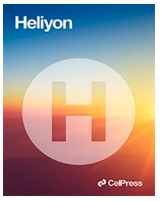
Heliyon 10 (2024) e32896

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

LINCATRA: Two-cycle method to amplify RNA for transcriptome analysis from formalin-fixed paraffin-embedded tissue

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ARTICLE INFO

Keywords:
Linear amplification
Formalin-fixed paraffin embedded
RNA-Seq
Degraded
Transcriptomic analysis

ABSTRACT

Whole transcriptome analysis (WTA) using RNA extracted from Formalin Fixed Paraffin Embedded (FFPE) tissue is an invaluable tool to understand the molecular pathology of disease. RNA extracted from FFPE tissue is either degraded and/or in very low quantities hampering gene expression analysis. Earlier studies described protocols applied for cellular RNA using poly-A primer-based linear amplification. The current study describes a method, LINCATRA (LINear amplifiCAtion of RNA for whole TRAnscriptome analysis). It employs random nonamer primer based method which can amplify short, fragmented RNA with high fidelity from as low as 5 ng to obtain enough material for WTA. The two-cycle method significantly amplified RNA at ~1000 folds ($p < 0.0001$) improving the mean read lengths ($p < 0.05$) in WTA. Overall, increased mean read length positively correlated with on-target reads (Pearson's $r = 0.71$, $p < 0.0001$) in both amplified and unamplified RNA-seq analysis. Gene expression analysis compared between un-amplified and amplified group displayed substantial overlap of the differentially expressed genes (DEGs) (log2 fold change cut-off < -2 and >2, $p < 0.05$) identified between lung cancer and asthma cohorts validating the method developed. This method is applicable in clinical molecular pathology field for both diagnostics and elucidation of disease mechanisms.

1. Introduction

High-throughput technologies including RNA-seq and microarrays continue to be robust methods to evaluate expression of multiple genes from a single sample [1–3]. Whole transcriptome analysis (WTA) gained importance lately allowing researchers to profile the functional network for various genes and establish disease specific biomarker [4,5]. However, the success of RNA-based methods to assess downstream transcriptomic analysis is largely dependent on the RNA yield and its integrity [6,7]. Furthermore, low RNA quantities compromise the end results, yielding poor RNA outcomes that often require labour and time to resolve.

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https://doi.org/10.1016/j.heliyon.2024.e32896
Received 23 February 2024; Received in revised form 21 May 2024; Accepted 11 June 2024
Available online 12 June 2024

Whole transcriptome analysis for formalin-fixed paraffin-embedded (FFPE) samples has emerged as a crucial tool in clinical diagnostics, revolutionizing our understanding of gene expression patterns in archived tissue specimens [8–10]. By analyzing the entire transcriptome, researchers can gain insights into the complex molecular signatures underlying diseases, enabling the identification of biomarkers and potential therapeutic targets [11]. FFPE samples are routinely used in pathology, but their RNA quality has traditionally posed challenges for comprehensive transcriptomic studies [12,13]. Several factors influence RNA integrity during FFPE preparation, such as the length of tissue fixation, age and storage conditions of the blocks, and the RNA extraction methodology [14–17]. All of these factors represent major challenges of RNA extraction from FFPE samples, leading to heavy RNA degradation, base modifications and extensive cross-linking to proteins [18,19]. Subsequently, these factors effect the transcriptomic analysis from FFPE samples as compared to fresh frozen tissue [12,20,21]. Advances in RNA extraction [22] and sequencing technologies [5] now allow researchers to overcome these limitations and unlock valuable information encoded in FFPE specimens.

Initially described by Phillips and Eberwine [23], cell specific gene expression using linear amplification have generated concentrations in micrograms of labelled cDNA through T7-based amplification from reactions starting at 5 ng of RNA [24]. A study described the use of a single RNA-based primer, Ribo-SPIA, designed for isothermal amplification to generate cDNA transcript products through priming with Ribo-SPIA at the poly A tail [25,26]. The results were associated to the T7-based linear amplification technique and produced reproducibility and a high correlation in the differential expression of the number of transcripts [27]. Reported earlier, a comparison study assessed the false positive rate for microarray spot intensties between unamplified RNA (uRNA) and mRNA extracted from bovine spleen and foetal ovary of diluted ranges from 2 μg to 500 pg, revealing that there is no significant change in the differentially expressed genes [28]. This shows that the linear amplification retains the fidelity of gene expression analysis with low degree of false positivity. In addition, the integration of linear amplification with gene profiling analysis has improved the sensitivity and accuracy, detecting a number of differential genes in arrays using amplified antisense RNA (aRNA) which is 1.5–2.5 times higher than uRNA [28–30]. Remarkably, the T7-based aRNA did not increase the false positive rate which suggested that linear amplification does not significantly alter the signal expression comparing to uRNA [31]. Therefore, efforts for linear amplification of RNA from potential application of T7-based amplification was investigated for use in gene expression profiling experiments where the amount of starting material is limited [32]. Alternative methods were developed to overcome challenges in nucleic acid amplifications such as 3' bias, low yield and non-specific amplification [33–36]. These methods were largely employed for either low quantity RNA from single cell or tissue and used a specific primer based approach to amplify the target RNA with known sequence. A table (Table 1) was presented below to summarise these alternative methods and their limitations were listed. In addition, most of the methods available so far for RNA-seq analysis from FFPE tissue used polyA-primer based approach for targeted mRNA sequencing [10,37,38]. As it is well established that FFPE RNA is heavily degraded, a polyA specific primer cannot amplify the fragmented RNA and result in loss of complete information on gene expression allowing bias in the data obtained.

Based on the earlier reports, reverse transcription using random-nonamer, dodecamer and pentadecamer [48] primers increased the yield and quality of the resulting cDNA as compared to random hexamerand the use of T7 and T3 RNA polymerase can be incorporated into the protocol to generate sense and anti-sense RNA [49,50].

Considering the factors mentioned and to circumvent the limitations of fragemented RNA and yield from FFPE, this study aims to develop novel method to amplify the RNA extracted from archival FFPE tissues and use the amplified RNA for whole transcriptome analysis using RNA-seq. Development of the methodology aims to use a T7 and T3 tagged random nonamer primers, together with T7/T3 polymerase based amplification ensuring that the amplification is of high fidelity. This method will enable access of transcirptomic data form archival FFPE material which can provide valuable information regarding the phenotype of the disease being studied.

Table 1
List of alternative amplification methods and their limitations.

Method	Description	Limitation
RT-PCR based method [35]	Enzymatic method; involves usage of oligo d(T) primer to detect 3′ end of mRNA; T7 based RNA polymerase is employed to obtain anti sense RNA.	Cannot be applied for short, fragmented RNA. RNA amplified in the end is complementary to the original RNA
Nucleic acid sequence-based amplification (NASBA) [33, 39–41]	Three enzymes are involved in RNA amplification during the NASBA reaction: RNase H, T7 RNA polymerase, and Avian Myeloblastosis Virus RT. Together, two distinct oligonucleotide primers-one of which has a bacteriophage T7 RNA polymerase promoter site—amplify RNA target sequences by a factor of more than 10^{12}.	Needs specific primers, cannot amplify unknown targets, needs longer than 120 bp fragments to amplify.
Transcription-mediated amplification (TMA) [42–45]	Involves isothermal amplification of ribosomal RNA, especially involved in diagnostics of retroviral RNA virus	Targets mainly ribosomal RNA, needs specific primers.
Ribo-Single Primer based Isothermal Amplification (SPIA) [25,46]	Uses chimeric RNA/DNA primers and amplifies by strand displacement.	Specialised kit and reagents are required
rolling circle transcription (RCT) [34,47]	This method involves amplification of circularised reverse transcribed RNA to cDNA and multiple rounds of amplifcation using Phi29 DNA polymerase and specific primer	An additional step of circularisation is required, and special primer design is involved

2. Materials and methods

2.1. Study cohort and ethical statement

The linear amplification method developed in this study was applied to 17 FFPE samples prepared from asthmatics, non-small cell lung cancer and asthmatic lung cancer patients obtained from Rashid Hospital, Dubai. The study was approved by the Dubai Scientific Research Ethical Committee (DSREC), Dubai Health Authority (DSREC-SR-03L2019_01) and all participants signed an informed consent agreement. All methods performed in this study were in accordance with the ethical standards of the 1964 Helsinki Declaration and its later amendments. Diagnostic information, age and sex of patients included in the study was presented in Supplemental Table S1.

2.2. Macrodissection and RNA extraction

FFPE tissue was sectioned into 10 sections at 3 μm thickness ensuring minimal overlap between different layers of the tissue. Tissue macrodissection was performed using a 30 gauge sterile needle tip with 0.076 mm thickness and the dissected tissue from five sections was stored in a sterile tube. The total RNA was extracted using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Invitrogen kit (Ambion, Carlsbad, USA) according to manufacturer's instruction. To eliminate genomic DNA (gDNA) contamination, the extracted RNA was digested using Turbo DNase kit (Invitrogen, USA). The use of Turbo DNase in solution is efficient at eliminating all traces of gDNA thereby providing gDNA-free RNA. The gDNA digested RNA was quantified using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA) with one replicate to avoid sample loss from the precious FFPE samples However, researchers are encouraged to estimate in triplicates if they have enough material. Samples with low RNA yield were concentrated using SpeedVac vacuum pump concentrator (ScanVac, Labogene, Denmark) and re-measured. Samples with organic contamination were purified using RNA Clean and Concentrator kit (Zymo Research, CA, USA). Based on the yield, the samples with low quantities were further subjected to linear amplification.

2.3. Linear RNA amplification

Samples with low RNA yield following clean-up and concentration were subjected to linear amplification. Various amounts (5–20 ng) of RNA from FFPE tissue was amplified. The amplification was carried out using in-house constructed T7 and T3 promotor

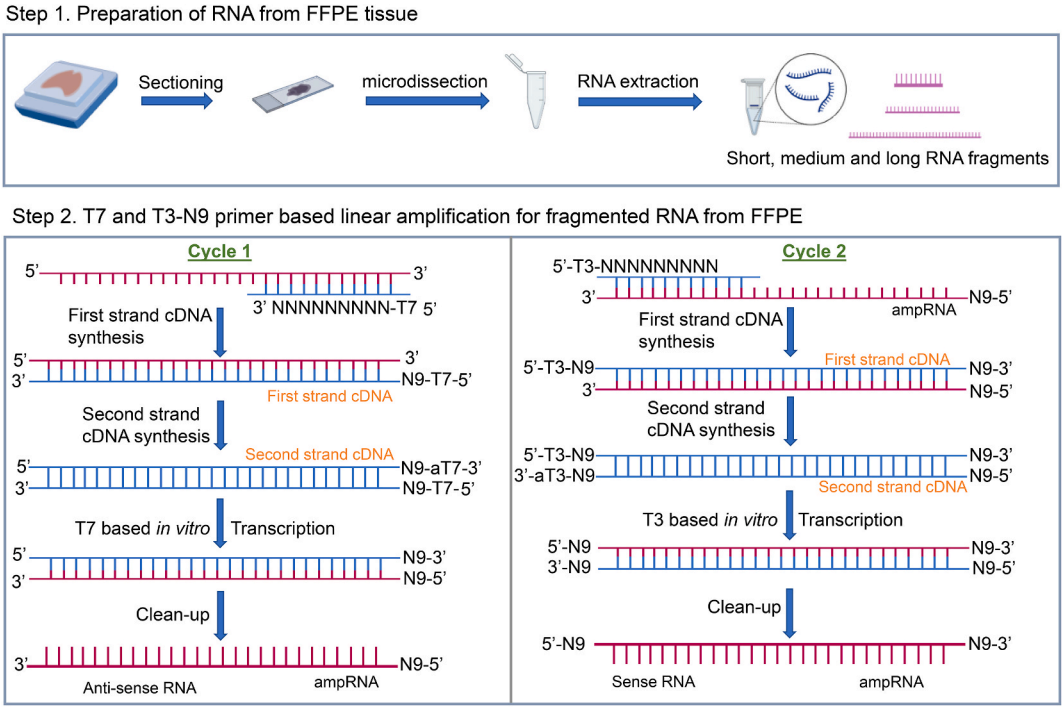


Fig. 1. Schematic representation for the methodology followed in the study to achieve linear amplification of RNA obtained from FFPE. Step 1 includes sectioning and macrodissection of diseased tissue from formalin-fixed paraffin-embedded (FFPE) blocks of bronchial biopsies. Total RNA with variable fragments was extracted from macrodissected samples. Step 2 includes preparation of sense RNA amplified from fragmented low amounts of RNA in a two-cycle process using T7 and T3 based in vitro transcription (IVT) and random-nonamer based linear amplification. N9 = random-nonamer; aT7/aT3 = anti-sense of T7/T3 promoter sequence; ampRNA = amplified RNA.

sequence tagged to random nonamer (N9) primers using the approach of synthesizing double stranded complementary DNA (cDNA), and the subsequent generation of amplified antisense and sense RNA. The random nonamer primer sequence comprises of a nine degenerate nucleotides sequence synthesized with different combinations of A, T, G and C nucleotides, hence, represented as N9 (Supplemental Table S2). Initially, first cycle of double stranded cDNA synthesis was performed using T7 tagged random nonamer (T7-N9) primers from (5–20 ng) RNA where the first strand cDNA was synthesized using Invitrogen™ SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, USA) enzyme and double stranded DNA was generated using DNA polymerase I enzyme in SuperScript™ Double-Stranded cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RNA in sense form would be converted into ds cDNA with T7-N9. Subsequently, in vitro transcription of antisense RNA was conducted using MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific, USA) that utilizes RNA polymerase which recognizes T7 promotor region to transcribe antisense RNA from the dscDNA segments generated earlier.

A second cycle of double stranded cDNA synthesis was carried out using T3 tagged random nonamer (T3-N9) primers from amplified antisense RNA from the previous cycle. First and second strand DNA was prepared as described for the first cycle followed using in vitro transcription of sense RNA using MEGAscript™ T3 Transcription Kit (Thermo Fisher Scientific, USA) that utilizes RNA polymerase which recognizes T3 promotor region to transcribe sense RNA from the dscDNA segments. A detailed stepwise methodology is described in supplementary data and a schematic of the method is shown in Fig. 1.

2.4. Whole transcriptomic analysis

Whole transcriptome library was prepared from both unamplified and amplified RNA using the Ion AmpliSeq human gene expression on S5 system (Thermo Fisher Scientific, USA) as previously described [51]. For each FFPE sample, ~10 ng of gDNA-free RNA was used to prepare barcoded libraries using Ion AmpliSeq transcriptome human gene expression kit (Thermo Fisher Scientific, USA). Purified barcoded libraries were quantified using TaqMan library quantitation kit (Applied Biosystems). The libraries were diluted to 100 pM, pooled together, amplified using emulsion PCR on the Ion One Touch 2 (OT2) instrument, and enriched using the Ion One Touch ES as per manufacturer's instructions. RNA-sequencing of the libraries was performed using Ion S5 XL Semiconductor sequencer on Ion 540 Chip (Thermo Fisher Scientific, USA) as previously described [51].

2.5. Bioinformatic analysis

2.5.1. Next generation sequencing

RNA-seq data were analyzed using Ion Torrent Suite software version 5.5. Ion AmpliSeq Human gene expression panel used in library preparation amplifies targeted regions of approximately 21,000 genes. Base called and aligned sequences were normalized using Fragments per kilobase million (FPKM) normalization according to Hammoudeh et al. [51]. In general, libraries prepared from FFPE samples result in large amounts of short fragment sequences. To eliminate such noise, four different alignment algorithms in the order of; Burrows-Wheeler Aligner (BWA)-short [52] BWA-long [53] sequence Search and Alignment by Hashing Algorithm (SSAHA) [54]and Super-maximal Exact Matching (SMEM) [55] the TMAP suite were implemented. TMAP is optimized for Ion Torrent™ sequencing data for aligning the raw sequencing reads against a custom reference sequence set containing all transcripts targeted by the AmpliSeq kit. The Ion AmpliSeq Transcriptome Human Gene Expression Panel allows for the simultaneous measurement of the expression levels of over 20,800 human RefSeq genes in a single pool. The panel targets 18,574 coding genes and 2228 non-coding genes based on UCSC hg19 annotation. This panel contains 20,802 amplicons made up of 41,604 primers. The reference genome is the human transcriptome, and the targeted amplicon regions (20,802 amplicons) are available in standard BED file format on the ThermoFisher portal (hg19_AmpliSeq_Transcriptome_21 K_v1. bed). The final mapped reads were obtained, and the data was normalized using FPKM according to Trapnell et al. [56]. From the total number of mapped reads, the reads aligned on the target regions are computed by ampliseqRNA plugin as On-target percentage and percentage of genes on the Ion AmpliSeq panel that had a read count $\geq$10 were presented as percentage of targets detected. The samples that displayed mapped read counts >1 million and mean read length >80bp were considered for further analysis (Supplemental Table S3).

2.6. Differential gene expression analysis

Differential gene expression analysis for unamplified group and amplified group were separately performed using DESeq2 in R/Bioconductor package. In each analysis, the read counts from lung cancer patients and/or asthmatic lung cancer patients were compared to severe asthma patients. Based on the cut-off value for log2 fold change >2 and <-2, with significant $p < 0.05$, genes were considered either upregulated or downregulated respectively for each comparison. Further, to validate and understand the bias that may have occurred due to amplification, the DEGs obtained from each comparison were evaluated for any overlap. The common upregulated and downregulated genes were assessed using Interactivenn, web-based tool (http://www.interactivenn.net/) [57]. The significance of the extent of overlap between the groups was checked by an online tool http://nemates.org/MA/progs/overlap_stats.html [58] which computes the representation factor by the formula x/expected number of genes. Expected number of genes = (n * D)/N where n is number of genes in group 1 and D is number of genes in group 2. N is the total number of genes tested which is 20,800 in our study.

Table 2
Concentration of RNA before and after linear amplification for 10 FFPE samples.

Diagnosis	Sample ID	Total RNA concentration (ng)			Fold increase in concentration
		Initial	First cycle of amplification	Second cycle of amplification	
Lung cancer	4	6	1091	16219	2703X
	7	5.8	102.9	5008	863X
	9	9.3	1148	17802	1914X
	10	6	580	6061	1010X
Asthma & lung cancer	15	9	1864	15129	1681X
	13	21.4	816	9454	442X
	16	10.2	1556	11881	1165X
Severe asthma	14	7	784.7	15017	2145X
	18	16.4	893.4	1190	73X
	20	8.6	NA*	1008	117X
Mean±SD		9.97 ± 5.1		9877 ± 6268	$p < 0.0001$

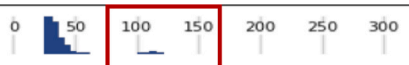
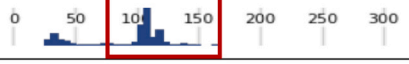
2.7. Gene ontology tools

To evaluate the functional pathway enrichment in each group, DEGs from each comparison were subjected to gene ontology enrichment using metascape (https://metascape.org/) [59]. The common genes identified from Interactivenn tool were also assessed for functional pathway enrichment to compare the similarities and differences in the outcome of unamplified and amplified methods.

2.8. cDNA synthesis and gene expression analysis using quantitative real-time PCR for validation

The amplified RNA was subjected to cDNA synthesis using high-capacity cDNA synthesis kit (Invitrogen), that utilizes both random primers and oligo dT, Reverse Transcriptase enzyme and dNTPs. Approximately, 1 μg RNA from each amplified RNA sample was used to prepare cDNA. Quantitative real-time PCR was performed using SYBR Green master mix (Thermo Fisher). The gene expression analysis was performed in triplicates for each sample where each reaction contains; master mix, 250 nM forward and reverse primers, and 50 ng cDNA sample. The primer sequence used is described in Supplemental Table S2 qRT-PCR was performed in a Quant Studio 3 system in the following conditions: Hold stage 50 °C for 2min, 95 °C for 10 min; 40 PCR cycles: 95 °C for 15 s, 60 °C for 1min, 95 °C for 15 s; Melt Curve stage: 60 °C for 1 min, 95 °C for 1 s. The Ct-value of the gene of interest was normalized against the expression of the house keeping gene (18S) from each sample, and the relative gene expression (2-ΔΔCt) was calculated using severe asthma cases as controls. The eight genes identified from bioinformatic analysis (*BCL3*, *STAT1*, *LUM*, *POSTN*, *MYC*, *FOSB*, *CD44* and *PPARD*) from our previous study [60] were used to assess the efficiency of amplified RNA in understanding the gene expression using qRT-PCR.

a

Sample ID	Diagnosis	Mean read length (bp)	Read length Histogram
13	Asthmatic Lung cancer	38	
15	Asthmatic Lung cancer	35	
16	Asthmatic Lung cancer	93	

b

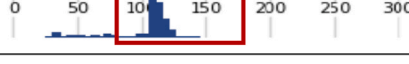
Sample ID	Diagnosis	Mean read length (bp)	Read length Histogram
13	Asthmatic Lung cancer	80	
15	Asthmatic Lung cancer	110	
16	Asthmatic Lung cancer	100	

Fig. 2. Screenshot from next generation sequencing run report displays the difference in the mean read length for the same three samples before (a) and after (b) the amplification. The differences in the mean read lengths was highlighted using a red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Differential RNA-seq parameters for amplified and unamplified RNA.

	Unamplified RNA (mean ± SD)	Amplified RNA (mean ± SD)	*p* value
Total Number, N	N = 11	N = 10	
RNA concentration, ng/μl	20.2 ± 14.8	9877 ± 6268	**<0.0001**
Mean read length, bp	76 ± 27	100 ± 11	**0.019**
On target reads (%)	49.44 ± 30.1	61.38 ± 26.7	0.348
Mapped reads, x million	6.36 ± 5.42	3.14 ± 2.66	0.105

2.9. Statistical analysis

Unpaired *t*-test was used to analyze the differences in RNA-seq parameters between unamplified and amplified groups. Pearson's correlation (r) was used to check the association between RNA concentration, mean read length, mapped reads and On-target reads. SPSS version 23 (IBM, USA) was used to perform the analysis. GraphPad Prism version 9 was used to generate box plots and heatmap for gene expression.

3. Results

3.1. A two-cycled linear amplification method significantly enhanced RNA quantity thereby improving mean read length in RNA-seq analysis

Total RNA quantity was significantly enhanced in all the RNA subjected to two-cycle linear amplification (Table 2). Mean start material of 9.97 ng total RNA was significantly amplified to an average of 9.8 μg RNA ($p < 0.0001$) towards the end of second cycle with a fold increase of around 990. Increase in the concentration of RNA substantially improved the mean read length in RNA-seq analysis. The three asthmatic lung cancer samples with low mean read length (<40 bp) before amplification (Fig. 2A) displayed increase in the mean read length (>80 bp) upon linear amplification (Fig. 2B).

3.2. Increased mean read length positively associated with on-target reads in amplified and unamplified RNA

RNA-seq data for both unamplified and amplified groups were assessed from the sequencing run parameters (Table 3). Mean read length was significantly improved in the group subjected for linear amplification (100 bp, $p < 0.05$) as compared to unamplified group (76 bp). However, no significant change in mapped reads and on target reads was observed in the amplified group.

To understand the importance of read length on other sequencing parameters, a correlation matrix was generated using Pearson's correlation analysis (Supplemental Table S4) for the entire cohort of samples. The matrix showed a positive correlation between mean read length and RNA concentration with $r = 0.46$, $p < 0.05$ (Fig. 3A) as well as On-target reads $r = 0.71$, $p < 0.0001$ (Fig. 3B). As expected, the mapped reads per million and on-target reads showed positive correlation ($r = 0.668$, $p < 0.001$) validating the observations.

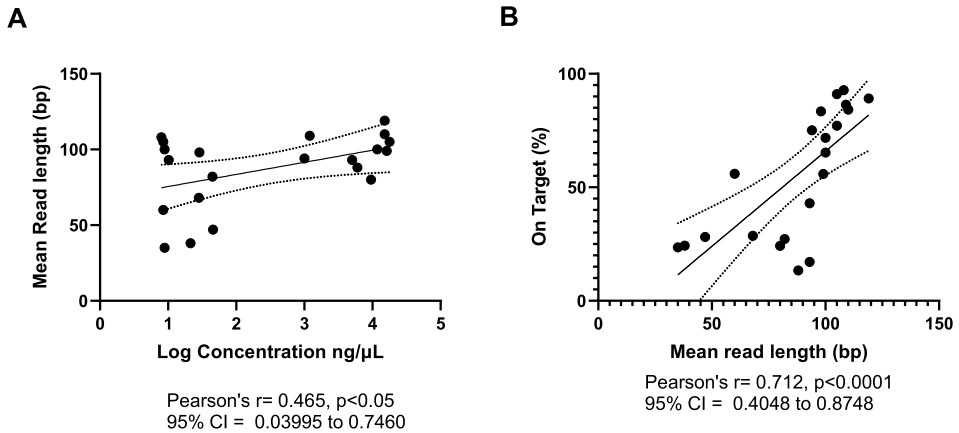


Fig. 3. Correlation plot between mean read length and A) log concentration for RNA and B) % of On-Target reads for all the FFPE samples from the study. A positive correlation with both the variables was observed where Pearson's $r = 0.48$; $p = 0.03$ (95 % CI = 0.03995 to 0.7460) for (A) and Pearson's $r = 0.712$; $p < 0.0001$(95 % CI = 0.4048 to 0.8748) for (B).

3.3. Gene expression analysis from RNA-seq data informs a substantial overlap in the findings between unamplified and amplified groups

Differential gene expression analysis was performed for both the groups by DEseq2. Based on the absolute read parameters (Supplemental Table S3), samples with read length <80 bp and mapped reads <1 million were excluded from further analysis. This resulted in only one sample in asthmatic lung cancer cases (AC). Further gene expression analysis was performed between lung cancer (LC) and asthmatics (AS) to assess the DEGs which resulted in 979 genes downregulated and 715 gene upregulated in lung cancer patients with log2 fold change <-2 and >2 respectively with significant $p < 0.05$ (File S1) among unamplified group.

Similarly, the amplified group comprised of 3 LC and 2 AS after curation based on read length and mapped reads. DEG analysis between lung cancer patients and asthmatics resulted in 342 downregulated and 1165 upregulated genes with cut off log2 fold change <-2 and >2 respectively with $p < 0.05$ (File S1).

In order to estimate the effect of amplification on expression analysis, a comparison between the findings from both the groups was performed. An overlap of almost 90 genes in upregulated DEGs and 82 genes in downregulated DEGs were commonly identified amongst both the groups (Fig. 4A and B) with representation factor >1 in both cases (Supplemental Table S5). In fact, the heatmap generated by DESeq analysis showed better clustering of lung cancer and asthma patients in amplified cohort (Supplemental Fig. S1). These data showed that the RNA linear amplification method does not confer bias to the gene expression analysis and substantially overlaps with findings from analysis of unamplified RNA.

In addition, the asthmatic lung cancer patients were compared with asthmatics in amplified group identifying 631 genes from upregulated and 694 genes from downregulated DEGs (Supplemental File S1).

3.4. Both the amplified and unamplified RNA-seq resulted in identification of similar pathways enrichment amongst lung cancer cases compared to severe asthma patients

Another important outcome from RNA-seq data is to understand the biological processes overrepresented by the DEGs in patient samples. Gene ontology analysis for the DEGs from both groups displayed important molecular pathways enriched by both the

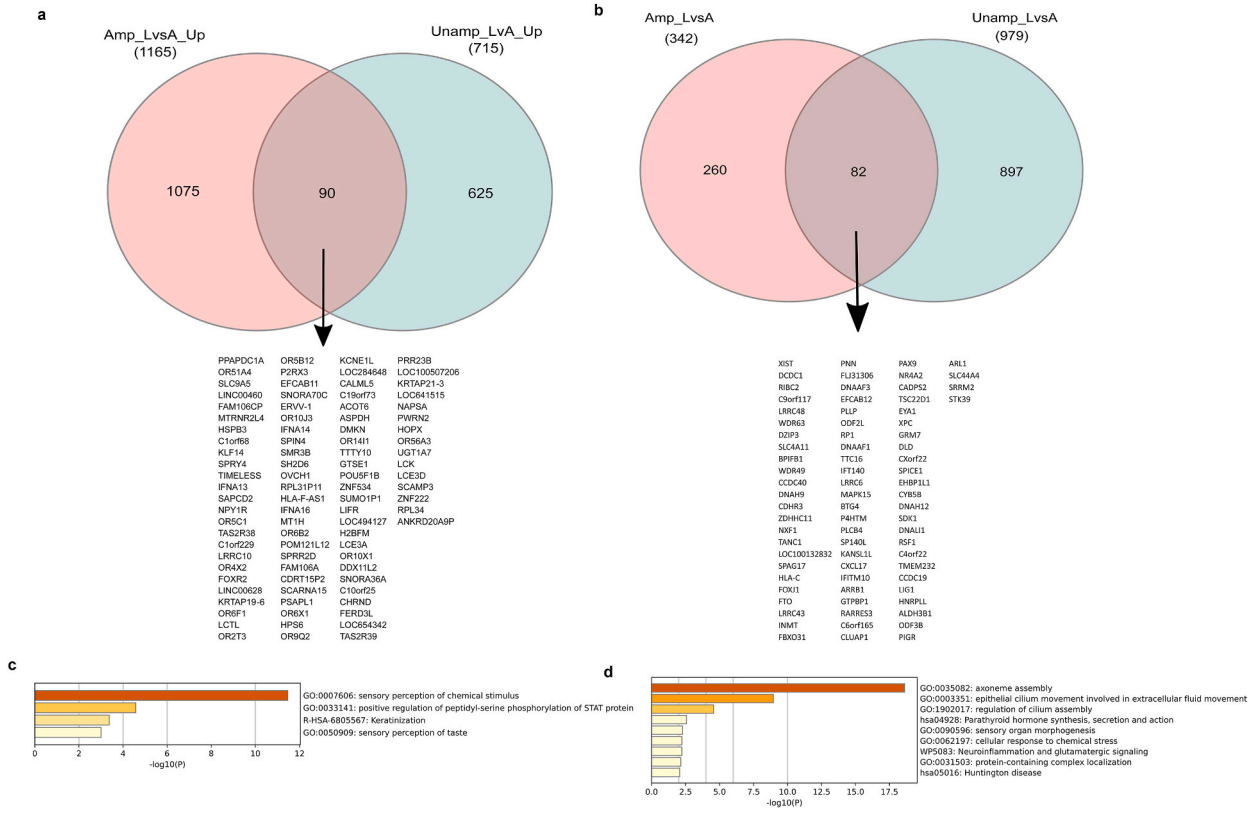


Fig. 4. Common genes differentially expressed between Lung cancer and severe asthma patients from unamplified and amplified cohort A) Upregulated B) Downregulated. The common pathways corresponding to upregulated (C) and downregulated (D) genes in both the cohorts. The venn diagram in (A) shows overlap of 90 upregulated genes and in (B) shows 82 downregulated genes in both comparisons. Upregulated genes were enriched in sensory perception and STAT phosphorylation pathways as seen in (C) and downregulated genes were enriched in epithelial cilium movement and axoneme assembly as in (D). C and D present the bar graphs for enriched terms across the input genes with colour code based on p-values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

upregulated and downregulated genes in lung cancer patients compared to asthmatics (Supplemental Figs. S2A–D). The common DEGs identified from both the analysis showed enrichment in molecular pathways such as keratinization, sensory perception and positive regulation of peptidyl-serine phosphorylation of STAT protein for upregulated genes (Fig. 4C) and axoneme assembly, epithelial cilium movement and regulation of cilium assembly for downregulated genes (Fig. 4D). Moreover, the same pathways were identified in independent analysis among both the cohorts (Supplemental Fig. S2).

3.5. Gene expression analysis using RNA-seq and qPCR validates the linear amplification method

Furthermore, to validate the findings obtained from RNA-seq and to validate the linear amplification method proposed in this study, gene expression analysis for eight genes identified from our previous *in silico* study [39] was performed. The genes *BCL3*, *PPARD*, *LUM*, *CD44*, *MYC* and *STAT1* were known to be over-expressed in lung cancer patients and *POSTN* and *FOSB* were known to over-express in asthmatics. To validate these findings in the current cohort, the fold change expression values for the eight genes in both unamplified and amplified groups between lung cancer and asthma patients were retrieved and heatmap was generated. Clustering based on fold change values in the heatmap showed that amplified group correlated with earlier findings with most of the genes upregulated in lung cancer patients (Fig. 5). In addition, most of these genes were significantly dysregulated ($p < 0.05$) only in the amplified group (Supplemental Table S6). These data suggest that the linear amplification method is useful in extracting important molecular information from FFPE samples.

In addition, differential expression analysis of these genes was performed using qRT-PCR to validate the findings from RNA-seq. Gene expression from asthmatic patients was considered as baseline as in RNA-seq analysis which resulted in higher expression for most of the eight gene panel in lung cancer patients. As the sample size per group was less than three, no significant value could be generated. However, a trend towards upregulation was noticed based on the boxplots (Fig. 6). *POSTN*, *LUM*, *PPARD* and *BCL3* showed higher expression in asthmatic lung cancer patients.

4. Discussion

Several isothermal amplification methods reported [33,34,42,61] to amplify nucleic acids and used as diagnostic tools [61–63]; having their own advantages and disadvantages as described in a recent review [31]. The main limitation of the current methods is either they are inapplicable to low quantities of fragmented RNA or involve challenges related to primers and linkers that underpin the methodology. The goal of this study, is to develop a method that produces sense RNA from small quantities of total or poly-(A) + RNA extracted from archival FFPE tissue. The current protocol developed to amplify low starting amounts of RNA from FFPE tissue is useful in unlocking the molecular information present in patient FFPE samples. Its main novelty is that it faithfully amplifies sense RNA with limited bias overcoming the main problem of low yield and degraded RNA obtained form FFPE archival tissue. Additional advatnages include the protocol developed within this study avoids the need for PCR steps and requires two primers only. Moreover, the protocol is cost effective, efficient, and technically simple to perform.

The in-house method uses a T7 tagged random nonamer primer to generate a double stranded cDNA from the 3-prime end, followed

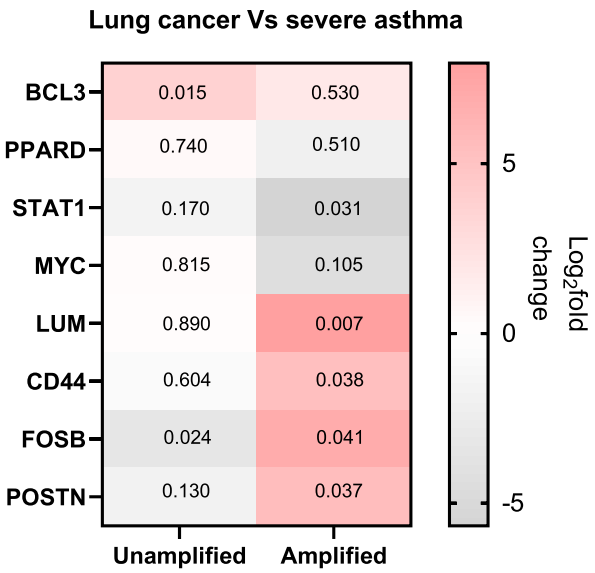


Fig. 5. Heatmap showing the differences in fold change calculations in unamplified and amplified groups for lung cancer vs severe asthma patients. Grey colour indicates negative fold change and dark pink indicates positive fold change. The p-values are presented in each box for every analysis with $p < 0.05$ being significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

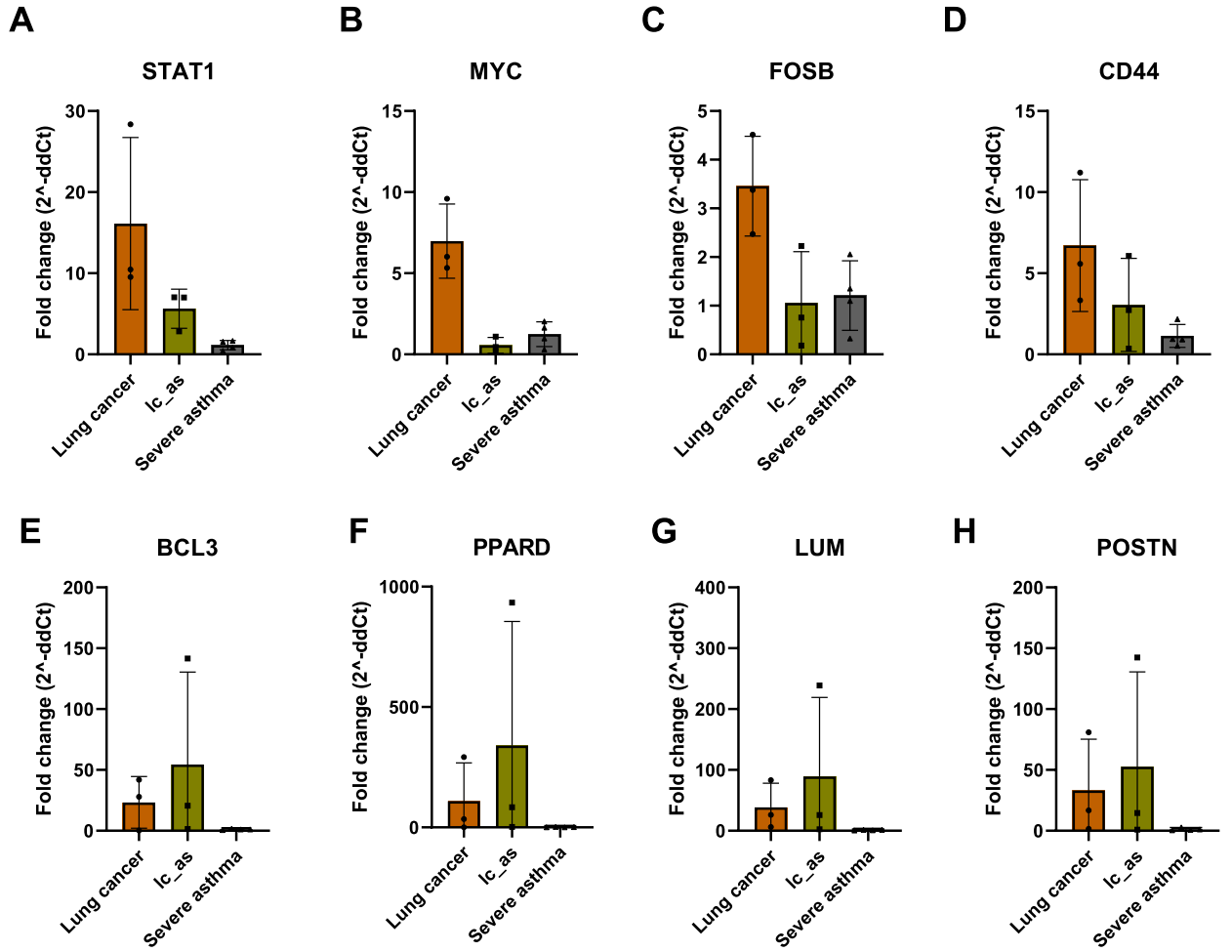


Fig. 6. Boxplots for gene expression for eight genes A. *STAT1*, B. *MYC*, C. *FOSB*, D. *CD44*, E. *BCL3*, F. *PPARD*, G. *LUM*, H. *POSTN* in amplified group between lung cancer, severe asthma and patients diagnosed with lung cancer and asthma. The two-tailed *t*-test computed for every comparison resulted with $p > 0.05$, hence not significant.

by T3 tagged random nonamer primers to generate a second cycle of double stranded cDNA. This mitigated the problems associated with amplifying degraded RNA and allowed small amounts of RNA to be amplified faithfully for downstream expression analysis using next generation sequencing. This allows the amplification of sense RNA (the native form of RNA) infinitely through multiple amplification rounds. The combination of T7 and T3 based primers on 3′ and 5′ ends respectively allows the amplification of RNA at both ends avoiding bias on either end. Most of the currently employed amplification methods lack such uniform amplification due to the usage of primers specific to a single end of the RNA [36,64]. A study comparing the cDNA synthesis using random primers and oligo d (T) described the advantage of using random pentadecamer over hexamer to amplify short, fragmented RNA to avoid 3' bias [48]. To enable the capture of heavily degraded RNA during the fixation of FFPE samples, the present study used a T7 and T3 tagged random nonamer primers resulting in the amplification of RNA, thereby improving the mean read lengths significantly. Along with mean read lengths, another important parameter in RNA-seq is the number of mapped reads with target region covered. Accordingly, the results from this study showed that amplified RNA used for whole transcriptomic analysis displayed comparable percentage of on-target reads with unamplified RNA. The short read lengths were curated using bioinformatic approach according to earlier method described by Hammoudeh et al. where a four-step alignment process reduced the noise due to fragmented RNA sequencing [51].

Single cell sequencing is a robust method with wide applications in precision cancer therapy [65]. It has been used in monitoring drug responses, identifying reliable biomarkers, and detecting rare subpopulations of cells. As the method described here deals with macrodissected samples, it can be applied even for studies based on single cell RNA sequencing. Results from recent studies exhibited success in T7-based linear amplification, generating higher yields of RNA for synthesis, with no significant bias in the fidelity of detection of genes [31,66]. Similarly, the present study showed detection of DEGs in both unamplified and amplified groups with substantial overlap which reflected dysregulation of similar pathways in gene ontology studies between lung cancer and severe asthma patients. LINCATRA method improved the detection of upregulated genes and provided better clustering in DESeq analysis as compared to unamplified group, however, it did not confer bias due to linear amplification as there was substantial overlap between

the amplified and unamplified groups in terms of commonly dysregulated genes and pathways.

A similar comparison conducted on the FFPE samples from lung cancer patients and epithelial brushings from severe asthma patients using *in silico* approach identified six transcripts *BCL3*, *STAT1*, *LUM*, *FOSB*, *PPARD* and *CD44* significantly differentially expressing in both cohorts and two genes *MYC* and *POSTN* upregulated in lung cancer and severe asthma respectively [60]. Genes like *STAT1*, *BCL3* and *FOSB* were upregulated in asthmatic lung cancer patients as compared to severe asthmatics. However, the unamplified cohort displayed variation in the expression of these genes largely from the expected trend. This could be explained based on the decreased percentage of on-target reads in sequencing resulting in the loss of information of these transcripts in expression analysis. To verify whether LINCATRA introduces bias in gene expression analysis, qPCR was performed for all the markers mentioned above using amplified RNA which showed similar results validating the linear amplification method proposed in this study. Whilst the review by Oliveira et al. [62] described the limitation of various linear amplification methods, LINCATRA proved advantageous in amplifying low quantity degraded RNA to be be used for whole transcriptomic analysis. Most of the currently available methods are applied for RNA extracted from cells or tissues and are mainly based on T7 based amplification of mRNA specific libraries [21,32,67]. These methods may not be successful in the context of single cell transcriptomics or FFPE based studies as the afore mentioned methods require high quality RNA [10].

The main limitations of the present study include lack of direct comparison with existing amplification methods. Since the tissue samples were limited and low RNA quantity were obtained from these samples, they were not included in unamplified group as the RNA would not meet the required quality/quantity parameters for whole transcriptomic analysis. To eliminate the issue of paired samples, tissue from similar pathological background were included in both amplified and unamplified cohorts. In addition, a comparison with *in silico* analysis performed on FFPE samples from lung cancer patients [68] and epithelial brushings of severe asthma patients [69]showed significant differential expression from genes between the two cohorts validating the findings from this approach.

LINCATRA's novelty is the combined use of T7 and T3 tagged random nonamers to amplify the RNA. Through integration of our method of generating double stranded cDNA, at the 3-prime end using T3-based amplification; our results have significantly improved the concentration of the total RNA, enabling discrete cell populations with low RNA yields to be analyzed. Hence, the method can be applied to studies based on single cell analysis from FFPE tissue which in general yield highly degraded low quantities of RNA.

5. Conclusions

The study described a high fidelity method to successfully carry out 1000 fold amplification from low starting amounts of RNA (~5 ng) extracted from FFPE tissue obtained from bronchial biopsies. The key advantage of the method is that it amplifies sense RNA with limited bias overcoming the main problem of low yield and degraded RNA extracted form FFPE archival tissue. The method uses T7 and T3 tagged random nonamer primer based linear amplification approach resulting in amplified sense RNA for downstream whole transcriptome sequencing analysis. This method can be applied in clinical pathology such as diagnostic biomarkers discovery, identification of novel drug targets, and elucidation of molecular mechanisms of diseases.

Funding

R.H. and B.M. are funded by the University of Sharjah (grant code: 22010902103).

Institutional review board statement

The study was conducted in accordance with the Dec-laration of Helsinki and approved by Dubai Scientific Research Ethical Committee (DSREC), Dubai Health Authority (DSREC-SR-03L2019_01). Informed consent was obtained from all subjects involved in the study.

Data availability statement

The datasets generated and analyzed during the current study are available in the GEO repository (GSE228217).

CRediT authorship contribution statement

Poorna Manasa Bhamidimarri: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Laila Salameh:** Writing – review & editing, Methodology. **Amena Mahdami:** Validation, Methodology. **Hanan Abdulla:** Writing – original draft, Validation. **Bassam Mahboub:** Writing – review & editing, Supervision, Resources. **Rifat Hamoudi:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32896.

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