Method development for extraction and purification of dermal RNA from FVB/N mice treated with environmental PAH mixtures

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Oil Plume Is Not Breaking Down Fast, Study Says

By JUSTIN GILLIS and JOHN COLLINS RUDOLF
Published: August 19, 2010

New research confirms the existence of a huge plume of dispersed oil deep in the Gulf of Mexico and suggests that it has not broken down rapidly, raising the possibility that it might pose a threat to wildlife for months or even years.

Mapping a Plume

Scientists mapped the location of an underwater oil plume stretching more than 20 miles from the site of the Macondo well during surveys conducted June 19-28.

The study, the most ambitious scientific paper to emerge so far from the Deepwater Horizon spill, casts some doubt on recent statements by the federal government that oil in the gulf appears to be dissipating at a brisk clip. However, the lead scientist in the research, Richard Camilli, cautioned that the samples were taken in June and circumstances could have changed in the last two months.

The paper, which is to appear in Friday's issue of the journal Science, adds to a welter of recent, and to some extent conflicting, scientific claims about the status of the

Overview

Introduction to PAHs

- Structure and properties
- Mechanisms of toxicity
- Regulation and risk assessment

Method development and results

- Objectives
- Initial method and results
- Modified method and results

Discussion

- Analysis of results
- Comparison of methods

Conclusions, future directions

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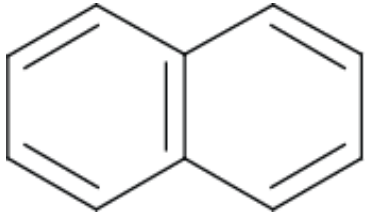
Polycyclic Aromatic Hydrocarbons (PAH)s

Structure

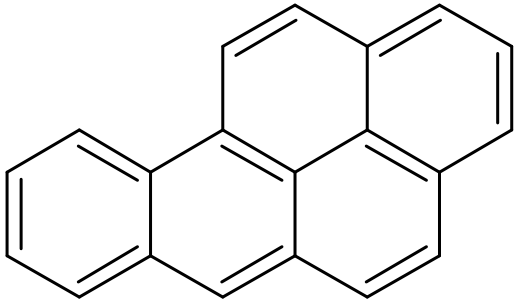
- Fused aromatic rings
- Planar
- Classified by molecular weight (low or high)



Napthalene

Sources

- Incomplete combustion of biofuels
  - Coal tar, diesel exhaust, forest fires

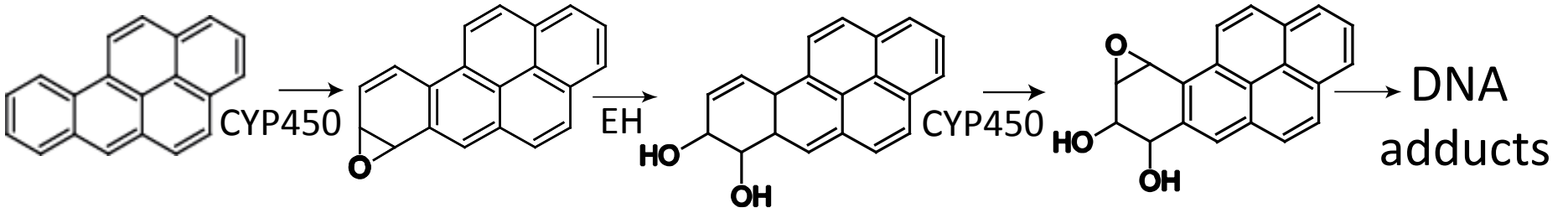


Benzo[a]pyrene
- B[a]P

Environmental Fate

- Lipophilic, persistent in the environment
- Differences in distribution, persistence

Mechanisms of B[a]P Toxicity

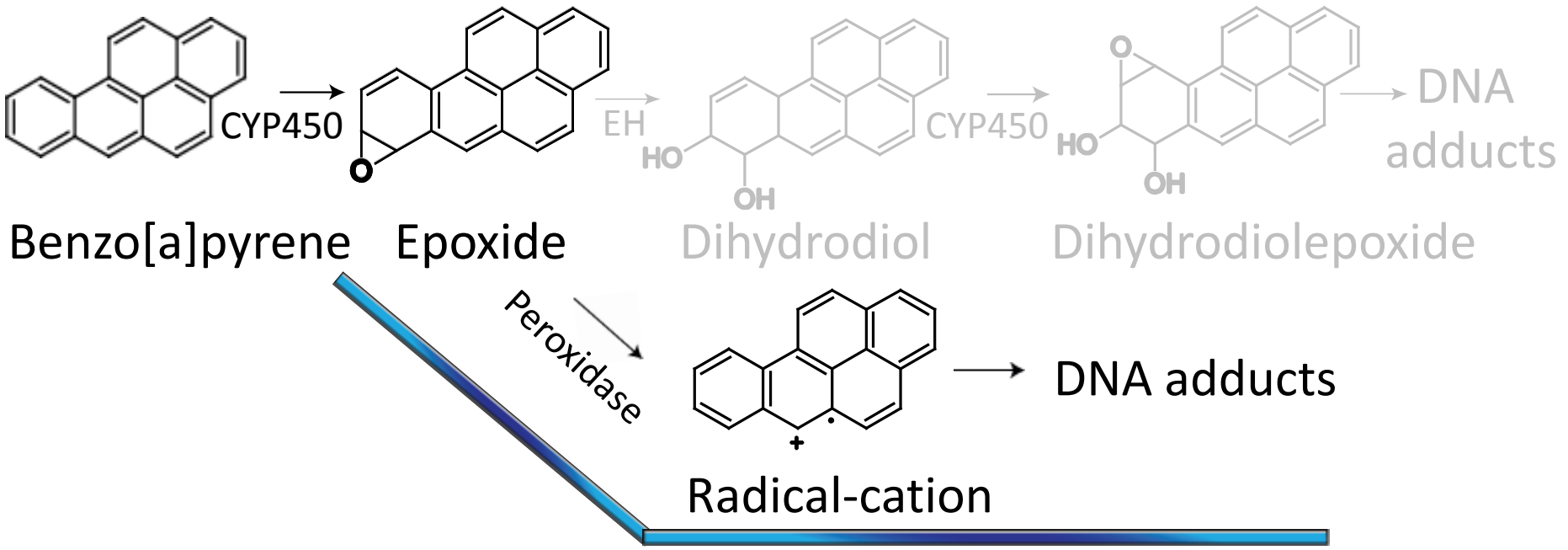


Benzo[a]pyrene Epoxide Dihydrodiol Dihydrodiolepoxide

1. Formation of a dihydrodiol epoxide

Mechanisms of B[a]P Toxicity

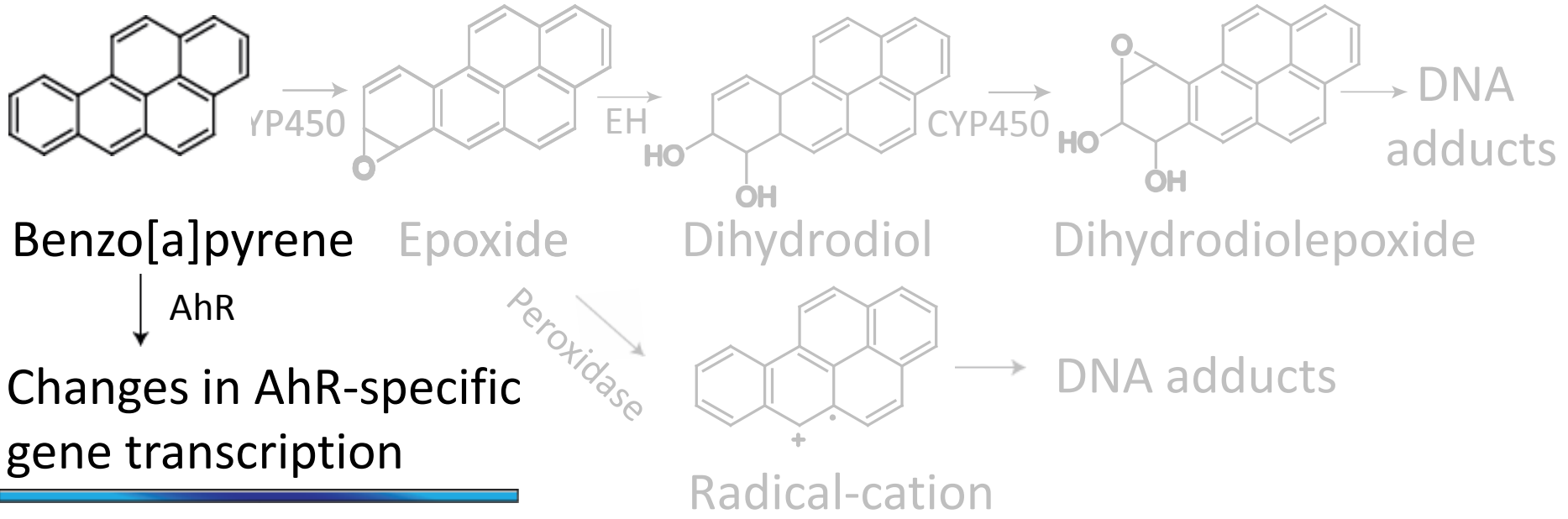
1. Dihydrodiol epoxide



2. Free radical cation formation

Mechanisms of B[a]P Toxicity

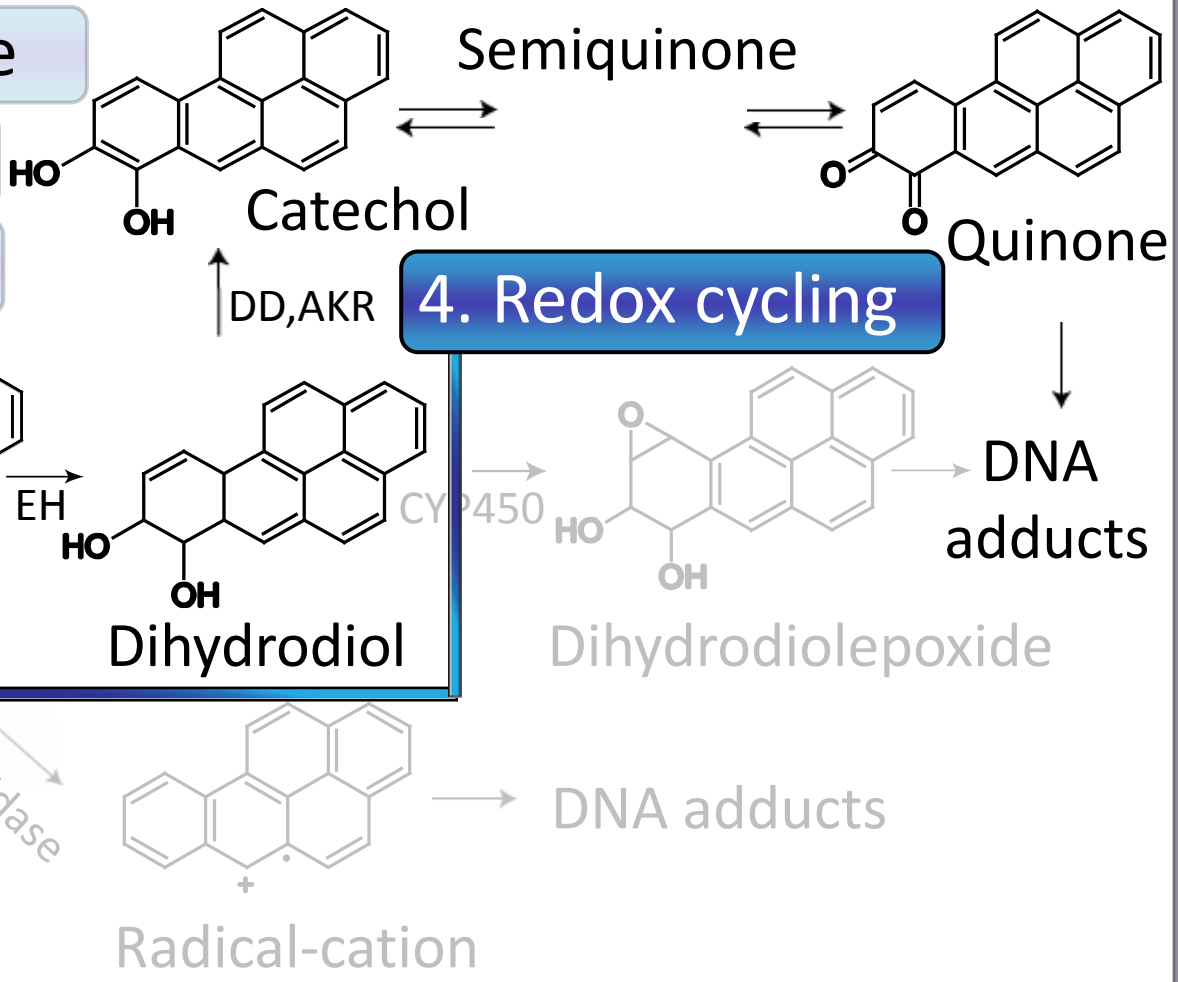
1. Dihydrodiol epoxide
2. Radical cation



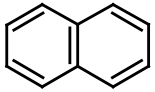
3. Binding to the aryl hydrocaron receptor (AhR)

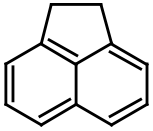
Mechanisms of B[a]P Toxicity

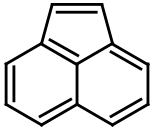
1. Dihydrodiol epoxide
2. Radical cation
3. Binding to AhR

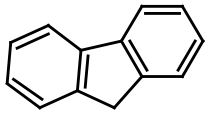


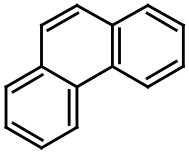
High Priority PAHs

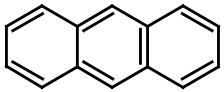

Napthalene

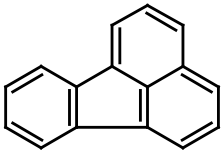

Acenaphthylene

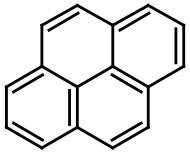

Acenaphthene

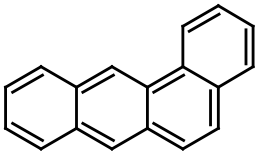

Fluorene

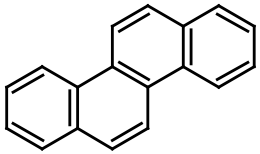

Phenanthrene

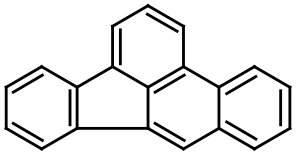

Anthracene

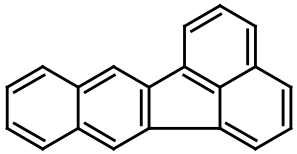

Fluoranthene

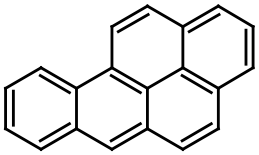

Pyrene

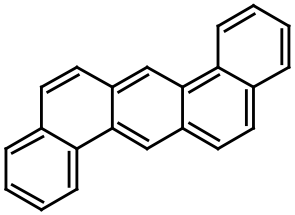

Benzo(a)anthracene

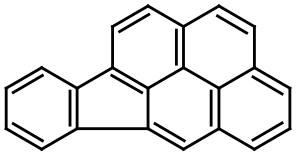

Chrysene

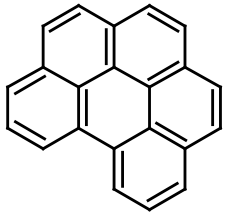

Benzo(b)fluoranthene


Benzo(k)fluoranthene


Benzo(a)pyrene


Dibenzo(a,h)anthracene

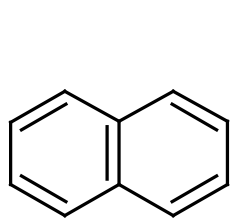

Indeno(1,2,3-cd)pyrene


Benzo(ghi)perylene

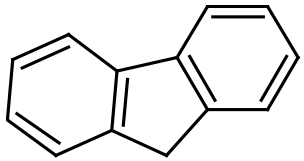
Priority PAHs in Cigarette Smoke Residue

PAH components	Concentration (µg/920 mg RES)
Napthalene	1300
Fluorene	860
Phenanthrene	780
Pyrene	350
Chrysene	80
Benzo[a]pyrene	50
Total	3420

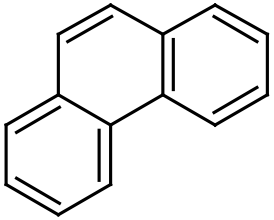
Rodgman *et al.*, 2000



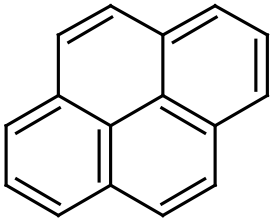
Napthalene



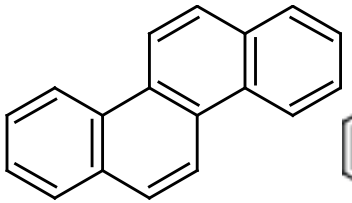
Fluorene



Phenanthrene



Pyrene



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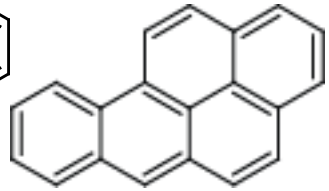
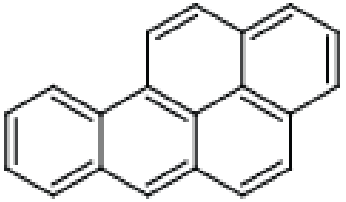


Benzo(a)pyrene

Benzo[a]pyrene equivalents (B[a]P_{eq}s)



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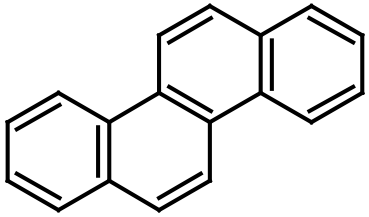
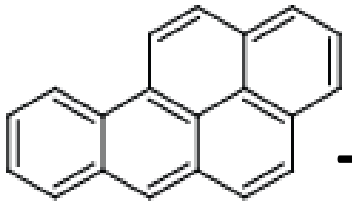
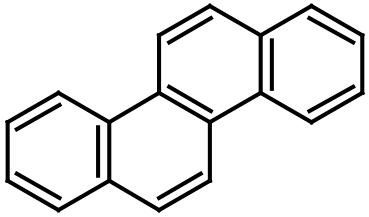


Chrysene

↓ 1[C] B[a]P_{eq}

↓ 0.0044[C] B[a]P_{eq}

Toxic Equivalent (TEQ)

$$TEQ = \sum$$  $+$  $+ \ldots + n$

Benzo(a)pyrene Chrysene

$$= \sum 1[C]\ B[a]P_{eq} + 0.0044[C]\ B[a]P_{eq} + \ldots + X_n\ B[a]P_{eq}$$

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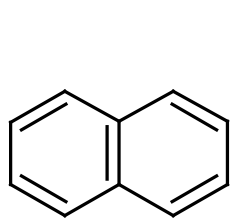

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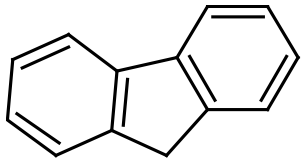
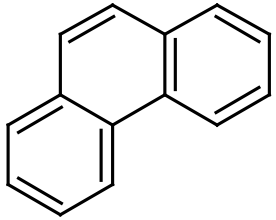
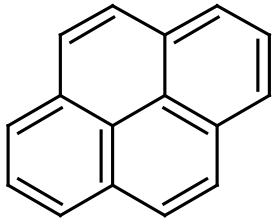


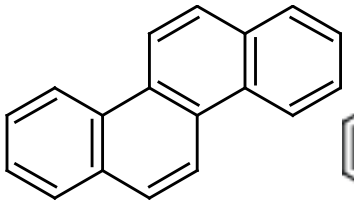
Fluorene



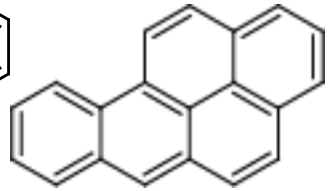
Phenanthrene



Pyrene



Chrysene



Benzo(a)pyrene

$$X_1\ B[a]P_{eq} + X_2\ B[a]P_{eq} + X_3\ B[a]P_{eq} + X_4\ B[a]P_{eq} + X_5\ B[a]P_{eq} + X_6\ B[a]P_{eq}$$

Why Study PAHs?

TEQs are not ideal

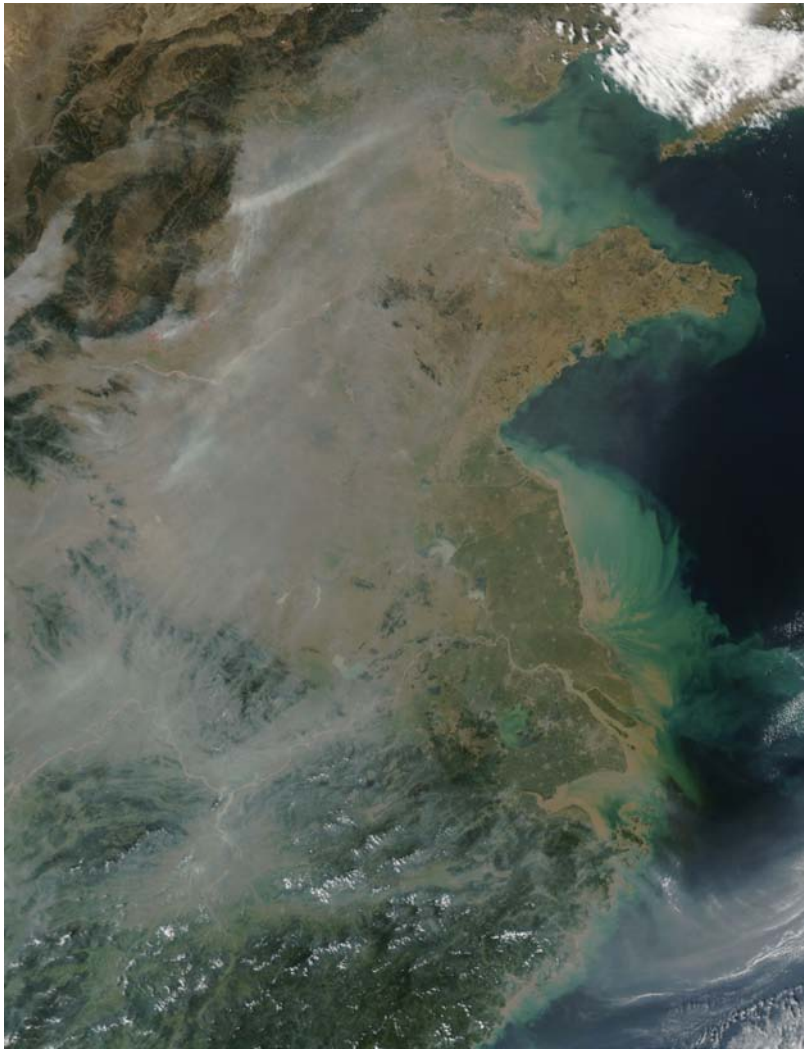
- Based on studies of individual PAHs
- Differences between regulatory agencies

EPA - 2002

- Study PAHs “as they occur together in common mixtures”
- Initiated study - results still not available

President’s Cancer Panel –2010

- Risk of environmentally induced cancer has been “grossly underestimated”



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Objectives

Elucidate the molecular mechanisms by which PAH mixtures induce basal cell and squamous cell carcinogenesis

- Develop method for harvesting epidermal RNA from mice treated with PAH mixtures
- Compare RNA expression levels between control mice and mice exposed to PAH mixtures

Hypothesis

Mice treated with PAH solutions will undergo changes in gene expression that promote tumor initiation, specifically at the site of application, the epidermal layer

Changes in gene expression in mice treated with environmental PAH mixtures differ from changes in gene expression in mice treated with PAH standards

Method Overview

- Treat mice with environmental PAH mixtures
- Harvest skin, isolate epidermal layer
- Extract and purify RNA
- RNA quality control analysis
- Synthesize cDNA
- Focused PCR microarrays
- Gene expression analysis

Method Overview

Treat mice with environmental PAH mixtures

Harvest skin, isolate epidermal layer

Extract and purify RNA, quality control

Synthesize cDNA

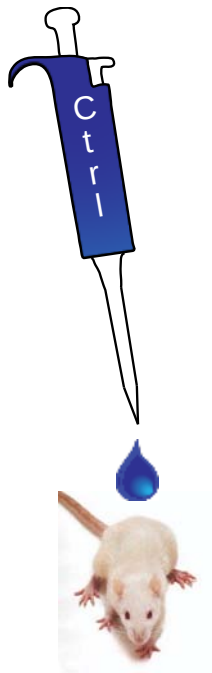
Perform gene expression analysis

Animal Preparation

- FVB/N strain
  - Inbred
  - Susceptible to carcinoma genesis
  - Resting phase of hair growth (7-8 weeks old)
- Application area shaved 48 hours before treatment

Animal Treatment

Control	PAH standards		Environmental PAH mixtures		
Ctrl	B[a]P	DBC	Mix1	Mix2	Mix3
200 µL Toluene	200 µL Toluene 400 nm B[a]P	200 µL Toluene 4 nm DBC	200 µL Toluene 1 mg DPE	200 µL Toluene 1 mg DPE 1 mg CTE	200 µL Toluene 1 mg DPE 1 mg CTE 2 mg CSC



CSC – cigarette smoke condensate
CTE – coal tar extract
DBC – dibenzo(*def,p*)chrysene
DPE – diesel particulate extract

Epidermal Cell Harvest

- Mice euthanized 12 h after treatment
- Area of application cleaned and excised
  - Gauze pads, Milli Q water, NAIR
- Skin placed into heat bath (58°C), then ice bath
  - Separates epidermal layer from dermal layer
- Epidermal layer scraped off, stored in Trizol
  - Inactivated endogenous RNases

RNA extraction

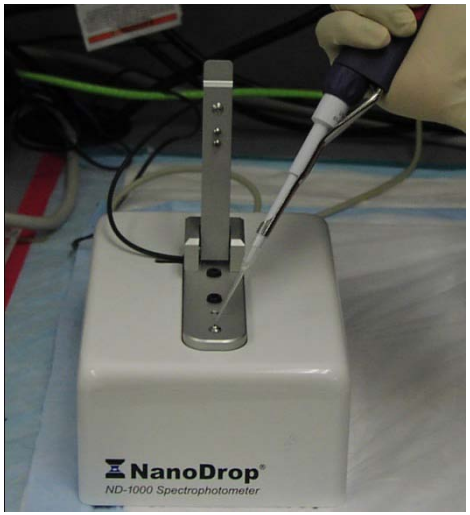
- Epidermal cells homogenized
- RNA separated from DNA and protein using chloroform and centrifugation
- Ethanol washes

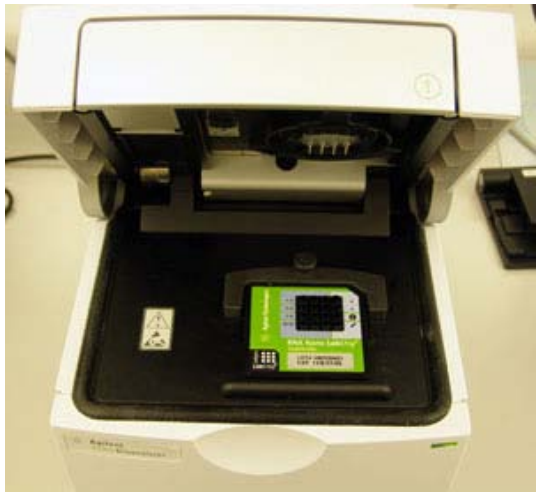
RNA purification

- RNeasy mini prep kit
  - Samples loaded onto column
  - Wash sample with buffers, centrifuge samples in between washes
  - Elute RNA from column using RNAse-free water and centrifugation

RNA quality control analysis

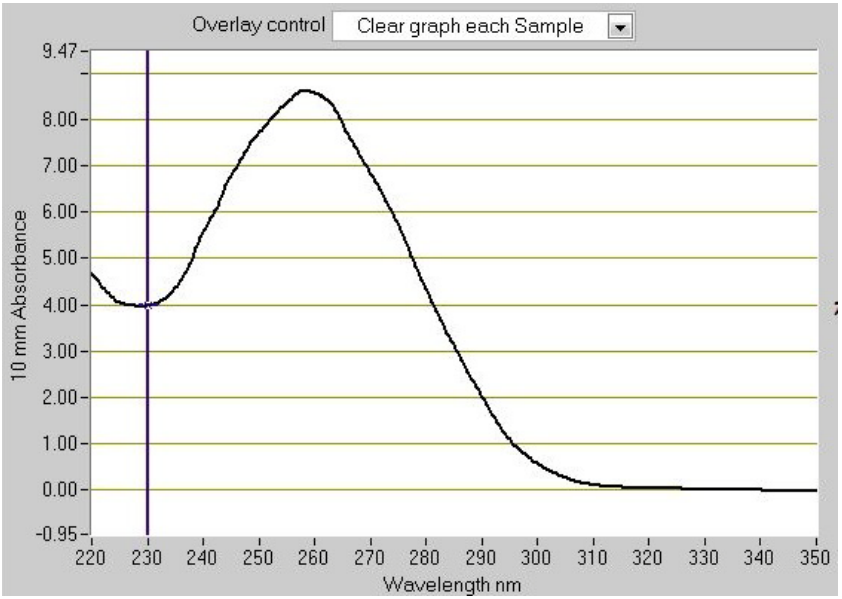
- Nanodrop Spetrophotometry
  - Nucleic acid concentration > 100ng/µL
  - 260/280 ratio ≥ 1.9
- Bioanalyzer analysis
  - RNA integrity analysis
  - RNA integrity number (RIN)
  - Scale of 1-10: 10 correlates with intact RNA
  - 18s and 28s rRNA





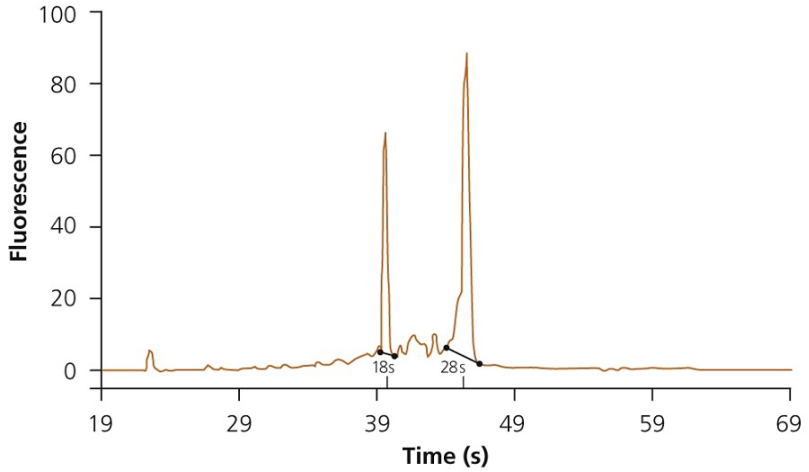
Results - Nanodrop

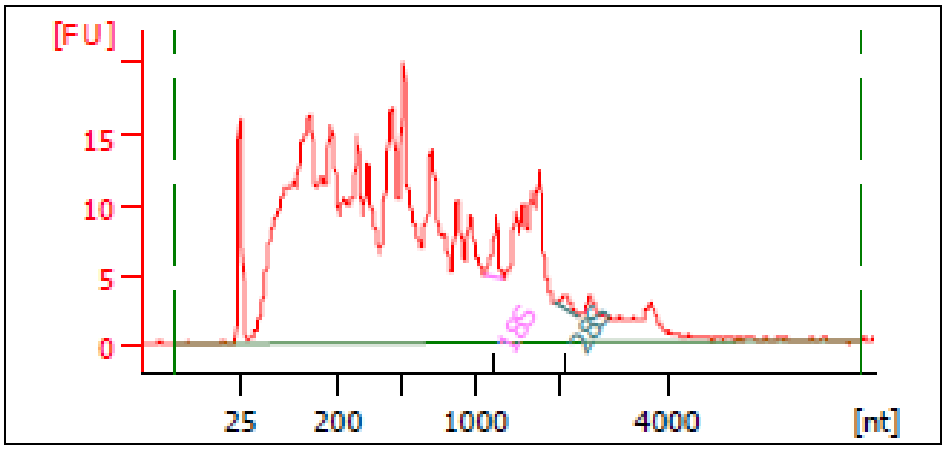
- 30 samples analyzed
  - 28 samples 260/280 ≥1.9
  - 2 ctrl samples < 1.9
- B[a]P samples < 100ng/ µL
  - Rotary evaporator



Results - Bioanalyzer

- 28 samples submitted
- RNA integrity numbers
  - range: 2.4-6.3
  - mean: 3.5
  - significant degradation





Possible sources of RNA degradation

- RNase activity during epidermal harvest
- Chemical degradation – NAIR
- Thermal degradation during heat treatment
- Contamination of stock solution
- Sample contamination during RNA extraction/purification

Cell harvest method modifications

- Dermal rather than epidermal cell harvest
  - Eliminate thermal degradation
- Forego NAIR application
- Snap freeze samples in liquid nitrogen

Dermal cell harvest method variations

group	1	2	3	4
animal preperation	none	none	shaved	shaved
cleaning solution	ethanol	ethanol	ethanol	RNA-zap
snap freezing conditions	no solution	no solution	Trizol	Trizol
thaw conditions	15-20 min no solution	Trizol	Trizol	Trizol

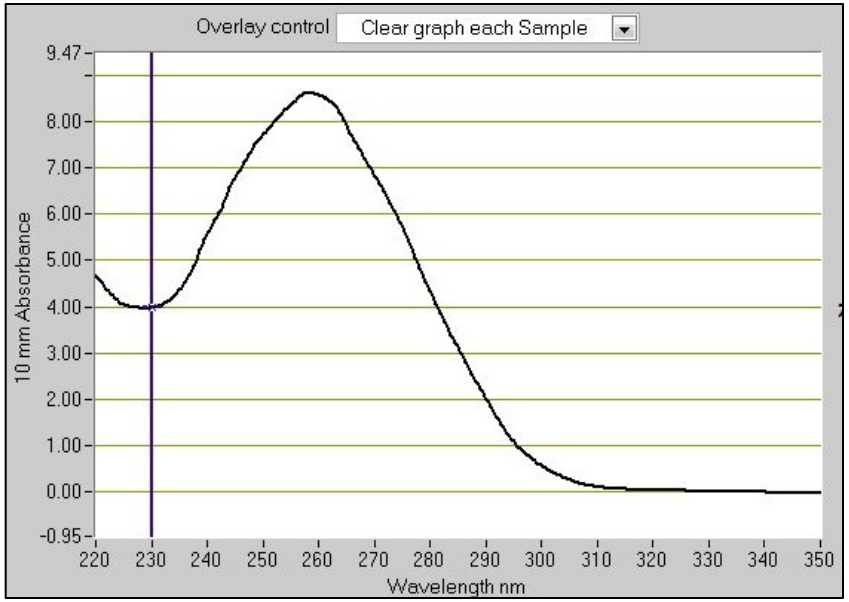
- Animal Preparation: shaved vs none
- Cleaning solution: ethanol vs. RNA-zap
- Snap freezing conditions: no solution vs. Trizol
- Thaw conditions – 15-20 min no solution vs. Trizol

RNA extraction

- Samples homogenized
  - 2 aliquots taken from each sample
- RNA separated from DNA and protein using chloroform and centrifugation
- Ethanol washes

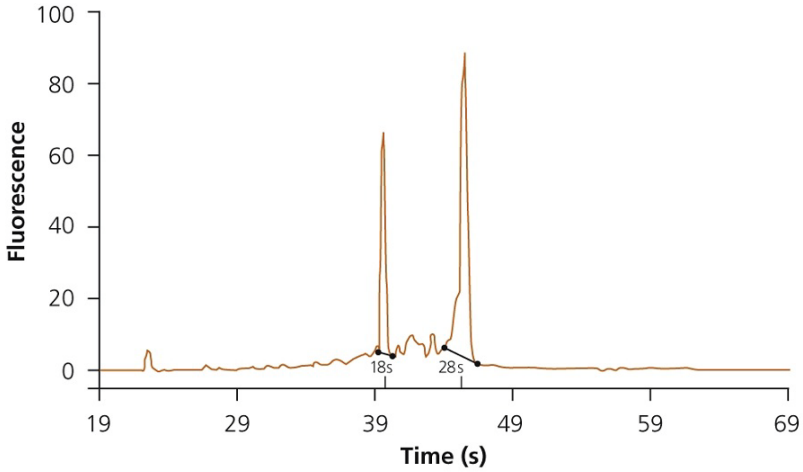
Results - Nanodrop

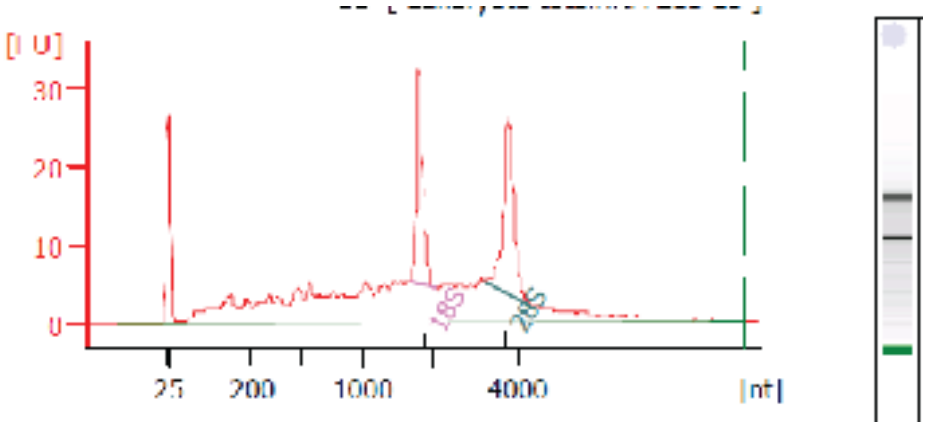
- 12 samples
- 9 samples 260/280 ratios ≥ 1.9
  - 2 samples from group 2 < 1.9
    - Limited analysis
  - 1 sample from group 3 <1.9



Results – Bioanalyzer

- 11 samples submitted
- 6 RNA integrity numbers
  - range: 6.3 - 9.2
  - mean: 7.6





G2S1A1

Discussion

Introduction to PAHs

- Structure and properties
- Mechanisms of toxicity
- Regulation and risk assessment

Method development and results

- Objectives
- Initial method and results
- Modified method and results

Discussion

- Analysis of results
- Comparison of methods

Conclusions, future directions

Interpreting RIN

Minimum RNA integrity levels

- No improvement RIN > 5 (Mueller *et al*. 2004)
- RIN ≥ 7 widely accepted
- RIN ≥ 6 widely accepted with RT-qPCR follow up

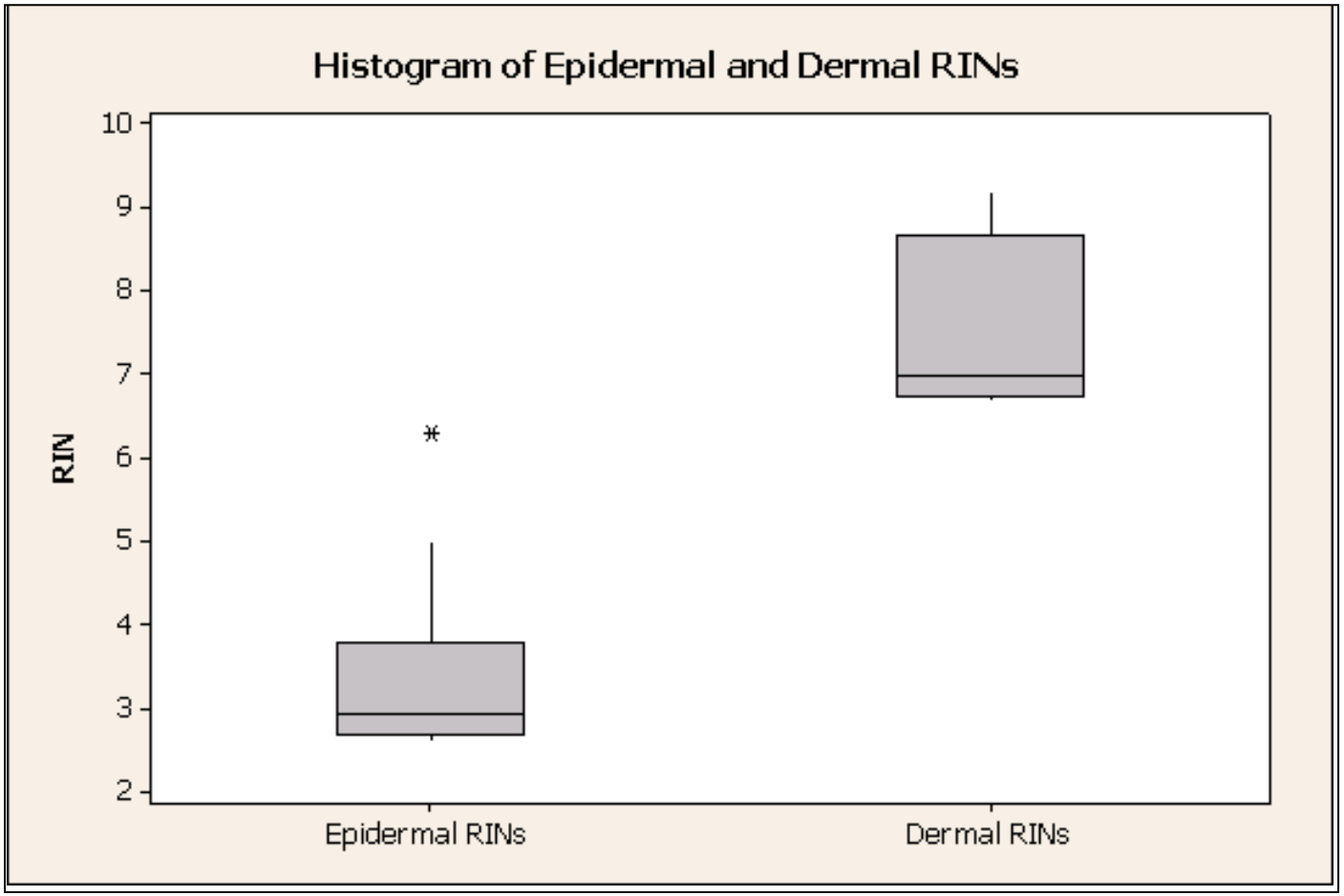
Epidermal RNA samples

- 1 out of 28 RIN ≥ 6

Dermal RNA aliquots

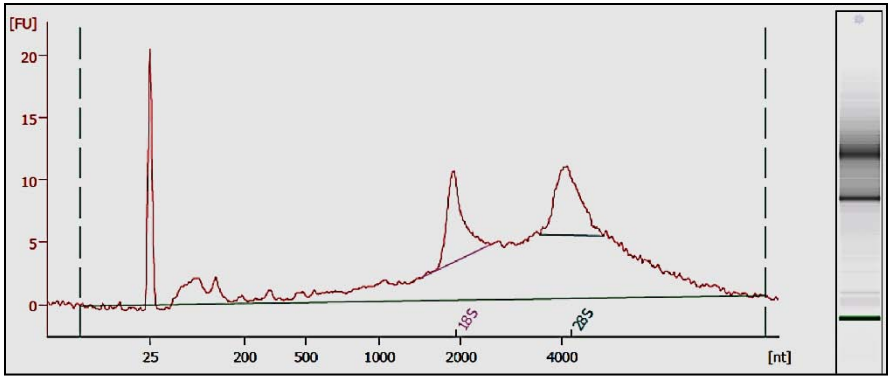
- 6 out of 6 RIN ≥ 6

Comparing epidermal and dermal RIN

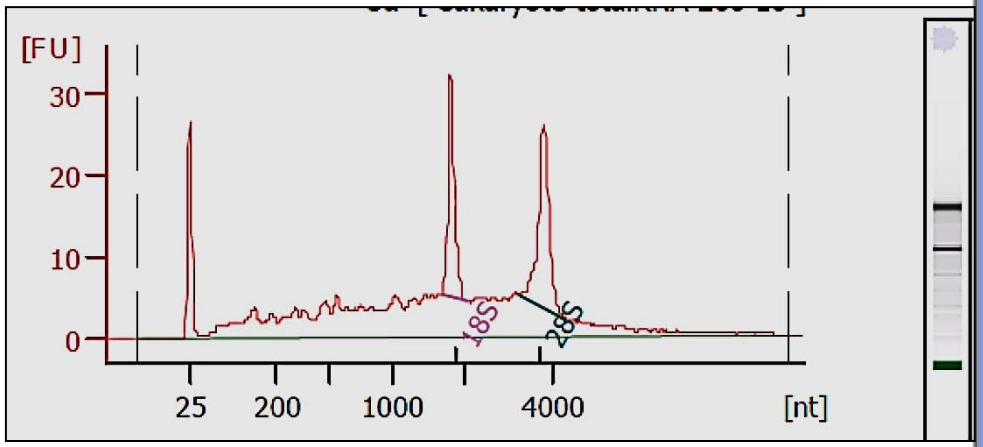


Statistically significant difference between epidermal and dermal RINs ($p = 0.005$)

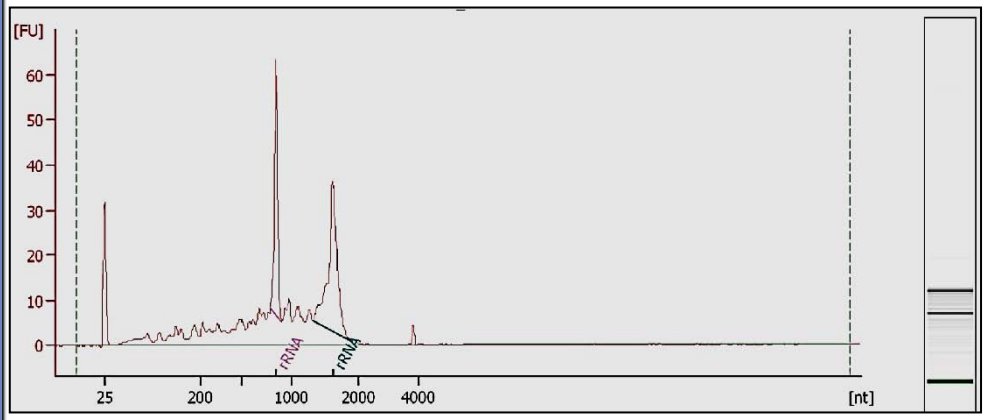
Comparing dermal method variations



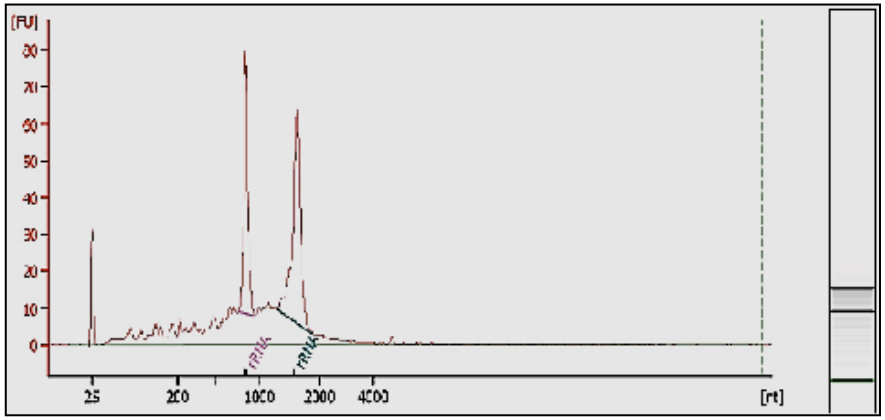
Group 1



Group 2

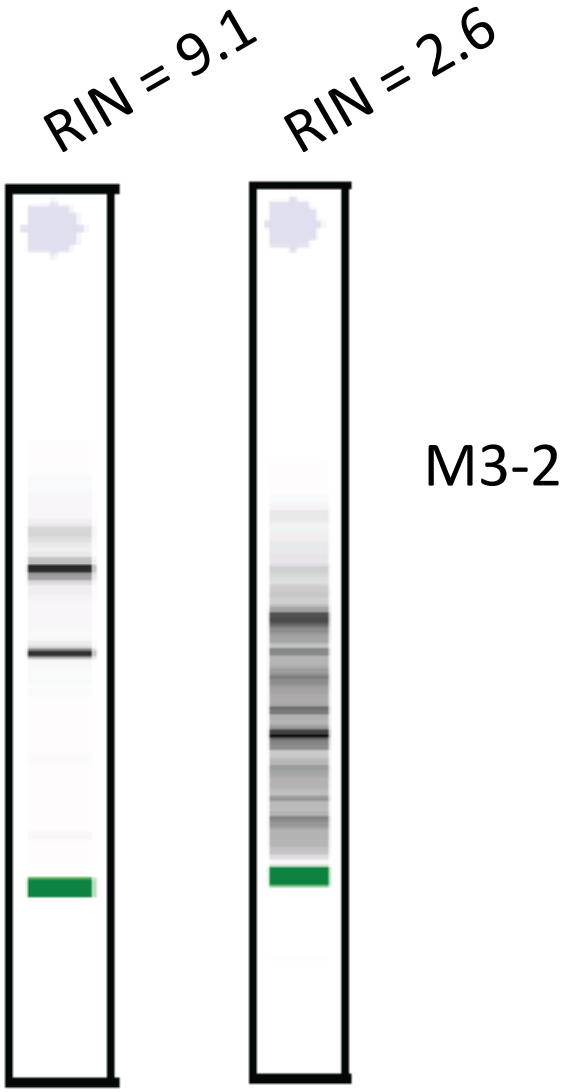


Group 3

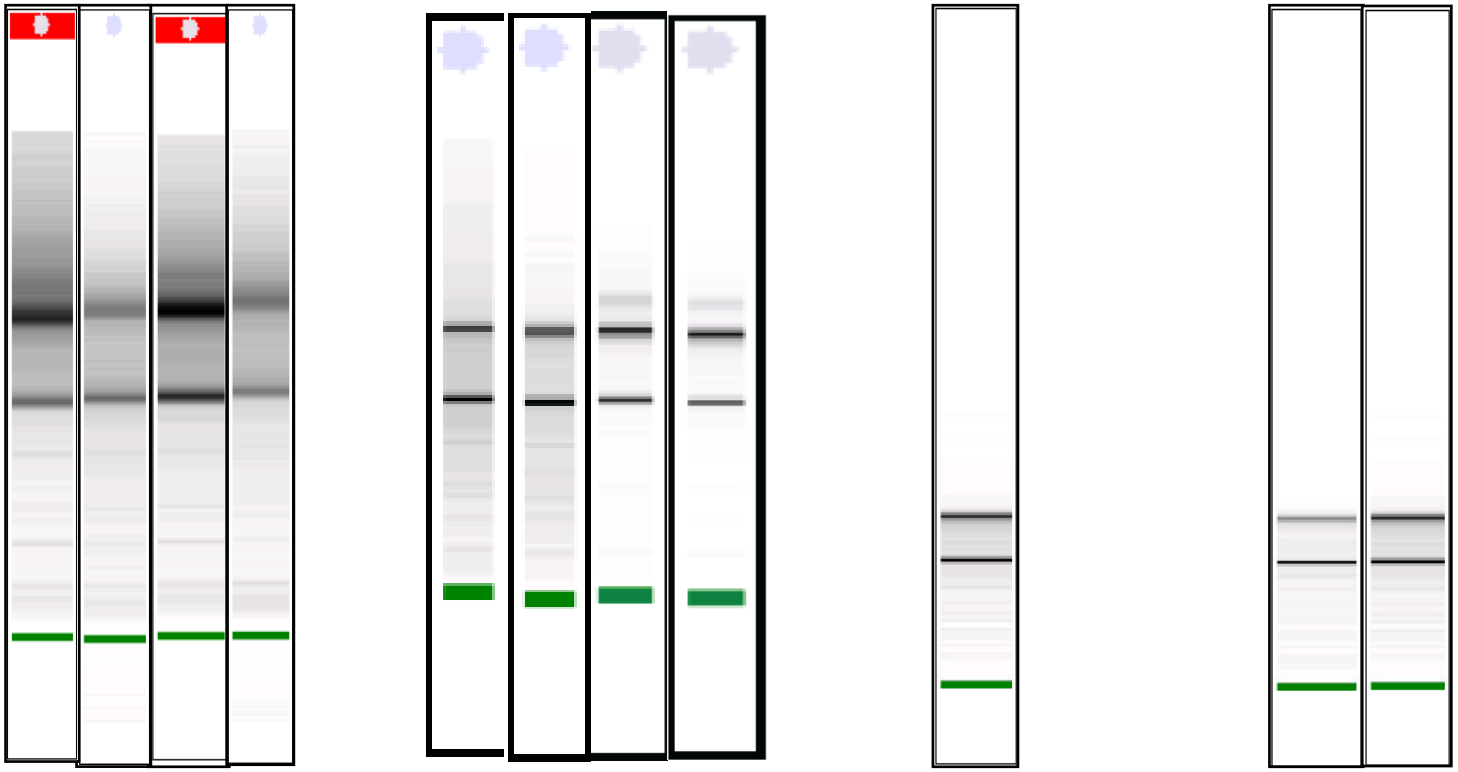


Group 4

Comparing dermal method variations



Comparing dermal method variations



Group 1 < Group 2 ≤ Group 3 = Group 4

Epidermal vs. dermal RNA

Epidermal RNA

- Detect small fold changes in gene expression in the epidermal layer
- Does not elucidate changes in surrounding tissues
- More difficult to harvest intact RNA

Dermal RNA

- Gene expression in the entire dermal layer
- Established methods to harvest intact RNA
- Miss small fold changes in gene expression in epidermis

Conclusions, future directions

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Discussion

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Conclusions, future directions

Conclusions

Initial attempts to harvest epidermal RNA from mice resulted in degraded RNA samples (RIN mean 3.5)

Sources of RNA degradation were identified, including RNase activity and heat treatment

Method to harvest dermal RNA produced dermal RNA samples with acceptable integrity (RIN mean 7.6)

Rnase activity contributes to epidermal and dermal RNA degradation - storing samples in Trizol limits RNA degradation

Future directions

Epidermal RNA harvesting

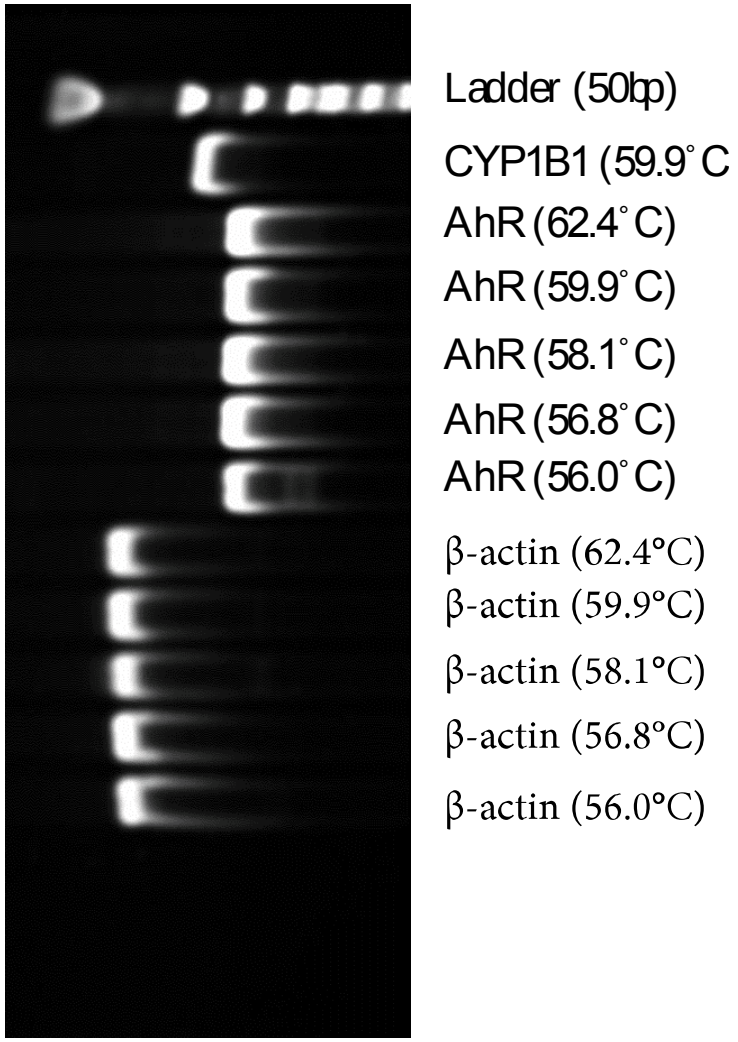
- Ammonium thiocycanate

RT-qPCR analysis of B[a]P-induced genes

- CYP1A1, CYP1B1, AhR

Protein expression in skin tumors

- CYP1A1, CYP1B1, AhR



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