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ARCTURUS

APPLICATION NOTE #6

A Novel Process for Gene Expression Profiling of Rat and Mouse Tissues from Formalin-Fixed Paraffin-Embedded Sections Using Microarrays

Abstract

This application note describes a method for extracting and isolating total RNA from rodent tissues that have been fixed in formalin and embedded in paraffin, and the subsequent amplification of mRNA from these tissues for use in microarray experiments. We have used this methodology to demonstrate that robust and reliable methods do exist for retrieval of high quality nucleic acids from formalin-fixed, paraffin-embedded (FFPE) tissues and that these results display a high level of concordance as compared to frozen sample counterparts.

Introduction

Large numbers of putative drug compounds have been studied in mouse and rat models for their effects on diseases, as well as for their toxicological effects. Enabling high-throughput gene expression analysis of tissues from these models will revolutionize disease diagnoses and drug discovery. Limiting this approach, however, is the fact that these tissues are commonly preserved by Formalin-Fixation and Paraffin-Embedding. Since the macromolecules in FFPE tissues are cross-linked, efficiently extracting and isolating RNA of adequate quality for microarray analysis from such samples poses a significant technical challenge. Arcturus has developed and optimized a novel process that integrates efficient isolation of total cellular RNA from FFPE sections, linear amplification of transcripts from isolated RNA, and synthesis of labeled cRNA for microarray analysis, and incorporated them together in the Paradise™ Reagent System. Results of a comparison between matched frozen and formalin-fixed samples in order to study the fidelity of gene expression ratios derived from both samples will be presented within this application note.

Equipment, Materials and Reagents

This protocol requires the following reagents for completing the study. For additional reagent and equipment requirements for slide preparation, RNA extraction, RNA isolation and amplification, please refer to the appropriate sections of the Paradise Reagent System (FFPE tissues) and PicoPure® RNA Isolation Kit (frozen tissues) User Guides.

Equipment:

- ❑ Cryostat with Disposable Blades
- ❑ Fume Hood
- ❑ -70ºC Freezer
- ❑ Desiccator
- ❑ Rotary Microtome
- ❑ Oven
- ❑ Tissue Flotation Water Bath
- ❑ Ice-Bath or Cold Block (4ºC)
- ❑ Pipettors
- ❑ Automatic Tissue Processor *(optional)*
- ❑ Nanodrop® ND-1000 Spectrophotometer
- ❑ Agilent 2100 BioAnalyzer
- ❑ Affymetrix GeneChip® Scanner 3000
- ❑ Affymetrix GeneChip® Operating Software (GCOS v1.1.1)
- ❑ Applied Maths Genemaths® XT Software (v2.01)

Materials:

- ❑ Disposable Gloves
- ❑ Cryomold (VWR, Cat. # 25608-916)
- ❑ Scalpels
- ❑ Tweezers
- ❑ Cover Glass Forceps
- ❑ Microslide Box – Plastic (VWR, Cat. # 48444-004)
- ❑ Dry ice
- ❑ Kimwipes® or Similar Lint-Free Towels
- ❑ Desiccant (VWR, Cat. #22890-900)
- ❑ RNase AWAY (Life Technologies, Cat.# 10328-011)
- ❑ Detergent (Fisher Scientific, Cat. #04-355)
- ❑ Pipette Tips, Nuclease Free
- ❑ 0.5 mL or 0.2 mL RNase-Free
- ❑ Microcentrifuge Tubes
- ❑ 2 mL lidless Tube (PGC Scientific, Cat. # 16-8101-06)
- ❑ Slides, Silane Coated (Sigma, Cat. # S4651)
- ❑ GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Cat. # 900496)
- ❑ GeneChip® Rat Genome 230 2.0 Array (Affymetrix, Cat. # 900506)

Reagents:

- ❑ 10% Neutral Buffered Formalin (NBF) (VWR, Cat. # EM-FX419-1)
- ❑ Paraffin, Paraplast X-tra (VWR, Cat. # 15159-486)
- ❑ Tissue-Tek® OCT compound (VWR, Cat. # 25608-903)
- ❑ Histogene® Frozen Section Staining Kit and User Guide (Arcturus, Cat. # KIT0401)
- ❑ PicoPure RNA Isolation Kit and User Guide (Arcturus, Cat. # KIT0204)
- ❑ Paradise Reagent System and User Guide (Arcturus, Cat. # KIT0311)
- ❑ GeneChip® IVT Labeling Kit (Affymetrix, Cat. # 900449)
- ❑ 2-Methylbutane / Isopentane (VWR, Cat. # JTQ223-7)

RNase-free Technique

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout the experiment:

- Always handle RNA in a manner that avoids introduction of RNases.
- Wear disposable gloves and change them frequently to prevent the introduction of RNases from skin surfaces.
- After putting on gloves, avoid touching surfaces that may introduce RNases onto glove surfaces.
- Do not use reagents not supplied with the Paradise Reagent System. Substitution of reagents or kit components may adversely affect yields or introduce RNases.
- Use only new plasticware that is certified nucleic acid-free.
- Use only new, sterile, RNase-free pipette tips and microcentrifuge tubes.
- Clean work surfaces with commercially available RNase decontamination solutions prior to performing reactions.

RNA Input Recommendations

The Paradise Reagent System RNA Amplification reagents are designed to amplify total RNA isolated using the Paradise Extraction/Isolation Reagents. Arcturus recommends the following input of formalin-fixed RNA:
Minimum: 5 ng
Recommended: 10 ng
This amount should yield enough aRNA for duplicate array hybridizations.

Methods

1. For this study, tissues were harvested from Sprague Dawley rats (brain and testes) and C57/Bl mice (brain and ovaries).
2. All tissues were bisected (except ovaries) with one half fixed in 10% neutral buffered formalin for paraffin sections (FFPE) and the other half embedded in OCT for frozen sections. To achieve high quality RNA, all tissues were processed immediately after harvesting (see sections 2.1 and 2.2).

2.1 Frozen Sections:

a. Dry ice was placed in an appropriate container.
b. Isopentane was poured slowly into the container with the dry ice, filling until the isopentane level was just above the layer of dry ice.

⚠ **Note: Isopentane has a very low flash point and should be kept away from open flames. Perform procedure in a fume hood or a well-ventilated space.**

c. Bubbling of the isopentane will occur upon its addition to the dry ice. Once this had subsided the isopentane was ready for use.
d. Specimens were identified on cryomolds using a sharpie.
e. A thin layer of OCT was placed on the bottom of the cryomold.
f. Dissected tissue specimens were placed onto the layer of OCT in the cryomold in the desired orientation
g. OCT was carefully added until the specimen was completely covered and the cryomold was filled.
h. The prepared cryomold was placed into the cooled isopentane.
i. The OCT was allowed to completely solidify. While freezing down each specimen, those already processed were kept in a separate container on dry ice only.
k. 8-µm sections were cut using a cryostat and mounted on silane coated slides. Frozen specimens not immediately used for slide preparation were stored in the cryomold in a –70°C freezer. In this study, two sections were prepared per slide, except for mouse ovaries where 3 sections were mounted per slide. All slides were held in a microslide box stored on dry ice.

⚠ **Note: Do not allow slides to come to room temperature after mounting section(s).**

l. Slides were immediately stained with the HistoGene LCM Frozen Staining Kit. Those not stained immediately were stored at –70°C until use.

2.2 Paraffin Sections:

a. Tissues no greater than 5mm in thickness were fixed in 10% NBF for 24 hours at room temperature.
b. After fixation was completed, the tissues were immediately processed into paraffin blocks by taking them through a series of graded ethanols, followed by xylene and finally infiltrated with paraffin using an automatic tissue processor.
c. Once the tissue was embedded into paraffin block, 7 µm thick sections were cut using a rotary microtome. The sections were floated briefly on a heated water bath containing nuclease free water and mounted onto glass slides.
d. After air-drying the sections at room temperature for at least 1 hour, the slides were taken through the Paradise staining protocol or stored in a dessicator for up to 1 week prior to staining. Do not place slides in oven to dry.

⚠ **Note: Fixation times will vary based on the overall tissue size with a minimum fixation time of 6 hours at room temperature for small tissue samples. Tissues should not be fixed at 4°C but at room temperature. Tissues should not be stored in any type of solution after fixation is completed and prior to processing into paraffin blocks.**

3. Tissue scrapes were performed, taking a 1 cm^2 area from each stained section and processed in 100 µl of Extraction Buffer (PicoPure RNA Isolation Kit) for frozen sections or 100 µl of Proteinase K solution (Paradise Reagent System) for FFPE sections. If needed, multiple tissue sections were processed together to achieve a total 1 cm^2 tissue area.
4. Final elution of total RNA was done with 22 µl of elution buffer.
5. Total RNA quantity was determined using the NanoDrop Spectrophotometer (Table 1) and RNA quality was assessed using the Agilent 2100 Bioanalyzer (Figure 1).
6. 5 ng of total RNA from the FFPE samples and 1ng of total RNA from the frozen samples were amplified following the Paradise Reagent System Amplification protocol. Both frozen and FFPE samples were amplified using the same process (Paradise Reagent System) after RNA isolation in order to maintain similar processing of the tissues to minimize variability.
7. After the first round of amplification, double stranded cDNA was generated following the Paradise protocol and this material was taken into the GeneChip® IVT labeling Kit (Affymetrix).
8. Biotin labeled aRNA was quantitated using the NanoDrop Spectrophotometer. See Tables 2a and 2b for aRNA yields.
9. 16 µg of aRNA was fragmented in 250 µL of hybridization cocktail (10 min fragmentation for FFPE samples and 15 min for frozen) and 200 µL was hybridized onto each array, following the Affymetrix protocol.
10. After hybridization, the arrays were scanned using the GeneChip® Scanner 3000 (Affymetrix) and the data was analyzed using GCOS v1.1.1 (Affymetrix) and Genemaths 2.01 (Applied Maths) software.
11. The resulting chip performance data is shown in Table 3.

Results

Efficient extraction and isolation of good quality RNA from both frozen and FFPE samples is necessary for generating high quality gene expression data. Table 1 shows RNA yields from frozen and FFPE samples demonstrating that adequate amounts of RNA can be obtained from these samples to enable linear amplification. In addition to quantity, quality of the isolated material is also an important factor. The Agilent 2100 bioanalyzer plots shown in Figure 2 are used to help qualify the samples prior to amplification. Even though the profiles generated from FFPE samples may seem to indicate that quality of RNA may be compromised, they simply illustrate the effect of formalin fixation on the RNA. These profiles further illustrate the need for a robust method of amplification to overcome the challenges of working with such formalin-fixed samples.

After the quantity and quality of the total RNA isolated from the frozen and FFPE samples are assessed, the RNA is then used for linear amplification with the Paradise Reagent System, which has been optimized to provide robust amplification of FFPE samples as mentioned above. In the first round of amplification, the Paradise Reagents are used to generate antisense RNA (aRNA) which is then converted to double stranded cDNA. This double stranded cDNA becomes the template in a second round of amplification (IVT) using the GeneChip IVT Labeling Kit. This step is necessary for incorporating biotinylated nucleotides into the aRNA, a necessary step to enable labeling after hybridization. Tables 2a and 2b summarize the yields of biotinylated aRNA obtained from samples after two rounds of amplification. These yields, as indicated in the table for both rat (Table 2a) and mouse (Table 2b), are more than sufficient for hybridization to GeneChip Probe arrays.

GeneChip array performance data shown in Table 3 demonstrates that percent present (%P) calls and average signal from the array are similar between frozen and FFPE material. Gene expression intensities show a high level of concordance between frozen and FFPE samples (Figure 2a-b) Furthermore, when 30 probe sets from both the rat and mouse arrays that show a high level of differential gene expression were studied, a high level of concordance was seen in differential gene expression ratios between the frozen and FFPE samples (Figure 3a-b).

Conclusions

The data shown in Table 1 indicate that the Paradise Reagent System provides a robust method of extraction and isolation of rodent tissues fixed in formalin. RNA yields from frozen samples were lower than their FFPE counterparts because the extraction process using Paradise is optimized for large area extractions, while PicoPure process is optimized for relatively smaller tissue input. This leads to higher total RNA yields with the FFPE samples when equal amounts of large areas are extracted. The RNA extracted is of quality suitable for linear amplification and microarray hybridization, as shown in Figure 1. Linear amplification yields microgram amounts of aRNA (Table 2) suitable for hybridization to catalog GeneChip Arrays from Affymetrix, resulting in high quality gene expression data (Table 3). Further, the results from arrays show a high level of concordance between FFPE and frozen samples, both in raw intensities as well as gene expression ratios (Figures 2 and 3).

This study demonstrates that we have developed an enabling process for obtaining high quality differential gene expression data from formalin-fixed rodent tissues. The data obtained bears a high level of concordance with similar studies conducted using frozen tissues and shows how this process can be used to generate biologically relevant global gene expression data from formalin-fixed rodent tissues using microarrays.

Table I. RNA Yield from FFPE and Frozen Samples: Total RNA yields from FFPE and frozen tissues of rat and mouse specimens measured using the NanoDrop Spectrophotometer.

Tissue	Sample	FFPE Sample Total RNA Yield (ng)	Frozen Sample Total RNA Yield (ng)
Rat Brain	005	172.7	40.92
	006	195.4	117.26
Rat Testis	005	396.0	212.3
	006	473.0	224.4
Mouse Brain	007	167.2	95.2
	008	400.4	119.2
Mouse Ovary	007	398.2	136.4
	008	283.8	158.1

Figure 1. RNA quality as measured using the Agilent 2100 BioAnalyzer. Top panel illustrates RNA quality of rat FFPE samples. 18s and 28s peaks are visible as an indicator of RNA quality. However, the quality of FFPE samples are not as high as their frozen counterparts (bottom panel) where 18s and 28s peaks are much more pronounced.

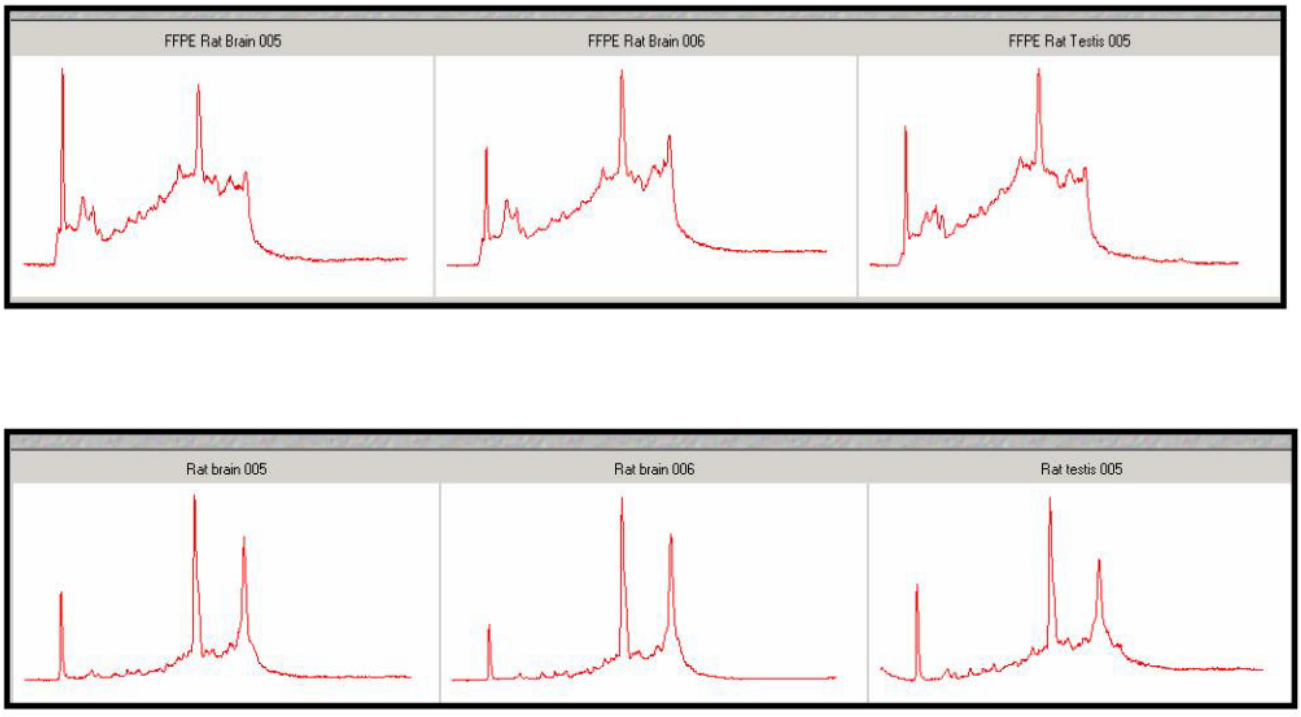


Table 2a. aRNA yield from Frozen and FFPE rat tissue samples.

	Frozen		FFPE	
Sample	Yield Biotin aRNA (µg)	Average Yield (µg)	Yield Biotin aRNA (µg)	Average Yield (µg)
Rat Brain 005	81.26	**70.94**	51.01	**46.60**
	60.61		42.19	
Rat Testis 006	80.73	**70.13**	46.49	**45.10**
	59.54		43.72	
Mouse Brain 005	79.47	**82.91**	55.90	**52.42**
	86.35		48.94	
Mouse Ovary 006	93.01	**93.91**	63.44	**60.38**
	94.82		57.32	

Table 2b. aRNA yield from Frozen and FFPE mouse tissue samples.

	Frozen		FFPE	
Sample	Yield Biotin aRNA (µg)	Average Yield (µg)	Yield Biotin aRNA (µg)	Average Yield (µg)
Mouse Brain 007	38.72	**37.29**	15.94	**17.95**
	35.87		19.95	
Mouse Brain 008	65.48	**63.23**	29.17	**22.80**
	60.98		16.42	
Mouse Ovary 007	20.63	**19.53**	19.38	**17.35**
	18.42		15.33	
Mouse Ovary 007	35.32	**31.43**	21.36	**20.59**
	27.54		19.82	

Table 3. Genechip array performance data for frozen and FFPE mouse and rat tissues.

Rodent / Tissue Type		Background:	SF	Noise (RawQ):	Ave Signal (P):	Sig(3'/5') GAPDH	Sig(3'/5') Beta-actin	% P
Rat Brain	FFPE	50.55	1	1.76	346.7	6.77	9.21	46.0%
		49.72	1	1.91	270.9	7.61	49.93	36.4%
		51.28	1	2.11	310	10.86	23.68	43.2%
	Frozen	52.76	1	1.73	435.8	12.57	42.09	49.6%
		52.81	1	1.59	360.2	16.76	8.1	45.7%
		55.75	1	1.83	426.2	20.21	70.44	48.8%
Rat Testis	FFPE	46.37	1	1.51	242.5	17.47	174.23	35.4%
		50.35	1	2.03	234.5	3.17	16.54	26.3%
		49.17	1	1.61	286.5	2.68	20.15	37.2%
	Frozen	52.11	1	1.71	401.2	7.81	61.89	40.0%
		58.3	1	2.55	351.2	4.2	39.36	35.4%
		51.99	1	1.68	377.5	7.09	50.47	39.4%
Mouse Brain	FFPE	39.96	1	1.14	226	29.7	20.11	26.6%
		45.75	1	1.43	263.1	26.86	23.51	27.4%
		42.49	1	1.26	199.2	61.5	23.07	26.2%
	Frozen	45.47	1	1.38	312.8	28.8	300.95	36.2%
		49.19	1	1.48	363.6	41.85	389.09	37.9%
		50.56	1	1.42	339.7	34.82	243.84	35.9%
Mouse Ovary	FFPE	38.2	1	1.17	192.6	11.46	225.37	27.7%
		44.29	1	1.3	285.4	17.76	72.54	26.9%
		40.3	1	1.22	231.6	17.95	87.66	26.6%
	Frozen	42.12	1	1.48	223.6	15.06	73.25	35.3%
		46.13	1	1.32	276.9	32.69	95.01	34.5%
		43.83	1	1.3	293.4	25.65	84.89	35.2%

Figure 2a. Correlation plots of raw intensities between frozen and formalin-fixed rat tissues.

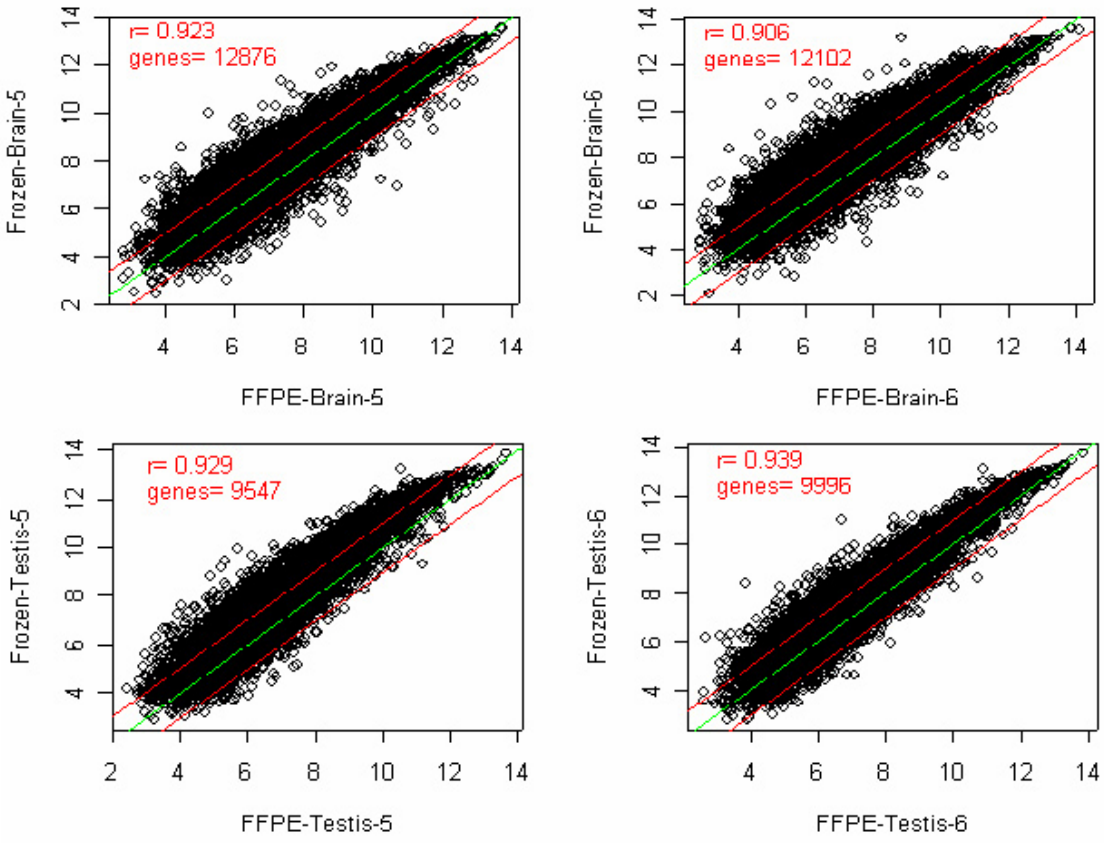


Figure 2b. Correlation plots of raw intensities between frozen and formalin-fixed mouse tissues.

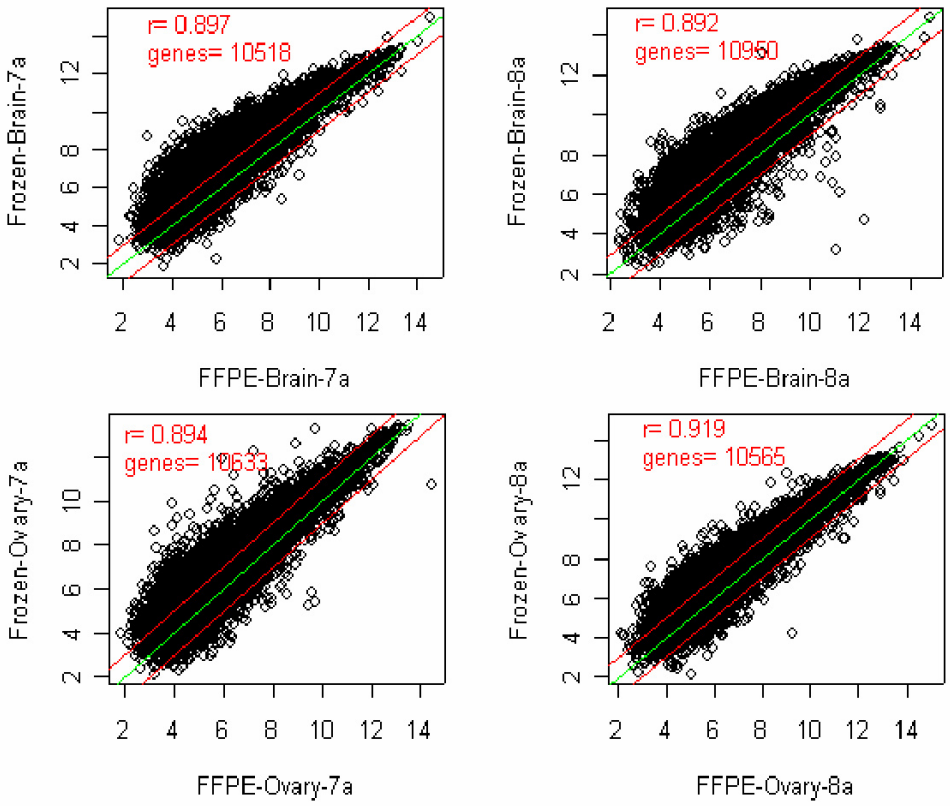


Figure 3a. Gene Expression Ratios from 30 probe sets exhibiting high differential expression: Two tissue types from rat were studied, both represented as frozen as well as FFPE.

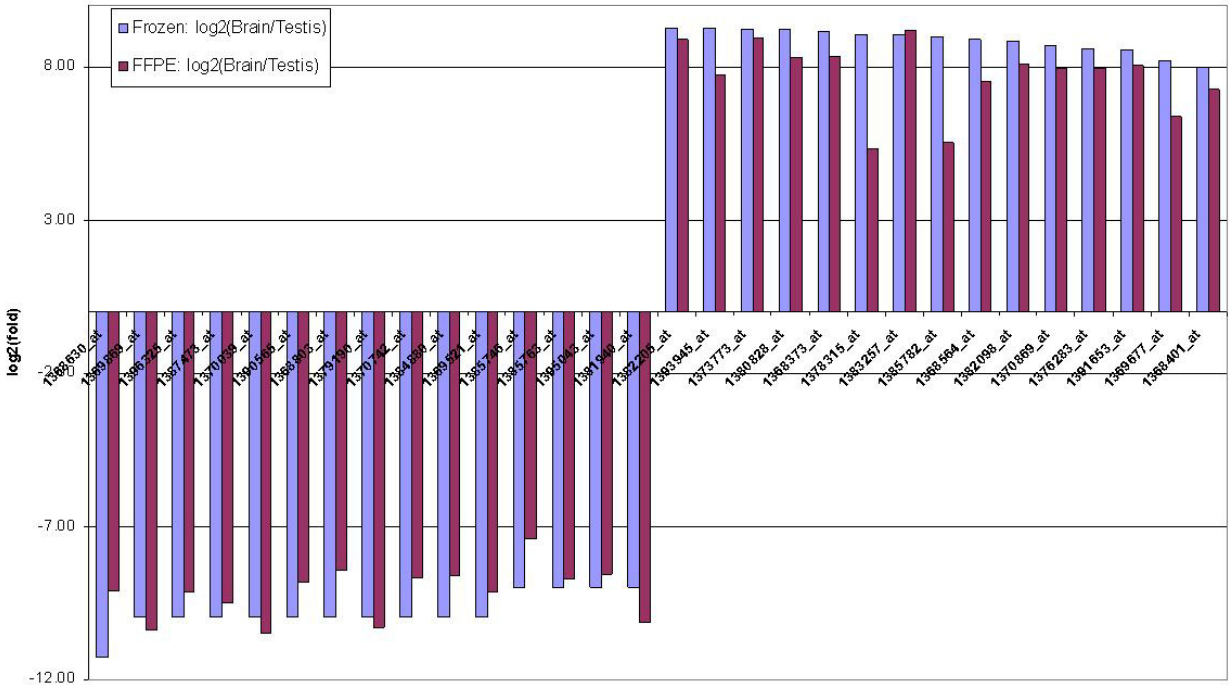
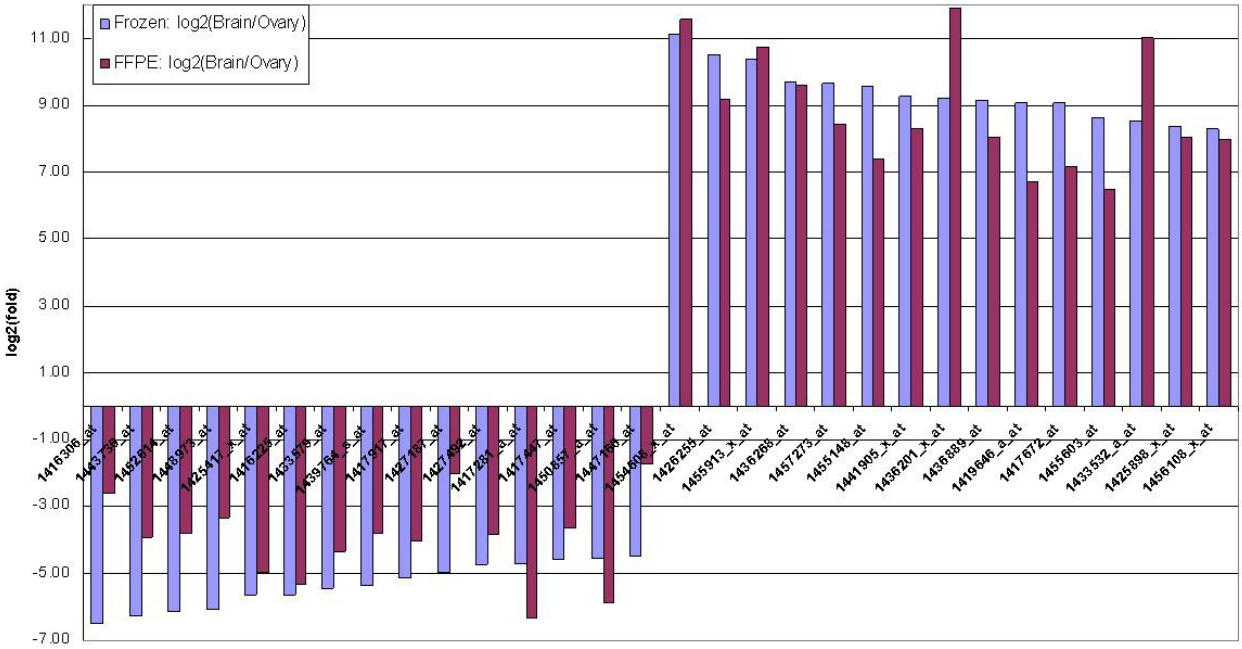


Figure 3b. Gene Expression Ratios from 30 probe sets exhibiting high differential expression: Two tissue types from mouse were studied, both represented as frozen as well as FFPE.



References

1. Affymetrix GeneChip Expression Analysis Technical Manual (Cat# 701021 Rev. 5)
2. Arcturus PicoPure RNA Isolation Kit User Guide (Cat# 12313-00 Version D)
3. Arcturus Paradise Reagent System User Guide (Cat# 12872-00 Version

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