Transcriptional biomarkers for chemical hazard screening: From gene sequence to biology

Bhaja K. Padhi and Guillaume Pelletier

Hazard Identification Division, Environmental Health Science and Research Bureau, HECSB, Health Canada, Ottawa, ON, Canada

"Science has no homeland, because knowledge is the heritage of humanity."

Louis Pasteur

1. Chemical Toxicity Testing: The Changing Strategies

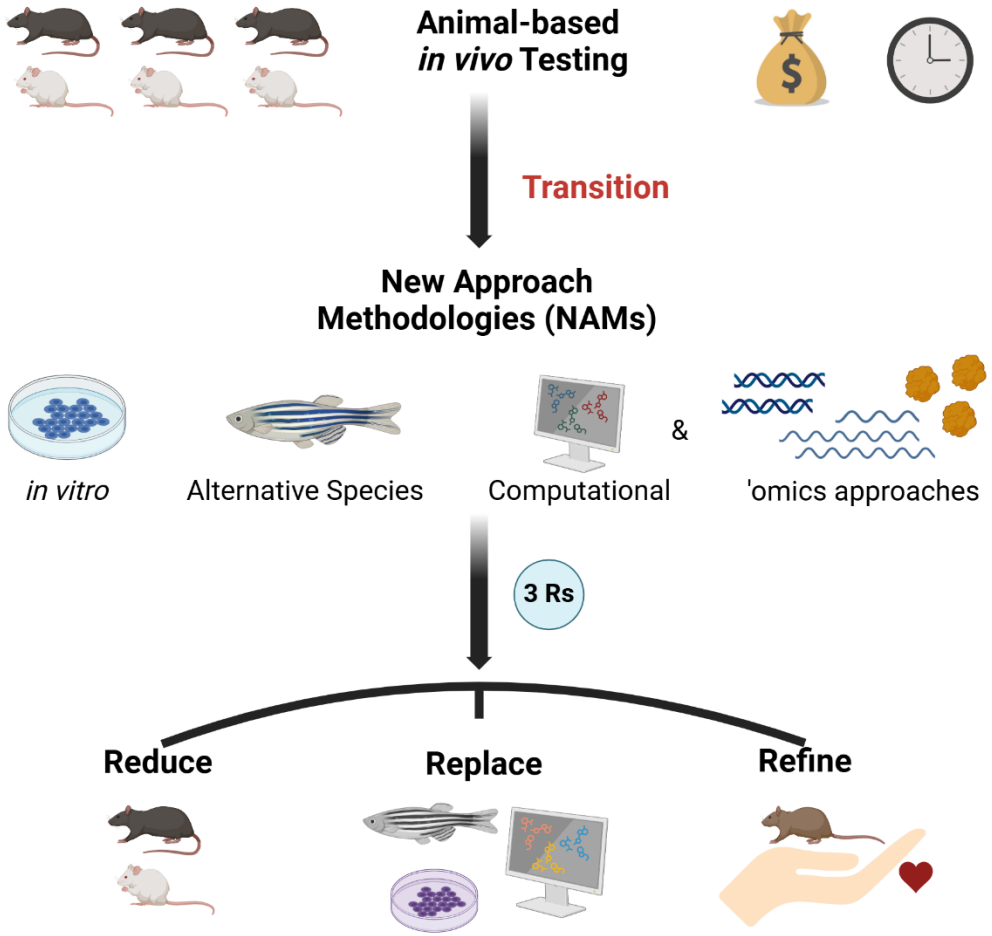


Figure 1

- Toxicity testing is transitioning from animal-based *in vivo* studies into the development of "New Approach Methodologies" (NAMs).
- Many regulatory agencies world-wide are promoting the use of NAMs, and adoption of omics-based approaches including **gene expression (mRNA) biomarkers** for assessing the biological effects of chemical exposure.
- Our laboratory aims to implement NAMs-based approach that includes *in vitro* system and mRNA biomarkers as the readout for expediating screening chemicals for their toxicity potential.

2. BACKGROUND

- Gene expression, primarily measured by mRNA levels, can indicate different cellular states and provide valuable insights into the biological functions and molecular pathways affected by toxicants.

- Measuring mRNA levels can offer practical and standardizable molecular readouts to assess potential perturbations resulting from chemical exposure.

- Studies show that changes in gene transcript (mRNA) level can occur before actual adverse effects manifest, with substantial evidence linking gene expression alterations to adverse outcomes (EPA, 2024).

2.1. What is a Biomarker? Why Measure mRNA Biomarkers?

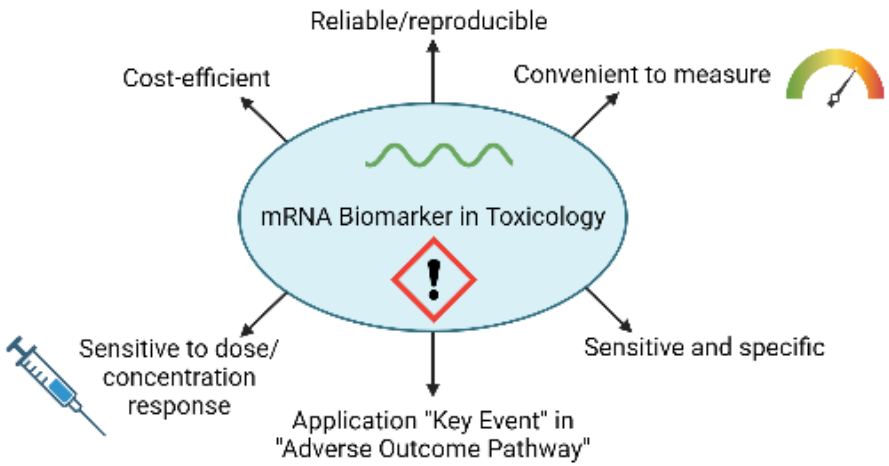
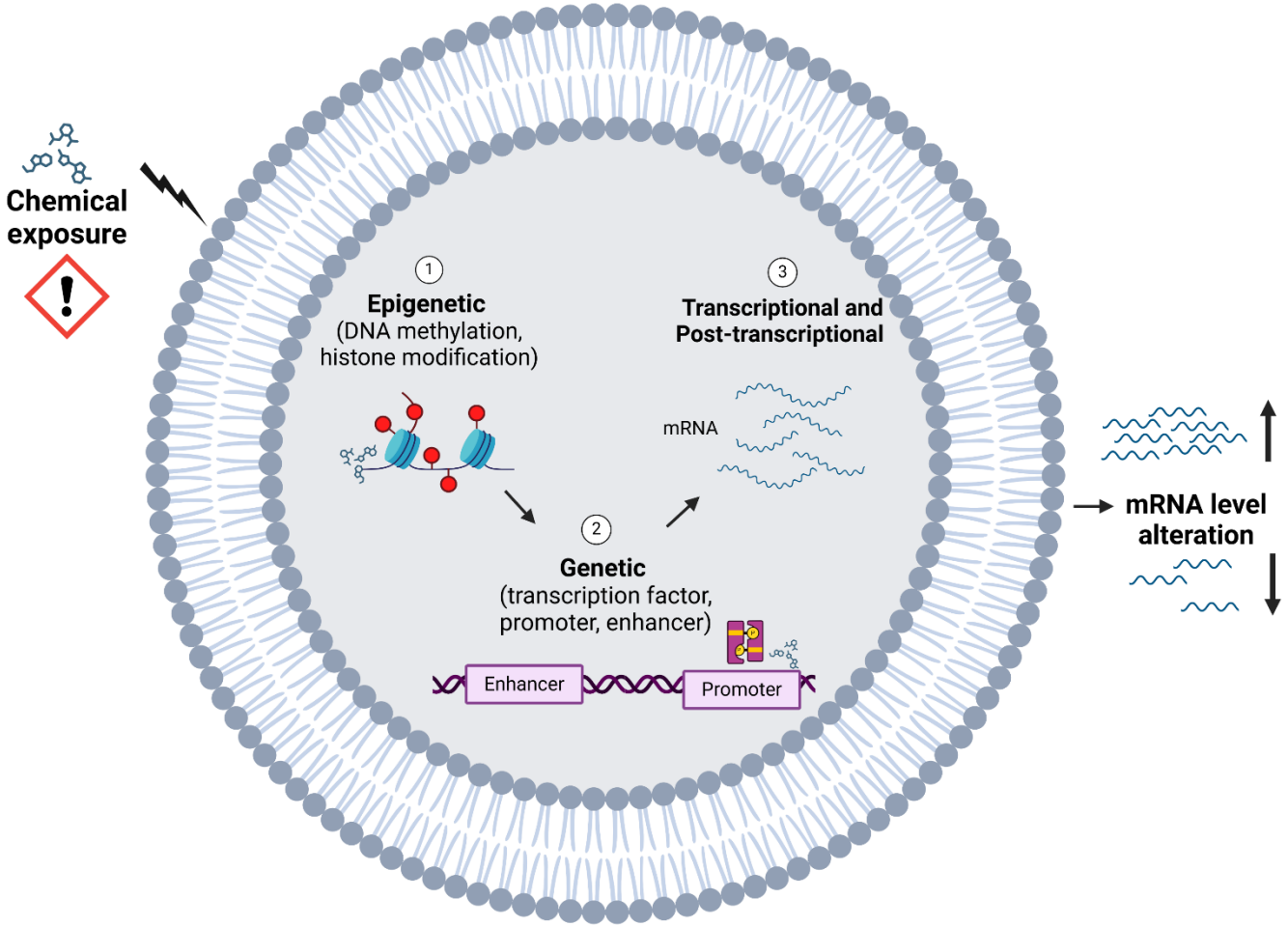


Figure 2 Utility of mRNA biomarkers

- Biomarkers are quantifiable indicators of both normal and disrupted biological activity.
- mRNA biomarkers are advantageous because they are convenient to measure, cost-effective, sensitive to dose and concentration responses, among other benefits.
- *However, the application of gene expression data in regulatory decision-making has been relatively limited to date.*
- Our overall goal is to characterize and develop translational mRNA biomarkers for screening the developmental neurotoxicity (DNT) potential of chemicals in rat primary neuronal cells differentiating *in vitro* (Padhi et al., 2022).

2.2. What Happens After Chemical Exposure at Molecular Level?



- Chemical exposure can impact different layers of gene regulation activity: epigenetic, genetic or gene transcription.
- Technically speaking the alteration in biomarker gene's mRNA level is most amenable to measurement.
- The decreased mRNA level is called down-regulation (↓), and increased expression is called up-regulation (↑).

Figure 3: A simplified view of complex molecular processes underlying toxicant-induced alteration of mRNA levels

2.3. How Do We Measure Alteration in Gene Transcript Levels: Top-down vs Bottom-up Approaches

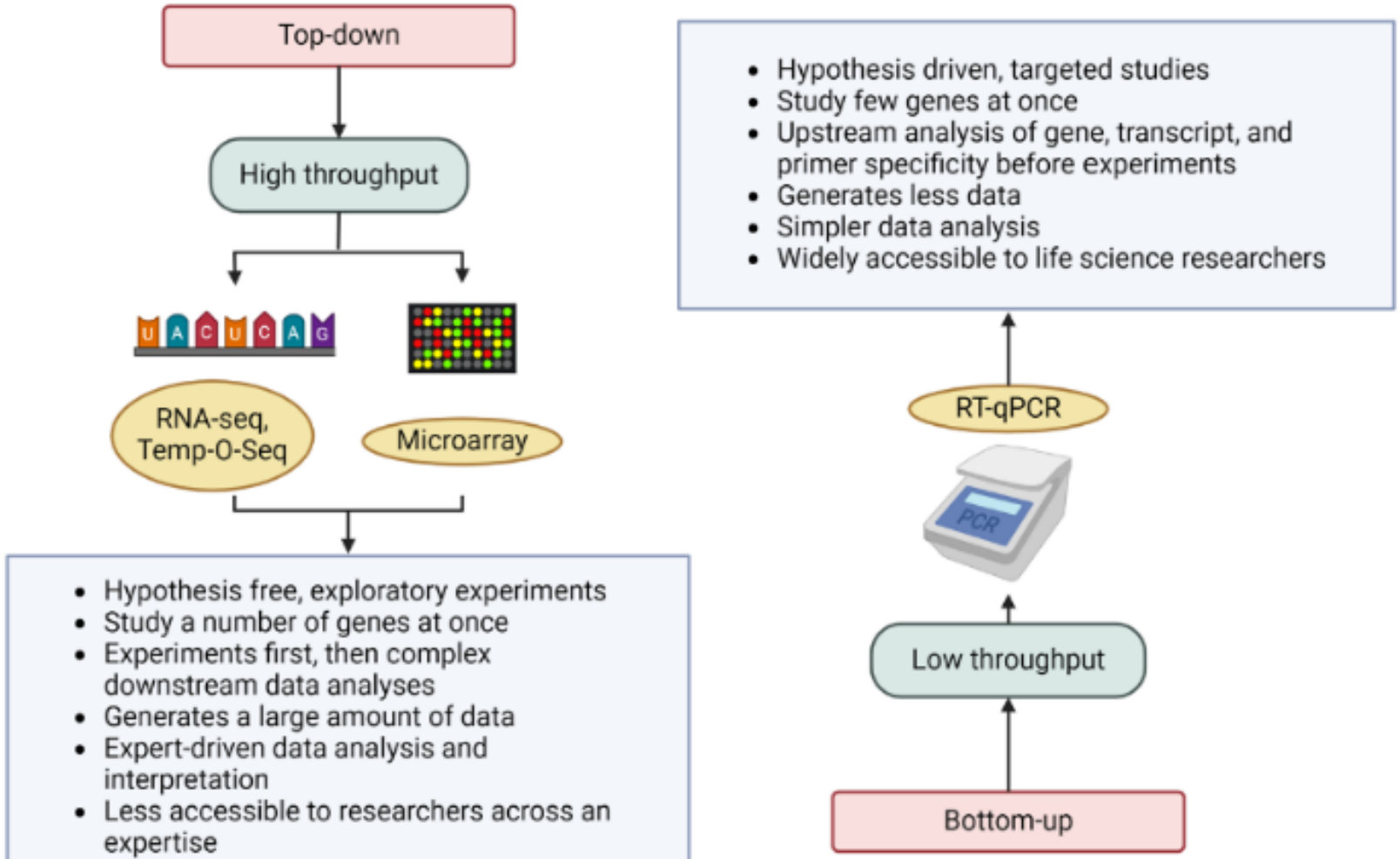


Figure 4

These two gene expression measurement methods are fundamentally different, but they complement each other

2.4. mRNA Biomarker Development and Characterisation for Chemical Toxicity Screening

- We hypothesize that a small set of mRNA biomarker genes could be valuable for screening the potential chemical toxicity in cell-based assays using a bottom-up approach.
- However, developing reliable and reproducible mRNA biomarkers can present significant challenges.
- To address this, our laboratory developed a **scientific framework for the development and characterisation** of messenger RNA (mRNA) biomarkers.
- This presentation aims to provide methodological approaches and strategies for developing and characterising mRNA biomarkers using evidence from our published studies and scientific literature to screen chemicals for developmental neurotoxicity potential.

2.5. KEY CHALLENGES

There are several key challenges of developing mRNA biomarkers for toxicological assays:

Challenge 1: Incomplete annotation of splice variants

Challenge 2: Comparability of mRNA biomarker data between rodents and humans

2.5.1. Challenge 1: Alternative Splicing

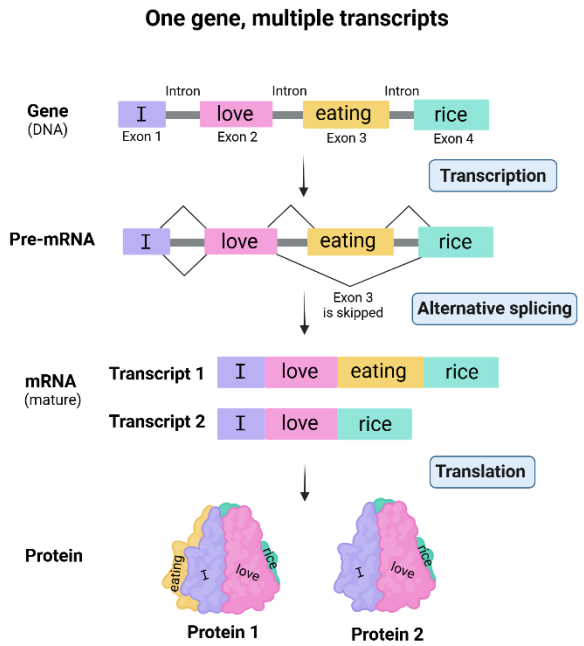


Figure 5: One gene can generate multiple distinct mRNAs by alternative splicing.

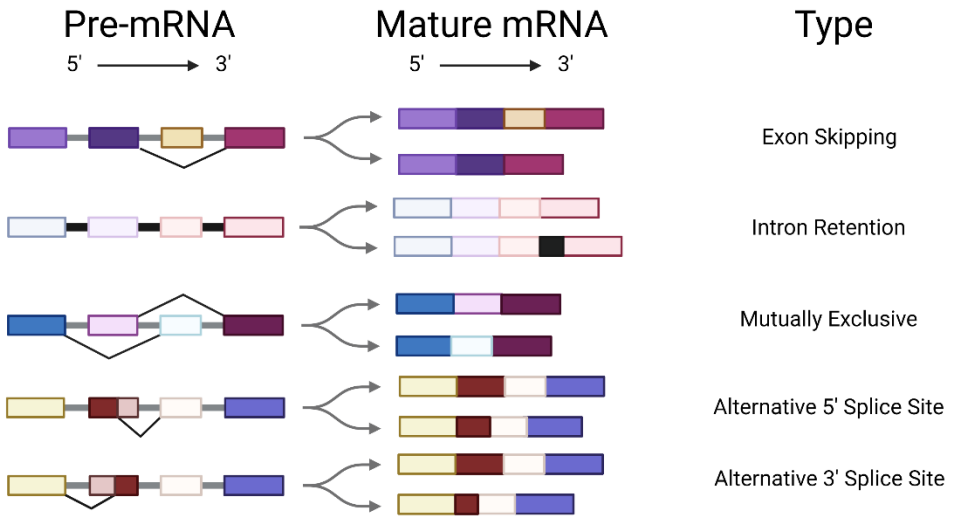


Figure 6: Splice variant types

- Gene splice variant annotation is far from complete, although continuously improving in major public genomic databases such as NCBI and Ensembl (Padhi and Pelletier, 2024).
- Unannotated splice variants can interfere with reliable mRNA biomarker measurement.

2.5.2. Challenge 2: Inter-species Data Comparability

- There are morphological, genetical, and behavioural differences between rodents and humans. Thus, extrapolating toxicology data from the rodents to humans is challenging.
- However, there is a commonality on certain biological processes which are evolutionarily conserved across rodents and humans (Fig 3).
- The use of biomarker genes linked to evolutionarily conserved biological processes can enhance comparability of gene expression data across different species (Padhi et. al., 2022; Padhi and Pelletier, 2024).

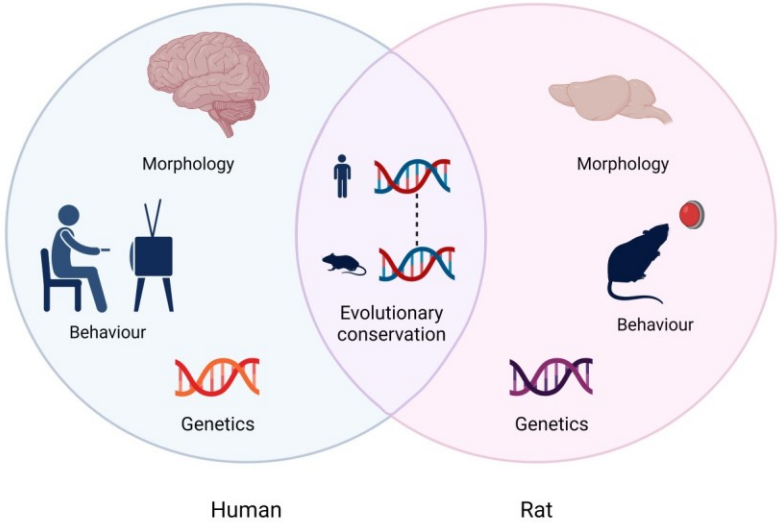


Figure 7: Evolutionary conservation is the core principle of mRNA biomarker gene selection.

3. METHODOLOGY

Principles and framework of mRNA biomarker development and characterisation

3.1. Framework for mRNA Biomarker Development and Characterisation

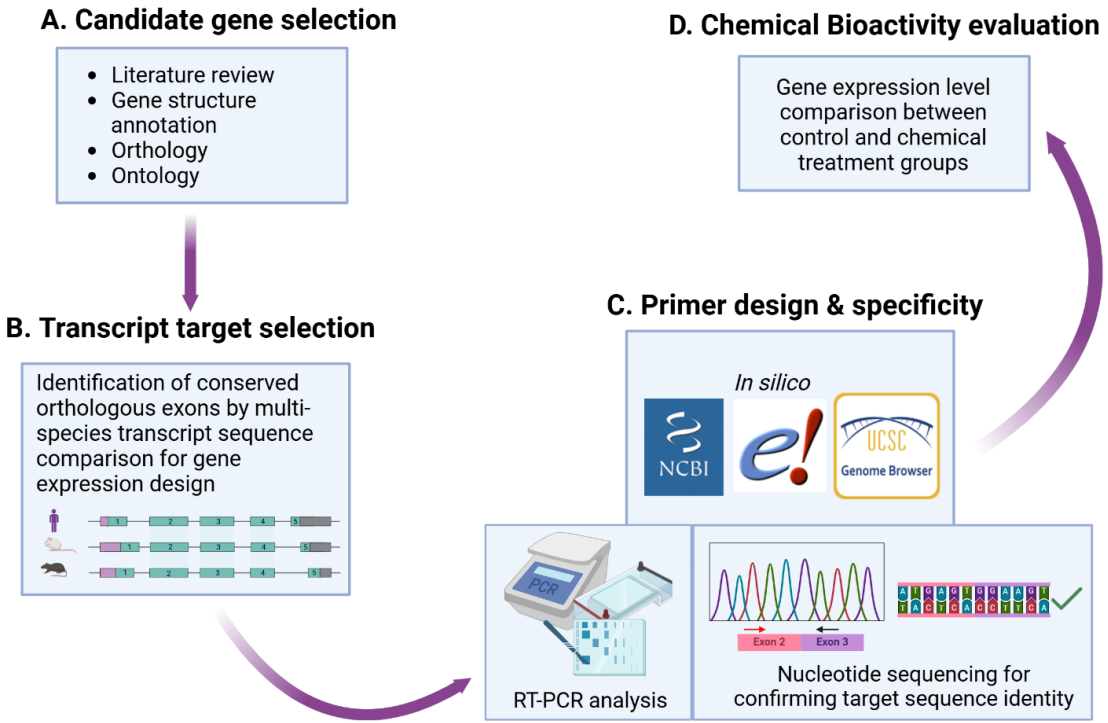


Figure 8: Identification and characterisation of biomarker genes and target sequences: An overview (Padhi *et al.*, 2022; Padhi and Pelletier, 2024).

3.1.A. Gene Orthology and Ontology Constitute the Fundamental Theme of Candidate Gene Selection

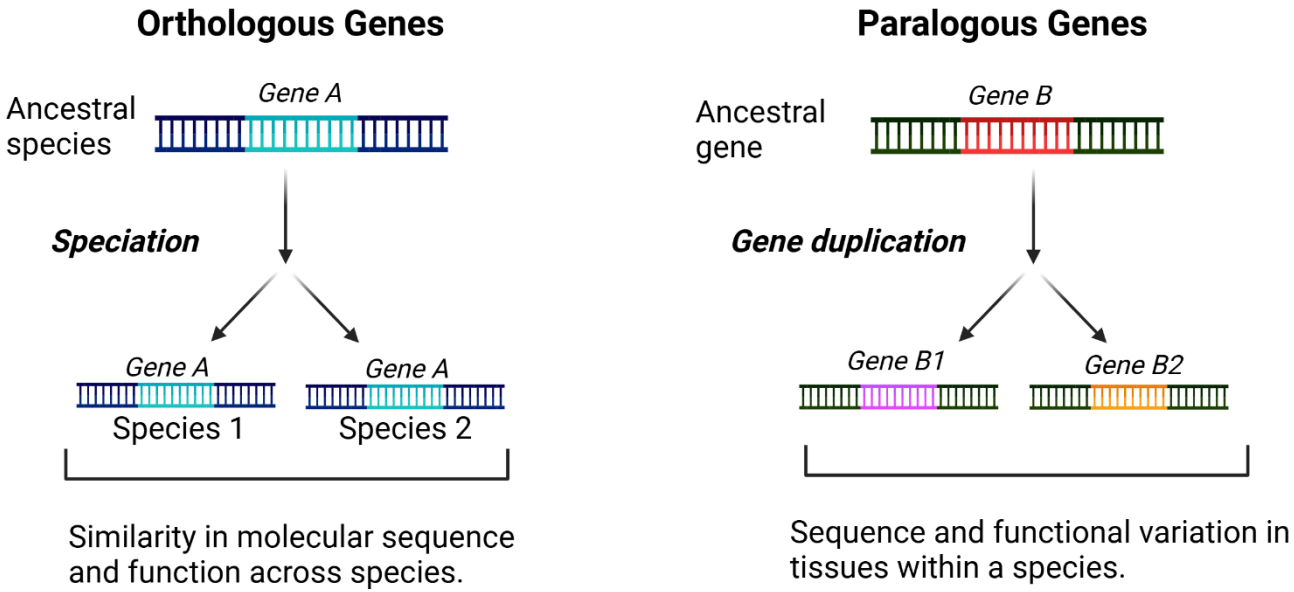
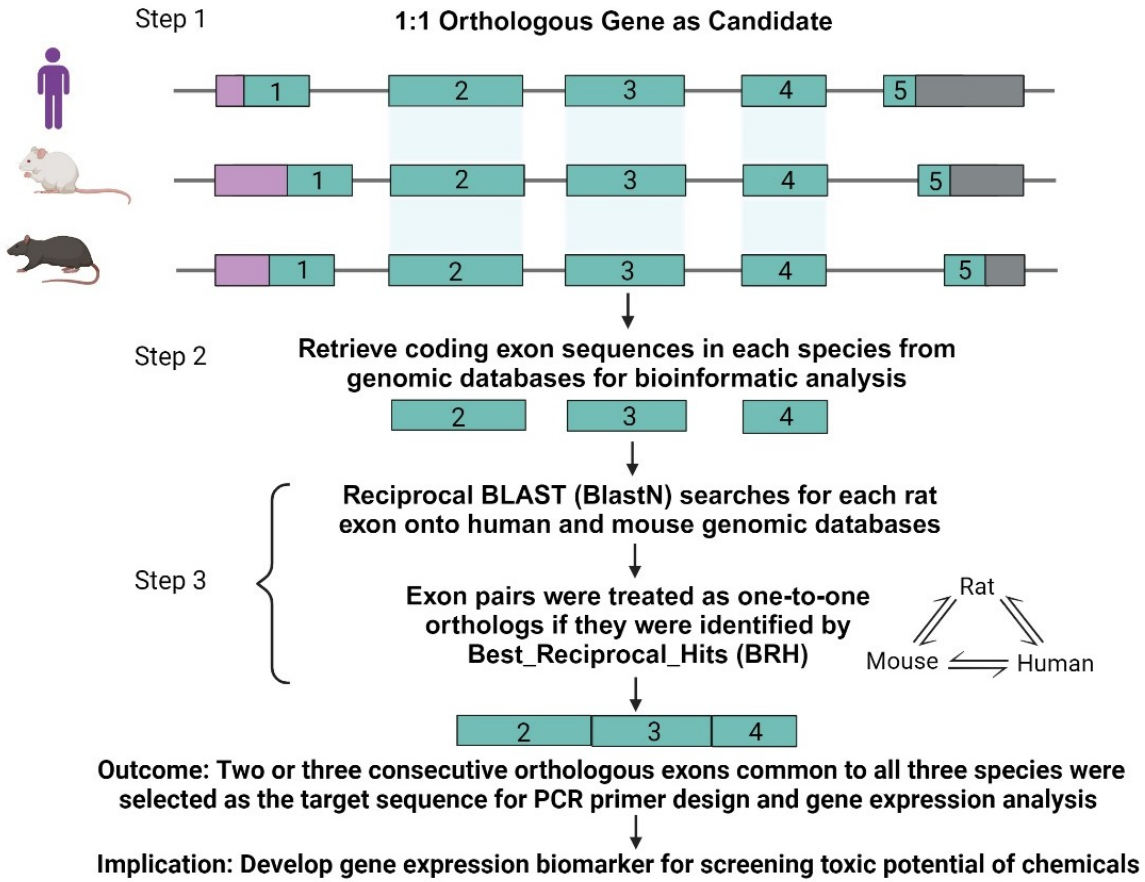


Figure 9

- Genes conserved between species are referred to as orthologs. These are sequences that evolved from a common ancestral gene and have retained the same function during the course of evolution.
- In principle, the expression profiles 1:1 orthologous can be comparable between species, especially when they are derived from matching cells/organs and developmental stages.
- Gene ontology provide specific functional information about molecular functions, biological processes, and cellular compartments to facilitate high quality gene annotation.

3.1.B. Evolutionarily Conserved Target Sequence for mRNA Biomarker Measurement



The RT-qPCR measurement of orthologous constitutive exonic sequences, which are evolutionarily conserved across rat, mouse, and human, should facilitate comparability of gene expression data across species

Figure 10: Identification of orthologous exons by multi-species transcript sequence comparison

3.1.C. A Workflow for mRNA Biomarker Target Sequence Validation

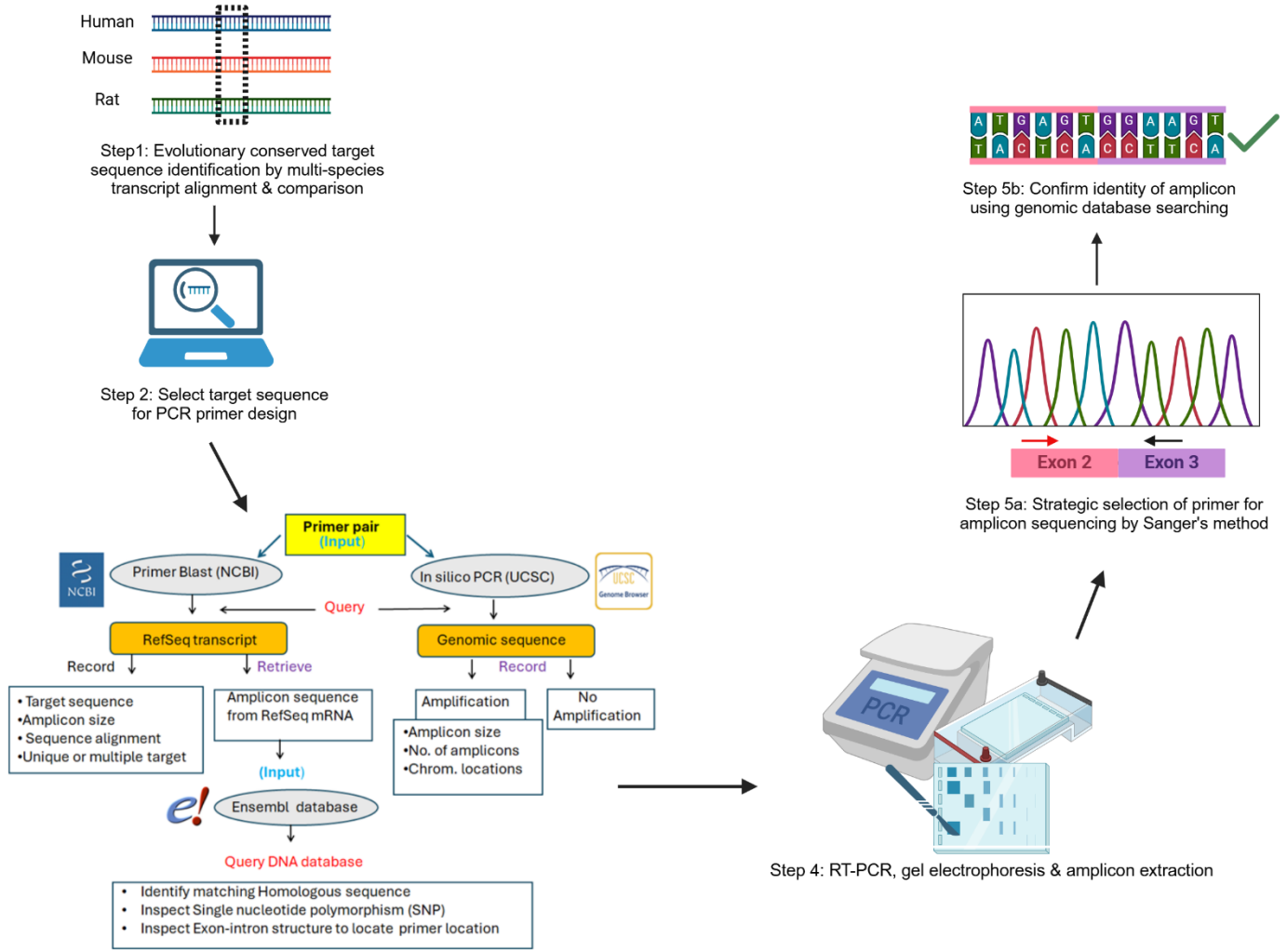


Figure 11

Padhi *et al.*, 2020; 2022; Padhi and Pelletier, 2024.

4. Results And Discussion

Application of the mRNA biomarker framework in *in vitro* developmental neurotoxicity (DNT) studies

4.1. *Grin2b* as an example: Evolutionarily Conserved Target Exonic Sequences across species

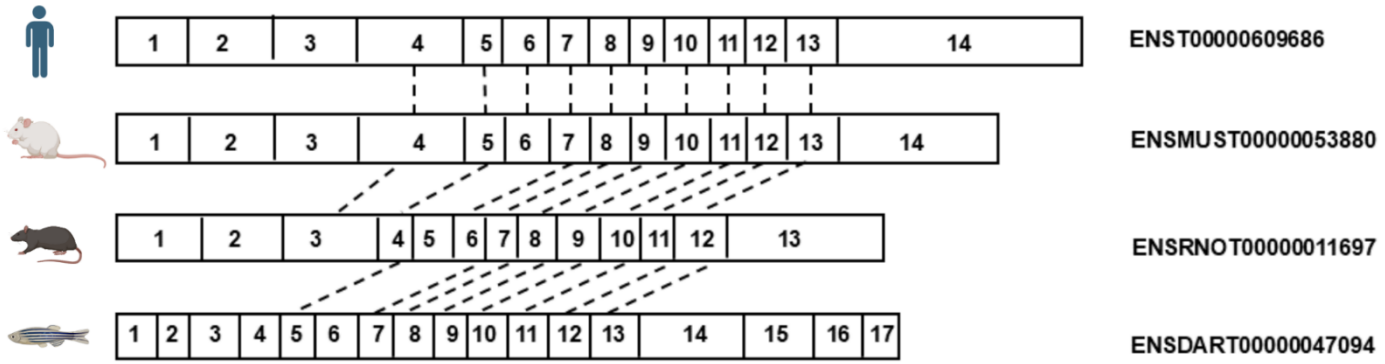


Figure 12: Comparative transcript analyses across species

- The evolutionarily conserved target sequences in biomarker genes, with 1:1 orthology, can facilitate the comparability of gene expression data across species.

- Bioinformatics analyses of gene transcript sequences across species helped to identify evolutionarily conserved, consecutive, and constitutive exons as target sequences, which are suitable for PCR primer design.

4.2. *Grin2b* as an Example: Expression and Amplicon Sequencing Analysis

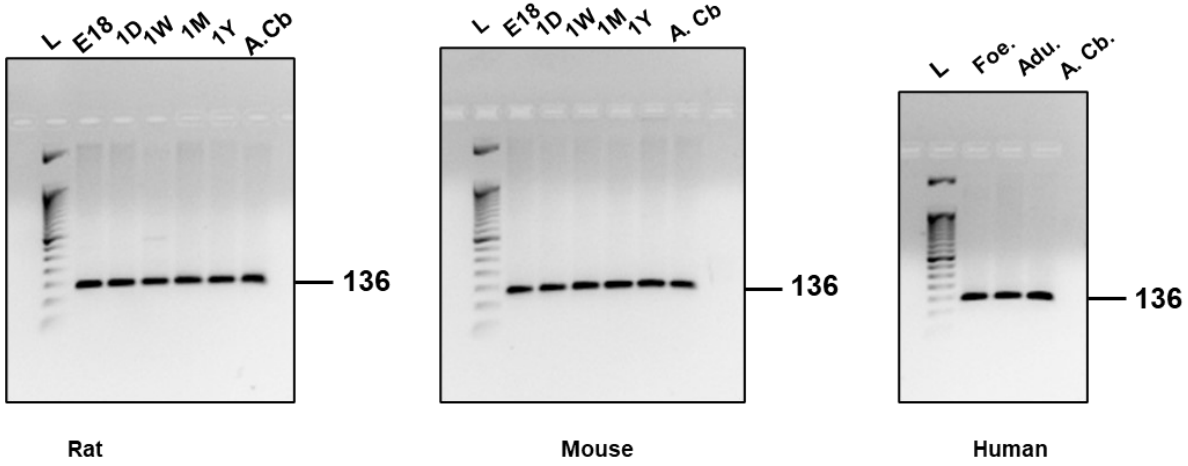


Figure 13: Expression analyses

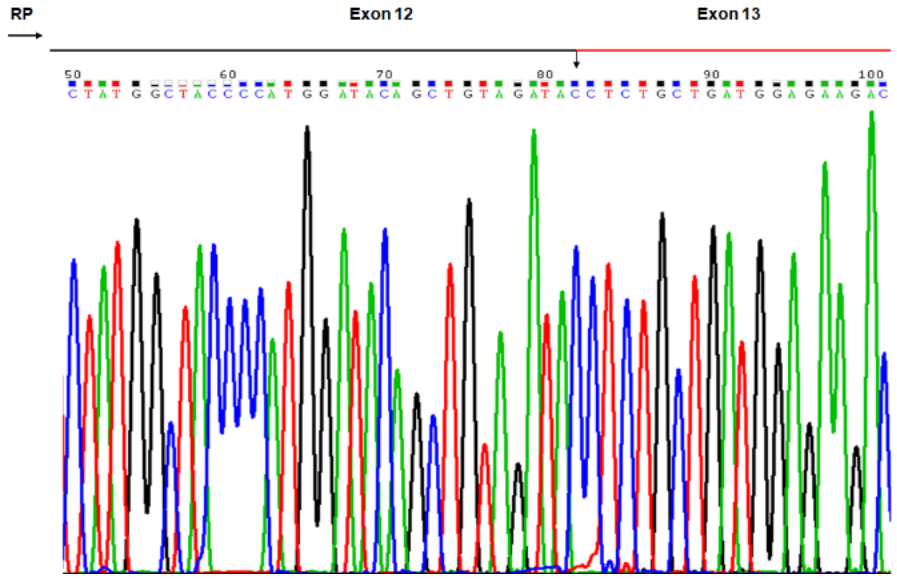
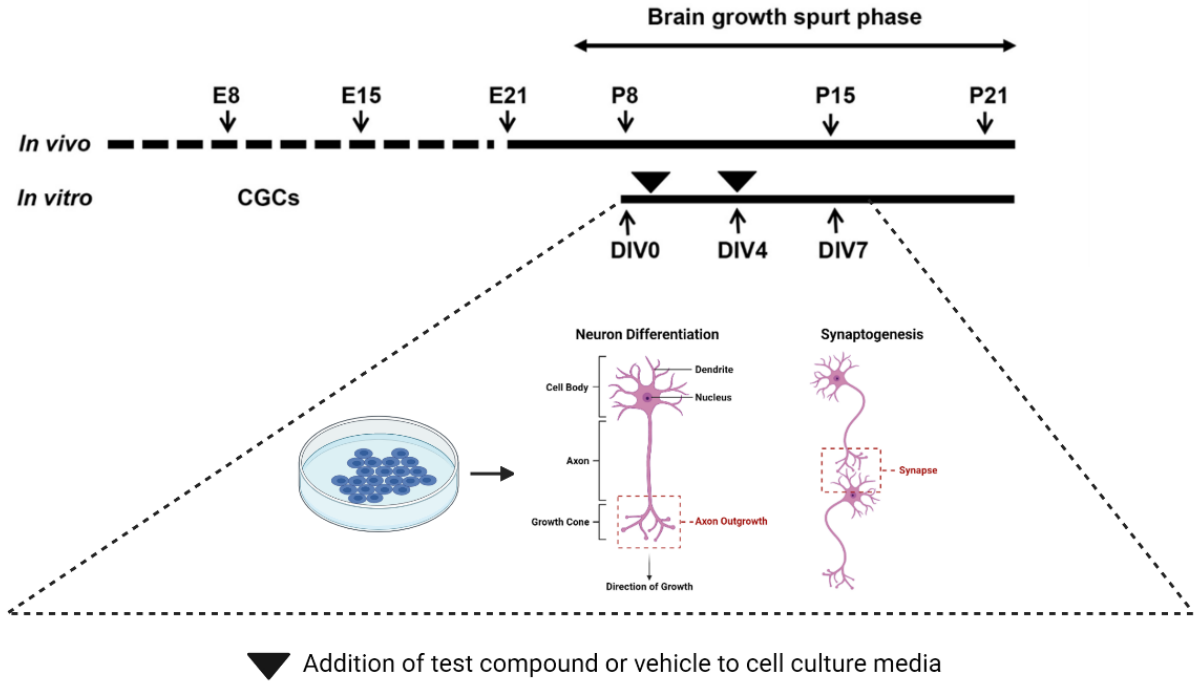


Figure 14: Sanger sequencing of PCR amplicon

RT-PCR analyses of exons 12-13 region in developing and adult brains in rat, mouse, and human generated a single amplicon (unimpacted by splicing event(s)) of the expected size, confirming bioinformatics predictions (Padhi and Pelletier, 2024). (Zebrafish primers are not experimentally tested).

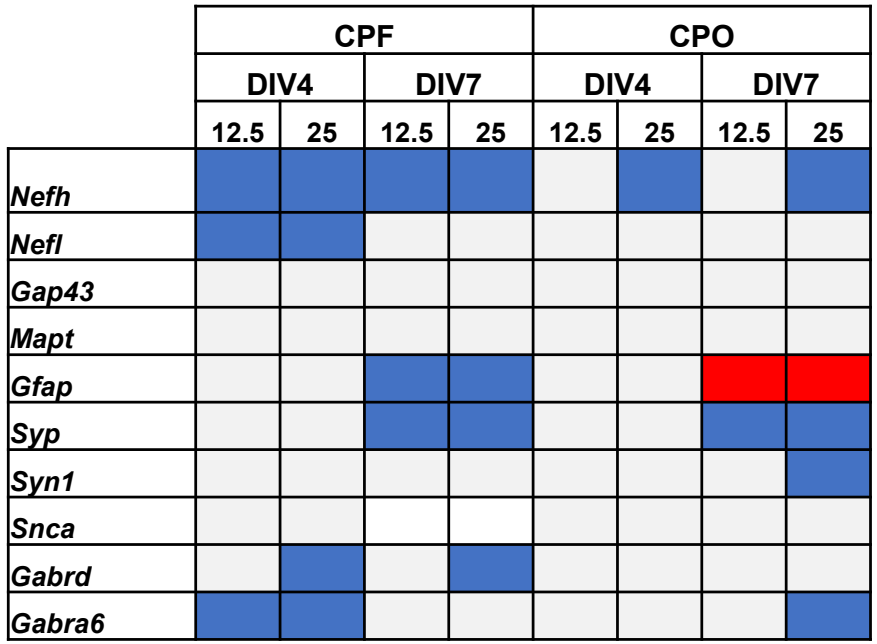
4.3. *In vitro* DNT Assay Design for Screening Toxicity Potential of Chemicals at Critical Stage of Neurodevelopment



- Primary rat CGCs are easy to grow and can mimic neurodevelopmental processes observed in vivo over a few days of cell culture.
- The brain growth spurt phase which corresponds to *in vivo* post-natal days 7-21 is particularly sensitive to interference resulting from exposure to xenobiotics.

Figure 15 *In vitro* model: Primary Rat Cerebellar Granule Cells (CGCs)

4.4. Using a Panel of Neurodevelopmental Biomarker Genes Reveal Gene Expression Perturbation in Toxicological Studies

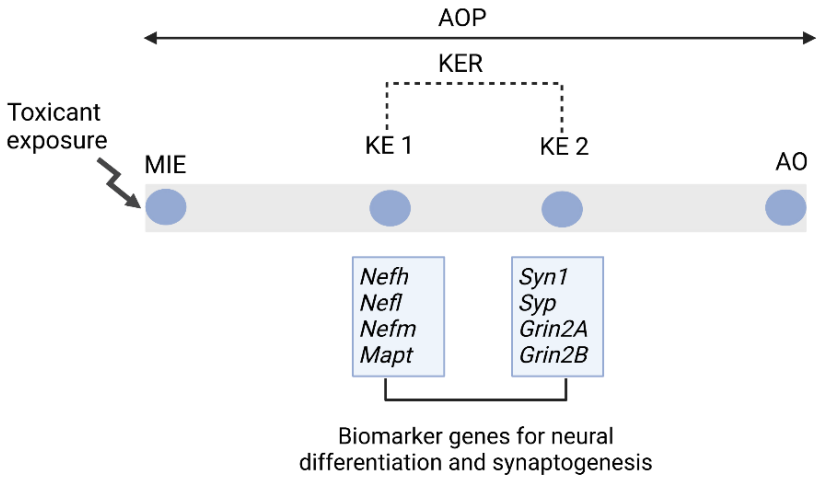


	CPF				CPO			
	DIV4		DIV7		DIV4		DIV7	
	12.5	25	12.5	25	12.5	25	12.5	25
Nefh								
Nefl								
Gap43								
Mapt								
Gfap								
Syp								
Syn1								
Snca								
Gabrd								
Gabra6								

Figure 16: A summary of differential gene expression in rat CGCs. Blue cells indicate down-regulation and red cells up-regulation.

- Summary of the impacts of Chlorpyrifos (CPF) and its metabolite Chloropyrifos oxon (CPO) administered at 12.5 µM (12.5) and 25 µM (25) on the expression of selected genes in CGCs at DIV4 and DIV7. No differential expression observed at 6.25 µM (not presented) (Padhi et. al., 2022).
- The expression of most of the selected evolutionarily conserved genes involved in neuronal differentiation and maturation were significantly affected in rat CGCs following exposure to non-cytotoxic concentrations of known neurotoxicants.
- CPF had a greater impact on gene expression than CPO.
- Monotonic dose-responses were observed.
- This proof-of-concept study agrees with our hypothesis

4.5. Adverse Outcome Pathway (AOP) Framework Can Help With Interpreting mRNA Biomarker Data



AOP=Adverse Outcome Pathway; MIE=Molecular Initiating Event;

KE=Key Event; KER=Key Event Relationship; AO=Adverse outcome

Figure 17: Linking mRNA biomarkers to key events.

- Changes in mRNA biomarker levels observed in cell-based studies suggest that chemical exposure has an effect.
- The Adverse Outcome Pathway (AOP) approach, which acts as a framework for integrating chemical toxicity data, will greatly help in the integration and interpretation of biomarker data.
- The mRNA biomarkers can capture the key events within the AOP framework, allowing for the interpretation of data from New Approach Methodologies (NAMs).

6. IMPLICATIONS AND OUTCOMES

- This framework for the analysis of mRNA biomarkers can contribute to the production of data that is both more reproducible and easier to interpret.
- Our laboratory will use this approach for the screening of chemicals to evaluate their bioactivity in the context of developmental neurotoxicity (DNT).
- The evaluators can utilize these data for benchmark modeling to identify Points Of Departure (POD) and for *in vitro* to *in vivo* extrapolation (IVIVE) in human health risk assessments.
- This approach can serve as a prototype for developing and characterising gene expression biomarkers for other organ/tissue types such as the liver and kidneys, and for ecotoxicology and preclinical studies.
- Reliable and well-characterised biomarkers can help lower experimental costs and enhance the interpretation of NAM data. The OECD (2023a) also supports the development of biomarkers to contribute to AOP.

7. This Presentation is Built Upon Years of Research Efforts

Received: 30 July 2024 | Revised: 19 September 2024 | Accepted: 21 September 2024
DOI: 10.1002/ctd2.70009

CLINICAL AND TRANSLATIONAL DISCOVERY — WILEY

SHORT COMMUNICATION

Developing messenger RNA biomarkers: A workflow to characterise and identify transcript target sequences unaffected by alternative splicing for reproducible gene transcript quantification by reverse transcriptase quantitative polymerase chain reaction

Bhaja Krushna Padhi | **Guillaume Pelletier**

Regulatory Toxicology and Pharmacology 133 (2022) 105211

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A bioinformatics framework for targeted gene expression assay design: Application to *in vitro* developmental neurotoxicity screening in a rat model

Bhaja K. Padhi *, Manjeet Singh, Guillaume Pelletier

Hazard Identification Division, Environmental Health Science and Research Bureau, HECSB, Health Canada, Ottawa, ON, Canada

Biochemical and Biophysical Research Communications 532 (2020) 528–534

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of the rat *Acetylcholinesterase readthrough* (*AChE-R*) splice variant: Implications for toxicological studies

Bhaja K. Padhi [a, *], Guillaume Pelletier [a], Manjeet Singh [a], Sunil Kulkarni [b]

[a] *Hazard Identification Division, Environmental Health Science and Research Bureau, HECSB, Health Canada, Ottawa, ON, Canada*
[b] *Existing Substances Risk Assessment Bureau, HECSB, Health Canada, Ottawa, ON, Canada*

Regulatory Toxicology and Pharmacology 111 (2020) 104575

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

A bioinformatics workflow for the evaluation of RT-qPCR primer specificity: Application for the assessment of gene expression data reliability in toxicological studies

Bhaja K. Padhi[a,*], Guillaume Pelletier[a], Philip S. Shwed[b]

[a] *Hazard Identification Division, Environmental Health Science and Research Bureau, HECSB, Health Canada, Tunney's Pasture, Ottawa, Ontario, K1A 0K9, Canada*
[b] *Mechanistic Studies Division, Environmental Health Science and Research Bureau, HECSB, Health Canada, Tunney's Pasture, Ottawa, Ontario, K1A 0K9, Canada*

Biomolecular Detection and Quantification 15 (2018) 18–23

Contents lists available at ScienceDirect

Biomolecular Detection and Quantification

journal homepage: www.elsevier.com/locate/bdq

Research Paper

A PCR-based quantitative assay for the evaluation of mRNA integrity in rat samples

Bhaja K. Padhi[a,*], Manjeet Singh[a], Marianela Rosales[a], Guillaume Pelletier[a], Sabit Cakmak[b]

[a] *Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario, K1A 0K9, Canada*
[b] *Population Studies Division, Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario, K1A 0K9, Canada*

genesis 52:378–386 (2014)

Analytical Biochemistry 494 (2016) 49–51

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

A PCR-based approach to assess genomic DNA contamination in RNA: Application to rat RNA samples

Bhaja K. Padhi*, Manjeet Singh, Nicholas Huang, Guillaume Pelletier

Hazard Identification Division, HECSB, Health Canada, Tunney's Pasture, Ottawa, Ontario, K1A 0L2, Canada

NeuroToxicology 48 (2015) 223–230

Contents lists available at ScienceDirect

NeuroToxicology

Perinatal methylmercury exposure perturbs the expression of *Plp1* and *Cnp* splice variants in cerebellum of rat pups

Bhaja K. Padhi *, Marianela Rosales, Guillaume Pelletier

Hazard Identification Division, HECSB, Health Canada, Tunney's Pasture, Ottawa, Ontario K1A 0L2, Canada

RESEARCH ARTICLE

Expression Pattern of an Evolutionarily Conserved Splice Variant in the Rat *Tacc2* Gene

Bhaja K. Padhi,[1,2] J. Samuel Zigler Jr.,[1] Piyush Padhi,[1] Stacey Hose,[1] and Debasish Sinha[1]*

[1]Ophthalmology, Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland

[2]Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada, Ottawa, Ontario, Canada

Received 18 December 2013; Revised 6 March 2014; Accepted 31 March 2014

Toxicology Letters 213 (2012) 374–380

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Perturbation of *Myelin basic protein* (*Mbp*) splice variant expression in developing rat cerebellum following perinatal exposure to methylmercury

Bhaja K. Padhi, Guillaume Pelletier*

Hazard Identification Division, HECSB, Health Canada, Tunney's Pasture, Ottawa, ON, K1A 0L2, Canada

Selected References

EPA, 2024. EPA Transcriptomic Assessment Product (ETAP) https://www.epa.gov/etap

NIEHS, 2024. Validation, Qualification, and Regulatory Acceptance of New Approach Methodologies A Report of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Validation Workgroup. https://ntp.niehs.nih.gov/sites/default/files/2024-03/VWG_Report_27Feb2024_FD_508.pdf

OECD, 2023. OECD Omics Reporting Framework (OORF): Guidance on reporting elements for the regulatory use of omics data from laboratory-based toxicology studies Series on Testing and Assessment No. 390. https://one.oecd.org/document/ENV/CBC/MONO(2023)41/en/pdf

OECD, 2023a, Omics technologies in chemical testing Biomarker OECD temporary archive (Accessed 5 Mar 2025)

Padhi, B.K., Pelletier , G., 2024. Developing messenger RNA biomarkers: A workflow to characterise and identify transcript target sequences unaffected by alternative splicing for reproducible gene transcript quantification by reverse transcriptase quantitative polymerase chain reaction. Clinical and Translational Discovery, e70009, DOI https://doi.org/10.1002/ctd2.70009

Padhi, B.K., Singh, M., and Pelletier, G. 2022. A bioinformatics framework for targeted gene expression assay design: Application to *in vitro* developmental neurotoxicity screening in a rat model. Regulatory Toxicology and Pharmacology, 133:105211. DOI: 10.1016/j.yrtph.2022.105211

Padhi, B.K., Pelletier, G. and Shwed, P. **2020**. A bioinformatics workflow for the evaluation of RT- qPCR primer specificity: Application for the assessment of gene expression data reliability in toxicological studies. Regulatory Toxicology and Pharmacology, 111, 104575. DOI: 10.1016/j.yrtph.2020.104575

ACKNOWLEDGEMENTS

We thank the following co-op students for technical support:

Zuhaib Siddiqui
Samantha Maerten
Helene Decotignie
Beatrice Hetke
Peter Albanese

This study was funded by the Chemicals Management Plan, Government of Canada.

Thank you