Alternative Splicing Novel lncRNAs and Their Target Genes in Ovine Skeletal Muscles

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Abstract

Long non-coding RNAs (lncRNAs) compose a plentiful category of transcripts that have gained increasing importance because of their roles in different biological processes. Although the function of most lncRNAs remains unclear. They are implicated in epigenetic regulation of gene expression, including muscle development and differentiation. We aimed to identify the effect of novel lncRNAs (Alternatively spliced) and their target genes on two stages of sheep skeletal muscle growth and development. FastQC files have been used to examine the quality control and the Trimmomatic program for trimming low-quality reads from twelve longissimus dorsal muscle tissue samples (including six young and six old from Texel sheep). Hisat2, Cufflink, Cuffmerge, and Cuffdiff investigated the expression levels. Novel lncRNAs (Alternative spliced) were distinguished using NONCODE databases and Cuffcompare software. In addition, the lncRNA–mRNA interactions and regulatory network visualization were identified via RIsearch and Cytoscape software, respectively. Those 139 novel lncRNA (Alternative spliced) transcripts had been recognized, probably 65 lncRNAs interacted with their target genes and regulated sheep skeletal muscle growth and development. Three novel lncRNA transcripts (TCONS_00041386, TCONS_00050059, and TCONS_00056428) showed a strong association and five transcripts (TCONS_00055761, TCONS_00055762, TCONS_00055763, TCONS_00055764, and TCONS_00055770) had made complex network correlations with mRNAs. Our research provided more knowledge of the associated mechanisms with novel lncRNAs, which could play a role in regulating sheep skeletal muscle tissue development and growth.

Keywords: Regulatory network, Novel lncRNAs, Skeletal muscle, Alternative splicing, Gene expression

Introduction

Enhanced knowledge of the myogenesis molecular mechanisms of the livestock (especially ram and lamb) may help raise meat production (Relaix and Zammit, 2012, Rashidian et al., 2020). Texel is a breed of domestic sheep that originates from the island of Texel in the Netherlands. It is a well-muscled sheep, produces a lean meat carcass, and will pass on this quality to its offspring. It is currently the popular sheep in Europe, Australia, New Zealand, and the United States. The most notable feature of Texel is its significant muscle growth and mass (double muscling phenotype) (Clark et al., 2018). Texel Lambs show the advantage of having a full leg score among breed comparisons and less total carcass fat especially contact fat (Kinka and Young, 2019).

RNA-seq (RNA-sequencing) is a technique that can examine RNA quantity and sequences via next-generation sequencing (NGS). It analyzes the transcriptome profile of the cells (or gene expression patterns) in different groups or treatments to understand the related biological processes, such as skeletal muscle growth and development (Badday betti et al., 2022). Some genes can have several promoter regions a tissue-specific expression pattern (Ghanipoor-Samami and Javadmanesh, 2018). Alternative spliced (class-code"j") is a multi-exon with at least one junction match (Pertea and Pertea, 2020). Non-coding RNAs less than 200 bp are known as small ncRNAs and consist of small nuclear RNAs (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nucleolar RNAs (snoRNA), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs) and microRNAs (miRNAs). Non-

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coding RNAs with more than 200 bp length have been classified as long non-coding RNAs (Neguembor et al., 2014). The gene expression profiling and in situ hybridization investigations have discovered that lncRNA expression is developmentally controlled, can be cell- and tissue-type specific, and can differ temporally, spatially, or in response to the stimulants (Derrien et al., 2012). To date, only some lncRNAs have been identified in detail (Badday Betti et al., 2022).

Nevertheless, lncRNAs are believed to have a broad range of cellular and developmental functions and have been introduced as significant gene expression regulators. LncRNAs may perform either gene expression inhibition or activation via different mechanisms, complicating our understanding of genomic regulation. It is predestined that 25 – 40% of coding genes have overlapping antisense transcriptions. LncRNAs are differentially expressed through three developmental muscle periods in sheep, which might have vital functional roles in myogenic differentiation (Chao et al., 2016). LncRNAs such as H19 play a role in multiple biological processes, including negative regulation of body weight, cell proliferation, and embryonic growth control (Gabory et al., 2009; Gabory et al., 2010). LncRNAs have different classifications based on gene conservation or functions and play a role in chromatin modeling and genomic localization (Ramakrishnaiah et al., 2020). LncRNAs can regulate muscle growth and differentiation through cis-regulatory, trans-regulatory, or competitive endogenous RNAs, indicating that lncRNAs could be important muscle growth regulatory factors and potential valuable molecular marker regions for mutton sheep breeding (Ballarino et al., 2015). Ovine lncRNAs may be involved in skeletal muscle development in Texel and Ujumqin. These results revealed that lncRNAs like TCONS_00044801, TCONS_00008482, and TCONS_00102859 participate in muscle development (Li et al., 2018). A total of 39 differentially expressed lncRNAs were detected in mutton sheep. Subsequent bioinformatics analyses revealed that 29 lncRNAs were associated with muscle development, metabolism, cell proliferation, and apoptosis. Six lncRNAs noticed as hub lncRNAs, and four lncRNAs showed potential regulatory relationships (Chao et al., 2018). Consequently, in our study, we aimed to identify the effect of novel lncRNAs (Alternative spliced) and their target genes to improve knowledge of their roles in sheep skeletal muscle growth and development at early and adult stages. Also, it might provide a vision about the regulatory genes and put the foundation for selection programs to improve the

meat production policies in sheep.

Materials and Methods

Data Collection
We retrieved the RNA raw reads (paired-end) with accession numbers ERR4891 and ERR4892 for analysis based on the Ensemble database. Twelve samples represented at two diverse functional stages: six muscle tissue (longissimus dorsal) samples (ERR489116_1_fastqc to ERR489121_2_fastqc) of Texel juvenile 6-10 months, and six samples (ERR489188_1_fastqc to ERR489189_2_fastqc and from ERR489242_1_fastqc to ERR489245_2_fastqc) for adults (above one year). The sanger/Illumina 1.9 platform had been used for sequencing these samples. Sheep reference genome (Oar_v3.1) and annotated the GTF file (Oar_v3.1.96) based on the Ensemble database had been downloaded.

RNA Sequences Data Analysis

141079400 raw reads were generated by Illumina 1.9 platform with 2×151 bp paired-end reads before trimming was used, and the quality was analyzed by FastQC (v0.11.5) software (Andrews, 2010). Accordingly, we obtained clean data by removing contamination reads, including bases below quality reads, adapters, and low-quality reads using Trimmomatic software (v.0.36) (Bolger et al., 2014). Finally, reads with a minimum Phred quality score of 20 and 36 bp as a minimum length retained.

LncRNA Identification Pipeline
We used the pipeline in Figure S1 (in the supplementary part) to distinguish the candidate lncRNAs. The sheep genome (Oar_v3.1) was indexed in the first step, and the clean reads were aligned via Hisat2-build (Kim et al.,2015). We converted output SAM files from Hisat2 software to BAM files and sorted all BAM files using Samtools software v.1.9 (Li et al.,2009). The mapped reads had assembled using Cufflinks software (v2.2.1.OSX_x86_64) (Trapnell et al., 2010). All GTF files for 12 samples have merged, and the expression level of transcripts (Alternative spliced) had detected by using Cuffmerge software(v2.2.1) (Trapnell et al., 2012).

In the second step, candidate transcripts were identified with class code (Alternative spliced = j) using Cuffcompare software (v2.2.1) (Trapnell et al., 2010), based on related annotation GTF file (Oar_v3.1.96). The pipeline with the following criteria used to detect the potential novel lncRNAs

(Alternative spliced):

1-The transcripts with length ≥ 200 bp retained.
2-The transcripts with exon number < 2 had been removed.
3-The protein-coding potential of transcripts was filtered using two tools CPC2 (score > 0.5) (Kang et al., 2017) and PLEK (score > 0) (Li et al., 2014). The transcripts predicted as non-coding potential using the two tools above have remained.
4-We predicted the open reading frame (ORF) using the TransDecoder tool (v5.5.0) (https://transdecoder.github.io/), and ORF < 300 aa retained.
5-Novel lncRNAs (Alternative spliced) were distinguished using NONCODE databases (v.5) (Fang et al., 2018) http://www.noncode.org/.

Interactions of Novel lncRNAs with Potential Target Genes

The mRNA stability can be modulated by lncRNA (Ramakrishnaiah et al., 2020). We considered each "gene_biotype = mRNA" group in the annotation GTF file (Oar_v3.1.96) as potential target mRNAs. Interaction between lncRNAs–mRNAs had performed to investigate the target mRNA genes, which may organize by the novel lncRNAs using the RIsearch software (RNA Interaction search) (Wenzel et al., 2012) under base-pairing free energy no more than -50 agreement with (Yuan et al., 2020).

Construction of lncRNA-mRNA Network

Cytoscape software v.3.7.2 (Shannon et al., 2003) had been used to visualize the lncRNAs-mRNAs regulatory network

Result

RNA Sequencing and Mapping

Possible functional lncRNAs in parallel to alternatively spliced novel lncRNAs that may implicate the growth and development of sheep skeletal muscle tissue had been identified. Results for quality control, trimming, and mapping for 12 samples had been mentioned in (Table 1).

Table 1. The results for Quality control trimming and mapping had gained per 12 samples

Name of samples	stage	Raw reads before trimming	Raw reads after trimming	GC content (%)	Aligned concordantly exactly %	Overall alignment rate %
ERR489116_1_fastqc / ERR489116_2_fastqc	young	8647125	6622025	44	42.20	83.14
ERR489117_1_fastqc / ERR489117_2_fastqc	young	13368944	10132560	45	51.08	94.46
ERR489118_1_fastqc / ERR489118_2_fastqc	young	13756065	10454415	45	51.17	94.38
ERR489119_1_fastqc / ERR489119_2_fastqc	young	8685734	6641245	44	43.72	84.72
ERR489120_1_fastqc / ERR489120_2_fastqc	young	13374250	10156487	45	51.11	94.52
ERR489121_1_fastqc / ERR489121_2_fastqc	young	13831915	10539372	45	50.79	94.49
ERR489188_1_fastqc / ERR489188_2_fastqc	adult	19697023	15023092	46	63.77	94.04
ERR489189_1_fastqc / ERR489189_2_fastqc	adult	19792913	10350142	46	63.94	94.22

ERR489242_1_fastqc / ERR489242_2_fastqc	adult	9016506	7220274	45	50.03	91.87
ERR489243_1_fastqc / ERR489243_2_fastqc	adult	5771315	4633682	45	48.80	89.71
ERR489244_1_fastqc / ERR489244_2_fastqc	adult	9285314	7445816	45	50.25	91.85
ERR489245_1_fastqc / ERR489245_2_fastqc	adult	5852296	4703164	45	48.11	89.99

Total raw reads before trimming =141079400
Total raw reads after trimming = 103922274
Total of low-quality reads was removed = 37157126
Average aligned concordantly exactly = 51.2475%
Average overall alignment rate = 91.44917%
Average GC content = 45%

Identification of Novel lncRNAs in Skeletal Muscle of Sheep

Five criteria had used to distinguish the novel lncRNAs (Alternative spliced) using pipeline (Figure S1) at the two stages (young and adult) of skeletal muscle tissue in sheep. First, we filtered 649300 transcripts with class code = j (Alternative spliced) using Cuffcompare software. The remaining transcripts (368099) had classified as class code = j (Alternative spliced). Approximately 56% of assembly transcripts were discarded by choosing class code = j. 358072 transcripts had removed by submitting the retrained transcripts (368099) for filtering length < 200bp and exon number < 2. A total of 10027 remaining transcripts had subjected to predict coding potential using two tools CPC2 (score > 0.5) (Kang et al., 2017) and PLEK (score > 0) (Li et al. 2014). We obtained 2910 coding and non-coding transcripts, only 310 transcripts had considered as a non-coding potential, and any coding transcript in one of the two tools was removed. The non-coding transcripts had submitted to remove ORF ≥ 300 aa using the TransDecoder tool (v5.5.0). We subjected 308 of the remaining non-coding transcripts to identify the novel lncRNAs (Alternative spliced) using NONCODE databases (v.5). At last, we introduced 139 potential novel lncRNAs.

Interaction Between Novel lncRNAs and Target mRNA Genes

For predicting the binding positions between the novel lncRNAs (Alternative spliced) and targeted mRNA genes, 139 sequences of novel lncRNAs (Alternative spliced) (query) with 10921 sequences of mRNA genes (target) were interacted using RIsearch software (Wenzel et al., 2012) with a threshold of the base-pairing free energy no more than -50. Our findings demonstrated that 65 novel lncRNA transcripts had targeted 263 mRNA genes (Table S1). We have revealed that three novel lncRNA transcripts, TCONS_00041386, TCONS_00050059, and TCONS_00056428, had a strong relationship with targeted mRNA genes, and five novel lncRNA transcripts TCONS_00055761, TCONS_00055762, TCONS_00055763, TCONS_00055764, and TCONS_00055770 had created complicated network correlations with targeted mRNA genes. Moreover, we noticed that six of novel lncRNA transcripts, TCONS_00050059, TCONS_00056428, TCONS_00055761, TCONS_00055762, TCONS_00055763, and TCONS_00055764, had more expression in the young than in the adult stages except for two transcripts, TCONS_00041386 and TCONS_00055770, which had less expression level at the early stage depending on values of transcript expression as fragments per kilobase per million (FPKM).

Construction of lncRNA-mRNA Network

All of 65 novel lncRNA transcripts were selected to construct the interaction network between novel lncRNAs and targeted mRNA genes using Cytoscape software v.3.7.2 (Shannon et al., 2003) (Figure S2). Our results from the regulatory network demonstrated that among 65 novel lncRNA transcripts, TCONS_00041386 connect with 48 mRNA genes, TCONS_00050059 connect with 28 mRNA genes, TCONS_00056428 connect with 20

mRNA genes, and 5 novel lncRNA transcripts (TCONS_00055761, TCONS_00055762, TCONS_00055763, TCONS_00055764 and TCONS_00055770) connect with 146 mRNA genes to construct complex network correlations. We noticed that the *BEST2* and *MFSD4B* genes connection with 18,12 novel lncRNAs, respectively, to form a strong correlation.

(Yuan et al., 2020). In the present study, comprehensive RNA-seq analysis was used to identify the effect of novel lncRNAs (Alternative spliced) and their target mRNA longissimus dorsi muscle tissue samples of sheep in two functional stages.

Discussion

Accumulating evidence has indicated that long non-coding RNAs (lncRNAs) play vital roles in differentiation, development, and human disease by regulating gene expression (Saliani et al., 2021 and 2022). The lncRNAs with multiple mechanisms have been classified into six main paradigms: R Loop, miRNA decoy or sponge, scaffolds, tripartite helix, stabilizing mRNA, and guides (Ramakrishnaiah et al., 2020). Increasing evidence indicates that lncRNAs can modulate nearly every cellular process through their association with mRNAs, DNAs, miRNAs, and proteins (Li et al., 2019; Badday Betti et al., 2022). Therefore, binding position detections and interaction analysis between novel lncRNAs and targeted mRNA, RIsearch software (Wenzel et al., 2012) were used in which free energy of base binding is no more than -50
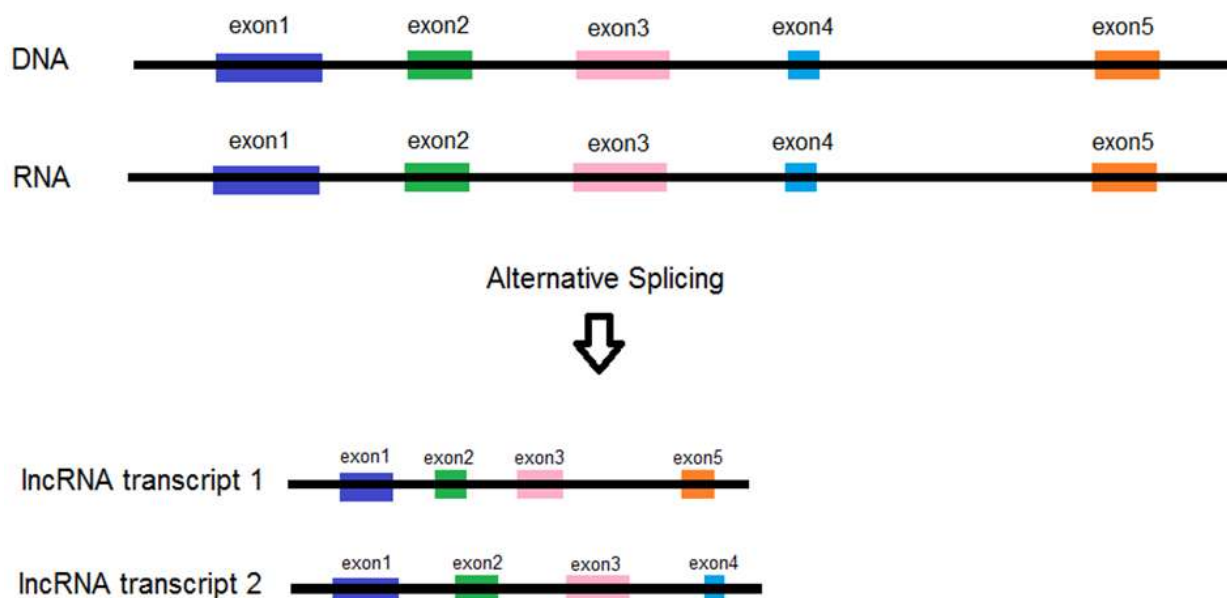
Figure 1. Schematic image of alternative splicing of lncRNA

The results implied the correlation between novel lncRNA transcripts with differential expression levels and muscle tissue in young and adult individuals. mRNA genes targeted by novel lncRNA transcripts extracted from the annotation GTF file for ovine muscle tissue were mentioned in many previous studies in which they play pivotal roles in skeletal muscle growth and development. Our results showed that 65 novel lncRNA transcripts had binding locations with 263 targeted mRNA genes (Yuan et al., 2020, Li et al., 2018 and 2019). Three novel lncRNA transcripts are strongly associated with their targeted genes. For example, TCONS_00041386 targeted 48 genes, TCONS_00050059 targeted 28 genes, and TCONS_00056428 targeted 20 genes. Clark et al. in 2017 showed that only 31 of 48 genes, 14 of 28 genes, and 9 of 20 genes, respectively, had shown differentially expressed in skeletal muscle between Texel (purebred) and T x BF (hybrid Texel x Scottish Blackface) (Clark et al., 2017). In addition, they reported that TCONS_00055769, novel lncRNA, targeted 29 genes and made a complex network, but only 19 out of 29 genes had appeared differentially expressed in skeletal muscle sheep between two breeds (Clark et al., 2017). In agreement with the previous study, TCONS_00047742, TCONS_00035416, and TCONS_00042104 (each separately) interacted with 10 genes but only 5 of 10 genes, 2 of 10 genes, and 3 of 10 genes, respectively, had demonstrated differentially expressed in sheep skeletal (Clark et al., 2017). TCONS_00042100, TCONS_00042102, and TCONS_00042103 interacted with the same 7 genes, but only 2 of 7 genes had mentioned differentially expressed in sheep skeletal muscle (Clark et al., 2017).

The other five lncRNA transcripts correlated with 146 mRNA genes and created complicated networks. For instance, TCONS_00055761 and TCONS_00055763 interacted with the same 27 genes, TCONS_00055762 interacted with 34 genes, TCONS_00055764 interacted with 30 genes, and TCONS_00055770 interacted with 28 genes, among them 18, 21, 18, and 17 genes, respectively also mentioned by Clark et al. to be differentially expressed in sheep skeletal muscle. Additionally, we observed some of these genes such as ENSOARG00000001608, targeted by TCONS_00055762, TCONS_00055770 and TCONS_00055764, ENSOARG00000016908 targeted by TCONS_00055762 and TCONS_00055764, ECI1, and

ENSOARG00000015179, ANPEP, ENSOARG00000018514 targeted by TCONS_00041386, NRAP targeted by TCONS_00056428 had higher expression levels in sheep skeletal muscle (fold change (FC) ≥ 2) (Clark et al., 2017). We found the ABL2 gene targeted by novel transcript TCONS_00055764, also reported by Yuan et al., as one of the differential expression genes in the longissimus dorsi muscle of sheep (Yuan et al., 2020). HSP90AA1, CXCL14, and NRAP genes targeted by novel transcripts TCONS_00041386, TCONS_00050059, and TCONS_00056428, respectively. Lobjois et al. have mentioned HSP90AA1 gene was involved with the myogenic differentiation and cell proliferation in pig longissimus dorsi muscle (LDM) (Lobjois et al., 2008). It was found that the CXCL14 gene had a vital role in the cell differentiation of chicken muscles (Nihashi et al., 2019). Noce et al. have shown that the NRAP gene was expressed in five breeds of sheep longissimus dorsi muscle (LDM) (Noce et al., 2018). Other genes such as PCK1, IRX3, CCND3, COLGALT2, KCNN1, UBE2Q1, CCDC88C, and SLC25A13 have been targeted by some of the novel transcripts such as TCONS_00041386, TCONS_00050059, TCONS_00055762, TCONS_00055764, TCONS_00055770, and TCONS_00055761 reported with differential expression patterns in skeletal muscle and high and low production mutton sheep.

Our study demonstrated six novel lncRNA transcripts, including TCONS_00050059, TCONS_00056428, TCONS_00055761, TCONS_00055762, TCONS_00055763, and TCONS_00055764, had high expression levels during the early stage compared with an adult. This result agrees with the article reported by Yuan et al. in 2020, which found there are time-specific lncRNA expressions through the pregnancy and after birth stages. Furthermore, they noticed those essential modifications in skeletal muscle development through gestation and newborn stages. In conclusion, we detected three novel lncRNA transcripts (TCONS_00041386, TCONS_00050059, and TCONS_00056428) had a strong relationship with targeted mRNA genes, and five novel lncRNA transcripts (TCONS_00055761, TCONS_00055762, TCONS_00055763, TCONS_00055764, and TCONS_00055770) created complex network correlations with targeted mRNA genes. Six novel lncRNA transcripts (TCONS_00050059, TCONS_00056428, TCONS_00055761, TCONS_00055762,

TCONS_00055763, and TCONS_00055764) had high expression in the young stages than in the adult. Finally, our findings proposed that novel lncRNAs (Alternative spliced) may play a critical role in regulating sheep skeletal muscle growth and development and improving mutton sheep breeding programs. Moreover, it is important to note that finding exact regulatory functions of lncRNAs need more investigation and research.

Conflict of Interest
The authors declare that there are no conflicts of interest.

Supplementary Information
Figure S1. Flow chart of the pipeline used to detect the novel lncRNAs (Alternative spliced)

Table S1. The results of the interaction between novel lncRNAs-mRNAs (Separate file as supplementary file).

Figure S2. Interaction network between novel lncRNAs and their target mRNA genes; red arrows denote lncRNAs, and green circles represent mRNAs. Lines represent the interaction between lncRNAs-mRNAs (Separate file as supplementary file).

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Supplementary Information:
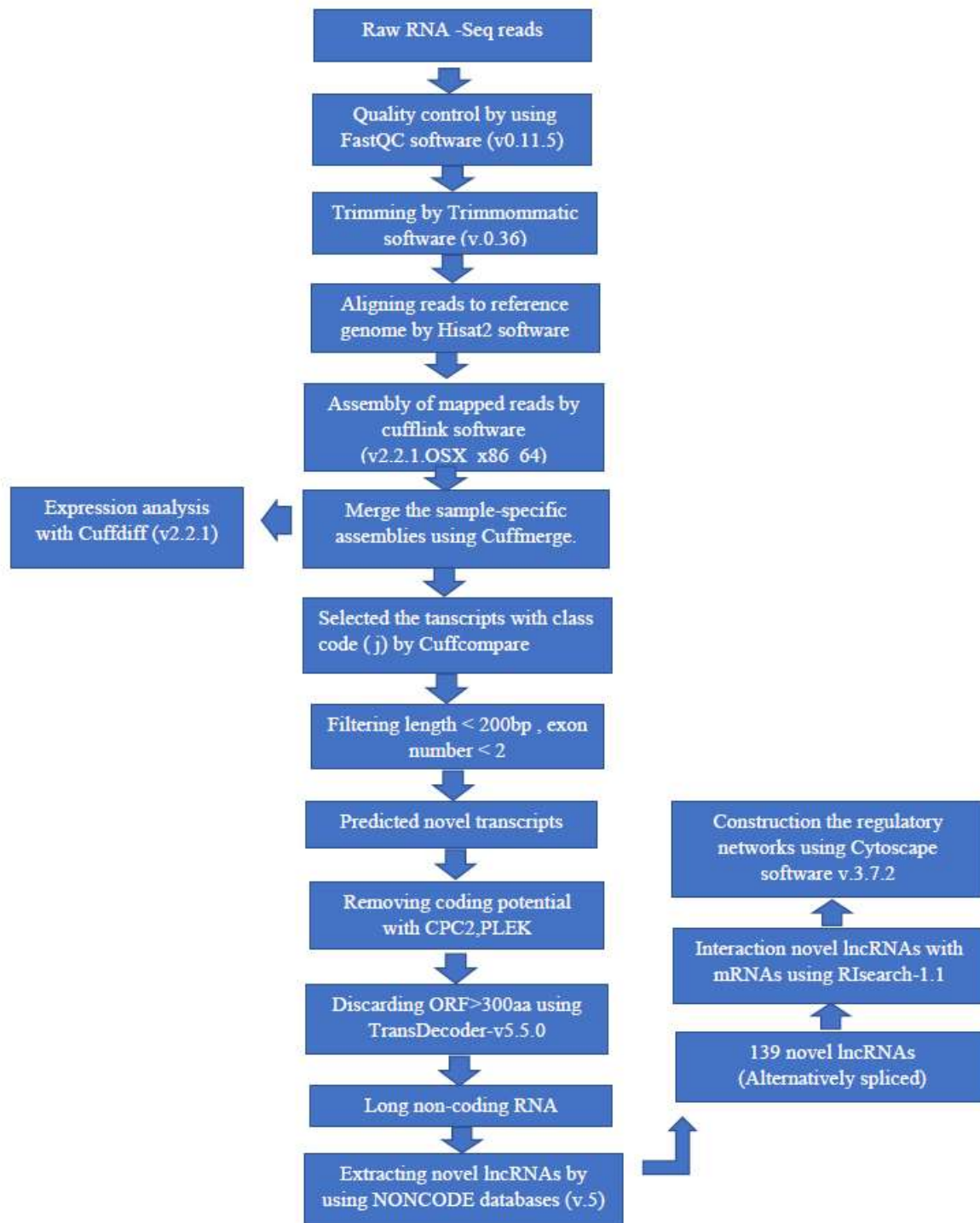
Figure S1. Flow chart for the pipeline was used to detect the novel lncRNAs (Alternative spliced)