Peer-Review

Ramesh, Shreya, Karthik Mangu, and Bhumandeep Kaur. 2025. “Circulating LncRNA Biomarker Variability in Early Cancer Detection: Implications of Standardization and SUMOylation-Associated Extracellular Vesicle Packaging.” *Journal of High School Science* 9 (4): 448–505. https://doi.org/10.64336/001c.154816

this review leaves out a lot of content. I read just one paper in this field from 2016, it has significantly more content (what are the challenges of detection?) (there may be more). https://doi.org/10.1186/s12943-016-0524-4 , even if points 1 through 9 are addressed, the Journal still requires an original idea or a new way of thinking to solve some or one of these challenges.

1.using equal amounts of input rather than equal amounts of RNA “…..In addition, many diseases, including cancer, may cause the release of nucleic acids into the circulation, leading to a significantly higher level of circulating RNA in cancer patients than in healthy subjects. Thus, it may be more accurate to use an equal volume of input rather than equal amounts of RNA when analyzing circulating biomarkers…….”

2.uniform detection by microarrays regardless of expiration date “….In addition, as circulating lncRNAs provide lower signals than do tissue lncRNAs, the increase in chip background as the expiration date approaches dramatically reduces the number of lncRNAs that can be detected……..”

3.internal controls or recovery metrics cannot mitigate variation introduced prior to RNA isolation “……Although spike-in synthetic RNAs may also be used, they cannot “remove” variation introduced prior to RNA isolation…….”

4.polymorphisms at lncRNA chromosome loci “…..differential lncRNA expression has been attributed to polymorphisms at lncRNA chromosome loci …….”

5.copy number variations may be polymorphism dependent but not necessarily disease specific “……..CNVs that cause lncRNA deregulation have been shown to play a role in the occurrence of various diseases [83–85], and this type of polymorphism might contribute to differences in lncRNA expression among individuals as well as in the levels of specific circulating lncRNAs……”

6.diet is a confounder “…..diet is a critical potential confounder in lncRNA studies [86, 87]: many of the lncRNAs contained in food could be largely indistinguishable from endogenous lncRNAs at the sequence and/or function level once they enter the circulation and could cause changes in lncRNA concentrations via homeostatic mechanisms that regulate circulating lncRNA-containing vehicles (including lipoprotein particles and exosomes)……..”

7.Intraindividual variability over time: …..measured lncRNA levels could represent a summary of individual behavior rather than of a specific disease state, such as diet and physical activity……."

8.Pharmacological treatments and chemotherapy affect lncRNA levels “……pharmacological treatments could have a profound influence on circulating lncRNA levels. ……..”

9.do levels represent cancer or the response of the body “……..In fact, because cancerous cells represent only a small fraction of the cells in the body, most of the changes observed in specific circulating lncRNAs are a result of indirect effects of the body’s response to cancer growth. ……..”

Please resubmit with all the challenges clearly articulated and discussed in depth. Then, present a scientific proposal or idea or a new way of looking at the detection and biomarker problem that has not yet been addressed in the literature.

Please also see the expectations required for a review manuscript here: https://jhss.scholasticahq.com/for-authors , types of manuscripts, review papers.

Dear Editor/Reviewers

Thank you for your valuable comments and suggestions on our manuscript. The suggestions have been incorporated into the revised manuscript to improve the overall quality of the paper. The revised changes are highlighted in yellow in the text. The authors have carefully rechecked and

corrected any grammatical/typographical errors in the whole manuscript. The detailed corrections are listed below, point by point:

Point-by-point response to the reviewers' comments

Comment 1:

This review leaves out a lot of content. I read just one paper in this field from 2016; it has significantly more content (what are the challenges of detection?) (there may be more, please perform a thorough search of the literature). https://doi.org/10.1186/s12943-016-0524-4, even if points 1 through 9 are addressed, the Journal still requires an original idea or a new way of thinking to solve some or one of these challenges.

Response: We appreciate this valuable feedback. In the revised version, we conducted a comprehensive and updated literature review extending beyond the 2016 reference, incorporating over 60 recent primary studies (2021–2025) to ensure completeness. We have also cited and critically compared Qi et al. (2016) with recent findings. We have added explicit subsections on the following challenges we missed before:

1. using equal amounts of input rather than equal amounts of RNA "…..In addition, many diseases, including cancer, may cause the release of nucleic acids into the circulation, leading to a significantly higher level of circulating RNA in cancer patients than in healthy subjects. Thus, it may be more accurate to use an equal volume of input rather than equal amounts of RNA when analyzing circulating biomarkers……."

Response: We have added a dedicated subsection (*"Input Volume vs RNA Amount in Circulating lncRNA Biomarker Analysis," p. 9–10*), elaborating on the rationale for using equal input volumes rather than equal RNA amounts. We integrated recent supporting studies (Brokāne et al., 2023; Northrop-Albrecht et al., 2024; exRNAQC Consortium, 2025) showing that volume normalization better reflects physiological RNA differences between cancer and control samples.

2. uniform detection by microarrays regardless of expiration date "….In addition, as circulating lncRNAs provide lower signals than do tissue lncRNAs, the increase in chip background as the expiration date approaches dramatically reduces the number of lncRNAs that can be detected……."

Response: We incorporated a comprehensive discussion (*p. 11–12, "Impact of Microarray Expiration and Background Noise"*) supported by recent reports (Gao et al., 2025; Minina et al., 2025) demonstrating the detrimental effect of chip aging and background increase on low-abundance circulating lncRNA detection. We referred Qi et al. (2016) for historical context and highlighted modern solutions, including a shift toward RNA-seq and digital PCR platforms.

3. internal controls or recovery metrics cannot mitigate variation introduced prior to RNA isolation "……Although spike-in synthetic RNAs may also be used, they cannot "remove" variation introduced prior to RNA isolation……."

   Response: A detailed explanation has been added (*p. 9*), citing Satake et al. (2024) , Zendjabil et al. (2024) and othersemphasizing that synthetic RNA spike-ins fail to correct pre-isolation variability and advocating for stringent pre-analytical control and SOP-based harmonization

4. polymorphisms at lncRNA chromosome loci "…..differential lncRNA expression has been attributed to polymorphisms at lncRNA chromosome loci ……."

Response: A new **subsection 5.5** titled "Polymorphisms at lncRNA Loci: Impact on Differential Expression and Biomarker Potential" (p. 15–16) has been included. It details recent genetic association studies (Senousy et al., 2024; Krishna et al., 2024; He et al., 2025) demonstrating SNP-dependent lncRNA expression variation and its implications for disease susceptibility and biomarker design.

5. copy number variations may be polymorphism dependent but not necessarily disease specific "……..CNVs that cause lncRNA deregulation have been shown to play a role in the occurrence of various diseases [83–85], and this type of polymorphism might contribute to

differences in lncRNA expression among individuals as well as in the levels of specific circulating lncRNAs……"

Response: We now explicitly address this issue in subsection **5.6,** "Copy Number Variation at lncRNA Loci: Expression Heterogeneity and Its Disease versus Non-Disease Specificity" (*p.16*), citing (Tyagi et al., 2025), (Lu et al., 2023), and more. We clarify that while CNV-induced deregulation is often observed in cancer, many CNVs are polymorphic and not disease-specific.

6. diet is a confounder "…..diet is a critical potential confounder in lncRNA studies [86, 87]: many of the lncRNAs contained in food could be largely indistinguishable from endogenous lncRNAs at the sequence and/or function level once they enter the circulation and could cause changes in lncRNA concentrations via homeostatic mechanisms that regulate circulating lncRNA-containing vehicles (including lipoprotein particles and exosomes)……."

Response: We added a new subsection 5.7, "Diet as a Critical Confounder in Circulating lncRNA Biomarker Research" (p. 17), discussing how diet-derived and host-modulated lncRNAs confound biomarker studies. We integrate emerging evidence from (Brandt & Kopp 2024), (Martino et al., 2024), and (Muse et al., 2025) and emphasize the need for dietary metadata in study design.

7. Intraindividual variability over time: …..measured lncRNA levels could represent a summary of individual behavior rather than of a specific disease state, such as diet and physical activity……."

Response: This is covered in Subsection **5.4**: "Intraindividual Temporal Variability" (p. 14–15), where we discuss how lifestyle, diet, and circadian factors modulate circulating lncRNA levels dynamically, referencing Roy et al. (2024) and Sandau et al. (2024). We propose longitudinal sampling as a solution to mitigate such confounding.

8. Pharmacological treatments and chemotherapy affect lncRNA levels "……pharmacological treatments could have a profound influence on circulating lncRNA levels. ……."

Response: An expanded Subsection **5.8:** (p. 17–18) now discusses drug-induced modulation of circulating lncRNAs, citing: Siahestalkhi et al., 2025, Ye et al., 2022, and more. We have differentiated between disease-related and treatment-induced transcriptomic changes and recommend accounting for medication history in biomarker validation.

9. do levels represent cancer or the response of the body "……..In fact, because cancerous cells represent only a small fraction of the cells in the body, most of the changes observed in specific circulating lncRNAs are a result of indirect effects of the body's response to cancer growth. ……."

Response: This key conceptual issue is now addressed throughout in separate **Section 6**: "Disentangling Circulating lncRNA Signals: Tumor-Derived Versus Host Response" (p. 18) by integrating evidence that many lncRNA level changes result from systemic responses (inflammation, immune activation) rather than direct tumor secretion. We highlight the need for pathway-level analysis to distinguish disease-specific from host-response signatures.

Comment 2:

Please resubmit with all the challenges clearly articulated and discussed in depth. Then, present a scientific proposal, or idea, or a new way of looking at the detection and biomarker problem that has not yet been addressed in the literature. To meet the Journal's requirement for originality and a new way of thinking:

Response: We thank the reviewer for highlighting the need to present a clearly defined and original conceptual framework. In the revised manuscript, we have extensively detailed all major analytical, pre-analytical, and biological challenges supported by recent evidence (2021–2025). Building upon these discussions, we now introduce an Integrated Detection and Validation Framework (illustrated

in Figure 4) and **Section 9** (p. 21) “Integrated Approach for lncRNA Discovery and Validation” that provides a new systems-level approach to overcoming reproducibility barriers in circulating lncRNA biomarker research. This model unifies experimental and computational pipelines into a single harmonized workflow that:
(1) *Standardizes sample input and normalization to control pre-analytical variability*, (2) *Selectively isolates tumor-derived exosomes using immunoaffinity capture for improved specificity*, *(3) Applies context-aware normalization incorporating metadata such as diet, medication, and circadian rhythm, (4) Integrates machine-learning-based–based noise filtration to reduce biological and technical confounders, (*5) *Validates candidate lncRNAs via CRISPR-Cas13 functional screening, linking molecular detection to biological causality,* and, *(6) Combines multi-omic layers (genomic CNVs, proteomics, immune transcriptomes) to distinguish tumor-derived signals from host responses.*
This multi-tiered framework represents a novel way of conceptualizing liquid-biopsy standardization, not merely detecting circulating lncRNAs, but systematically refining them through iterative experimental, computational, and functional validation. To our knowledge, such a unified, end-to-end model has not been previously proposed in the literature, and it directly fulfils the Journal’s requirement for originality and a new way of addressing the detection and biomarker reproducibility problem.

Thank you for your responses to my earlier review. However, the manuscript does not present any original technical ideas/concepts/actionable methods that are not yet available in the public domain. Proposing a harmonized workflow does not contribute to this deficiency. The manuscript is hence still a collection of information and content from the published literature and does not meet the Journal’s guidelines of what is expected of a review paper as seen here; https://jhss.scholasticahq.com/for-authors , types of manuscripts, review papers. As an example, points 6 through 9 , all that seems to have been addressed is that you acknowledge these deficiencies in the manuscript and present references. Can you suggest actual implementable procedures or concepts which will distinguish measured lncRNA levels as being representative of a specific disease state, rather than originating from individual behavior, pharmacotherapy, age, physical activity…or other confounders. You are -of course - free to think of other ideas.

Dear Editor/Reviewers
Thank you for your valuable comments and suggestions on our manuscript. The suggestions have been incorporated into the revised manuscript to improve the overall quality of the paper. The revised changes are highlighted in green in the text. The authors have carefully rechecked and corrected any grammatical/typographical errors in the whole manuscript. The detailed corrections are listed below, point by point:
Point-by-point response to the reviewers’ comments
Comment 1:
The manuscript does not present any original technical ideas/concepts/actionable methods that are not yet available in the public domain. Proposing a harmonized workflow does not contribute to this deficiency. The manuscript is hence still a collection of information and content from the published literature and does not meet the Journal’s guidelines of what is expected of a review paper. Can you suggest actual implementable procedures or concepts which will distinguish measured lncRNA levels as being representative of a specific disease state, rather than originating from individual behavior, pharmacotherapy, age, physical activity…or other confounders.

Response: We thank the reviewer for this important observation. In response, we have now incorporated a novel, mechanistically motivated hypothesis. It is explained under **Section 10: “Targeting SUMO-Modified Exosomal Cargo-Loading Machinery for Accurate Identification**

of Tumor-Derived Circulating lncRNAs". This study proposes that cancer cells imprint distinct SUMOylation patterns on RBP–lncRNA complexes within exosomes, enabling the discrimination of tumor-derived lncRNAs from those in the physiological background. We further outline a single-exosome nanosensor platform capable of detecting these SUMO-barcodes in real time. This constitutes a new, actionable, and technically implementable concept that goes beyond summarizing prior literature and provides a concrete direction for biomarker innovation, enabling more accurate clinical interpretation.

Thank you for addressing my comments. The manuscript is much improved from its original version.
I agree that the approach you propose is an extrapolation of existing concepts and satisfies the Journal's requirements for a review paper. However, I still need to see a more specific, actionable game-plan/roadmap to execute your approach medium-long term, as well as to present a more feasible analytical chip assay. To that efffect please incorporate the following concerns in the manuscript.

1.Temper the language or specifically mention limitations with regad to 1) Overstated certainty about SUMOylation patterns being "unique" or cancer-specific. 2) Assumption that SUMO-modified RBPs remain stably attached to lncRNAs inside exosomes.3) Assumption that exosomes preserve post-translational modifications with high fidelity. 4) Generality across cancer types. 5) assumption that a single analytical probe can discriminate 'cancer' versus 'non-cancer' exosome packaged contents (see point 4). Reframe your idea as a testable hypothesis, avoid strong assertions about universality, and acknowledge biological variability.
2.Include the following in the manuscript. any clinically useful SUMO-barcode classifier would require collecting single-exosome data from both cancer and non-cancer patients, plus carefully handling comorbidities. This reveals the multi-dimensional cancer signature. Hence, Propose cancer vs. healthy vs. comorbidity cohorts. Here's what a minimal, hypothesis-testing dataset could look like:
Cohort A: Healthy controls, 10–20 donors, low systemic inflammation, baseline SUMO-RBP–lncRNA exosome profiles
Cohort B: Cancer patients, 10–20 patients, a single cancer type (to reduce biological noise), matched stage
Cohort C: Non-cancer diseases with high stress/SUMO activation, Comorbidity controls (to ensure signal is cancer-specific): chronic inflammation (e.g., rheumatoid arthritis), neurodegeneration (Alzheimer's/Parkinson's), cardiovascular disease, viral infection
You only need 10–15 per group for initial signal detection.This is feasible for an exploratory study, not a massive consortia-scale effort.
This is so that the classifier can learn a pattern and/or signature; not necessarily a threshold. These patterns can be: SUMO1 vs SUMO2/3 distribution, monoSUMO vs polySUMO, specific RBP sites used (e.g., SUMO on hnRNPA2B1 K137), co-occurrence of m^6A-rich lncRNAs, frequency of SUMO-RBP–lncRNA complexes per sample, subpopulation size and heterogeneity.
3.Include the following in the manuscript. Initially, Measure hundreds to thousands of exosomes per patient. Use statistical metrics:Frequency of SUMO-RBP–lncRNA positive exosomes, Distribution of SUMO isoforms and chain lengths, Co-occurrence patterns. This creates a patient-level "SUMO-barcode profile", robust to sampling noise. However, even after enrichment, heterogeneity exists due to:Tumor stage / burden, Genetic and epigenetic differences, Comorbidities, Systemic stress response. This is why building a classifier (say using machine language) based on pattern recognition is significantly more useful and actionable than a single/or even multiple-value thresholds.
4.The analytical platform you have proposed is hence too simplistic. Propose the following (still based on chip detection)
A. Microfluidic nanoarrays / exosome-on-a-chip platforms, Exosomes are captured on a chip via antibodies (surface markers: CD63, CD81, or tumor-specific markers like EpCAM)., Allows immobilization of thousands of individual exosomes in defined positions. Examples: ExoView

(NanoView Biosciences) or custom micro/nano pillar arrays. Advantages: Single-exosome resolution, Compatible with multiplexed fluorescent detection, Amenable to downstream chemical labeling (SUMO, RNA)
B. Detection of SUMO-RBPs, (i. Antibody or nanobody labeling, SUMO-specific nanobodies for SUMO1 vs SUMO2/3, RBP-specific antibodies for hnRNPA2B1, YBX1, HuR, etc., Fluorescent conjugates for simultaneous detection. (ii. Chain length / site specificity, Use SUMO chain-sensitive nanobodies or engineered FRET probes that report mono vs polySUMO., Alternatively, enzymatic cleavage plus labeling (SUMO proteases + fluorescent tags) for chain resolution.
C. RNA detection. Molecular beacons or aptamer probes specific for the lncRNA of interest. In situ hybridization on-chip (smFISH-like) to detect RNA associated with each exosome. Optional: use modification-specific RNA probes (e.g., m^6A, m^5C) for added layer of information.
D. Multiplexed co-localization readout, Use multi-channel fluorescence microscopy or super-resolution imaging (STORM, DNA-PAINT) to resolve co-occurring signals. Each exosome is individually imaged for: Surface capture marker, SUMO isoform / chain length, RBP identity, RNA presence (and optionally PTM), Data is extracted per exosome, giving frequency and co-occurrence patterns.
E. Optional amplification strategies. Proximity ligation assay (PLA) or nanoPLA: Detect protein-protein or protein-RNA interactions with high sensitivity., Can differentiate SUMOylated vs non-SUMOylated RBP at single exosome., Rolling circle amplification for RNA detection enhances fluorescence signal.
5.Present a summary of this idea in the abstract and conclusion in the manuscript. Revise the title of the manuscript so that it better reflects this idea incorporation.

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Thank you for your valuable comments and suggestions on our manuscript. The suggestions have been incorporated into the revised manuscript to improve the overall quality of the paper. The revised changes are highlighted in yellow in the text. The authors have carefully rechecked and corrected any grammatical/typographical errors in the whole manuscript. The detailed corrections are listed below, point by point:

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Response: We thank the reviewer for this important and constructive comment. In response, we have carefully revised the manuscript to temper the language, explicitly acknowledge biological and technical limitations, and clearly frame the proposed SUMOylation-related concept as a testable hypothesis rather than an established mechanism.

Comment 2:
Include the following in the manuscript. any clinically useful SUMO-barcode classifier would require collecting single-exosome data from both cancer and non-cancer patients, plus carefully handling comorbidities. This reveals the multi-dimensional cancer signature. Hence, Propose cancer vs. healthy vs. comorbidity cohorts. Here's what a minimal, hypothesis-testing dataset could look like:

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Response: We thank the reviewer for this valuable suggestion. To address this point, we have incorporated a clearly defined exploratory clinical study framework outlining minimal yet feasible cohort design. The revised manuscript now explicitly proposes three cohorts: (i) healthy controls with low systemic inflammation, (ii) patients with a single cancer type and matched stage to reduce biological noise, and (iii) non-cancer disease controls characterized by elevated stress or SUMO activation (e.g., chronic inflammatory or neurodegenerative conditions). We emphasize that relatively small cohort sizes (10–15 individuals per group) are sufficient for initial hypothesis testing and signal detection, consistent with exploratory biomarker studies.

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Include the following in the manuscript. Initially, Measure hundreds to thousands of exosomes per patient. Use statistical metrics:Frequency of SUMO-RBP–lncRNA positive exosomes, Distribution of SUMO isoforms and chain lengths, Co-occurrence patterns. This creates a patient-level "SUMO-barcode profile", robust to sampling noise. However, even after enrichment, heterogeneity exists due to:Tumor stage / burden, Genetic and epigenetic differences, Comorbidities, Systemic stress response. This is why building a classifier (say using machine language) based on pattern recognition is significantly more useful and actionable than a single/or even multiple-value thresholds.
Response: We have substantially expanded the analytical framework to address this concern. The revised manuscript now explicitly states that hundreds to thousands of individual exosomes per patient should be analyzed to generate robust patient-level profiles. We describe specific quantitative metrics, including the frequency of SUMO-RBP–lncRNA-positive exosomes, SUMO isoform distributions, co-occurrence patterns, and subpopulation heterogeneity. We further discuss sources of biological heterogeneity, such as tumor burden, genetic background, and comorbidities, and explain why these factors undermine threshold-based biomarker strategies. Accordingly, we argue that pattern-recognition and classifier-based approaches are more suitable for interpreting multidimensional EV data. This section now clearly links single-exosome resolution to improved robustness, reproducibility, and clinical interpretability.

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E. Optional amplification strategies. Proximity ligation assay (PLA) or nanoPLA: Detect protein-protein or protein-RNA interactions with high sensitivity., Can differentiate SUMOylated vs non-SUMOylated RBP at single exosome., Rolling circle amplification for RNA detection enhances fluorescence signal.
Response: We appreciate the reviewer's detailed guidance and have revised the manuscript to include a state-of-the-art, technically feasible analytical platform. In response, we have substantially expanded and revised the analytical framework in the manuscript to incorporate a more realistic and experimentally feasible chip-based strategy. We outline multiplexed detection strategies incorporating SUMO-specific nanobodies, RBP-targeting antibodies, and lncRNA-specific molecular probes, with optional signal amplification via proximity ligation assays or rolling-circle amplification. We further discuss multiplexed fluorescence and super-resolution imaging approaches to enable vesicle-level co-localization analysis. Importantly, this platform is presented as an adaptation of existing technologies, rather than a speculative invention, and is explicitly linked to the analytical requirements.

Comment 5:
Present a summary of this idea in the abstract and conclusion in the manuscript. Revise the title of the manuscript so that it better reflects this idea incorporation.
Response: We have revised the abstract and conclusion to clearly summarize the proposed biological framework, analytical strategy, and clinical roadmap, while maintaining the manuscript's primary focus on standardization and variability in circulating lncRNA detection. We have also refined the title to better reflect the manuscript so that it better reflect the idea we incorporated.

Thank you for addressing my comments. Accepted.