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toolbox

Development and testing of a high-density cDNA microarray resource for cattle

Steven P. Suchyta,[1,2] Sue Sipkovsky,[2] Rachael Kruska,[1,2] Abra Jeffers,[1,2] Amanda McNulty,[1,2] Matthew J. Coussens,[1,2] Robert J. Tempelman,[1,2] Robert G. Halgren,[3] Peter M. Saama,[1,2] Dale E. Bauman,[4] Yves R. Boisclair,[4] Jeanne L. Burton,[1,2] Robert J. Collier,[5] Edward J. DePeters,[6] Theodore A. Ferris,[1,2] Matthew C. Lucy,[7] Mark A. McGuire,[8] Juan F. Medrano,[6] Thomas R. Overton,[4] Timothy P. Smith,[9] George W. Smith,[1,2] Tad S. Sonstegard,[10] James N. Spain,[7] Donald E. Spiers,[7] Jianbo Yao,[1,2] and Paul M. Coussens[1,2]

[1]*Department of Animal Science,* [2]*Center for Animal Functional Genomics,* [3]*Genomics Technology Support Facility, Michigan State University, East Lansing, Michigan 48824;* [4]*Department of Animal Science, Cornell University, Ithaca, New York 14853;* [5]*Department of Animal Sciences, University of Arizona, Tucson, Arizona 85721;* [6]*Department of Animal Science, University of California, Davis, California 95616;* [7]*Department of Animal Sciences, University of Missouri, Columbia, Missouri 65211;* [8] *Department of Animal and Veterinary Science, University of Idaho, Moscow, Idaho 83844;* [9]*United States Department of Agriculture, Agricultural Research Service, United States Meat Animal Research Center, Clay Center, Nebraska 68933; and* [10]*United States Department of Agriculture, Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, Maryland 20705*

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Suchyta, Steven P., Sue Sipkovsky, Rachael Kruska, Abra Jeffers, Amanda McNulty, Matthew J. Coussens, Robert J. Tempelman, Robert G. Halgren, Peter M. Saama, Dale E. Bauman, Yves R. Boisclair, Jeanne L. Burton, Robert J. Collier, Edward J. DePeters, Theodore A. Ferris, Matthew C. Lucy, Mark A. McGuire, Juan F. Medrano, Thomas R. Overton, Timothy P. Smith, George W. Smith, Tad S. Sonstegard, James N. Spain, Donald E. Spiers, Jianbo Yao, and Paul M. Coussens. Development and testing of a high-density cDNA microarray resource for cattle. *Physiol Genomics* 15: 158–164, 2003. First published September 16, 2003; 10.1152/physiolgenomics.00094.2003.—A cDNA microarray resource has been developed with the goal of providing integrated functional genomics resources for cattle. The National Bovine Functional Genomics Consortium's (NBFGC) expressed sequence tag (EST) collection was established in 2001 to develop resources for functional genomics research. The NBFGC EST collection and microarray contains 18,263 unique transcripts, derived from many different tissue types and various physiologically important states within these tissues. The NBFGC microarray has been tested for false-positive rates using self-self hybridizations and was shown to yield robust results in test microarray experiments. A web-accessible database has been established to provide pertinent data related to NBFGC clones, including sequence data, BLAST results, and ontology information. The NBFGC microarray represents the largest cDNA microarray for a livestock species prepared to date and should prove to be a valuable tool in studying genome-wide gene expression in cattle.

expressed sequence tag; bovine

MICROARRAYS HAVE PROVED to be valuable as an exploratory method for understanding biological pathways important in physiological processes. These resources have been used successfully in a myriad of research areas but primarily limited to rodent and human systems. Thus far, there has been a lack of publicly available microarray resources specifically for livestock species. The use of high-density microarray resources can be of particular benefit to livestock animals due to the variety of complex factors in these species that form the basis of disease states and production traits. To date, there are only two publicly available bovine cDNA microarrays (2, 10). Studies are beginning to show the usefulness of these resources in elucidating pathways important in bovine diseases (3, 4). Clearly, though there is a need for additional functional genomics resources, as currently available microarrays are limited in gene number and variety of tissue types used to generate the original cDNA libraries.

To properly address application of expressed sequence tag (EST) collections and cDNA microarrays to problems in cattle, a diverse group of scientists, representing several major animal agriculture research institutions in North America, collaborated to form the National Bovine Functional Genomics Consortium (NBFGC). The main objective of the NBFGC is to develop integrated functional genomics resources for cattle including high-density cDNA microarrays and web-accessible EST information. To meet this objective, the Center for Animal Functional Genomics (CAFG) at Michigan State University, in collaboration with the NBFGC has developed an EST collection and cDNA microarray containing >18,000 unique transcripts.

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Address for reprint requests and other correspondence: S. P. Suchyta, B215 Anthony Hall, Center for Animal Functional Genomics, Michigan State Univ., East Lansing, MI 48824 (E-mail: suchytas@msu.edu).

The cDNA clones used to form the NBFGC microarray were generated from more than 10 different tissue types, in many cases, different physiological or disease states represented within tissues collected (5, 6). The NBFGC cDNA microarray contains more than four times the number of transcripts of any currently available bovine microarray. Information for each NBFGC clone, including sequence data, BLAST results, and ontology information, is integrated into a publicly available database, which can be accessed via the NBFGC web site (**http://nbfgc.msu.edu**). The NBFGC microarray is a fully integrated functional genomics resource, which should be a great asset for assessing genome-wide gene expression changes in cattle.

MATERIALS AND METHODS

Selection of EST clones. The United States Department of Agriculture (USDA) Meat Animal Research Center (MARC) and the USDA Agricultural Research Service (ARS) Beltsville Agricultural Research Center (BARC) have previously constructed five cDNA libraries isolated from a wide variety of bovine tissue types (5, 6) (Table 1). All ESTs in the MARC and BARC libraries are represented in The Institute for Genomic Research (TIGR) Cattle Gene Index (BtGI), and are ordered according to sequence overlap in clusters (TIGR Clusters, TC). The TIGR BtGI (release 5.0) contained a total of 20,209 TCs, of which 18,263 contained at least one MARC or BARC clone. The MARC or BARC clone with the longest sequence read length was selected to represent each cluster through examination of the nucleotide sequence given in each clone's GenBank entry. Several Perl (v. 5.6.1) scripts were written to facilitate clone selection, and are available upon request. Selected MARC or BARC clones (18,263) were used to form the NBFGC bovine EST clone set.

Table 1. *Physiological and/or disease state of the bovine tissues in the MARC and BARC cDNA libraries used in selection of EST clones for the NBFGC EST collection and microarray*

Tissue	Physiological and/or Disease State
Lymph node	Mesenteric and hilar, heifer
Ovary	Post- and prepubertal
Fat	Kidney-associated and subcutaneous, heifer
Muscle	Semitendenosous and longissimus, fetal and 20-day-old calf
Hypothalamus	Heifer
Pituitary	20-day-old calf
Testis	48-h-old calf
Thymus	20-day-old calf
Adrenal	20-day-old calf
Pancreas	20-day-old calf
Endometrium	Pregnant cow
Bone marrow	20-day-old calf
Macrophage	Alveolar, heifer
Embryo	*Day 20* and *day 40*
Mammary fat pad	Prepubertal, with and without estradial treatment
Mammary epithelium	Prepubertal, with and without estradial treatment
Mammary gland	Mid and late gestation, lactating (with and without mastitis), 8 and 30 days postlactation

MARC, USDA Meat Animal Research Center, Clay Center Nebraska; BARC, USDA Beltsville Animal Research Center, Beltsville, Maryland; NBFGC, National Bovine Functional Genomics Consortium; EST, expressed sequence tag.

Ontology assignment of NBFGC EST clone set. Ontology information was generated for clones in the NBFGC EST set using a combination of ontology information derived from TIGR BtGI Release 7.0 and information generated using GeneSpring software (v. 4.1.5; Silicon Genetics, Redwood City, CA) and the "build simplified ontology" function. Sequence and ontology information from the BtGI were retrieved, and SAS (SAS Institute, Cary, IN) routines were developed to extract and classify the ontology information for each TC. There were 6,367 TCs (~20%) in the BtGI that had ontology information. A BLASTN analysis was then performed to match clones in the NBFGC EST collection with BtGI. We found that 2,799 NBFGC ESTs matched TCs with ontology information in the current TIGR BtGI. Clones in the NBFGC EST collection were also functionally classified using the GeneSpring (v. 4.1.5, Silicon Genetics) "build simplified ontology" function, which is based on the Gene Ontology Consortium classifications (1). Ontology classification assignment for each clone is based on the description line of the top BLASTX hit if available, otherwise it is based on the top BLASTN hit corresponding to functional gene ontology. We found that 2,140 NBFGC ESTs had ontology information from both sources when the ontology data was combined. For each of these 2,140 clones, the gene ontology source with more information (based on word count) was used. For example, if the existing ontology term was "peroxisome" and the term from the BtGI was "peroxisome targeting signal-1 receptor" then the latter would be used. UNIX *awk* scripts and SAS tools were used to process and insert gene ontology information for the 2,799 NBFGC clones into the NBFGC EST database. All UNIX *awk* and SAS programs are available upon request.

DNA sequencing and sequence analysis. To make the NBFGC EST clone set, 1 μl of liquid culture from each selected MARC or BARC clone was transferred into wells of 96-well plates that contained 150 μl luria broth (LB) plus 8% wt/vol glycerol and 100 μg/ml ampicillin using a Tecan Genosys 150 liquid handling robot (Tecan US, Research Triangle Park, NC). Plates were covered with foil and grown overnight at 37°C with constant shaking at 225 rpm. Replicates of the NBFGC library were made from the overnight cultures and stored at −80°C.

A random selection of 384 NBFGC clones and a random selection of 384 final insert amplicons used to construct the NBFGC cDNA microarray (as described below) were used for sequence verification via a commercial sequencing company (Genome Therapeutics, Waltham MA). NBFGC clones were sequenced using the SP6 promoter primer, and final insert amplicons were sequenced with the primer (5′ AATTCCCGGGATATCGTCGAC 3′), which is the forward primer used for amplification of NBFGC clone inserts. Vector and/or quality trimmed sequence data from selected NBFGC clones were compared with the original MARC and BARC EST clone sets via BLASTN. We found that 97% of NBFGC clones and 94% of final insert amplicons sent for sequencing matched with the original MARC or BARC clone sequence.

Production of cDNA microarrays. NBFGC plasmids were amplified from 1 μl of bacterial culture using the TempliPhi DNA Sequencing Template Amplification Kit (Amersham Biosciences, Piscataway, NJ) essentially as recommended by the manufacturer's protocol. NBFGC clone inserts were PCR amplified by adding 1 μl of the plasmid amplification reaction directly into PCR master mix [20 μM each dNTP, 0.5 μM forward primer, 0.5 μM reverse primer, 200 mM Tris-HCl

(pH 8.4), 500 mM KCl, and 2.0 units of *Taq* DNA polymerase] in 96-well plates. The forward primer (5′ AATTCCCGGGATATCGTCGAC 3′) and reverse primer (5′ TCGAGGGATACTCTAGAGC 3′) are immediately adjacent to the insertion site for cloning vector pSPORT 6 used for construction of the original BARC and MARC EST libraries (5, 6). Following a preheat step of 94°C for 2 min, PCR was performed in a PE 9700 Thermocycler (PerkinElmer, Palo Alto, CA) using the following conditions: 94°C for 30 s, 60°C for 1 min, and 72°C for 3 min; for 30 cycles. Based on agarose gel electrophoresis of purified amplicons (E-Gel 96; Invitrogen, Carlsbad, CA), our success rate with this protocol was >90%. Clones that did not amplify were taken through the TempliPhi amplification (Amersham Biosciences) and PCR amplification steps again, which substantially increased the overall success rate (>98%). Insert amplicons were purified using a Millipore Multiscreen PCR 96-well purification system (Millipore, Bedford, MA). PCR reaction products were added to corresponding wells of purification plates. The plates were placed on a Te-VacS vacuum manifold using a Tecan Genosys 150 liquid handling robot (Tecan US). A vacuum of 700 mbar was applied for 10 min to remove buffer and bind amplicon DNA. Final insert amplicons were resuspended in 35 μl of 3× saline sodium citrate (SSC). Approximately 2 μl of each purified insert amplicon was separated on Invitrogen E-Gel 96 1% agarose gels (Invitrogen) to ensure that amplicons for each clone were actually represented on final microarrays in approximately equal concentration. A total of 10 μl of each purified amplicon was transferred to one of forty-eight 384-well microarray source plates. An additional 384-well plate containing lambda Q (96), GAPDH (96), β-actin (96), and negative control (3× SSC) was also produced for a total of 49 NBFGC microarray source plates.

Microarray spotting was performed at the Genomics Technology Support Facility at Michigan State University (East Lansing, MI). Microarrays were spotted using a GeneMachines OmniGrid 100 (San Carlos, CA) microarray spotting robot affixed with TeleChem ChipMaker 3 (Sunnyvale, CA) pins. These pins produce a spot size of roughly 100 μm in diameter. The pin configuration yields microarrays consisting of 48 subarrays (patches) arranged in 4 columns and 12 rows. Each subarray contains 20 rows and 20 columns of spots spaced 210 μm center to center. Lambda Q is spotted in each corner surrounded by negative controls, establishing a landmark for subsequent microarray image orientation and analysis.

RNA extraction, labeling, and hybridization. Whole tissue samples of bovine adrenal and lymph were collected from a 3-mo-old Holstein steer at the time of slaughter. RNA was extracted from all tissues using TRIzol reagent (Invitrogen) as described previously (7). Quantity and quality of total RNA extracted from all tissues and cells was estimated by UV spectrophotometry and electrophoresis on 1.2% native agarose gels. To calculate the amount of false-positive signals found on the NBFGC cDNA microarray, a total of six self vs. self microarrays were performed, three directly comparing identical RNA samples from adrenal tissue and three directly comparing identical RNA samples from lymph tissue. For all experiments, total RNA (8 μg) was used as template in reverse transcription reactions, using the Atlas Glass PowerScript Fluorescent labeling system (BD Biosciences, Alameda, CA) with oligo $(dT)_{18}$ as primer. Synthesis of cDNA in this system incorporates an amino-modified dUTP into the cDNA. Following first-strand synthesis, cDNAs were labeled using *n*-hydroxysuccinate (NHS)-derivatized Cy3 and Cy5 dyes (Amersham Biosciences). Labeled cDNAs were purified to remove unincorporated dyes, then combined and concentrated to ~10 μl using Microcon 30 spin concentrators (Millipore). Final probes were combined with 100 μl of prewarmed (70°C) SlideHyb 3 (Ambion), and the probe solutions were applied to prewarmed microarray slides via the port of a GeneTAC Hybridization Station microarray hybridization chamber (Genomic Solutions, Ann Arbor, MI). Hybridizations were conducted for 18 h using step-down temperatures from 65°C to 42°C in sealed chambers of the GeneTAC HybStation hybridization chamber. Following hybridizations, the station applies three washes: two with medium stringency buffer, and one with high-stringency buffer (Genomic Solutions). Slides were finally rinsed briefly at room temperature in 2× SSC and 1× in double-distilled H_2O. Washed microarrays were dried by centrifugation at 1,000 rpm in a cushioned 50-ml conical centrifugation tube.

Dried microarrays were scanned immediately using a GeneTAC LS IV microarray scanner and GeneTAC LS software (Genomic Solutions). GeneTAC Integrator 4.0 software was then used to process array images, align spots, integrate robot-spotting files with the microarray image, and to export reports of spot intensity data. The final report was retrieved as raw spot intensities in comma-separated value files, compatible with Microsoft Excel and SAS analysis programs. Data for the six microarray experiments used in this report can be found in the NCBI Gene Expression Omnibus (GEO), with series accession number GSE436.

Microarray data analysis. As previously indicated, GeneTAC LS IV software (Genomic Solutions, Ann Arbor, MI) was used to provide Cy3 and Cy5 fluorescence intensity measurements. The measurement strategy is based on determining the sum of fluorescence intensities in pixels within a fixed target circle (89 or 121 pixels in area for each of the 6 arrays considered) that lie above median background in the four corners of a square that encloses the target circle. This total fluorescence is then corrected for median background and reported along with the area in pixels represented in that total for both dyes. On that basis, all measurements are strictly defined to be positive with blank spots on expectation having half of the target pixel area lying above background. The number of saturated spots (giving average fluorescence intensity readings beyond $2^{16} - 1$ being the upper limit for TIFF measurements) from the adrenal vs. adrenal microarray and lymph vs. lymph microarray experiments was determined for each array.

Fluorescence intensity data was log transformed and normalized for potential dye intensity bias using the LOESS smoothing procedure as advocated in Ref. 9. Subsequent statistical analysis was based on a two-stage mixed model approach essentially as described (8). In the first stage, a normalization model that included fixed effects of dye and random effects of subarray and source plate were fitted using REML estimation of variance components in SAS PROC MIXED. The saved residuals from this first-stage model were further standardized by dividing by the estimated array-specific residual standard deviation, as residual variances were highly heterogeneous across arrays. Since there were only two "treatments" being compared, arbitrarily choosing Cy3 as a treatment and Cy5 as the other, SAS PROC TTEST was used as the second-stage analysis based on a gene-specific paired *t*-test using the pair of normalized values for each of the three arrays within tissue. *P* values and mean differences antilogged to ratios and standard errors of mean differences were saved for each gene.

Table 2. *Frequency of genes with significant difference in gene expression in self vs. self microarray experiments*

	P-value ≤ 0.05	
Experiment:	Frequency	Percentage*
lymph vs. lymph	572	3.13
adrenal vs. adrenal	681	3.73
	P-value ≤ 0.01	
Experiment:	Frequency	Percentage*
lymph vs. lymph	106	.58
adrenal vs. adrenal	110	.60
	P-value ≤ 0.001	
Experiment:	Frequency	Percentage*
lymph vs. lymph	13	.07
adrenal vs. adrenal	12	.07

*Percent of genes out of 18263.

RESULTS

NBFGC EST characterization and database. A web-accessible resource was established (**http://nbfgc.msu.edu**) to provide information on all EST clones contained in the NBFGC microarray. The web database can be searched by cDNA clone name, ontology, or keyword (keyword based on BLAST information from the cDNA sequence). Ontological classification (molecular function, molecular component, and biological process, if known) for each EST is linked on the NBFGC web site (**http://nbfgc.msu.edu**) to AmiGO!, an HTML-based browser for Gene Ontology from the Gene Ontology Consortium (**http://www.geneontology.org**).

NBFGC microarray characterization. The NBFGC microarray contains a total of 18,263 gene spots, 96 *Bos taurus* β-actin spots, 96 *B. taurus* GAPDH spots, 120 lambda Q spots, 241 negative spots (3× SSC), and 384 blank spots. Thus the total number of spots on the 20 × 20 patch configuration is 19,200 (400 spots in each patch).

As a first step in ascertaining the reliability of gene expression data derived from the NBFGC cDNA microarray, it was important to determine the potential for false-positive expression changes. The false-positive rate was assessed by hybridizing differentially labeled (Cy3 and Cy5) aliquots of cDNA from the same sample of reference RNA. This process was replicated to produce three microarrays for each tissue. Since the Cy3 and Cy5 labeled samples on these microarrays were from identical cDNA sources, there should be no significant differences in gene expression, and we would anticipate microarray analysis to yield equal fluorescence intensities for both the Cy3 and Cy5 labels at every spot on the microarray. The combined data from three lymph vs. lymph microarray experiments gave rise to 3.13% of genes showing significant differences based on unadjusted tests ($P \leq 0.05$, Table 2), unadjusted in the sense that the multiple testing issues involving 18,263 genes are not considered. For the adrenal vs. adrenal microarray experiments, 3.73% of the genes showed a significant difference ($P \leq 0.05$, Table 2). Surprisingly, these proportions (<4%) are somewhat more conservative that expected ($P \leq 0.05$, 5% of genes); however, this discrepancy might be due to the fact that the 18,263 genes are not expected to be independent in terms of their expression.

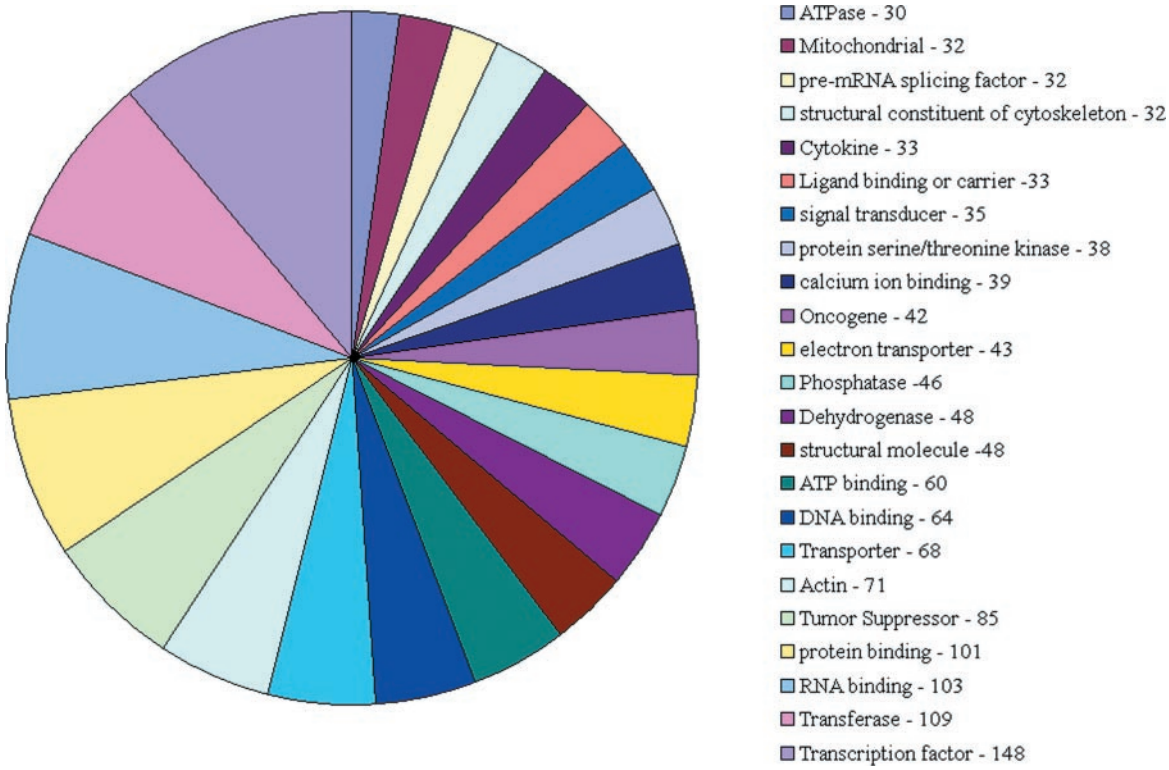

Fig. 1. Major molecular function ontology categories in the NBFGC EST collection and microarray.

For the three adrenal vs. adrenal microarray experiments, there was an average of 59 saturated spots for the Cy3 channel and 30 spots for the Cy5 channel. For the three lymph vs. lymph experiments, the average number of saturated spots was 39 for the Cy3 channel and 13 for the Cy5 channel. Hence, scanner saturation is not of appreciable concern in the NBFGC arrays and can be further controlled if necessary by adjusting the gain settings on the scanner.

DISCUSSION

A high-density cDNA microarray resource (NBFGC) has been developed containing 18,263 unique bovine gene spots. Bovine GAPDH and β-actin amplicons have been added to the cDNA microarray, which can be used for assessing RNA loading differences and potential dye effects. The negative control spots (3× SSC) can be used to further adjust for background effects on a global or patch-specific basis.

The NBFGC clone set is annotated in a web-accessible database (**http://nbfgc.msu.edu**) designed to assist in interpretation of microarray data. NBFGC clone data in our database allows retrieval of the top BLAST results for each clone against sequences in GenBank nonredundant (nr) database. Additional information, including clone sequence data, the

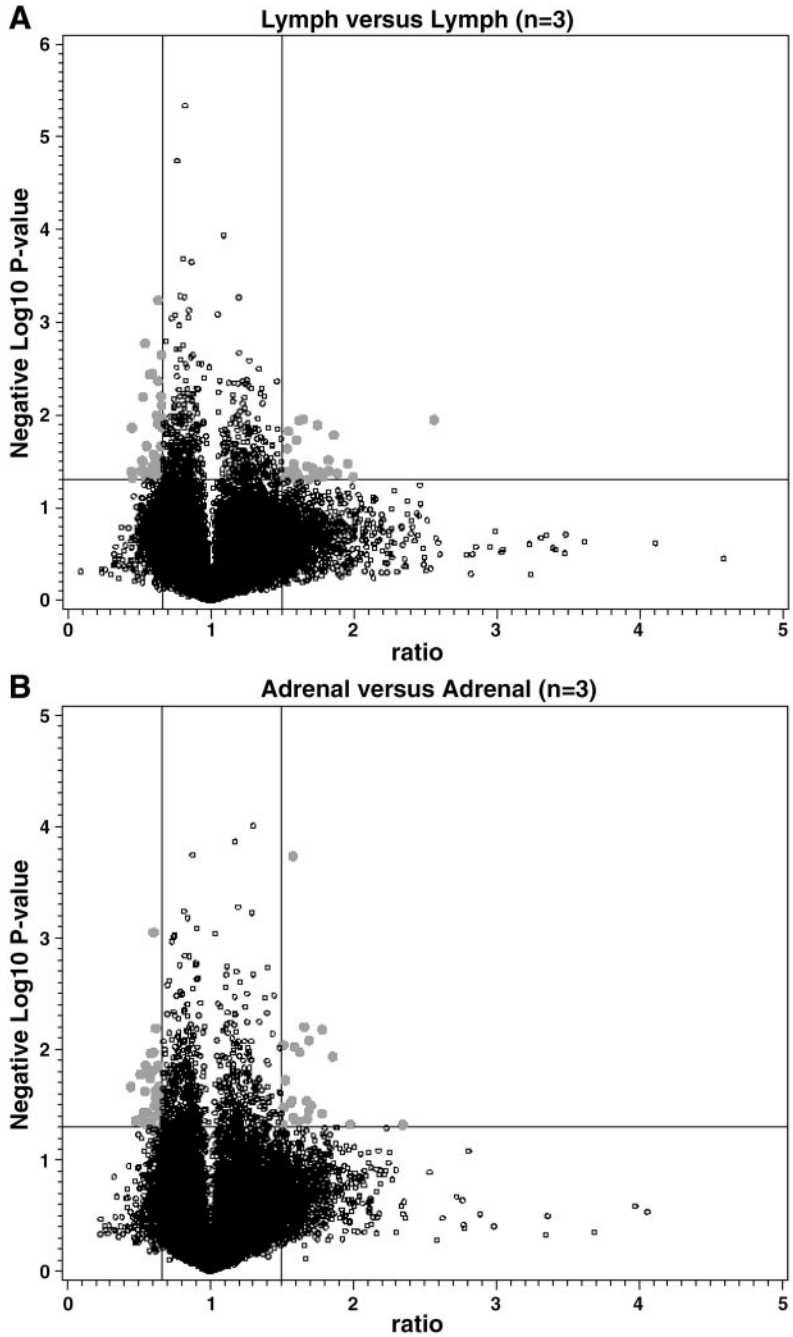

Fig. 2. Volcano plots of negative $\log_{10}$ *P* values vs. estimated ratios of differential expression for lymph (*A*) and adrenal (*B*) tissue for each of 18,263 genes. Vertical reference ratio lines are provided at 1.5 and 1/1.5. Horizontal reference line is provided at $-\log_{10}(0.05) = 1.30$. Red dots signify genes with expression ratios exceeding 1.5 or less than 1/1.5 and having an unadjusted *P* value of 0.05 or less.

TIGR TC number, and gene ontology information, is also displayed.

The NBFGC clone set and microarray contain clones are derived from a wide variety of tissue types and developmental states within tissue types. A large array of ontology classifications are found in the NBFGC EST set as would be expected considering the diverse set of tissue types represented (Fig. 1). Molecular function categories containing the most ESTs were transcription factors (148 ESTs), transferase (109 ESTs), RNA binding (109 ESTs), protein binding (103 ESTs), and tumor suppressor (85 ESTs) (Fig. 1). Considering the wide variety of tissue types and developmental states used to construct the MARC and BARC cDNA libraries (Table 1), it is not surprising that these categories represent a wide range of functional categories.

The NBFGC cDNA microarray was tested to determine the rate of false-positive signals. When two sets of three arrays hybridizing the same cDNA sample were analyzed, we found a slightly lower than expected rate

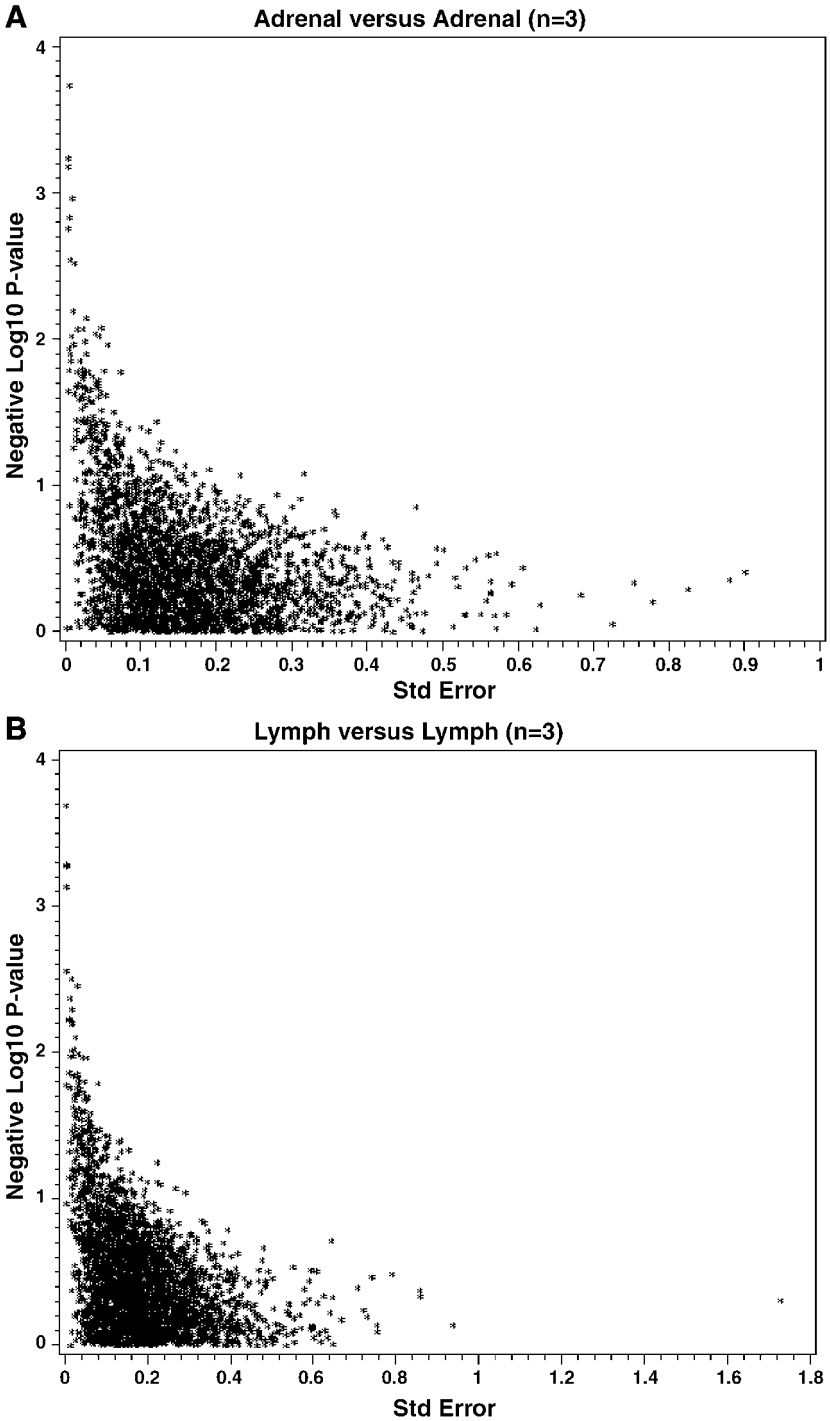

Fig. 3. Negative $\log_{10} P$ values vs. standard errors of mean differences ($\log_{10}$ scale) for adrenal (*A*) and lymph (*B*) tissue.

of unadjusted false positives overall (<4%). As can be seen by the volcano plots (8) in Fig. 2, the majority of genes showing significant difference ($P \leq 0.05$) between Cy3 and Cy5 channels in self vs. self microarray experiments are clustered around a ratio of 1. Additionally, most genes with significant P values ($P \leq 0.05$) have a very low standard error (Fig. 3). Therefore, some genes with ratios near 1 may be declared significant simply due to unusually low standard errors, which may occur simply due to chance. Taking into account a ratio cutoff and significance cutoff, the number of false positives is greatly reduced (Fig. 2). In fact, for the adrenal vs. adrenal arrays, 72 genes (0.4% of all genes) have P values less than 0.05 and expression ratios exceeding 1.5, and for the lymph vs. lymph arrays 56 genes (0.3% of all genes) have P values less than 0.05 and ratios exceeding 1.5 (Fig. 2). This result along with the low number of saturated spots demonstrates that design of the NBFGC microarray, combined with a basic statistical analysis, yields a low rate of false positives.

In summary, we have developed a high-quality high-density cDNA microarray (NBFGC) resource for studies of bovine physiology. Design of the NBFGC cDNA microarray produced a low incidence of false positives, and therefore gene expression differences observed using this resource should be highly reliable. The NBFGC EST set is fully integrated in a web-accessible database (http://nbfgc.msu.edu). Integration of EST clone information with putative gene identification and ontology is important when analyzing microarray results, since it is relatively simple to move from a list of differentially expressed clone names to identification of the transcript via web queries. The NBFGC cDNA microarray represents the largest microarray to date designed specifically for a livestock species and will allow for large scale screening of gene expression differences in cattle.

GRANTS

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