

Sample Quality Considerations

For Long Read Sequencing

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“Delivering internationally competitive genomic services to the research community”

Ramaciotti Centre Overview

*Delivering
internationally
competitive
genomic
services to the
research
community*

National Infrastructure Facility (NCRIS)



15 ARC LIEF Grants

>\$9M ARC

>\$15M Universities

>500 research groups
>1,000 individual researchers

Not for profit



Est. 1999

18 staff

Steering Committee:
UNSW, USyd, Macquarie,
UTS, Newcastle
Garvan, Kolling, VCCRI

**60% clients
external**

**85% income
is external**

Team RAMAC



Image obtained from: The Ramaciotti Centre for Genomics: *We confirm we have permission to use these images presented here*

Technology

Sequencing

Illumina:

NovaSeq 6000

NextSeq 500

MiSeq

Image obtained from: T

RSII

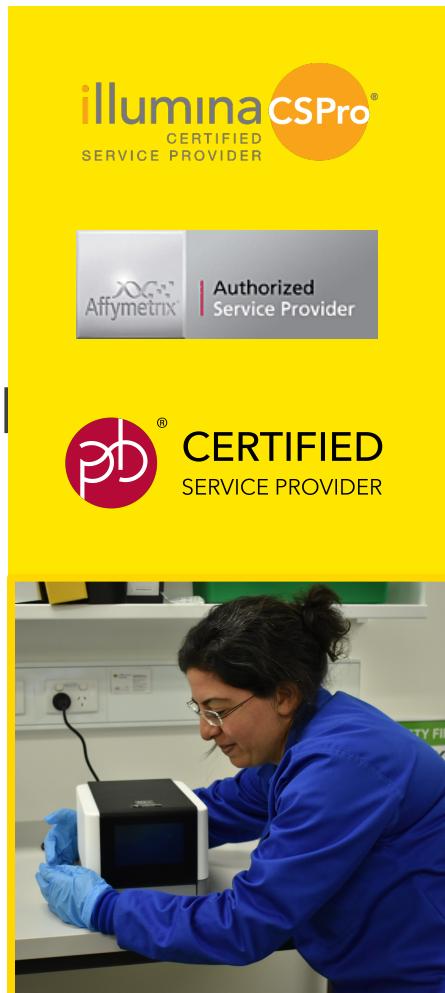
Sequel

Oxford Nanopore:

Minlon

Life Technologies:

AB3730 (Sanger)



Gene Expression & Genotyping

Affymetrix

Agilent

Fluidigm BioMark

NanoString

entre for Genomics

Single Cell

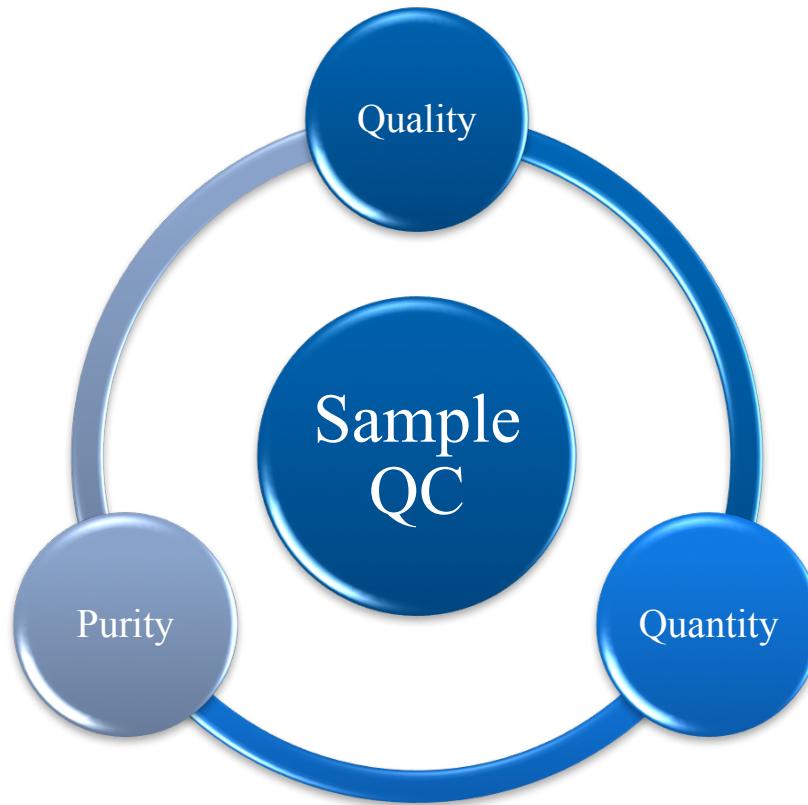
10X Genomics Chromium

Fluidigm C1 single-cell



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Three Aspects to Assessing Quality





Genomic Sample Quality

It is essential that DNA:

- Is double stranded.
- Has not been exposed to high temperatures (e.g.: > 65° C for 1 hour) or pH extremes (< 6 or > 9).
- Has not been exposed to UV or intercalating fluorescent dyes.
- OD260/280 ratio between ~1.8 and a OD260/230 ratio between 2.0-2.2.
- Does not contain insoluble material or RNA contamination
- Does not contain divalent metal cations, denaturants (e.g. guanidinium salts or phenol) or detergents (e.g. EDTA).
- Does not contain carryover contamination from the original organism/tissue (e.g. polysaccharides.)



RNA Sample Quality

It is essential that RNA:

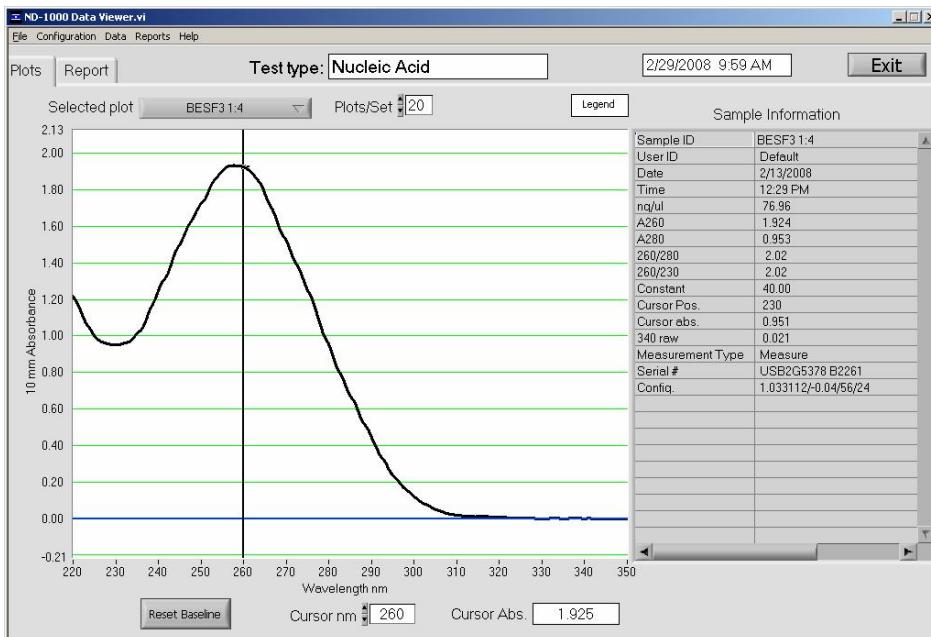
- Has not undergone multiple freeze-thaw cycles
- Has not been exposed to high temperatures (e.g.: > 65° C for 1 hour) or pH extremes (< 6 or > 9).
- OD260/280 ratio between 2.0-2.2 and a OD260/230 ratio between 1.8-2.2.
- Does not contain insoluble material.
- Does not contain DNA contamination.
- Does not contain denaturants (e.g. guanidinium salts or phenol) or detergents (e.g. SDS or Triton-X100).
- Does not contain carryover contamination from the original organism/tissue (e.g. heme, humic acid, polyphenols etc.)



Quantification/Purity: Nanodrop

NanoDrop ND-2000

Image obtained from:
<https://www.thermofisher.com/order/catalog/product/ND-2000>



Advantages

- Assess contamination
- No cuvette required
- Needs only 1-2 μ l of sample
- Broad spectral output
- DNA & RNA quantification

Disadvantages

- Only reliable >30ng/ μ l
- Quantification errors



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The Good!

Optimal Ratios

DNA

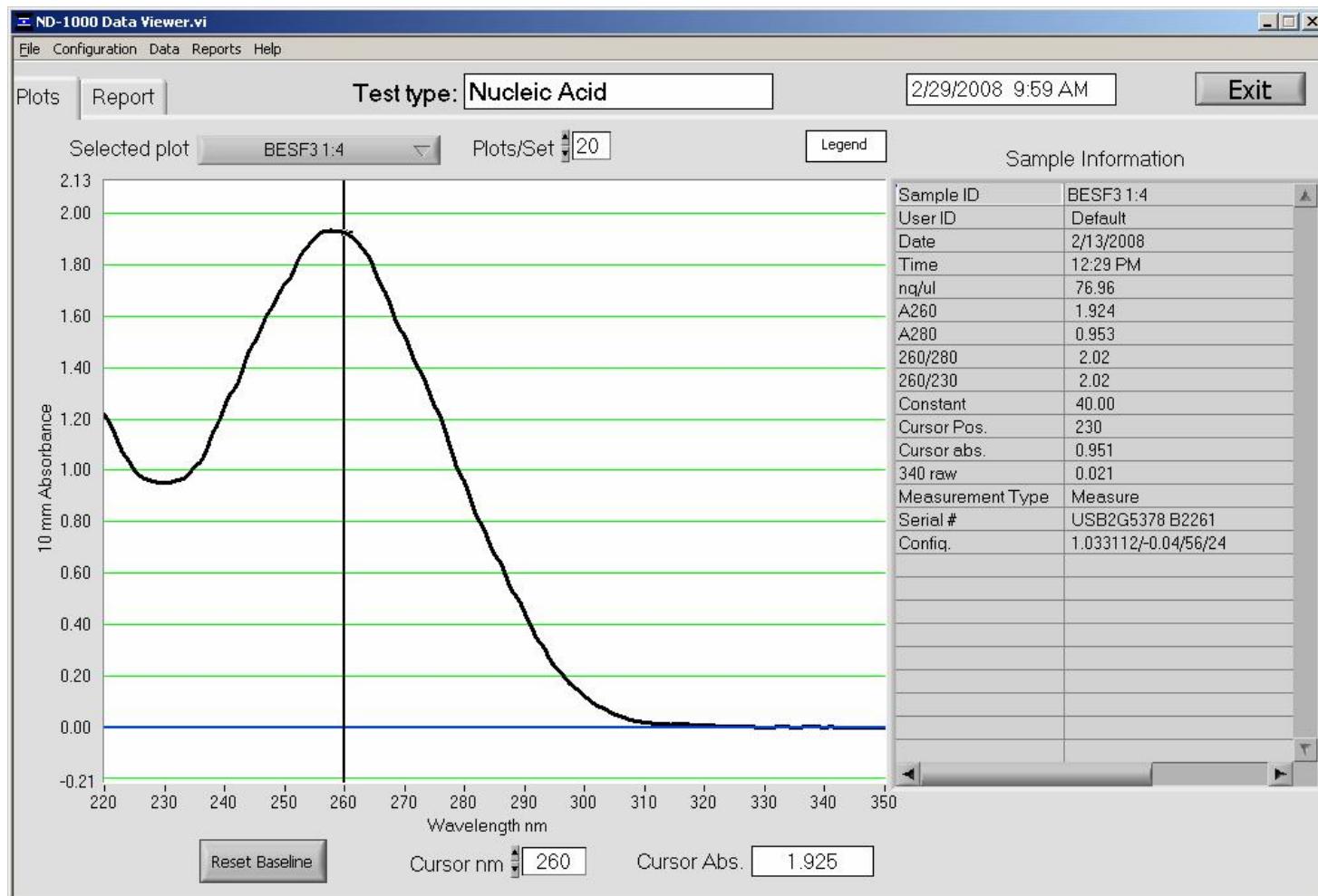
260/280 ~1.8- 2.0

260/230 ~ 2.0-2.2

RNA

260/280 ~2.0

260/230 ~ 2.0-2.2





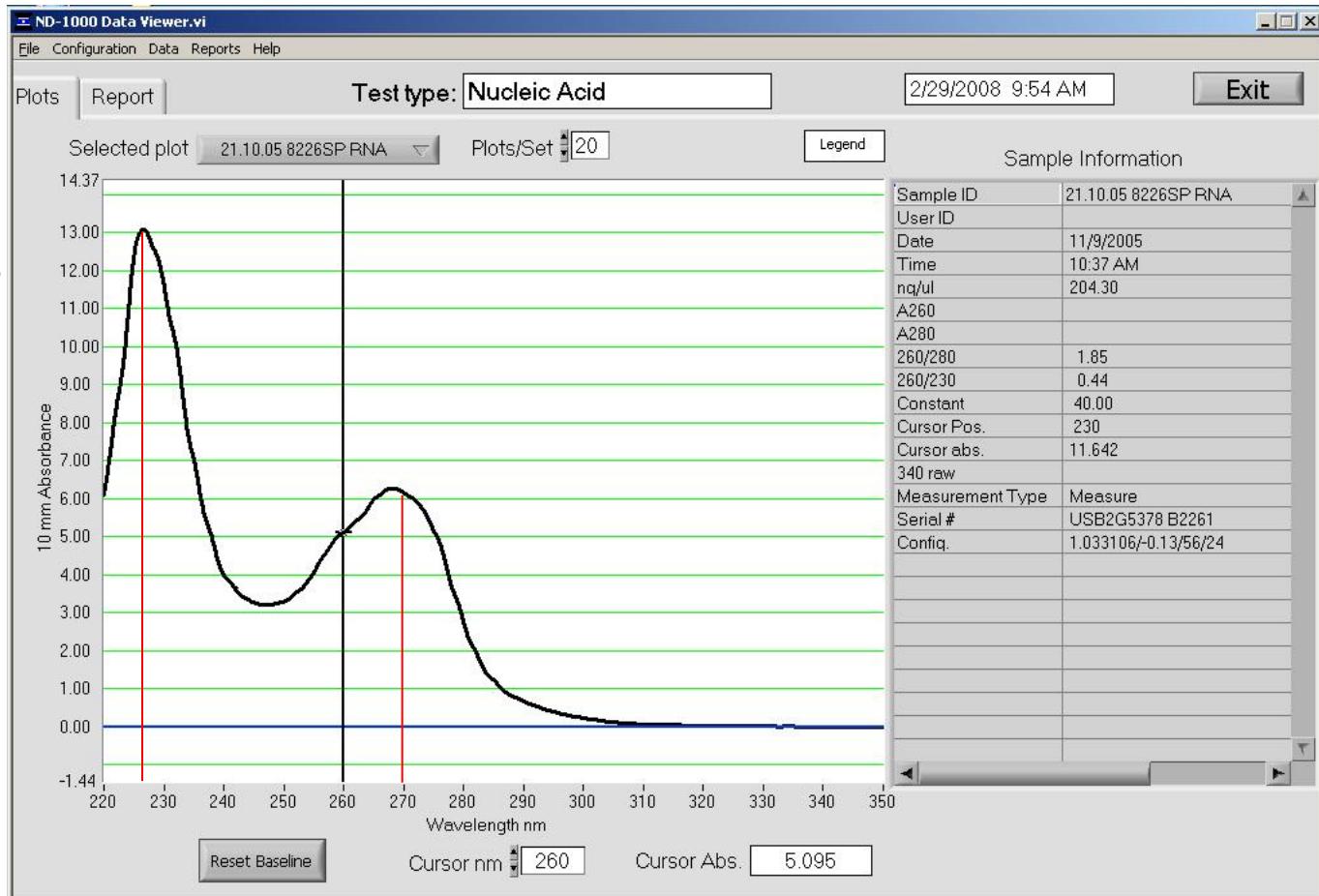
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The Ugly!

Low 260/230 ratios;
Presence of organic compounds

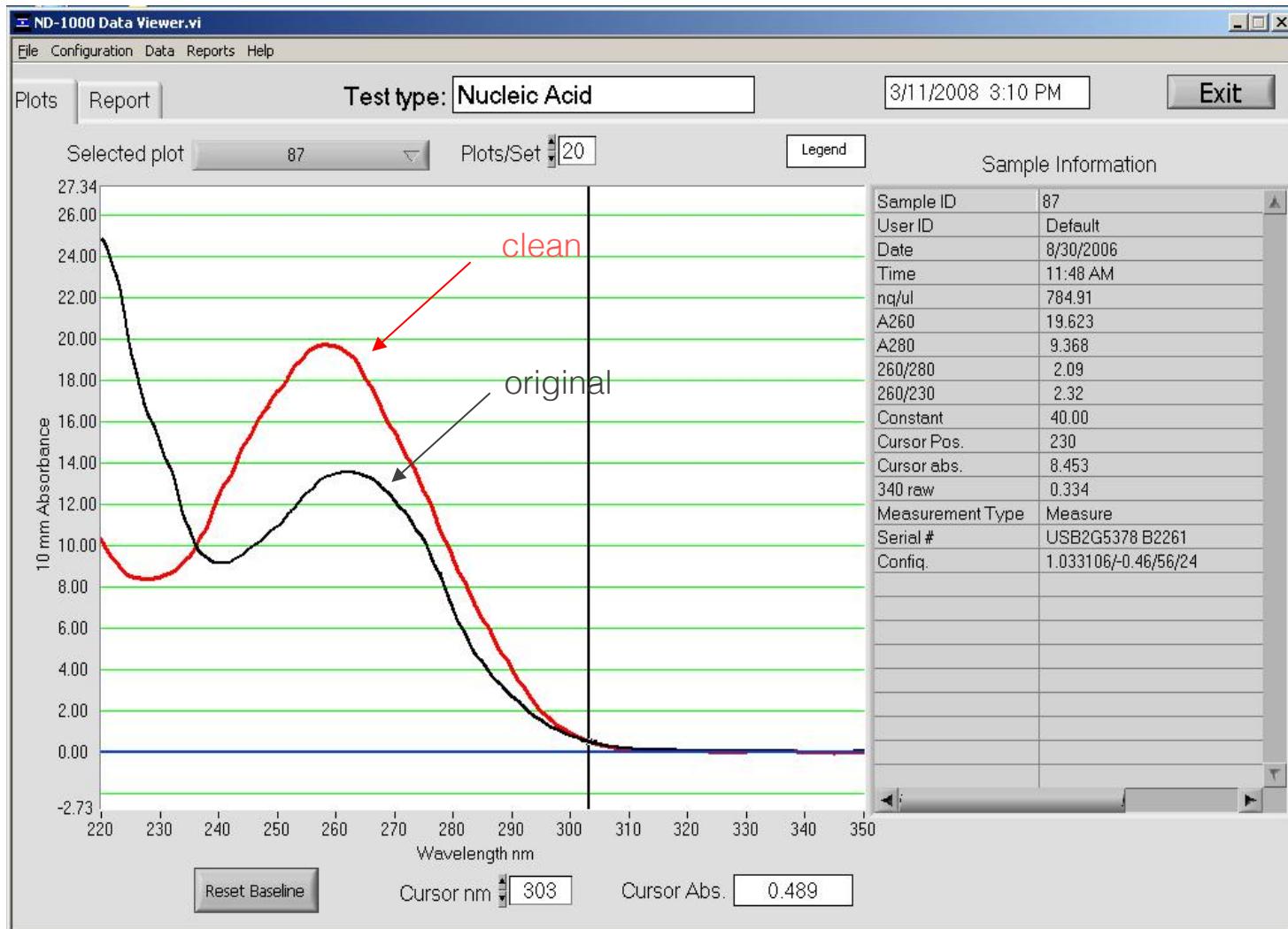
- Phenol
- Trizol
- Chaotropic salts

Low 260/280 ratios;
-Protein
-Guanidine
-Phenol





Sample Clean up





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Quantification/Purity:Trinean Xpose

Advantages

- Reads 16 samples in 1 minute
- Broad spectral output
- Spectral content profiling
- Specific quantification of DNA, RNA and protein fraction
- Subtraction of other impurities

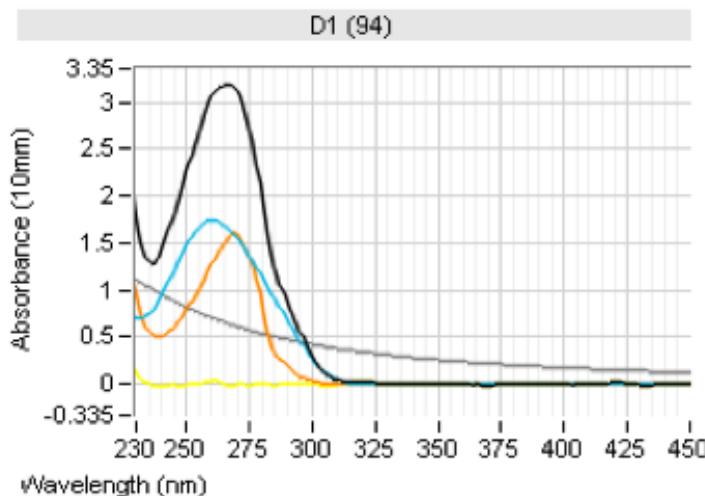
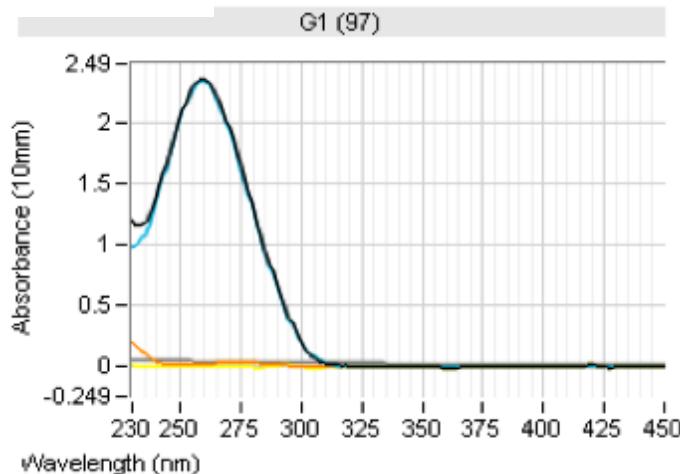


Image obtained from: <http://www.pltscientificinstruments.com/trinean-xpose.html>



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Quantification/Purity: Trinean Xpose





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Quantification – Fluorescence



Image obtained from:

<https://www.moleculardevices.com/systems/microplate-readers/absorbance-readers/spectramax-190-microplate-reader>

Methods for Quantifying Nucleic Acid

- Qubit assays for RNA and DNA
- RiboGreen for RNA
- PicoGreen for DNA



Image obtained from:

<https://www.thermofisher.com>

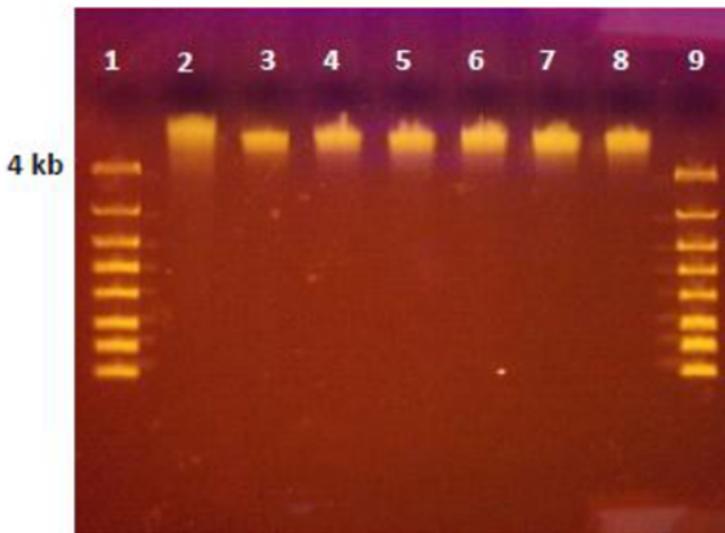


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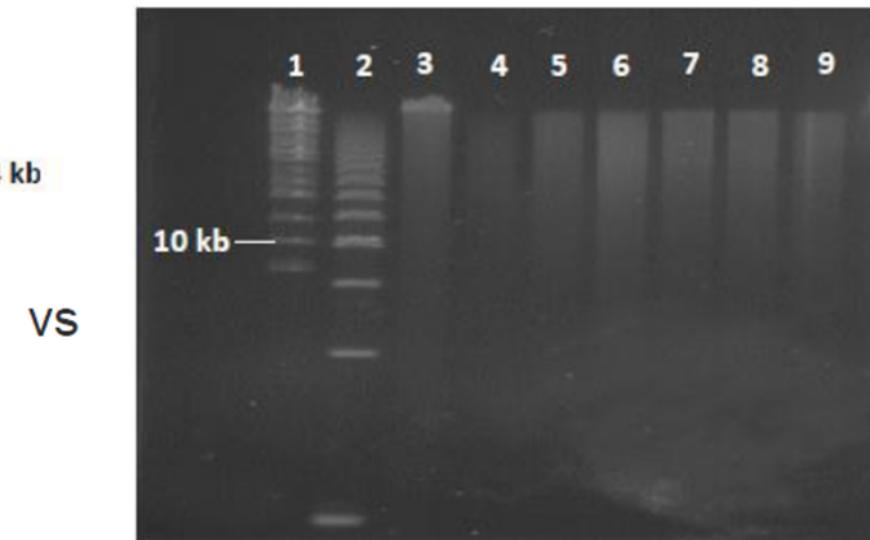
DNA Quality/Integrity

Is my agarose gel lying to me?

- Low percentage agarose gel can be misleading.
- Use Field Inversion or Pulse Field gel electrophoresis for accurate assessment of DNA integrity.



1.2% Lonza Gel



FIGE

Image obtained from: Pacific BioSciences: Guidelines for Preparing 20kb SMRTBell Templates
<https://www.pacb.com/wp-content/uploads/2015/09/User-Bulletin-Guidelines-for-Preparing-20-kb-SMRTbell-Templates.pdf>



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RNA Quality/Integrity- Bioanalyzer

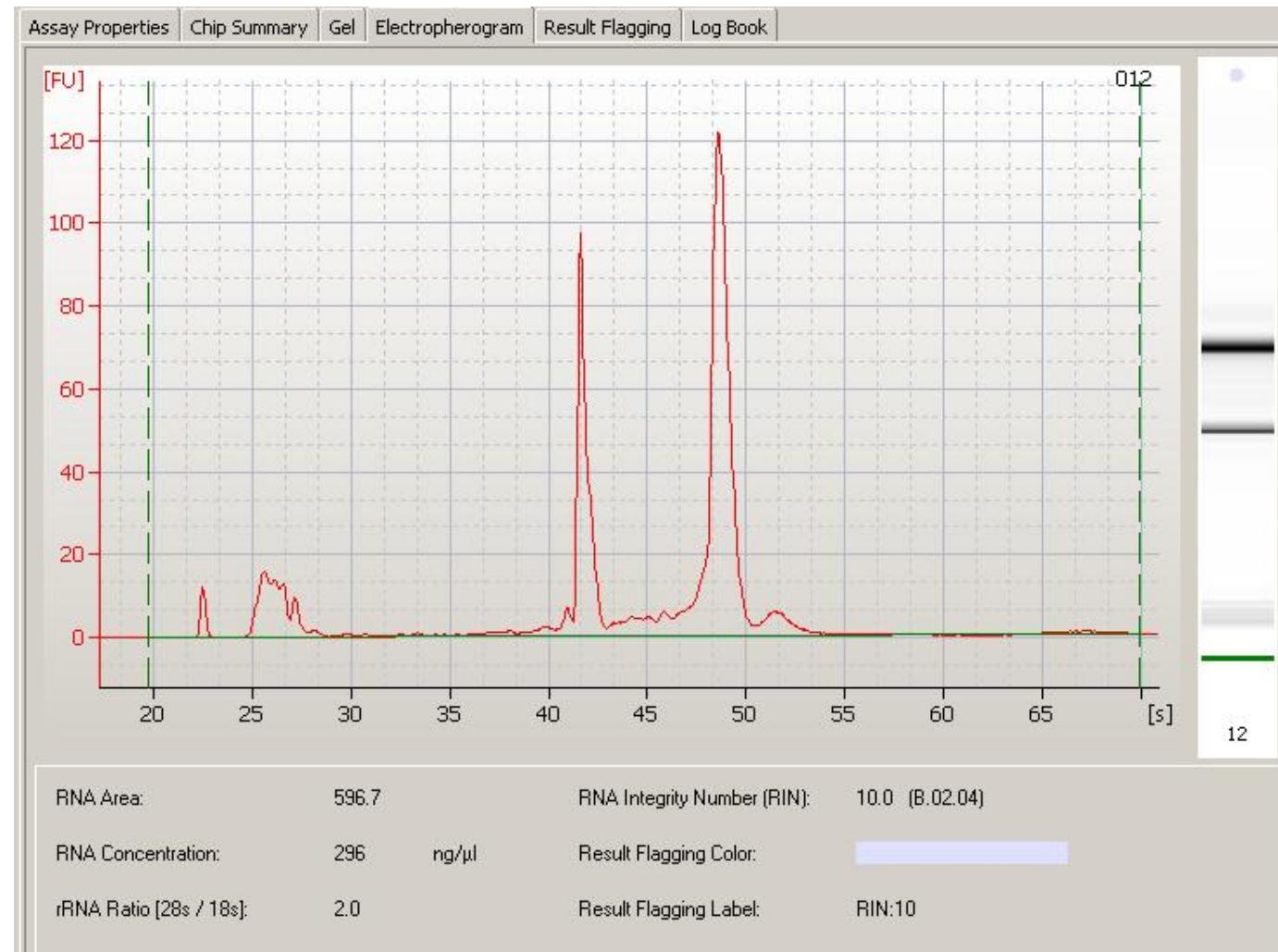
Intact RNA

RNA Integrity

Number (RIN): 9-10



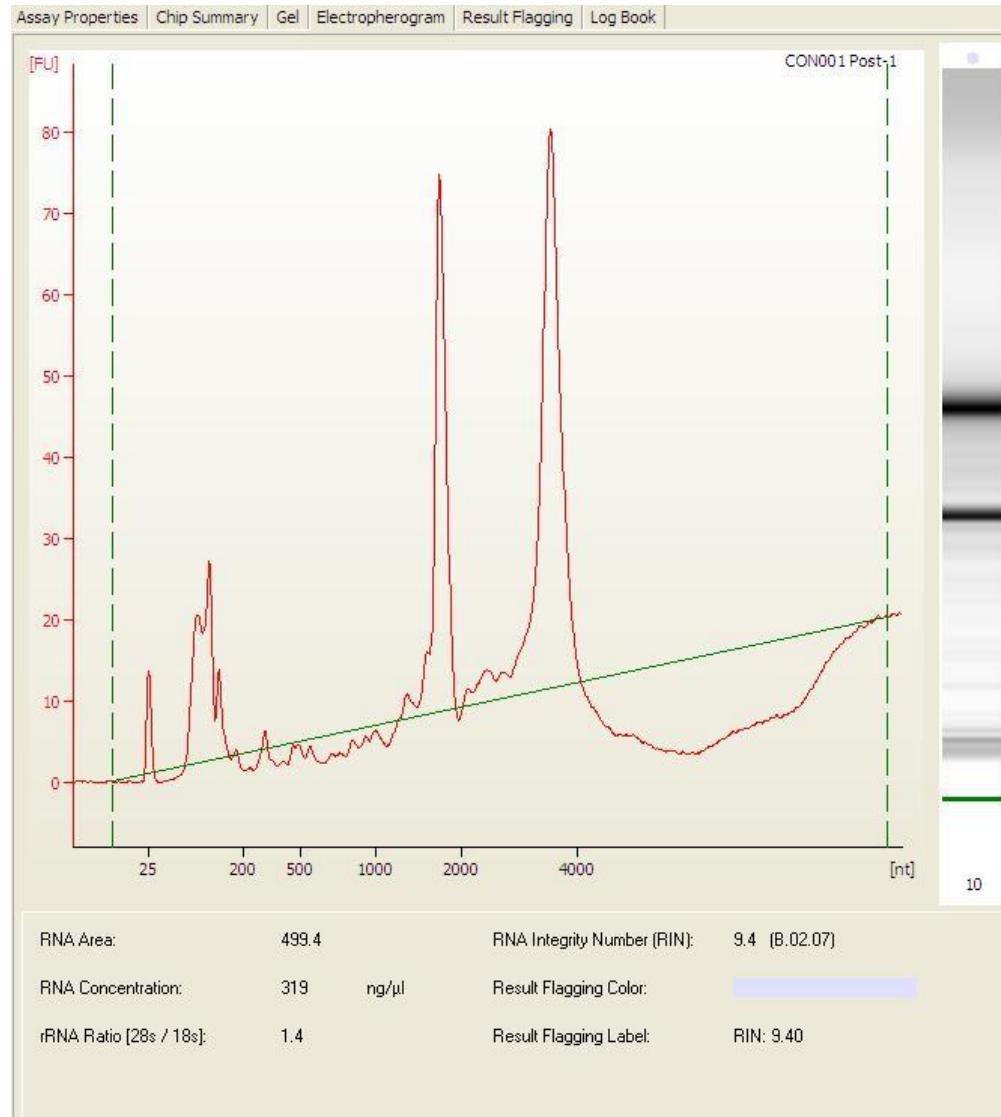
Image obtained from:
<http://www.integratedsci.com.au>





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DNA contamination of RNA





How to obtain Quality DNA/RNA

- ❶ Species dependent. May need multiple extraction methods.
- ❷ Do your research and be prepared for some trial and error.
- ❸ Some commercial extraction/clean up kit columns will shear DNA below 50kb.
- ❹ <https://www.protocols.io/> (Ben Schwessinger)
- ❺ Number of high molecular weight extraction kits on the market.
- ❻ Many Phenol/Chloroform protocols for long read sequencing on the internet.

DNA Extraction Methods

- ⌚ Standard Spin Column Kits: 20-50kb
 - ⌚ QIAgen DNeasy
- ⌚ Commercial high molecular weight kits: Up to ~150kb
 - ⌚ QIAgen MagAttract HMW
 - ⌚ QIAgen Genomic tip
- ⌚ Phenol/Chloroform methods: Up to ~200kb
 - ⌚ Must ensure DNA contains no remaining contaminates from extraction procedure.
- ⌚ Agarose plug: Up to ~1000kb
 - ⌚ Tricky procedure.



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Tips for Handling DNA/RNA

- ❶ Avoid too many freeze thaw cycles.
- ❷ Avoid vortexing: Flick mix or slow pipetting with wide bore tips.
- ❸ Avoid excessive heat exposure.
- ❹ Do not use UV/Ethidium Bromide for gel visualisation if you need to gel purify your DNA/RNA.
- ❺ Resuspend in a neutral buffer such as Tris HCL. Do not use EDTA.
- ❻ RNA: Maintain RNase free work conditions.

Thank You!



Image obtained from: UNSW Sydney