EPA Transcriptomic Assessment Product Study Overview

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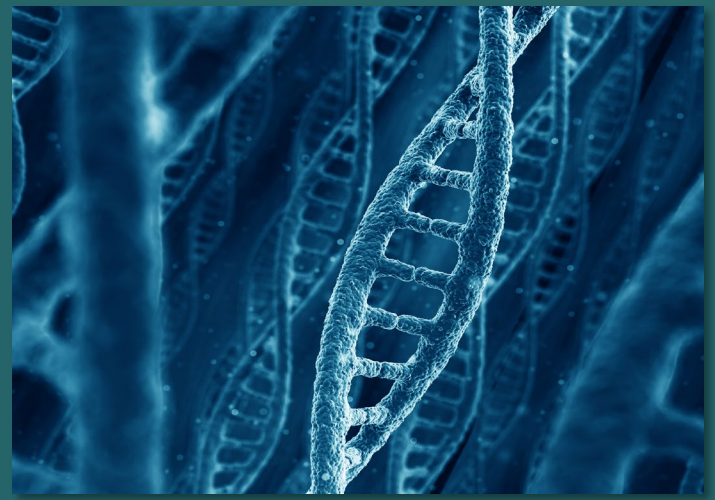

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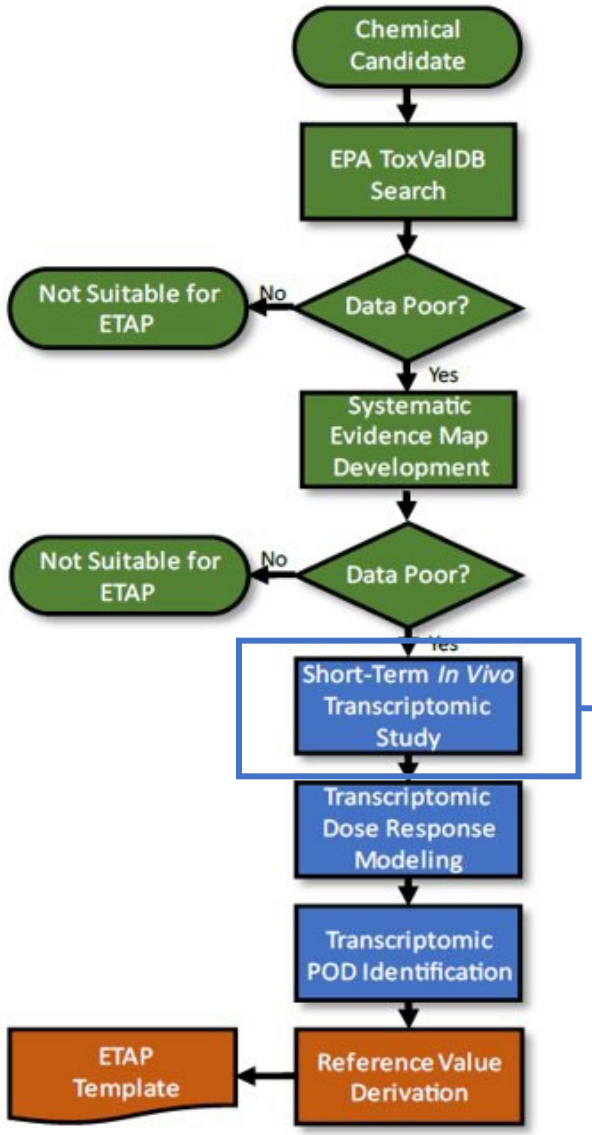
Outline

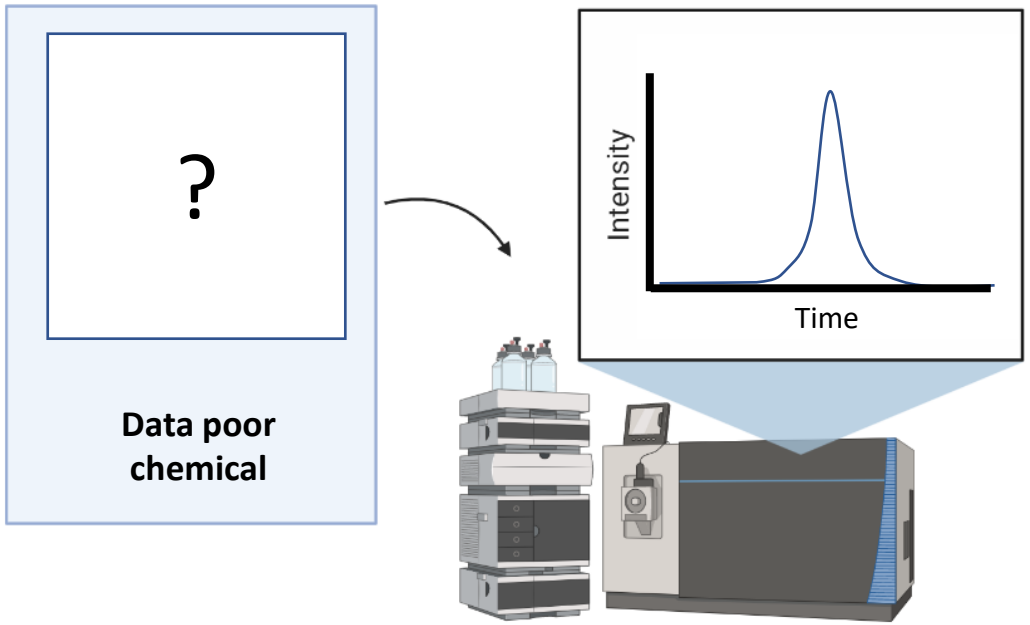


- Analytical chemistry
- Dose formulation and selection
- *In vivo* study design
- Tissue collection
- RNA isolation and sequencing

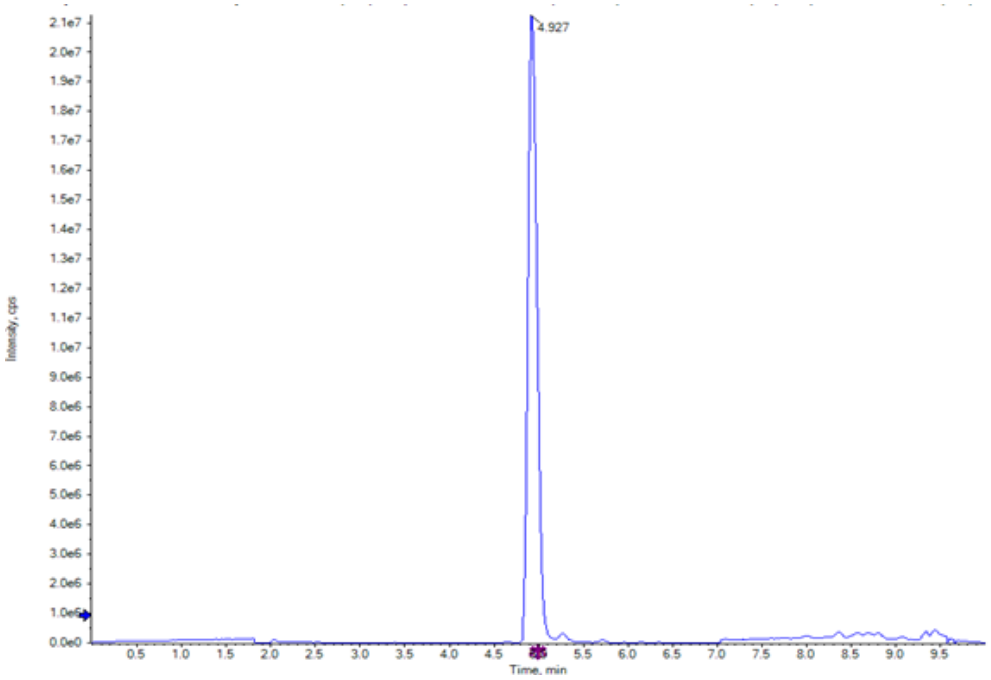
1. Confirm chemical purity at ≥ 95%

if purity <95%, document purity accordingly

Procure commercially, by synthesis, or reliable third party



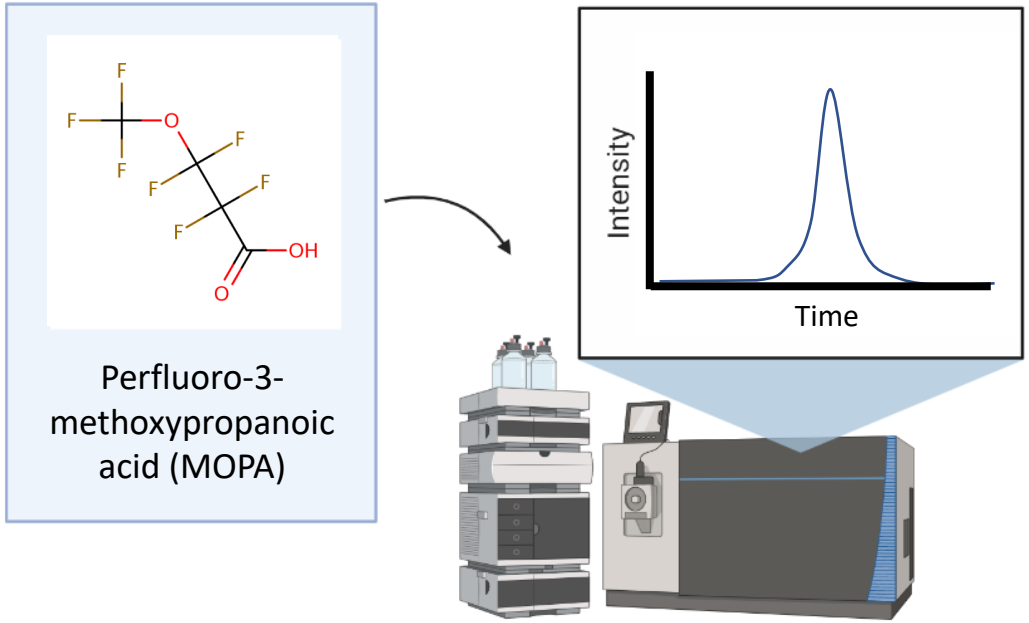
Verify purity by appropriate analytical method



1. MOPA purity verified at >98% by LC/MS/MS

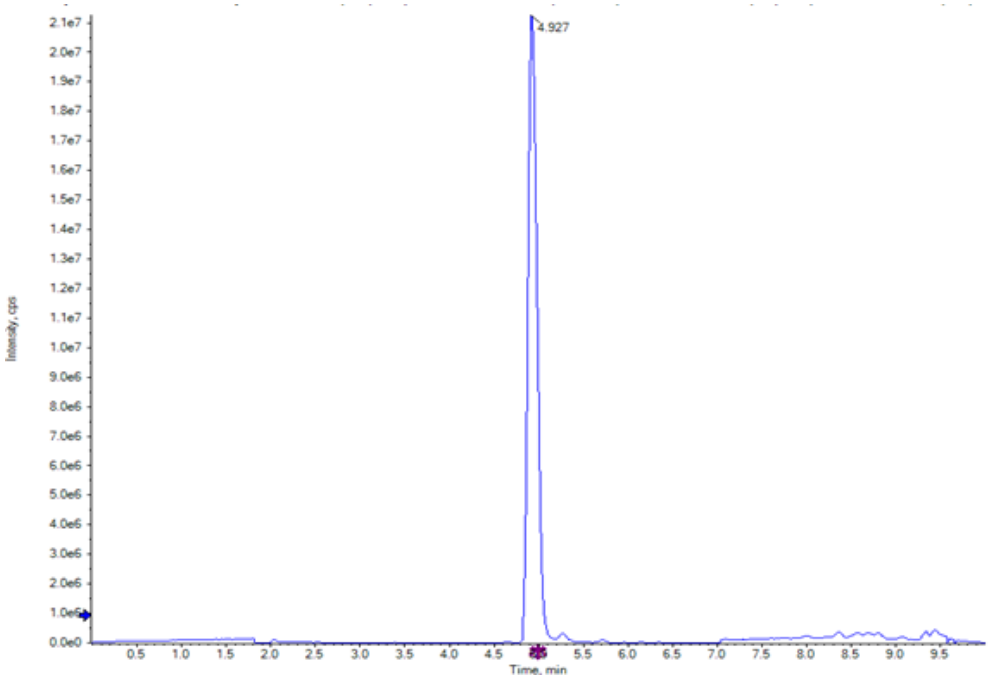
if purity <95%, document purity accordingly

Synquest Laboratories Inc.



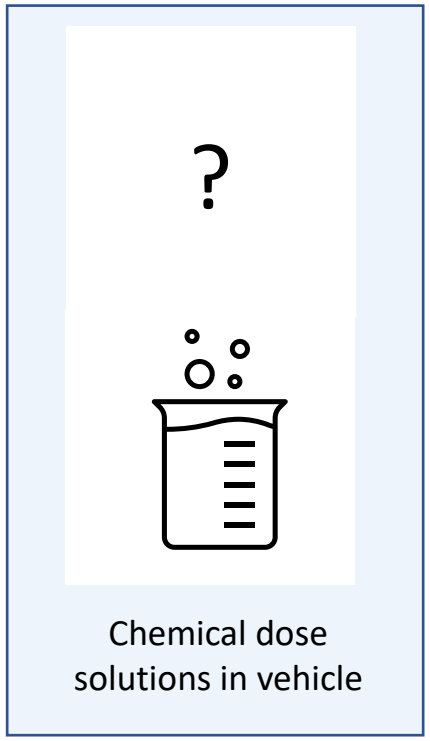
Perfluoro-3-methoxypropanoic acid (MOPA)

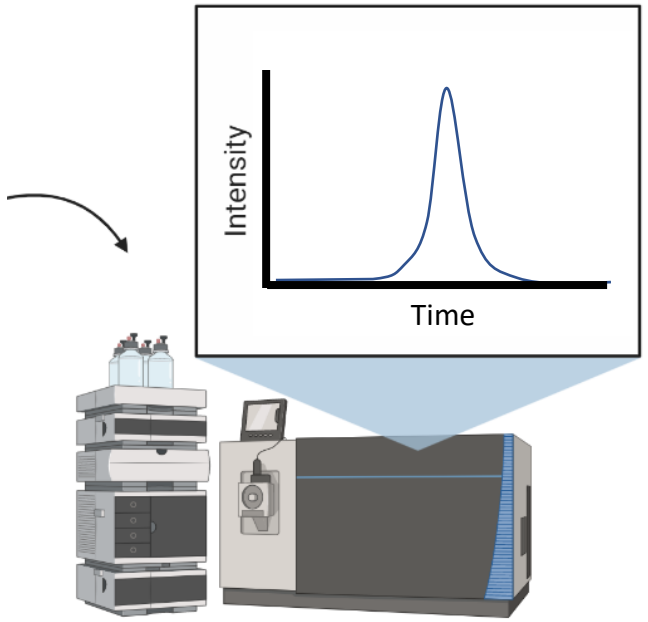
Verified purity by LC/MS/MS



Total ion chromatogram of a 100 µg/mL perfluoro-3-methoxypropanoic acid standard used for purity confirmation.

1. Assess Dose Formulation and Dose Selection

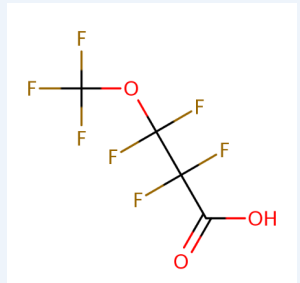




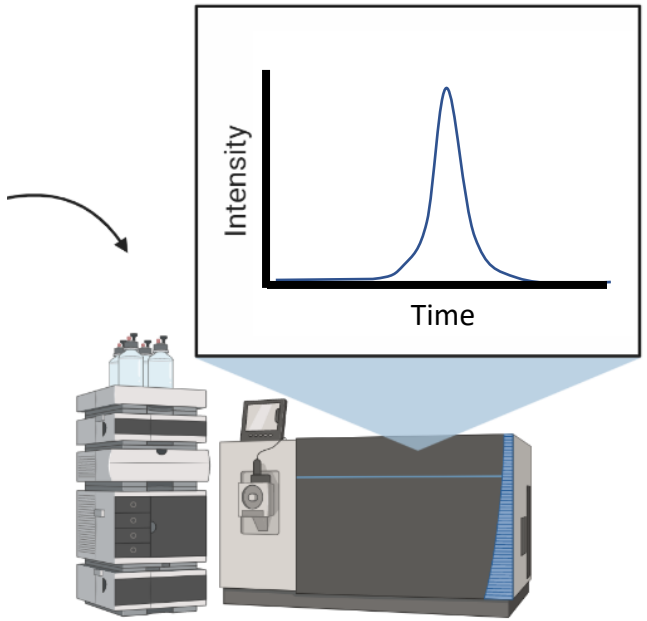
Vehicles: deionized (DI) water, DI water with ≤2% Tween® 80, corn oil, *etc.*

- Assess solubility and stability visually or analytically in vehicle
- Set high dose based on rat tolerability test or *in silico* prediction
- Recommend ≥ 5 dose-levels
- Use half-log spacing except the lowest positive dose, full log lower
- Test pH of aqueous solution

1. <10% Loss of MOPA in Dose Solutions Over Five Days



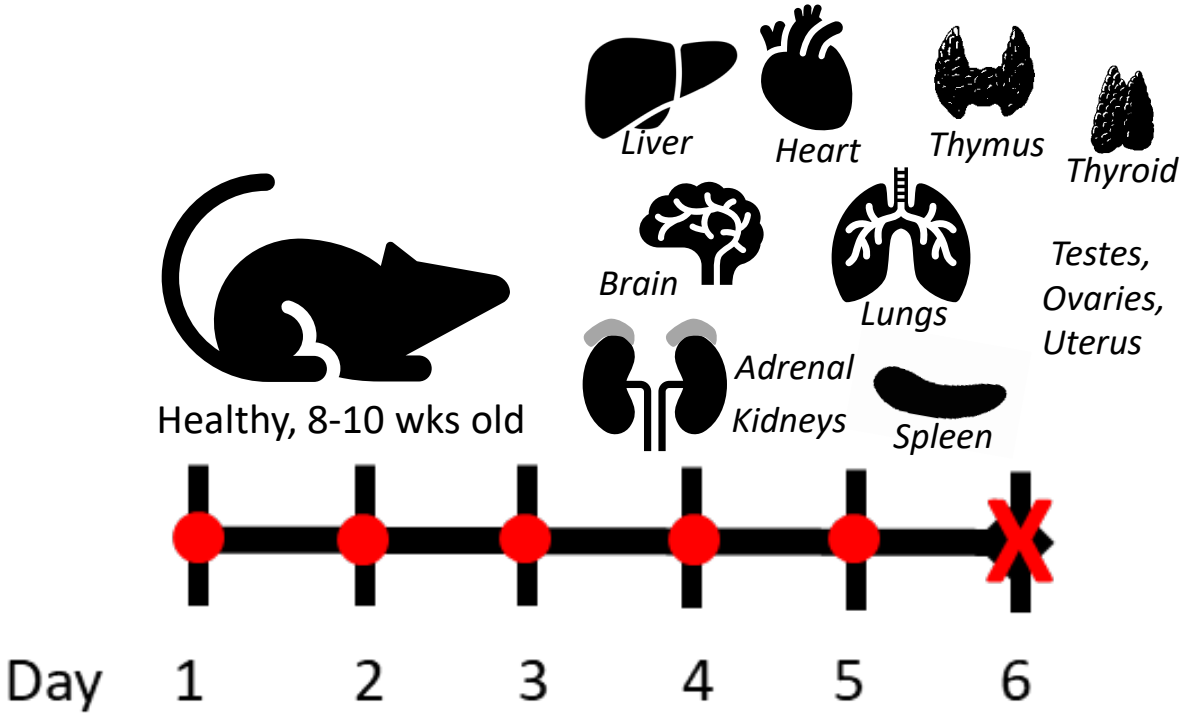
MOPA dose solutions in deionized water



Stability assessed by LC/MS/MS

- MOPA soluble in deionized water
- 60 mg/ml set highest concentration = 300 mg/kg
- Selected nine dose-levels 0.01, 0.1, 0.3, 1.0, 3.0, 10.0, 30.0, 100.0, 300.0 mg/kg-day
- pH adjusted to 4.5

2. Five-Day Repeated Dose Study



Housing conditions

- Water and food ad libitum
- Temperature 22°C (± 3°C)
- Relative humidity ideally 50-60%; not outside 30-70%
- Artificial 12 hours light, 12 hours dark
- 1-3 animals per cage
- Association for Assessment and Accreditation of Laboratory Animal Care accreditation required
- Follow Public Health Service animal care and use guidelines

Day -14 to 0

- Acclimatize
- Randomize
- At least 4 rats/sex/vehicle and dose level

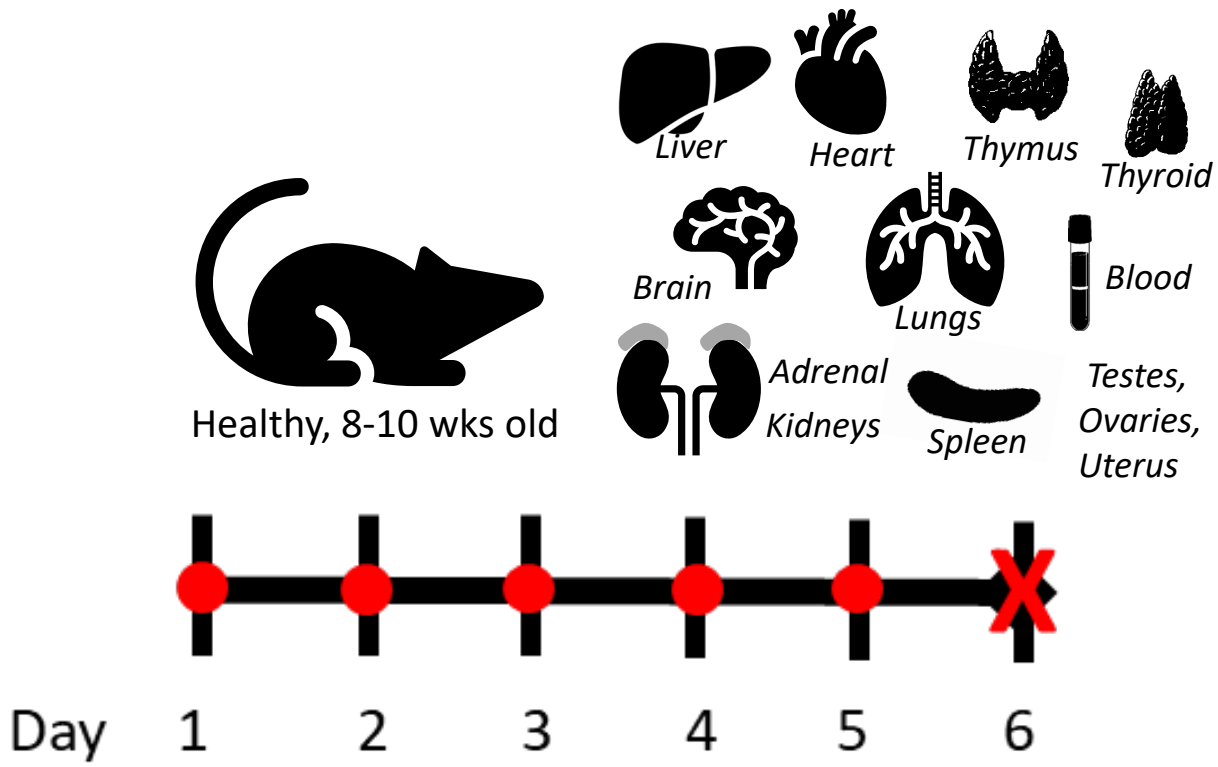
Day 1 to 5 ●

- Record body weights daily
- Oral gavage at 5 to 10 ml/kg
- Record morbidity and mortality twice daily
- Remove rats showing overt toxicity

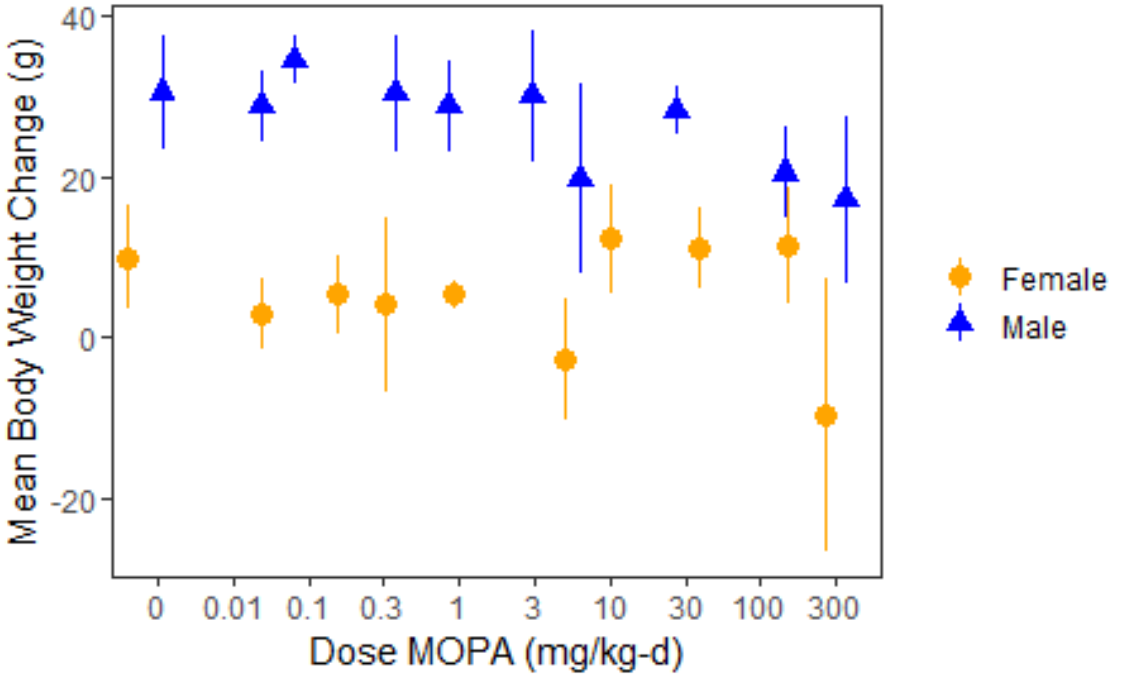
Day 6 X

- Record body weights
- Euthanize
- Cube (~5 mm^3) and collect 2 aliquots
  - 1 snap freeze, 1 RNALater

2. MOPA Five-Day Repeated Dose Results

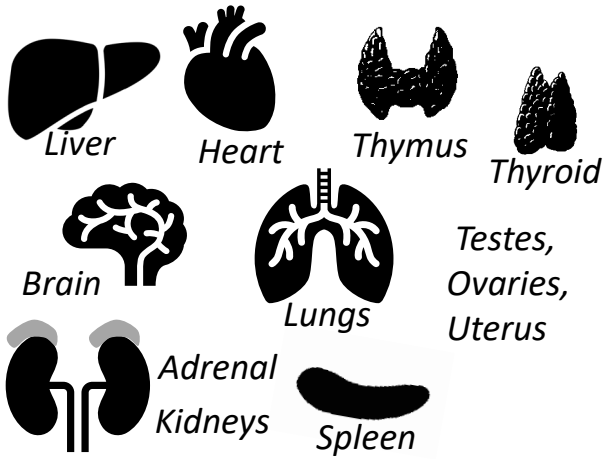


- Collected 3 aliquots
  - 2 RNALater, 1 snap frozen
- Collected 6 aliquots of blood

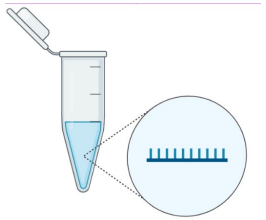


- n=5/sex/dose-level and 8/sex/vehicle
- Observations
  - 1 male and 1 female (30 mg/kg-day) and 1 female (300 mg/kg-day) died
  - 1 female (300 mg/kg-day) had abnormal breathing
  - Another female (300 mg/kg-day) had rales

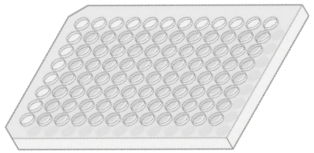
3. Aliquot Tissue, RNA Isolation/Purification, and Sequencing

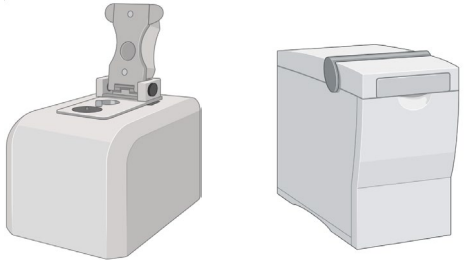


RNALater aliquot

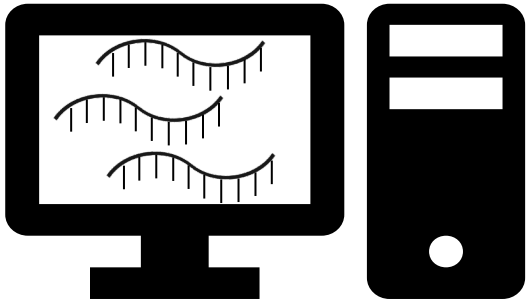


RNA isolation





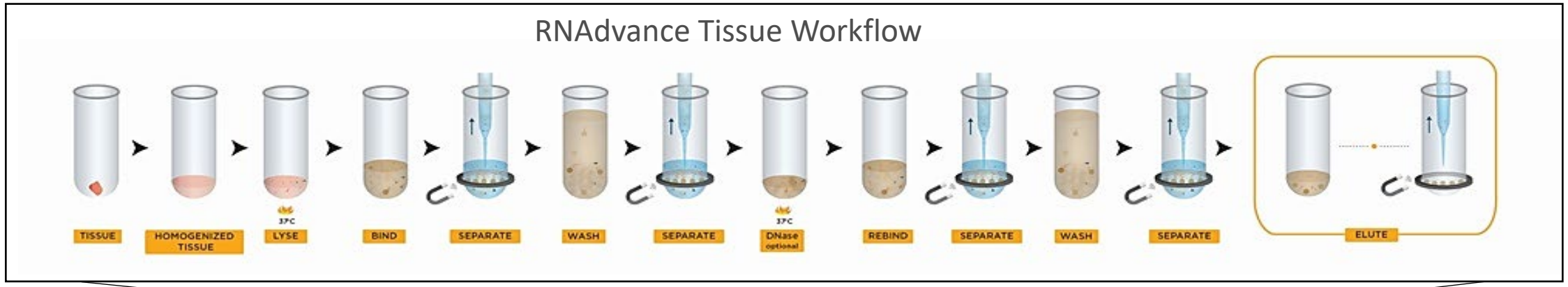
Evaluate RNA quantity & purity

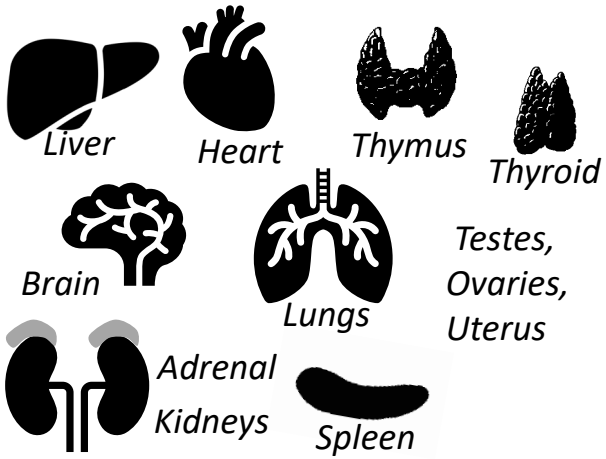


Targeted RNA-sequencing
BioSpyder TempO-Seq rat S1500+
According to manufacturer's directions
≥ 1 million mapped reads/sample

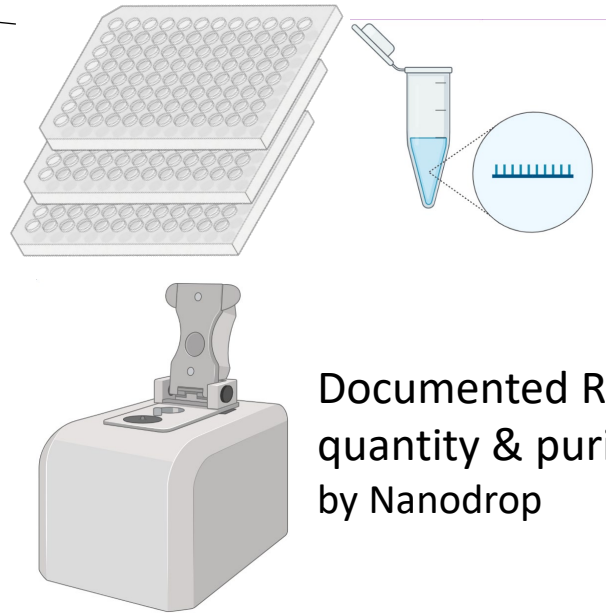
Note: No specific RNA purity or integrity criteria are applied

3. MOPA Tissue Preparation, RNA Isolation/Purification, and Sequencing

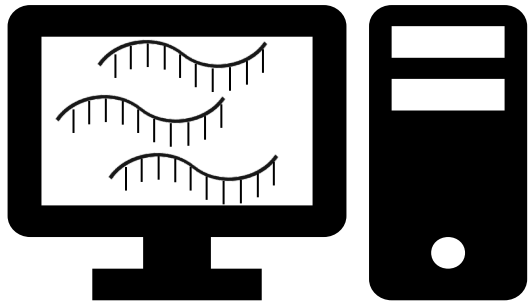




RNALater aliquot



Documented RNA quantity & purity
by Nanodrop



Targeted RNA-sequencing
BioSpyder TempO-Seq rat S1500+
≥ 1 million mapped reads/sample

Summary

- A standardized design was developed for the 5-day *in vivo* studies for the ETAP.
- As an example, MOPA was commercially acquired, and its purity verified at >98% by LC/MS/MS.
- MOPA solubility in deionized water was verified by LC/MS/MS with <10% loss over 5 days.
- MOPA was formulated in deionized water and delivered daily to male and female rats via gavage at nine dose levels for 5 days.
- Following 5 days of exposure, twelve tissues were removed, RNA isolated, and targeted RNA sequencing performed using the BioSpyder TempO-Seq rat S1500+.