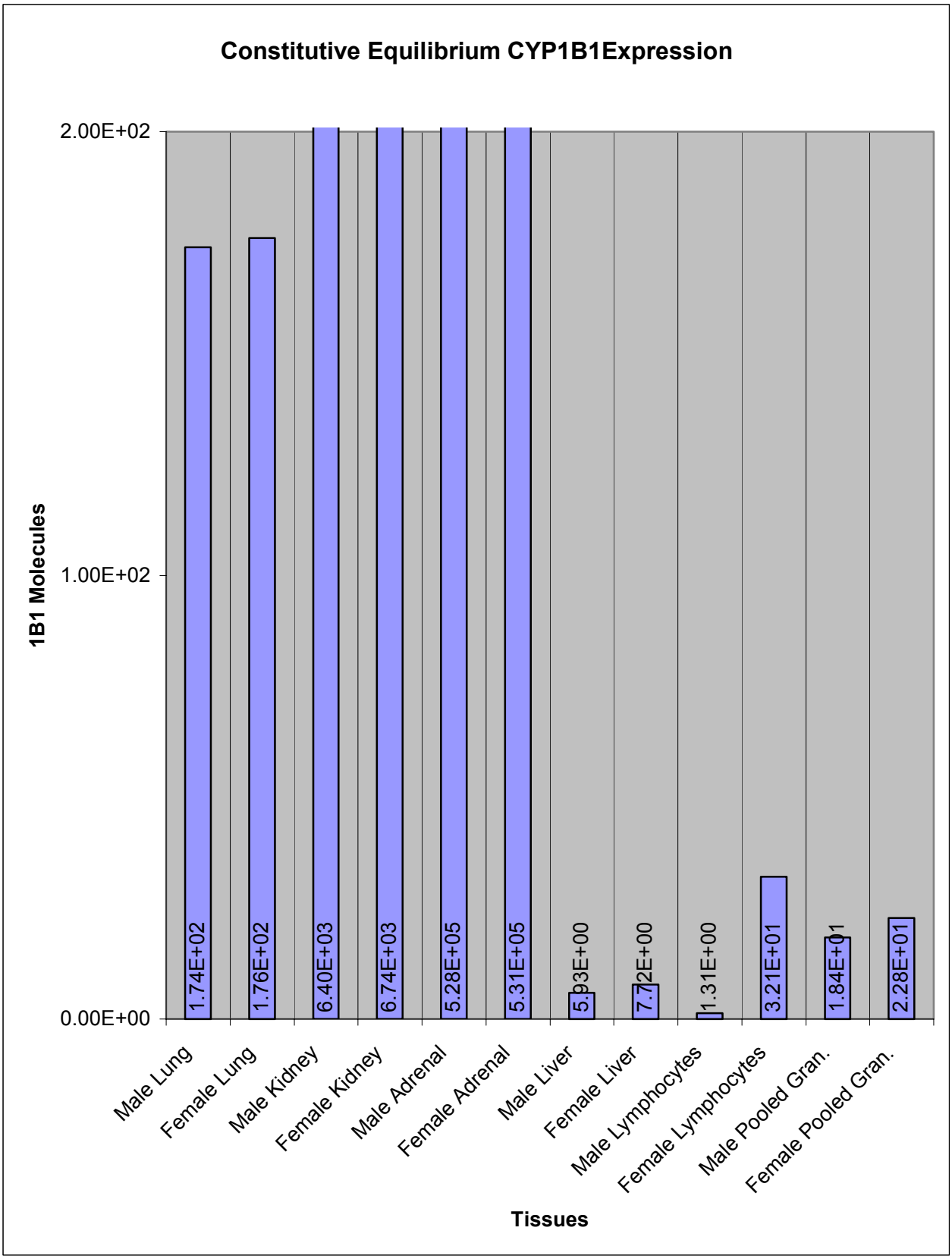

3 Day CYP1B1 Expression
1B1 Molecules
1.00E+03
9.50E+02
9.00E+02
8.50E+02
8.00E+02
7.50E+02
7.00E+02
6.50E+02
6.00E+02
5.50E+02
5.00E+02
4.50E+02
4.00E+02
3.50E+02
3.00E+02
2.50E+02
2.00E+02
1.50E+02
1.00E+02
5.00E+01
0.00E+00
2.25E+02
5.12E+02
6.54E+03
6.79E+03
4.98E+05
5.27E+05
2.48E+02
7.12E+00
1.63E+01
3.60E+01
2.88E+01
3.00E+01
Male Lung
Female Lung
Male Kidney
Female Kidney
Male Adrenal
Female Adrenal
Male Liver
Female Liver
Male Lymphocytes
Female Lymphocytes
Male Pooled Gran.
Female Pooled Gran.
Tissues

Figure 2

Table 2 Control CYP1A1 Expression in 3 Day Study

Sex	Treatment	Tissue	Avg. CYP1A1 molecules	STDEV	Coeff. Var.
Male	3 Day Control	Lung	1.95E+04	3.10E+03	15.95%
Female	3 Day Control	Lung	2.31E+04	2.59E+03	11.22%
Male	3 Day Control	Kidney	1.48E+05	3.21E+03	2.18%
Female	3 Day Control	Kidney	1.70E+05	1.33E+04	7.68%
Male	3 Day Control	Adrenal	1.46E+04	1.54E+03	10.54%
Female	3 Day Control	Adrenal	1.75E+04	1.40E+03	8.01%
Male	3 Day Control	Liver	3.23E+04	4.07E+03	12.61%
Female	3 Day Control	Liver	3.80E+04	3.82E+04	10.04%
Male	3 Day Control	Lymphocytes	1.17E+03	1.49E+02	12.77%
Female	3 Day Control	Lymphocytes	1.54E+03	2.26E+02	14.67%
Male	3 Day Control	Pooled Gran.	2.46E+02	3.27E+01	13.32%
Female	3 Day Control	Pooled Gran.	2.56E+02	2.76E+01	10.76%
Male	Equilibrium Control	Lung	5.19E+04	7.97E+03	15.34%
Female	Equilibrium Control	Lung	5.43E+05	2.08E+04	3.83%
Male	Equilibrium Control	Kidney	1.81E+05	2.54E+04	14.01%
Female	Equilibrium Control	Kidney	1.96E+05	1.51E+04	7.70%
Male	Equilibrium Control	Adrenal	1.99E+04	1.01E+03	5.05%
Female	Equilibrium Control	Adrenal	2.16E+04	2.16E+03	10.00%
Male	Equilibrium Control	Liver	1.47E+05	1.85E+04	12.59%
Female	Equilibrium Control	Liver	1.50E+06	1.96E+05	13.07%
Male	Equilibrium Control	Lymphocytes	1.32E+03	1.76E+02	13.33%
Female	Equilibrium Control	Lymphocytes	1.46E+03	1.06E+02	7.24%
Male	Equilibrium Control	Pooled Gran.	1.82E+04	3.11E+03	17.09%
Female	Equilibrium Control	Pooled Gran.	2.59E+05	8.49E+03	3.28%

Figure 3


Constitutive Equilibrium CYP1B1Expression
2.00E+02
1.00E+02
0.00E+00
1B1 Molecules
1.74E+02
1.76E+02
6.40E+03
6.74E+03
5.28E+05
5.31E+05
5.93E+00
7.72E+00
1.31E+00
3.21E+01
1.84E+01
2.28E+01
Male Lung
Female Lung
Male Kidney
Female Kidney
Male Adrenal
Female Adrenal
Male Liver
Female Liver
Male Lymphocytes
Female Lymphocytes
Male Pooled Gran.
Female Pooled Gran.
Tissues

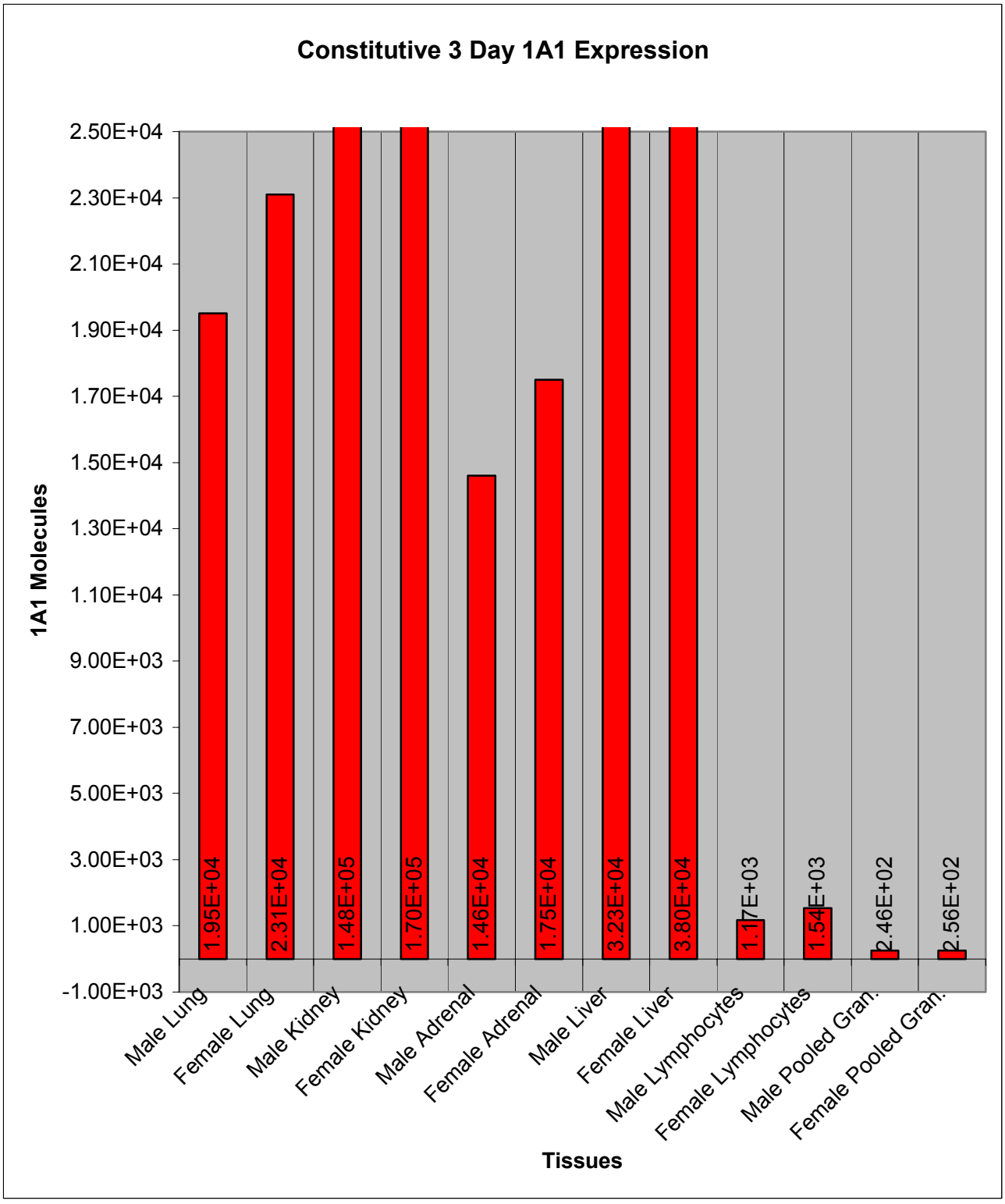

Constitutive 3 Day 1A1 Expression
1A1 Molecules
2.50E+04
2.30E+04
2.10E+04
1.90E+04
1.70E+04
1.50E+04
1.30E+04
1.10E+04
9.00E+03
7.00E+03
5.00E+03
3.00E+03
1.00E+03
-1.00E+03
1.95E+04
2.31E+04
1.48E+05
1.70E+05
1.46E+04
1.75E+04
3.23E+04
3.80E+04
1.17E+03
1.54E+03
2.46E+02
2.56E+02
Male Lung
Female Lung
Male Kidney
Female Kidney
Male Adrenal
Female Adrenal
Male Liver
Female Liver
Male Lymphocytes
Female Lymphocytes
Male Pooled Gran.
Female Pooled Gran.
Tissues

Figure 4

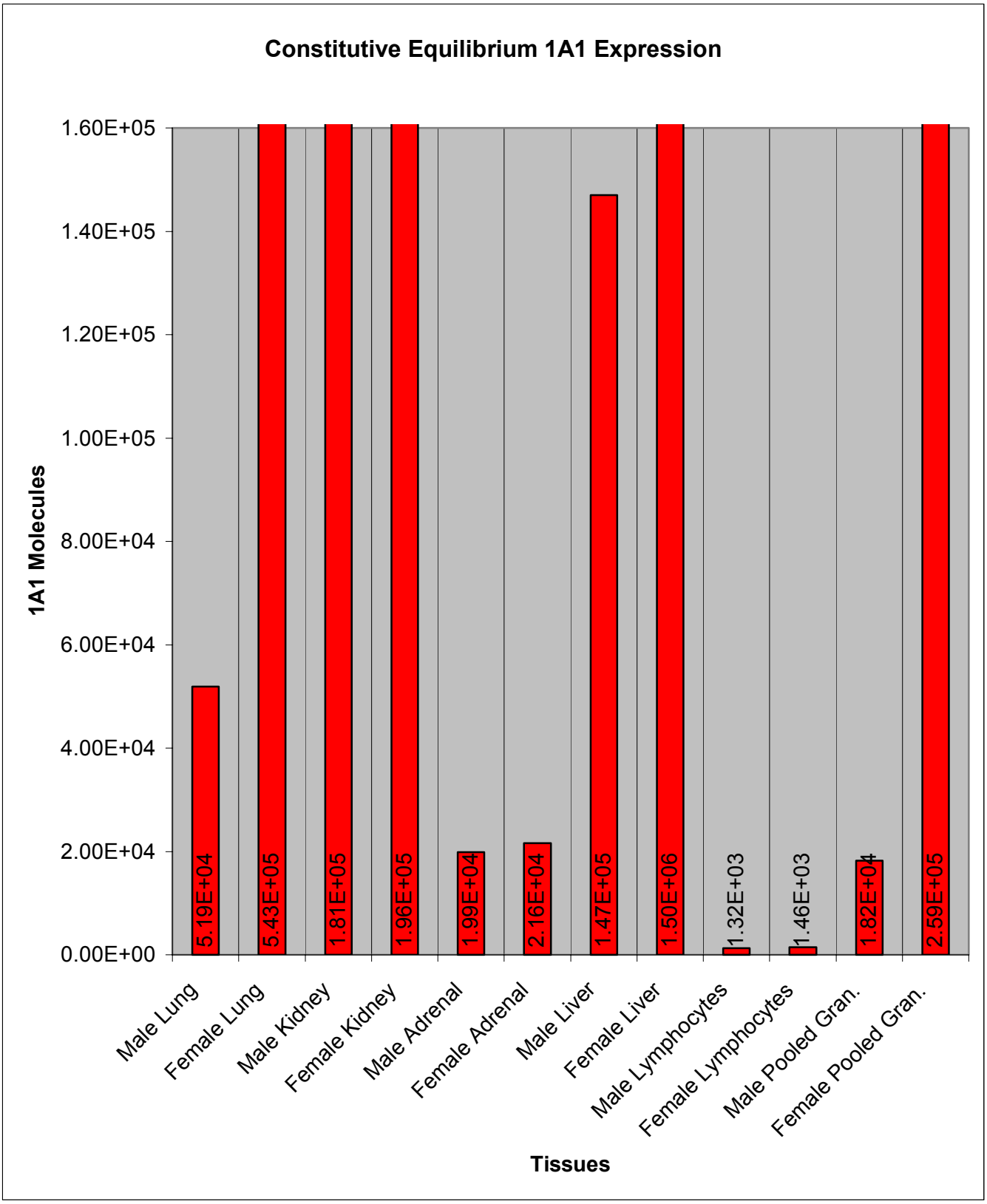

Constitutive Equilibrium 1A1 Expression
1A1 Molecules
1.60E+05
1.40E+05
1.20E+05
1.00E+05
8.00E+04
6.00E+04
4.00E+04
2.00E+04
0.00E+00
5.19E+04
5.43E+05
1.81E+05
1.96E+05
1.99E+04
2.16E+04
1.47E+05
1.50E+06
1.32E+03
1.46E+03
1.82E+04
2.59E+05
Male Lung
Female Lung
Male Kidney
Female Kidney
Male Adrenal
Female Adrenal
Male Liver
Female Liver
Male Lymphocytes
Female Lymphocytes
Male Pooled Gran.
Female Pooled Gran.
Tissues

Figure 5

Table 3 CYP1B1 Expression Levels for 3 Day Study

Sex	Treatment	Tissue	Avg. CYP1B1 molecules	STDEV	Coeff. Var.
Male	3 Day Control	Lung	2.36E+02	2.21E+01	9.36%
Female	3 Day Control	Lung	4.80E+02	6.18E+01	12.88%
Male	3 Day Control	Kidney	6.54E+03	5.66E+01	0.86%
Female	3 Day Control	Kidney	6.79E+03	2.69E+02	3.96%
Male	3 Day Control	Adrenal	4.98E+05	2.06E+04	4.12%
Female	3 Day Control	Adrenal	5.27E+05	2.83E+04	5.37%
Male	3 Day Control	Liver	2.48E+02	6.24E+01	25.16%
Female	3 Day Control	Liver	7.12E+00	4.82E-01	6.77%
Male	3 Day Control	Lymphocytes	1.63E+01	1.06E+00	6.53%
Female	3 Day Control	Lymphocytes	3.60E+01	5.59E+00	15.54%
Male	3 Day Control	Pooled Gran.	2.88E+01	2.83E-01	0.98%
Female	3 Day Control	Pooled Gran.	3.00E+01	1.41E+00	4.71%
Male	3 Day TCDD	Lung	2.46E+03	2.93E+02	11.91%
Female	3 Day TCDD	Lung	4.11E+03	2.89E+02	7.03%
Male	3 Day TCDD	Kidney	3.86E+04	1.27E+03	3.29%
Female	3 Day TCDD	Kidney	3.25E+04	2.72E+03	8.37%
Male	3 Day TCDD	Adrenal	7.36E+05	1.66E+04	2.26%
Female	3 Day TCDD	Adrenal	6.95E+05	2.32E+04	3.34%
Male	3 Day TCDD	Liver	4.25E+04	2.80E+03	6.59%
Female	3 Day TCDD	Liver	4.33E+04	2.15E+03	4.97%
Male	3 Day TCDD	Lymphocytes	4.04E+02	2.48E+01	6.14%
Female	3 Day TCDD	Lymphocytes	3.54E+02	2.80E+01	7.91%
Male	3 Day TCDD	Pooled Gran.	3.65E+01	1.16E+00	3.18%
Female	3 Day TCDD	Pooled Gran.	4.03E+01	1.68E+00	4.17%
Male	Equilibrium Control	Lung	1.74E+02	6.24E+00	3.59%
Female	Equilibrium Control	Lung	1.76E+02	6.11E+00	3.47%
Male	Equilibrium Control	Kidney	6.40E+03	2.12E+02	3.31%
Female	Equilibrium Control	Kidney	6.74E+03	2.62E+02	3.88%
Male	Equilibrium Control	Adrenal	5.28E+05	1.03E+05	19.55%
Female	Equilibrium Control	Adrenal	5.31E+05	2.33E+04	4.40%
Male	Equilibrium Control	Liver	5.93E+00	5.86E-01	9.88%
Female	Equilibrium Control	Liver	7.72E+00	2.25E-01	2.91%
Male	Equilibrium Control	Lymphocytes	1.31E+00	4.95E-01	3.79%
Female	Equilibrium Control	Lymphocytes	3.21E+01	3.29E+00	10.25%
Male	Equilibrium Control	Pooled Gran.	1.84E+01	1.63E+00	8.86%
Female	Equilibrium Control	Pooled Gran.	2.28E+01	1.06E+00	4.66%
Male	Equilibrium TCDD	Lung	2.34E+01	2.92E+00	12.48%
Female	Equilibrium TCDD	Lung	1.70E+01	1.53E+00	9.00%
Male	Equilibrium TCDD	Kidney	5.29E+04	3.10E+03	5.86%
Female	Equilibrium TCDD	Kidney	4.68E+04	2.91E+03	6.22%
Male	Equilibrium TCDD	Adrenal	8.08E+05	1.42E+04	1.76%
Female	Equilibrium TCDD	Adrenal	7.15E+05	2.84E+04	3.97%
Male	Equilibrium TCDD	Liver	2.83E+01	3.82E+00	13.50%
Female	Equilibrium TCDD	Liver	2.40E+01	9.98E-01	4.16%
Male	Equilibrium TCDD	Lymphocytes	2.44E+02	2.87E+01	11.76%
Female	Equilibrium TCDD	Lymphocytes	6.07E+01	8.49E+00	13.99%
Male	Equilibrium TCDD	Pooled Gran.	2.77E+02	2.25E+01	8.12%
Female	Equilibrium TCDD	Pooled Gran.	4.25E+02	2.93E+01	6.89%

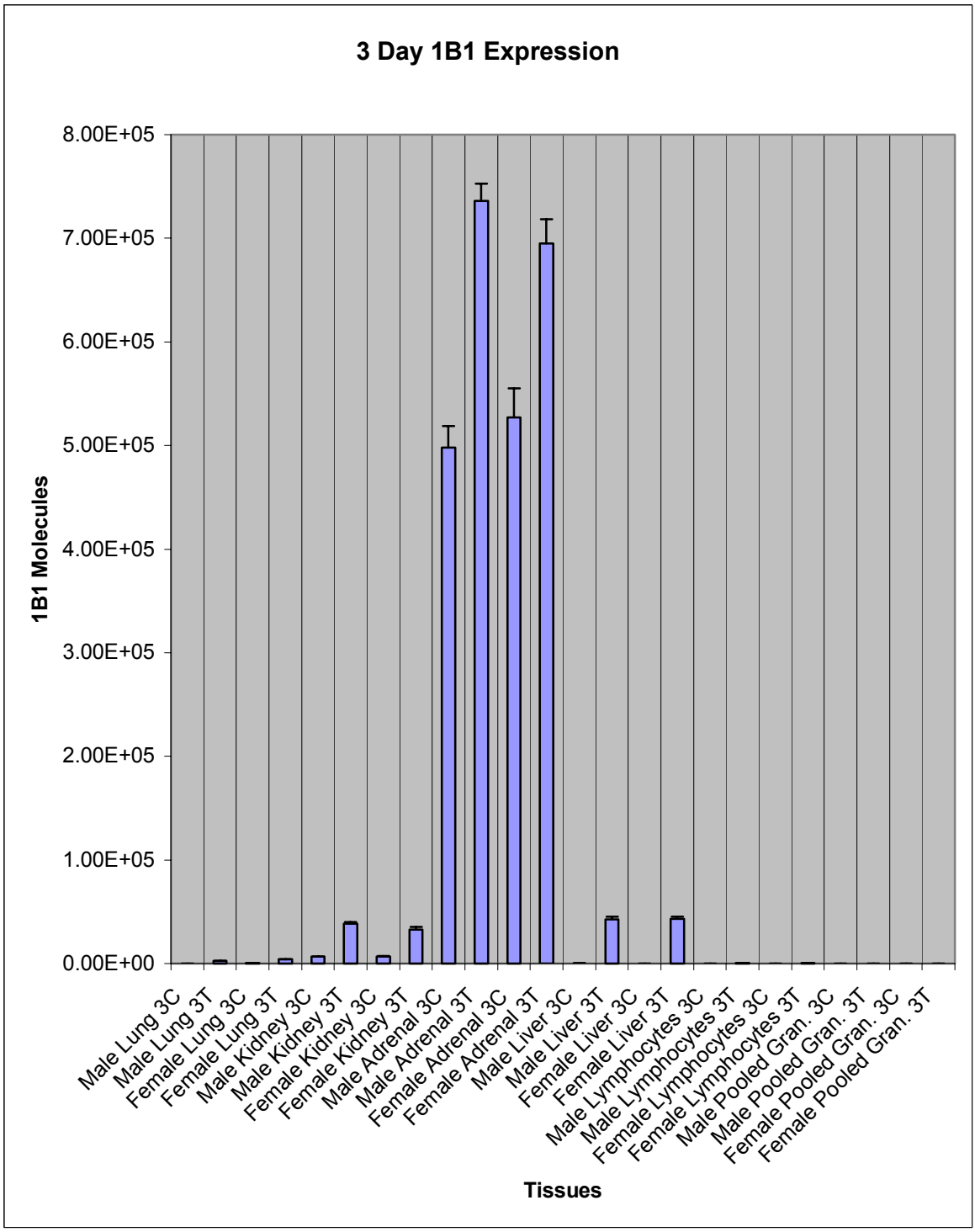


Figure 6

Figure 7

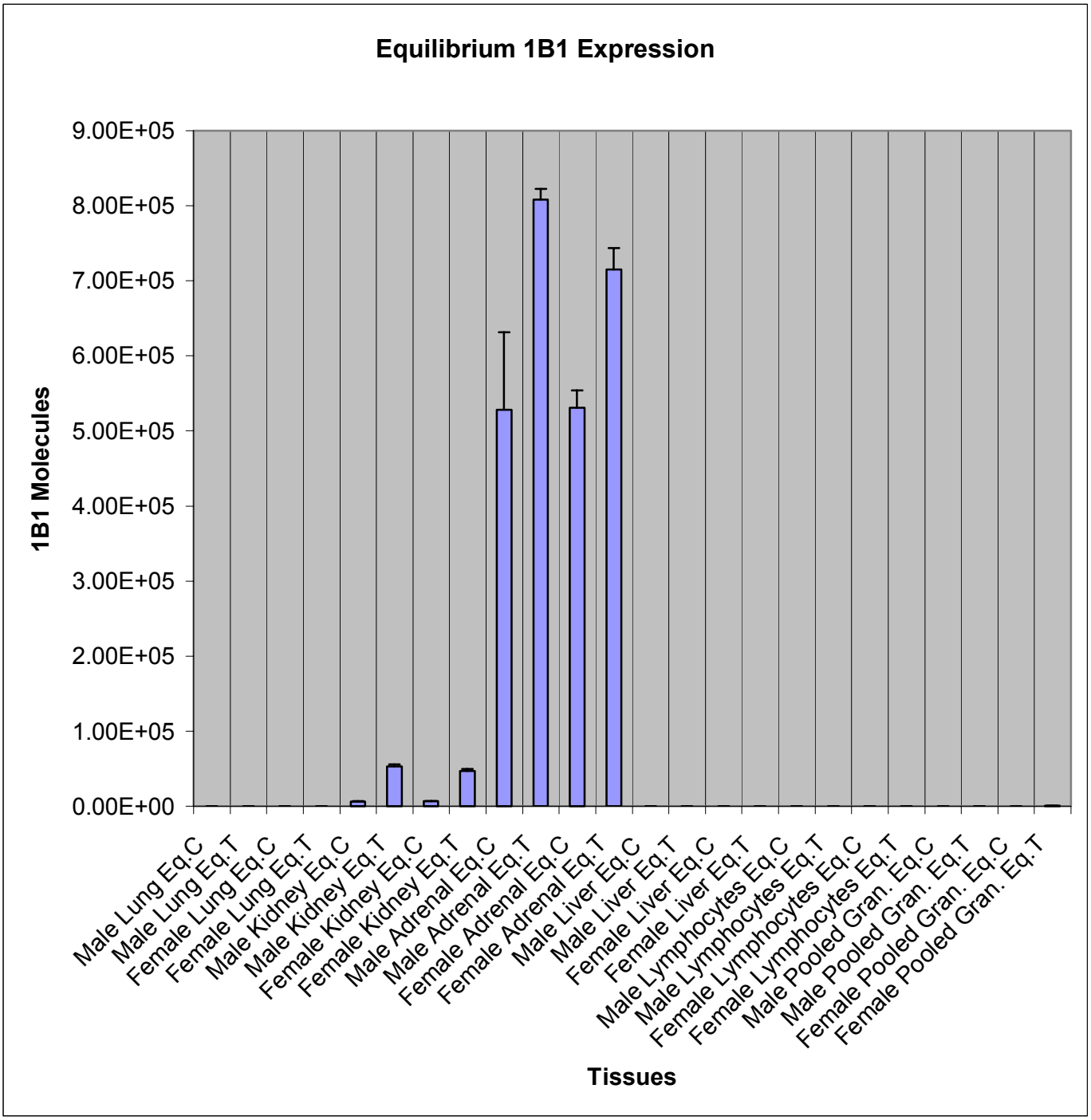


Table 4 CYP1A1 Expression 3 Day Study

Sex	Treatment	Tissue	Avg. CYP1A1 molecules	STDEV	Coeff. Var.
Male	3 Day Control	Lung	1.95E+04	3.10E+03	15.95%
Female	3 Day Control	Lung	2.31E+04	2.59E+03	11.22%
Male	3 Day Control	Kidney	1.48E+05	3.21E+03	2.18%
Female	3 Day Control	Kidney	1.70E+05	1.33E+04	7.68%
Male	3 Day Control	Adrenal	1.46E+04	1.54E+03	10.54%
Female	3 Day Control	Adrenal	1.75E+04	1.40E+03	8.01%
Male	3 Day Control	Liver	3.23E+04	4.07E+03	12.61%
Female	3 Day Control	Liver	3.80E+04	3.82E+04	10.04%
Male	3 Day Control	Lymphocytes	1.17E+03	1.49E+02	12.77%
Female	3 Day Control	Lymphocytes	1.54E+03	2.26E+02	14.67%
Male	3 Day Control	Pooled Gran.	2.46E+02	3.27E+01	13.32%
Female	3 Day Control	Pooled Gran.	2.56E+02	2.76E+01	10.76%
Male	3 Day TCDD	Lung	2.43E+07	3.21E+06	13.21%
Female	3 Day TCDD	Lung	2.81E+07	4.29E+06	15.27%
Male	3 Day TCDD	Kidney	7.19E+06	2.90E+05	4.03%
Female	3 Day TCDD	Kidney	6.95E+06	2.19E+05	3.15%
Male	3 Day TCDD	Adrenal	4.34E+04	2.62E+03	6.04%
Female	3 Day TCDD	Adrenal	4.70E+04	5.29E+03	11.26%
Male	3 Day TCDD	Liver	4.03E+05	3.06E+04	7.59%
Female	3 Day TCDD	Liver	2.87E+05	3.79E+04	13.21%
Male	3 Day TCDD	Lymphocytes	3.42E+04	3.49E+03	10.20%
Female	3 Day TCDD	Lymphocytes	2.87E+04	3.79E+03	13.21%
Male	3 Day TCDD	Pooled Gran.	2.34E+03	3.12E+02	13.33%
Female	3 Day TCDD	Pooled Gran.	2.31E+03	2.91E+02	12.60%
Male	Equilibrium Control	Lung	5.19E+04	7.97E+03	15.34%
Female	Equilibrium Control	Lung	5.43E+05	2.08E+04	3.83%
Male	Equilibrium Control	Kidney	1.81E+05	2.54E+04	14.01%
Female	Equilibrium Control	Kidney	1.96E+05	1.51E+04	7.70%
Male	Equilibrium Control	Adrenal	1.99E+04	1.01E+03	5.05%
Female	Equilibrium Control	Adrenal	2.16E+04	2.16E+03	10.00%
Male	Equilibrium Control	Liver	1.47E+05	1.85E+04	12.59%
Female	Equilibrium Control	Liver	1.50E+06	1.96E+05	13.07%
Male	Equilibrium Control	Lymphocytes	1.32E+03	1.76E+02	13.33%
Female	Equilibrium Control	Lymphocytes	1.46E+03	1.06E+02	7.24%
Male	Equilibrium Control	Pooled Gran.	1.82E+04	3.11E+03	17.09%
Female	Equilibrium Control	Pooled Gran.	2.59E+05	8.49E+03	3.28%
Male	Equilibrium TCDD	Lung	3.16E+06	4.56E+05	14.43%
Female	Equilibrium TCDD	Lung	2.89E+06	1.91E+05	6.61%
Male	Equilibrium TCDD	Kidney	3.30E+06	4.58E+05	13.88%
Female	Equilibrium TCDD	Kidney	2.44E+06	2.50E+05	10.25%
Male	Equilibrium TCDD	Adrenal	5.35E+04	3.10E+03	5.79%
Female	Equilibrium TCDD	Adrenal	4.77E+04	2.08E+03	4.36%
Male	Equilibrium TCDD	Liver	1.31E+08	1.76E+07	13.44%
Female	Equilibrium TCDD	Liver	6.41E+07	7.82E+06	12.20%
Male	Equilibrium TCDD	Lymphocytes	5.10E+04	1.99E+03	3.90%
Female	Equilibrium TCDD	Lymphocytes	4.90E+04	2.65E+03	5.41%
Male	Equilibrium TCDD	Pooled Gran.	1.91E+03	1.18E+03	6.17%
Female	Equilibrium TCDD	Pooled Gran.	2.18E+03	2.86E+02	13.12%

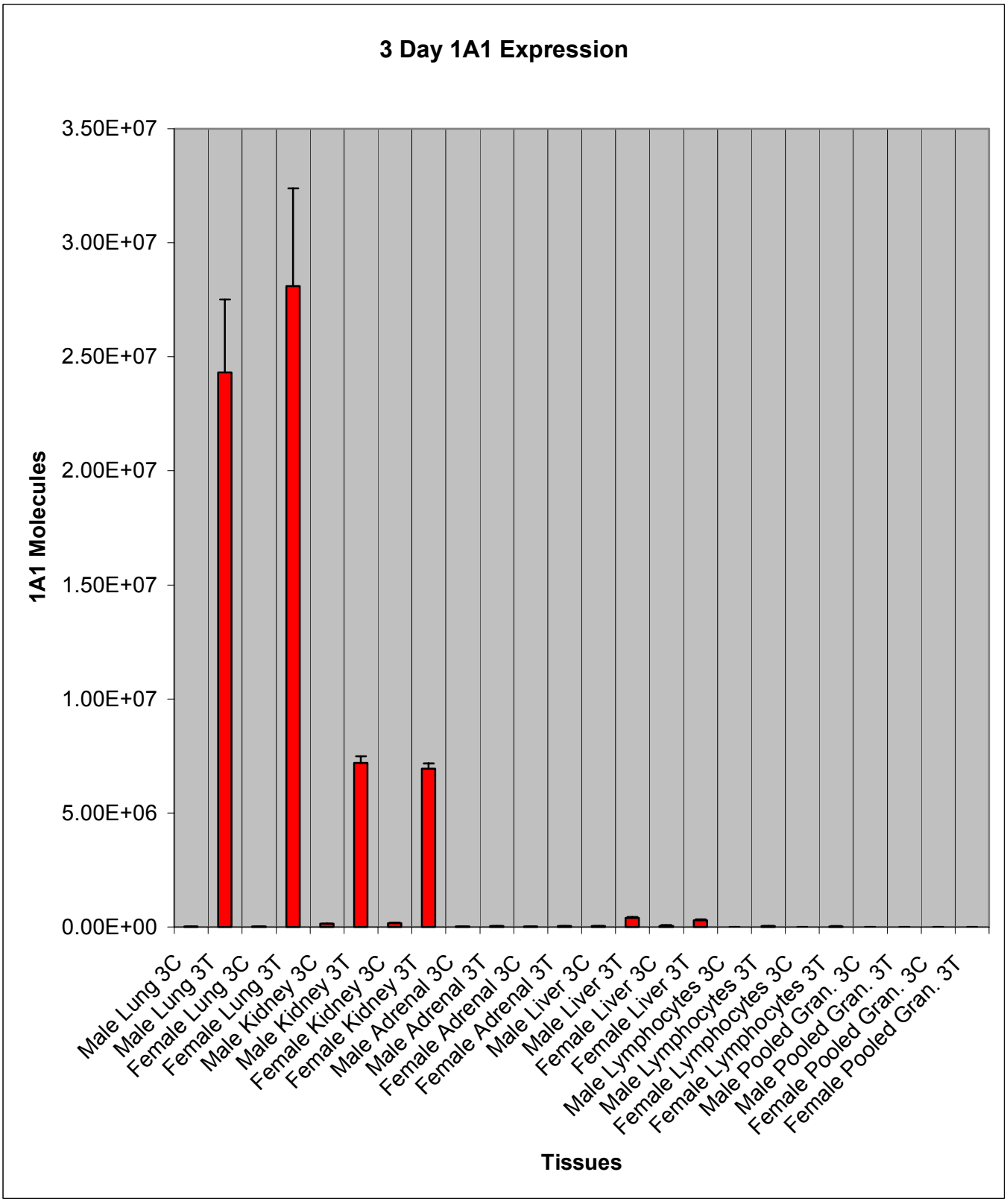

3 Day 1A1 Expression
3.50E+07
3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
0.00E+00
1A1 Molecules
Male Lung 3C
Male Lung 3T
Female Lung 3C
Female Lung 3T
Male Kidney 3C
Male Kidney 3T
Female Kidney 3C
Female Kidney 3T
Male Adrenal 3C
Male Adrenal 3T
Female Adrenal 3C
Female Adrenal 3T
Male Liver 3C
Male Liver 3T
Female Liver 3C
Female Liver 3T
Male Lymphocytes 3C
Male Lymphocytes 3T
Female Lymphocytes 3C
Female Lymphocytes 3T
Male Pooled Gran. 3C
Male Pooled Gran. 3T
Female Pooled Gran. 3C
Female Pooled Gran. 3T
Tissues

Figure 8

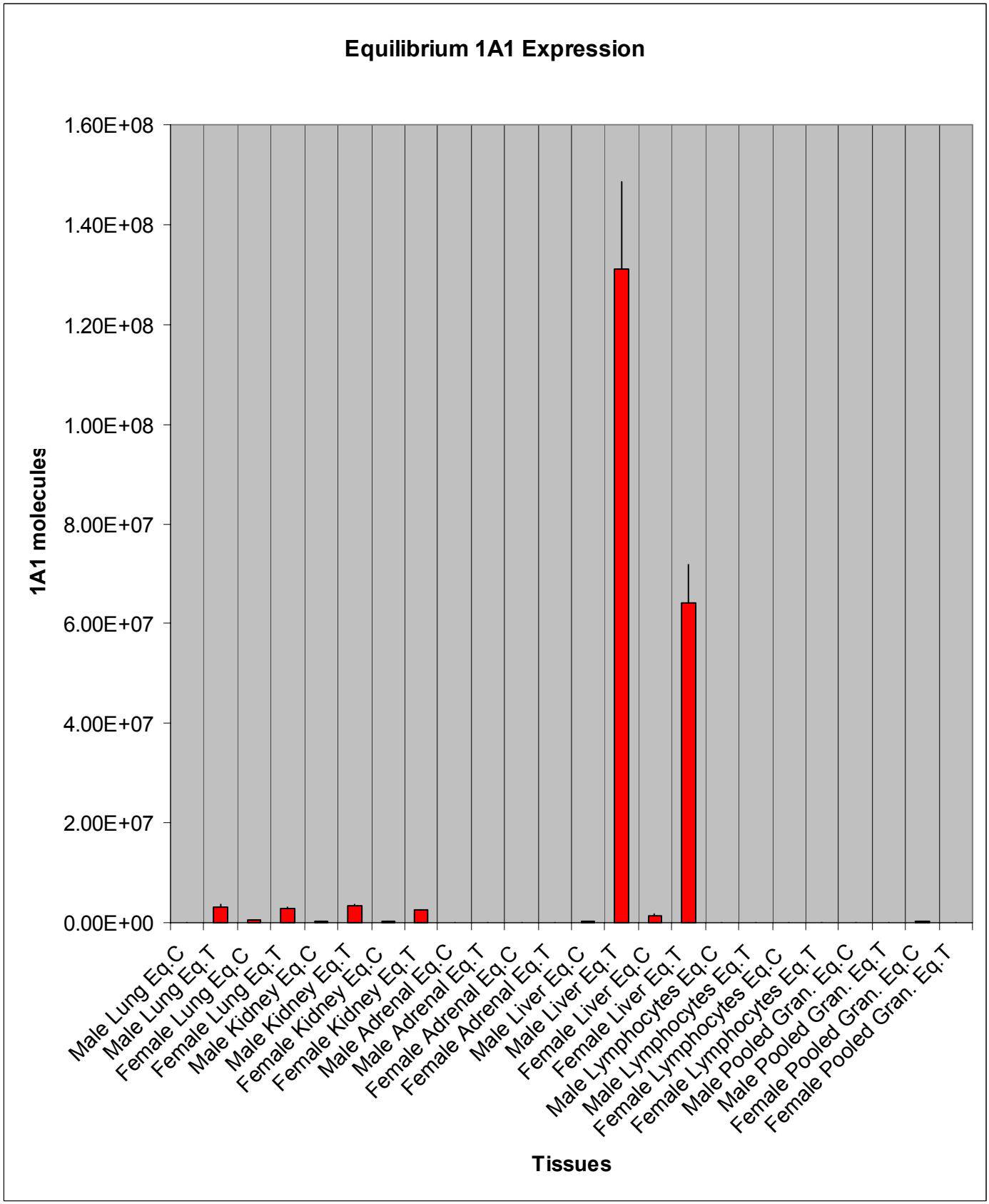

Equilibrium 1A1 Expression
1A1 molecules
1.60E+08
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
Male Lung Eq.C
Male Lung Eq.T
Female Lung Eq.C
Female Lung Eq.T
Male Kidney Eq.C
Male Kidney Eq.T
Female Kidney Eq.C
Female Kidney Eq.T
Male Adrenal Eq.C
Male Adrenal Eq.T
Female Adrenal Eq.C
Female Adrenal Eq.T
Male Liver Eq.C
Male Liver Eq.T
Female Liver Eq.C
Female Liver Eq.T
Male Lymphocytes Eq.C
Male Lymphocytes Eq.T
Female Lymphocytes Eq.C
Female Lymphocytes Eq.T
Male Pooled Gran. Eq.C
Male Pooled Gran. Eq.T
Female Pooled Gran. Eq.C
Female Pooled Gran. Eq.T
Tissues

Figure 9

Figure 10

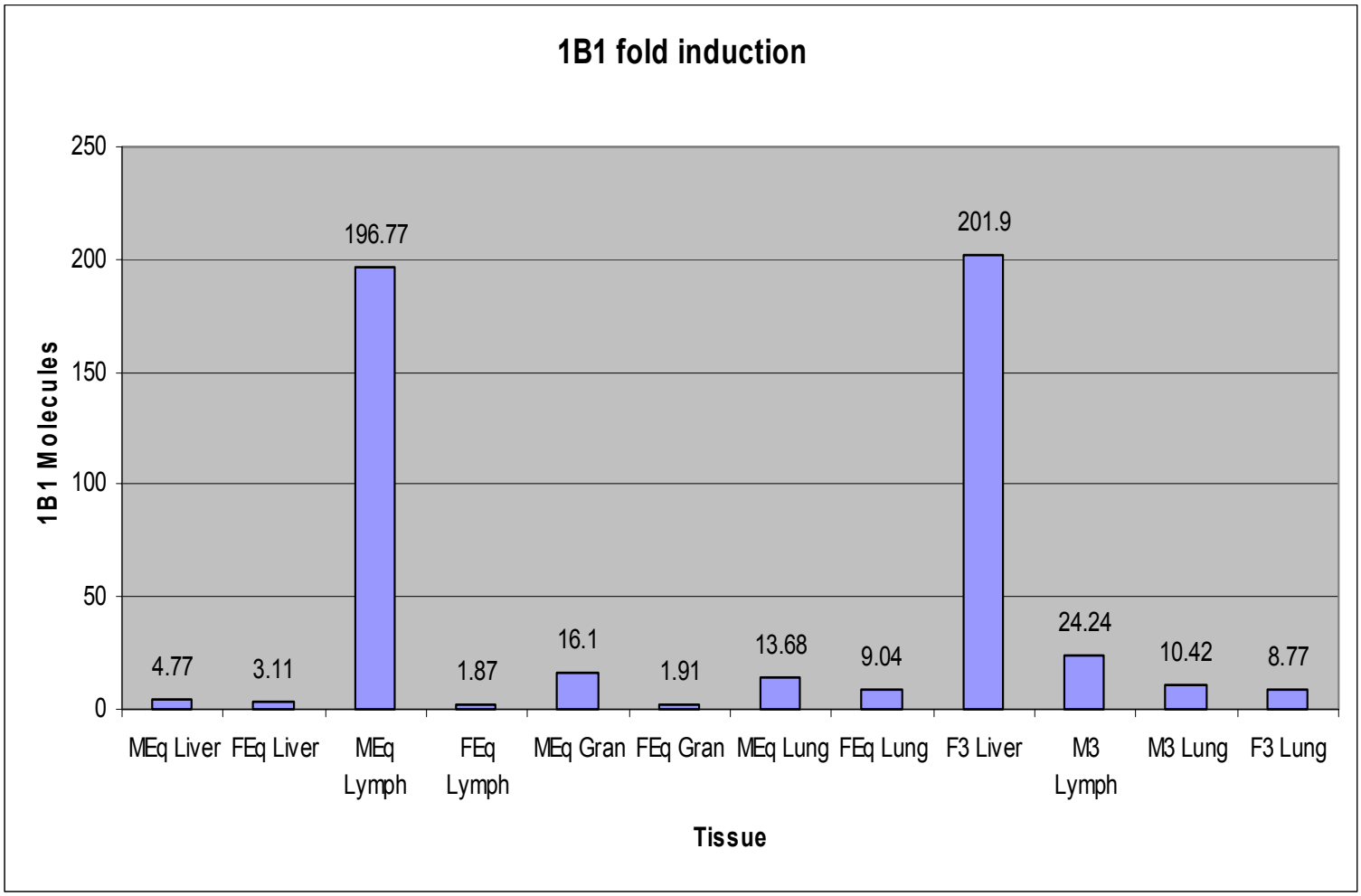
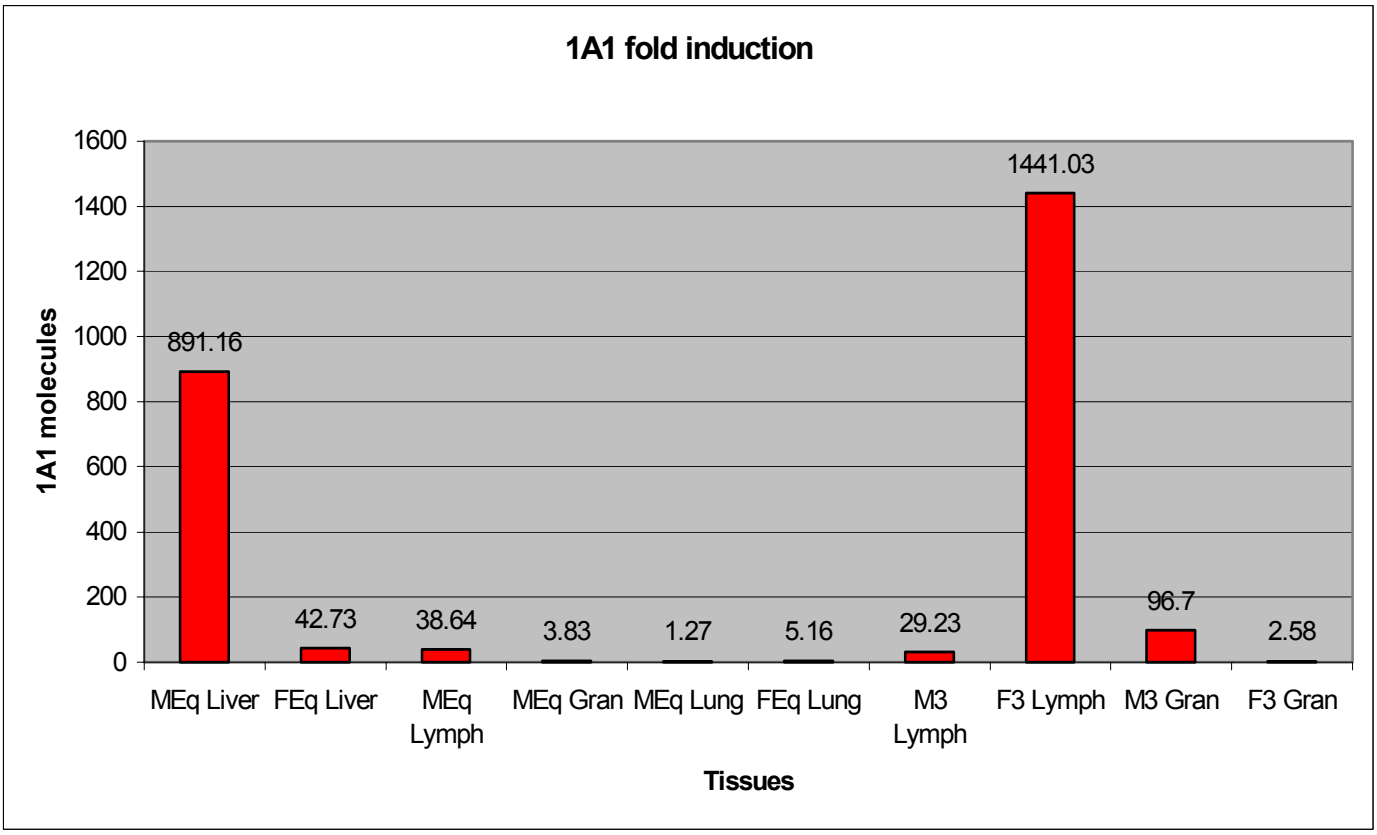

1B1 fold induction
1B1 Molecules
250
200
150
100
50
0
4.77
3.11
196.77
1.87
16.1
1.91
13.68
9.04
201.9
24.24
10.42
8.77
MEq Liver
FEq Liver
MEq Lymph
FEq Lymph
MEq Gran
FEq Gran
MEq Lung
FEq Lung
F3 Liver
M3 Lymph
M3 Lung
F3 Lung
Tissue

Figure 11


1A1 fold induction
1A1 molecules
1600
1400
1200
1000
800
600
400
200
0
891.16
42.73
38.64
3.83
1.27
5.16
29.23
1441.03
96.7
2.58
MEq Liver
FEq Liver
MEq Lymph
MEq Gran
MEq Lung
FEq Lung
M3 Lymph
F3 Lymph
M3 Gran
F3 Gran
Tissues