Preservation of milk in liquid nitrogen during sample collection does not affect RNA quality for RNA-seq analysis

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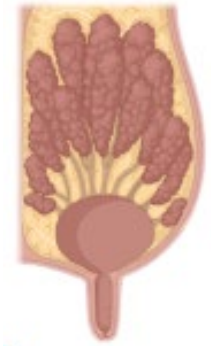
Session 97 "Biological mechanisms regulating inflammatory and energy metabolism in livestock species

INTRODUCTION

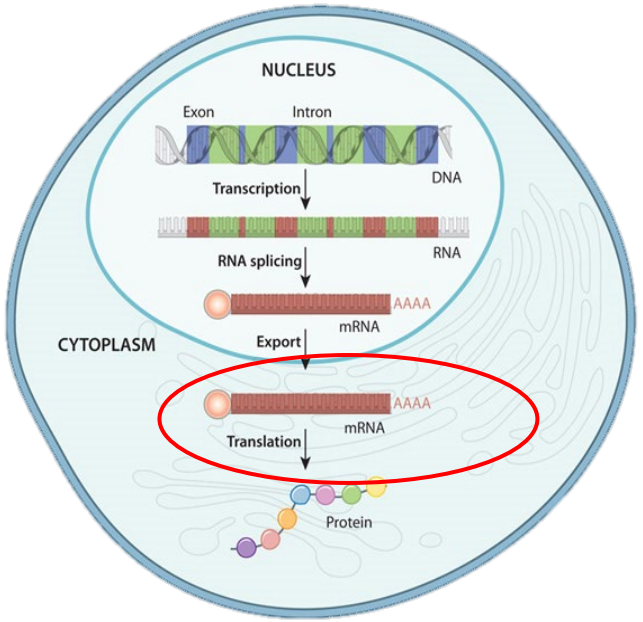
1.2. Why studying the mammary gland transcriptome?

MAMMARY GLAND

In charge of **synthesis** and **secretion** of **MILK**



TRANSCRIPTOMIC

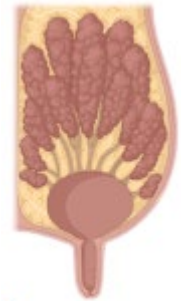


Will increase the BIOLOGICAL and PHYSIOLOGICAL knowledge of **LACTATION**

1.3. RNA sources for studying the mammary gland

From TISSUE samples

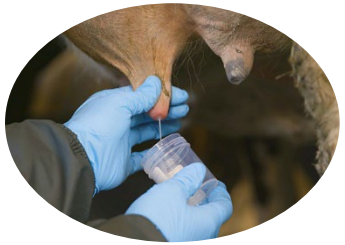
- **Mammary biopsies**
- **Laser microdissected mammary epithelial cells (LCMEC)**



✖ Damage
✖ No opportunities for multiple sampling
✖ Disrupts normal lactation process

From MILK samples

- **Milk Somatic cells (MSC)**
- **Antibody-captured milk mammary epitelial cells (mMEC)**
- **Milk Fat Globules (MFG)**



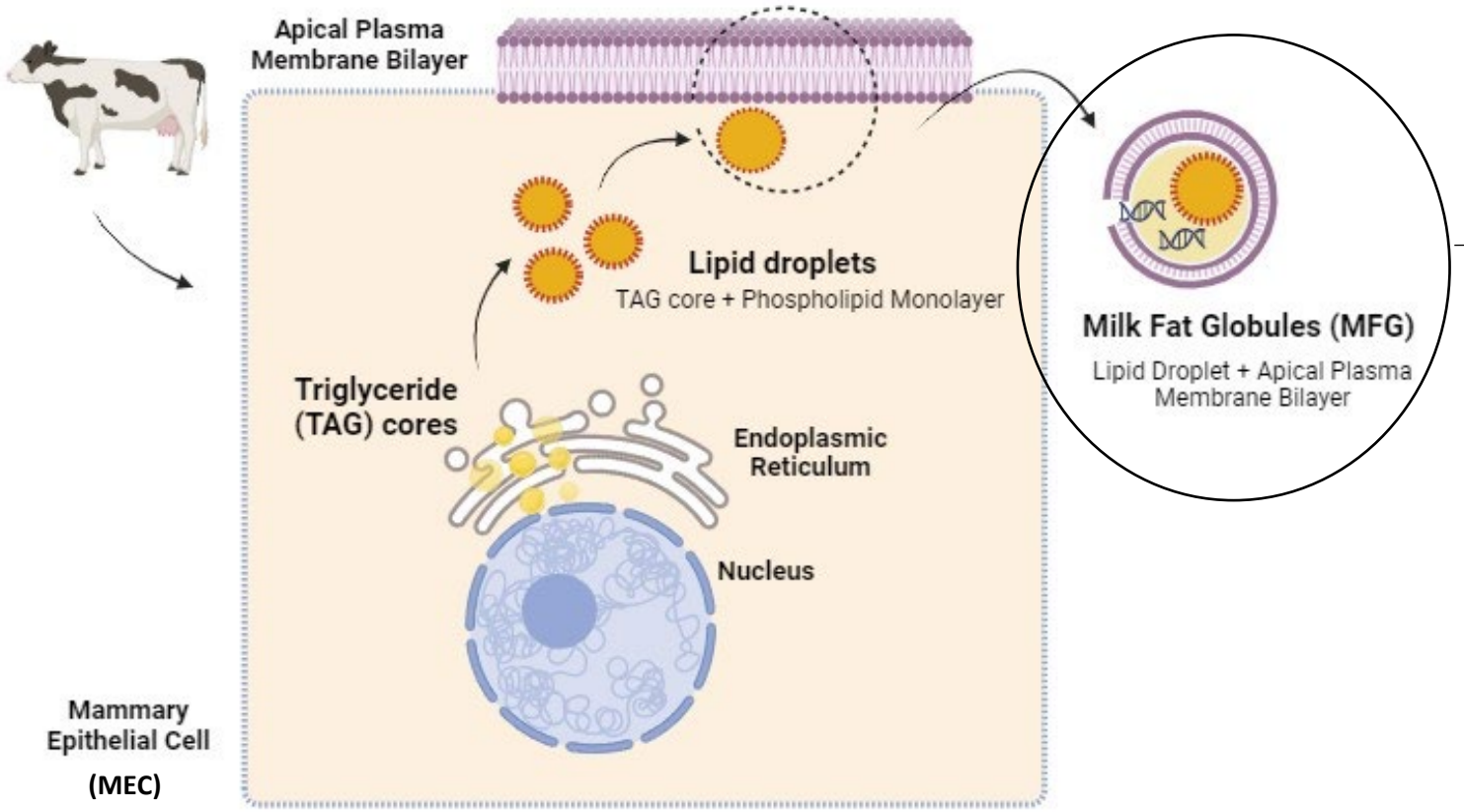
✖ **MSC:** only **2%** of the cells are **mammary epithelial cells (MEC)**

✖ **mMEC:** difficult to obtain

✔ **MFG** ➡ A good representation of the mammary gland transcriptome (Yang et al., 2015)

1.3. RNA sources for studying the mammary gland

MFG formation



The MFG envelop fragments of MEC (**cytoplasmic crescents**)

In which different types of **RNA** from MEC could be found

1.4. Milk sample collection for RNA-seq analysis

Standard procedures for **milk sample collection** in **transcriptome analysis**

ICE

- Require inmediate milk sample processing
- **MILK**: challenging matrix
  - High abundance of ribonucleases
  - Low quantity of RNA

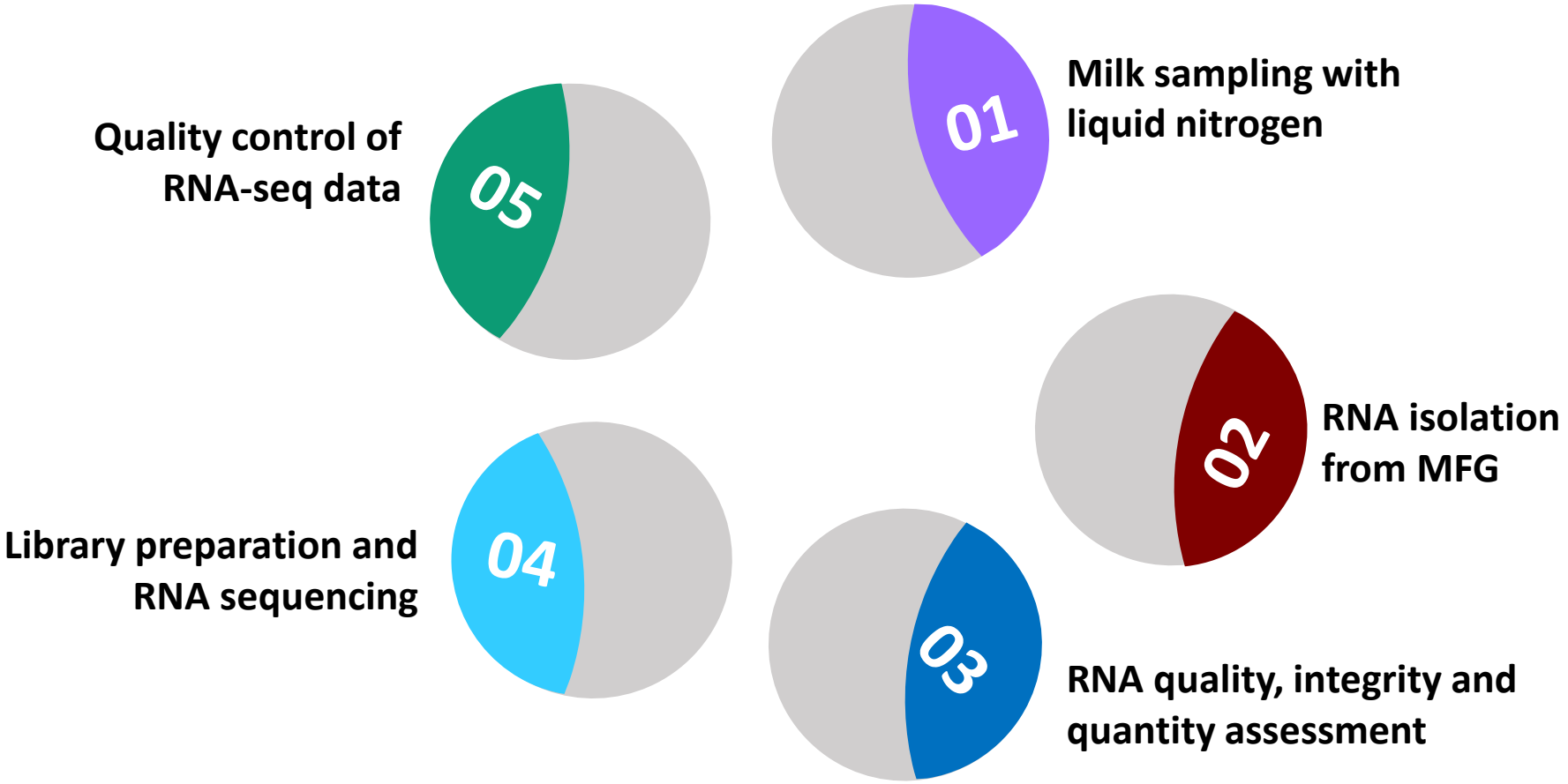
ALTERNATIVE MILK SAMPLE COLLECTION

- No inmediate milk sample processing
- Inactivation of ribonucleases

Liquid Nitrogen

OBJECTIVES

The applicability of a method for milk preservation with **liquid nitrogen** during sample collection, subsequent **extraction of total RNA from milk fat globules** and its sequencing by **RNA-seq**

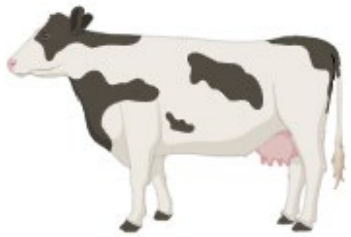


MATERIAL AND METHODS

3.1. Milk sampling with liquid nitrogen

❖ **Fifteen Holstein cows** from a commercial farm (Navarre, Spain) were selected for the study.

Milk was directly collected **from the udder of the cow**



…into **50 mL RNase-free** tubes…

…which were immediately snap-frozen in **LIQUID NITROGEN** and then **stored at -80°C** until further analysis.

3.2. RNA isolation from milk fat globules (MFG)

Milk sample pretreatment

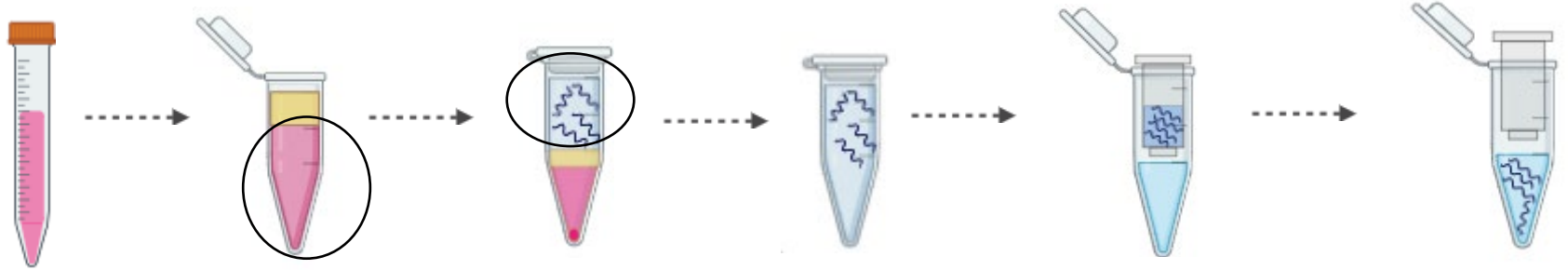
To enable **MFG collection**

- ✓ MFG fraction
- Skim milk
- Somatic cell pellet

RNA isolation

Using **Trizol LS Reagent**

Sample lysis → **Fat layer removal** → **Phase separation** → **RNA precipitation** → **Column-based method** → **RNA elution**



3.3. RNA integrity, quality and quantity assesment

Nanodrop 2000

- Concentration: $\mathbf{A_{260}}$
- Quality: $\mathbf{A_{260/280}}$
  - ✓ RNA: 1.9-2.1
- Quality: $\mathbf{A_{260/230}}$
  - ✓ RNA: 2.0-2.2

*A= Absorbances

TapeStation 4200 RNA Screentape

- Integrity: **RIN**
  - ✓ RNA ≥ 7

*RIN= RNA Integrity Number

Qubit 4 fluorometer

- Concentration: **RNA HS**
- Integrity: **RNA IQ**
  - ✓ RNA ≥ 9

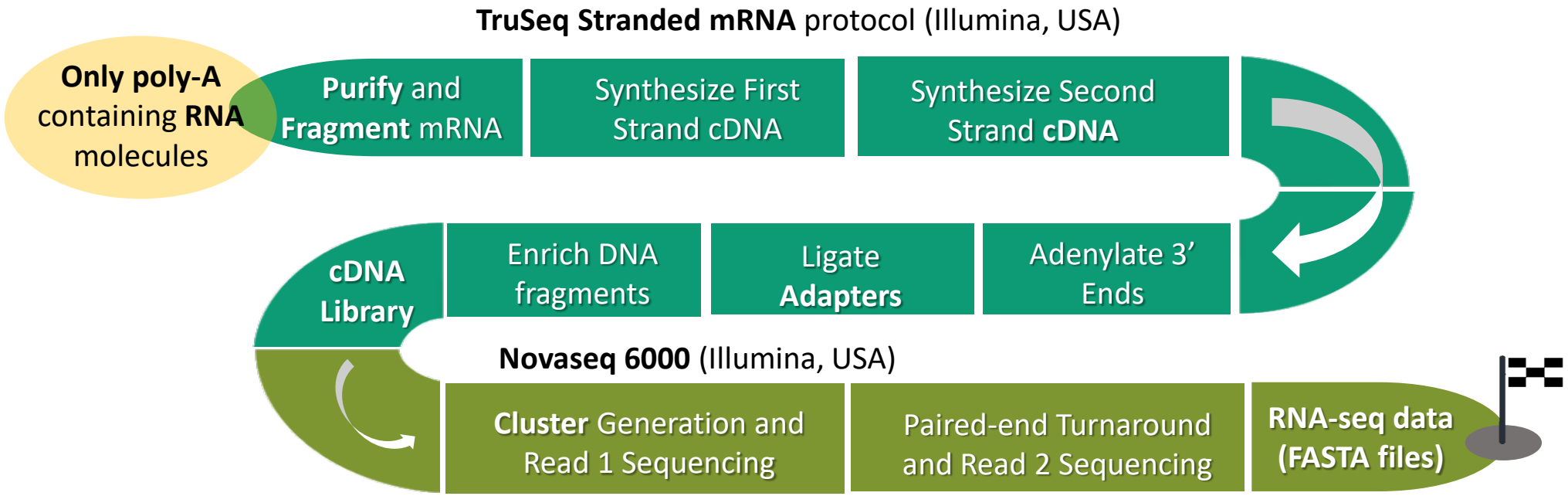
*HS=High Sensitivity; IQ= Integrity and Quality

Statystical analysis:

- $\mathbf{A_{260/280}}$: one sample equivalence test
- **RIN and RNA IQ :** one-sample non-inferiority test

3.4. Library preparation, mRNA sequencing and quality control

- **Library preparation and mRNA sequencing**



- **Quality control of RNA-seq data**
  - Base quality scores of RNA-seq reads: **Phred scale (Q)**

✔ Q = 30 – 40
✖ Q < 20: low quality

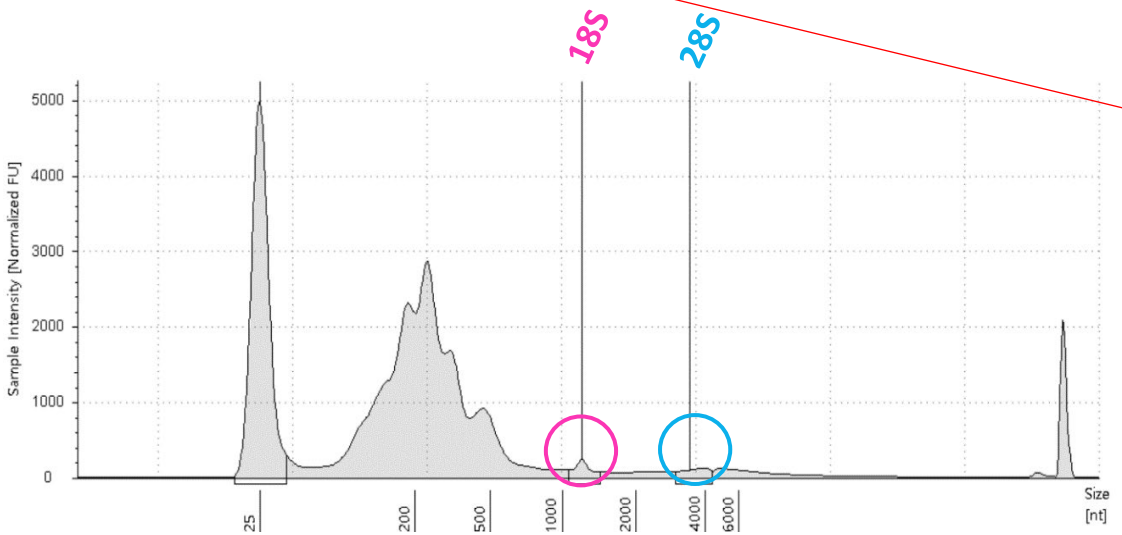
\+ FastQC Report

RESULTS AND DISCUSSION

4.1. RNA integrity, quality and quantity assesment

Quality and Integrity

Variable	Mean	SE	Reference value	Tested H_0	*P*-value
$A_{260/280}$	**2.03**	0.01	**1.9-2.1**	Mean-Reference ≤ -0.1 or Mean-Reference ≥ 0.1	$P_1 = 0.000$ / $P_2 = 0.000$
IQ	**9.51**	0.15	**> 9**	Mean-Reference ≤ 0	$P = 0.010$
RIN	**3.59**	0.27	**> 7**	Mean-Reference ≤ 0	$P = 1.000$



The **RNA from the MFG** contains a large amount of **low molecular weight RNA fragments** and a small amount of 28S and 18S rRNA (RIN).

4.1. RNA integrity, quality and quantity assesment

Quantity

Variable	Mean	SE	Minimum	Maximum	*n*
Nanodrop (ng/µL)	**120.43**	22.3	31.09	366.91	15
Qubit HS (ng/µL)	**102.87**	15.6	22.00	198.00	14

Same initial MFG quantity per animal (20-25 milk fat), but…

Low RNA concentration & High variability between animals

4.2. cDNA library quantification

Variable	Mean	Standard error	Minimum	Maximum	*n*
Library concentration (nM)	**8.64**	5.0	1.60	39.70	15

High variability among cDNA libraries

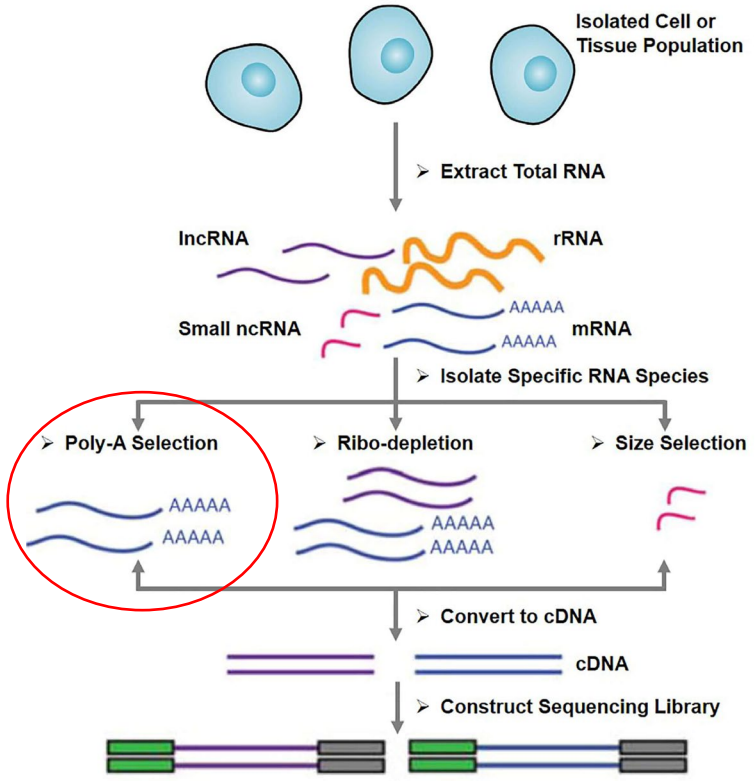
↓

Same initial input

(1µg of total RNA/animal)

Why?

Different quantities of **mRNA molecules** within **total RNA**



4.3. Quality control of RNA-seq data

A) Sequencing results

- A total of **791 million reads** were generated.
- An average read number of **52.7 million reads per sample.**

Q20 → The **98%** of the nucleotides were identified with an error LESS than 1/100

Q30 → The **94-95%** of the nucleotides were identified with an error LESS than 1/1000

Good quality of sequencing results

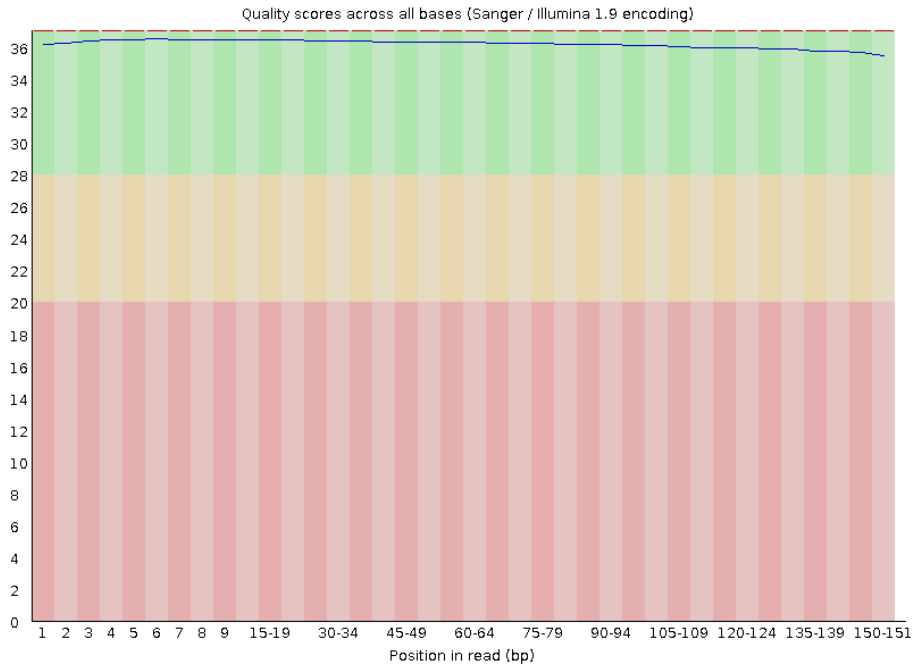
4.3. Quality control of RNA-seq data

FastQC Report

1. Introduction
2. Objectives
3. Materials and Methods
4. Results and Discussion
5. Conclusions

B) FastQC results

- Quality score per base ✓ Q= 36



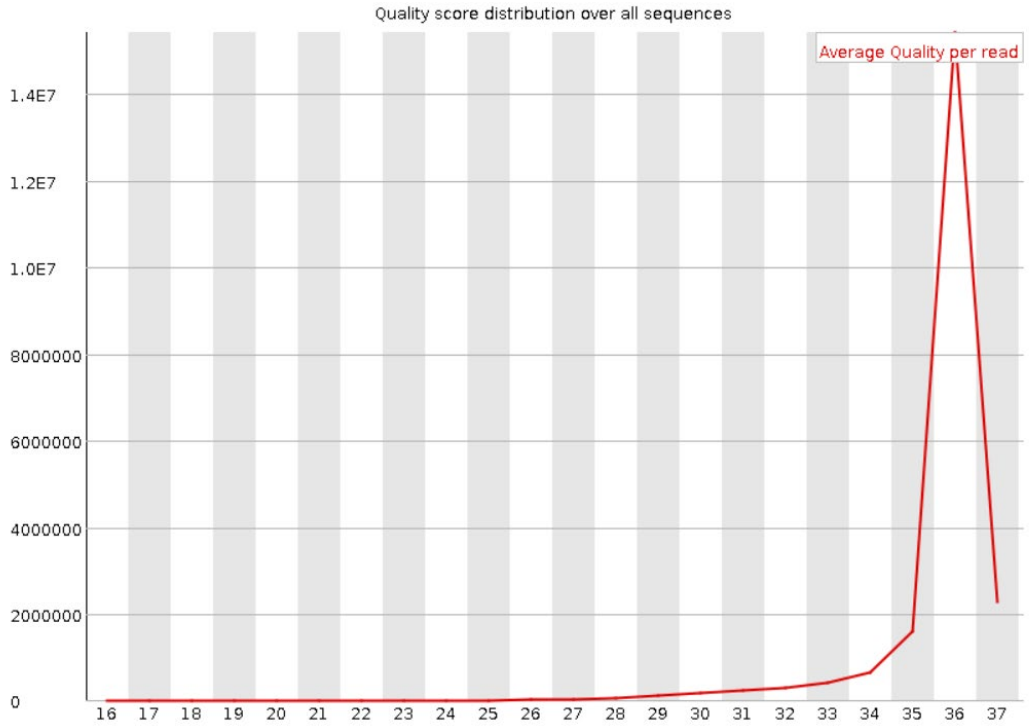
4.3. Quality control of RNA-seq data

FastQC Report

B) FastQC results

- Quality score per sequence ✔

Q= 36

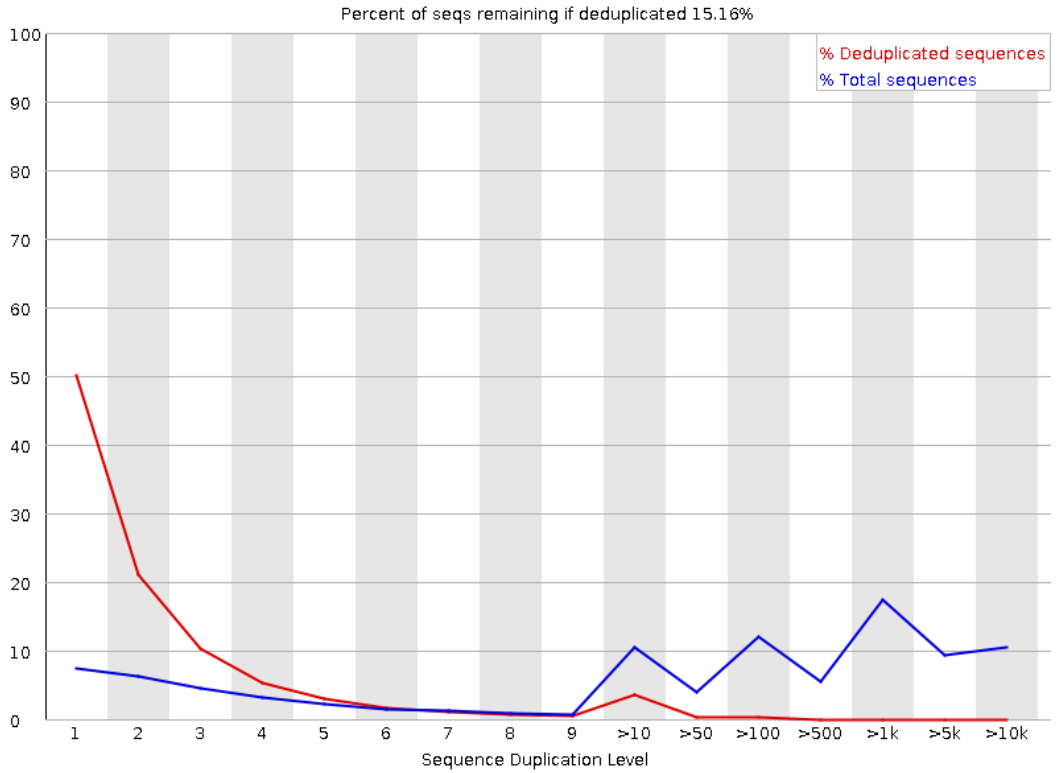


4.3. Quality control of RNA-seq data

FastQC Report

B) FastQC results

- Sequence Duplication levels ⟷ Overrepresented sequences



In a RNA-seq experiment is normal the presence of….

- **Biologically relevant transcripts** for this RNA matrix.

CONCLUSION

GENERAL CONCLUSIONS

- Results suggest that **milk preservation** using **liquid nitrogen** is a **suitable sample collection method** that prevents RNA degradation and overcomes the limitations of immediate sample processing required if using ice.

- The **quality, integrity and quantity of the RNA extracts isolated from MFG** were adequate allowing **successful downstream RNA-seq analysis.**

TAKE HOME MESSAGES

- This procedure could be considered a more **practical** and **non-invasive** means of **measuring the mammary epithelial cell transcriptome.**

- The **RNA isolated from MFG contained low molecular** RNA fragments and a very **low amount of 18S and 28S rRNA** due to the presence of small amounts of cytoplasmic material. Because of that **RIN values** are generally **below the benchmark of 7**.