To NanoDrop® or Not to NanoDrop®:
Choosing the Most Appropriate Method for Nucleic Acid Quantitation

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Presentation Outline

- Nucleic acid quantitation in the DNA/RNA workflow
- UV absorbance
- Fluorescent dye-based quantitation
- Real-time PCR
- Examples of challenging samples
DNA Workflow
Each Step Affects the Quality of the Final Data

- Purify
- Quantify
- PCR Amplify
- qPCR
- Cloning
- Whole Genome Sequencing
- Microarray
RNA Workflow
Each Step Affects the Quality of the Final Data

Purify → Quantify → Reverse Transcribe → qPCR, End-Point PCR, Array, RNA-Seq, Northern Blot, Nuclease Protection

Protect
Sample Quantitation is a Key Workflow Step
Key Challenges Include Sensitivity, Accuracy, and Nucleic Acid Specificity

**Challenges**

- **Sensitivity**
  - effectively measuring small nucleic acid amounts

- **Accuracy**
  - affected by purity
  - detection range

- **Specificity**
  - dsDNA vs ssDNA vs RNA
  - human vs non-human
Three Common Methods of Quantitation Utilize UV Absorbance, Fluorescent Dyes and qPCR

- **UV Absorbance**
  - Spectrophotometer
  - NanoDrop®/NanoVue™

- **Fluorescent Dye-based Quantitation**
  - Plate Reader
  - Hand-held Instruments

- **Real-Time PCR**

What information does each method give us......

What information does each method *not* give us......

What are the advantages and disadvantages of each method.....
UV Absorbance Spectroscopy Gives a Rapid Assessment of Concentration and Purity

Measure nucleic acid:
✓ Concentration
✓ Purity

**Spectrophotometer**
(various manufacturers)

**NanoDrop®**
(Thermo Scientific)

**NanoVue™**
(GE Healthcare)
UV Absorbance Measures Different Components with Distinct Wavelengths

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Measurement</th>
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</thead>
</table>
| 260nm      | Amount of nucleic acid present in a sample
            | $A_{260\text{nm}}$ of 1.0 = 50µg/ml for dsDNA
            | 40µg/ml for RNA
            | 33µg/ml for ssDNA |
| 280nm      | Amount of protein present in a sample |
| 230nm      | Amount of other contaminants present in a sample |
| 320nm      | Amount of light scattering components present in a sample; used for background subtraction |
NanoDrop® Measures UV Absorbance of Small Volumes with Good Sensitivity

**Features:**

- Measures the absorbance of small volume samples
- 0.5 – 2μl of sample required
- 190nm – 840nm wavelength range
- Wide detection range
  - 2ng/μl minimum (DNA)
  - 15,000ng/μl maximum (DNA)
- Measurements in less than 30 seconds
- No other reagents or accessories required
NanoDrop® is an Easy-to-use and Popular System

Choose Read Type: dsDNA, ssDNA, or RNA

Read:
- Water
- Blank
- Sample

Output:
- Spectra
- Table
  - Concentrations
  - Purity ratios
  - Absorbance readings
Contaminants such as Guanidine Thiocyanate Lower the $A_{260}/A_{230}$ Purity Ratio

<table>
<thead>
<tr>
<th>Purity Measurement</th>
<th>Acceptable Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{260}/A_{280}$</td>
<td>Generally 1.8 – 2.2</td>
</tr>
<tr>
<td>$A_{260}/A_{230}$</td>
<td>Generally &gt;1.7</td>
</tr>
</tbody>
</table>

Guanidine Thiocyanate Affects $A_{260}/A_{230}$ Ratio

**Pure RNA:**
- $A_{260}/A_{280} = 1.80$
- $A_{260}/A_{230} = 2.19$

**RNA + 0.01% GTC:**
- $A_{260}/A_{280} = 1.87$
- $A_{260}/A_{230} = 1.16$
Large Peaks at Wavelengths Lower than 260nm Can Influence the Measured Peak at 260nm

Optimal Spectra

Large peaks at wavelengths lower than 260nm can influence the measured peak at 260nm, and low absorbance levels often yield unreliable concentrations.
**A₂₆₀ Absorbance is Not Specific and Cannot Distinguish Between dsDNA, RNA, or ssDNA**

<table>
<thead>
<tr>
<th>Sample A</th>
<th>Sample B</th>
<th>RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**DNA** → **RNA**

<table>
<thead>
<tr>
<th>NanoDrop® (ng/µl)</th>
<th>QuantiFluor™ dsDNA (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>213.5</td>
</tr>
<tr>
<td>Sample B</td>
<td>87.5</td>
</tr>
</tbody>
</table>

260nm reading represents total amount of all nucleic acid present in sample

Cannot distinguish between dsDNA, ssDNA, or RNA
Disadvantages of Absorbance Include Lack of Specificity, Overestimation, and Lack of Integrity Information

✓ Lack of Specificity
  • Cannot distinguish between dsDNA, RNA or ssDNA
  • Nucleic acid contamination cannot be determined

✓ Overestimation of nucleic acid concentration due to contaminants
  • Many contaminants absorb at or around 260nm

✓ No information on integrity
  • Nucleotides and small nucleic acid fragments still contribute to the 260nm reading
Three Common Methods of Quantitation Utilize UV Absorbance, Fluorescent Dyes and qPCR

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- Fluorescent Dye-based Quantitation
  - Plate Reader
  - Hand-held Instruments

- Real-Time PCR

What information does each method give us......

What information does each method not give us......

What are the advantages and disadvantages of each method.....
Fluorescent Dye-based Quantitation is a More Sensitive Method

• Dye binds nucleic acid – the resulting conformation shift produces in fluorescence when excited

• Fluorescence is directly proportional to the amount of nucleic acid in the sample

• Higher signal = more nucleic acid present

• Unbound dye does not fluoresce

• Low background increases sensitivity

Easy Protocol: Add, Mix, Measure

- Incubate at room temp for 5 minutes
- 504nm Excitation
- Emits @ 531nm
- Unbound dye
Two Fluorescent Dye Formats to Match Your Workflow
Single Tube or Plate Assays

1. Dilute dye to make working solution
2. Prepare standards and unknown samples
3. Add dye to samples and standards – incubate
4. Measure fluorescence

Single tube format:
- Quantus™ Fluorometer

Microplate reader:
- GloMax® Discover Detection System
QuantiFluor® Dyes are Optimized for the Different Types of Nucleic Acids

- **QuantiFluor® dsDNA System**
  - Specific for dsDNA with minimal binding to ssDNA, RNA, protein and interfering compounds
  - Sensitivity down to 50 pg/ml in a microplate system (200µl volume)

- **QuantiFluor® One dsDNA System***

- **QuantiFluor® ssDNA System**

- **QuantiFluor® RNA System**
QuantiFluor® dsDNA System Exhibits High Sensitivity Down to 50pg

R² = 0.9998

200ng Maximum

50pg Sensitivity
The QuantiFluor® RNA System Shows High Sensitivity Down to 100pg

- **High Concentration RNA Standard Curve**
  - $R^2 = 0.9984$
  - 500ng Maximum

- **Low Concentration RNA Standard Curve**
  - **100pg Sensitivity**
  - $R^2 = 0.9963$
Low Concentration DNA Samples May Fall Outside of the Range of the NanoDrop®

Comparing Concentrations Obtained Using the NanoDrop® and the QuantiFluor™ dsDNA system

<table>
<thead>
<tr>
<th>FFPE Sample</th>
<th>NanoDrop®</th>
<th>QuantiFluor™ dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/µl</td>
<td>(A_{260}/A_{280})</td>
</tr>
<tr>
<td>Kidney</td>
<td>7.02</td>
<td>1.77</td>
</tr>
<tr>
<td>Liver</td>
<td>8.65</td>
<td>1.76</td>
</tr>
<tr>
<td>Spleen</td>
<td>9.86</td>
<td>1.65</td>
</tr>
<tr>
<td>Heart</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>4.26</td>
<td>1.72</td>
</tr>
<tr>
<td>Int. tumor 1</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Int. tumor 2</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Int. tumor 3</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>
The NanoDrop® Instrument Often Overestimates the Amount of DNA in Solution

- Accurate quantitation is critical for many downstream applications
- Many FFPE tissue sections are small, and isolated DNA samples have concentrations well below the limit of detection of traditional spectrophotometric assays
- Even with highly purified DNA, the NanoDrop® consistently overestimates the amount of DNA in solution
The Key Advantage of Fluorescent Dye-based Quantitation is Sensitivity

- Some sample types such as FFPE contain low levels of nucleic acid
- Absorbance methods (NanoDrop®) lack detection *sensitivity* as well as *target-specificity*
- Proper nucleic acid quantitation improves success in a variety of downstream assays such as PCR, cloning, next gen sequencing (NGS), transfections

Fluorescence is 40,000X more sensitive than NanoDrop® UV
Disadvantages of Fluorescent Dye-based Quantitation Include No Information on Purity or Integrity

✓ Must create standards

✓ No information on purity
  • Separate dye-based quantification systems are available for ssDNA, RNA and protein

✓ No information on integrity

✓ Lack of specificity with RNA or ssDNA dyes

✓ Fluorescent dyes are potentially hazardous
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- **Real-Time PCR**

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Real-Time PCR (quantitative PCR, qPCR) Quantitation Involves Detection of Product at Each Cycle

**What is end-point PCR?**

The amount of amplified product is typically determined only after a set number of amplification cycles is completed.

**What is Real-Time PCR?**

The amount of amplified product is measured after each PCR amplification cycle.

![Graph showing fluorescence over cycles](image)
The Amplification Curve Shows Accumulation of Product as PCR Progresses

- **baseline** – initial reporter fluorescence, measured before product formation can be detected
- **exponential phase** – stage of reaction when product is doubling with each cycle
- **plateau phase** – stage of reaction when rate of product formation is diminishing
Viewing the Data on a Log Scale Allows Different Phases of the Curve to Be Seen More Clearly

- **baseline** – initial reporter fluorescence, measured before product formation can be detected
- **exponential phase** – stage of reaction when product is doubling with each cycle
- **plateau phase** – stage of reaction when rate of product formation is diminishing
The Threshold Is Where Product Accumulation Can Be Distinguished from the Background

- **threshold** – the fluorescence level where product can be distinguished from background
- **C_q** – the cycle number at which amplification can be detected by change in fluorescence
- **ΔR_n** – the change in signal during amplification (compared to baseline)

![Graph showing detection threshold set above baseline, within exponential region of amp curve]
Absolute Quantitation can be Determining by Using a Standard Curve

- Standard curve is generated by plotting $C_q$ vs (log) concentration for known sample
- Concentrations of unknown samples are extrapolated from the standard curve, based on $C_q$
- Amplification efficiency is also derived from the standard curve (function of slope)
Amplification Efficiency is a Measure of PCR Reaction Quality

- If amplification is 100% efficient, product doubles with every cycle
- Slope of -3.3 = 100% efficiency
- Ideal range: ± 5%
- Acceptable range: ± 10%

Efficiency = \(10 \left( \frac{-1}{\text{slope}} \right) - 1\) \times 100%

Slope: -3.373  
R\(^2\): 0.9995  
Efficiency = 97.9%
Relative Quantitation is Used to Determine Relative Template Levels in Test Samples vs. Controls

Relative quantitation is based on the premise that the amount of product doubles with each cycle.

This relationship also works in reverse: two samples with a $C_q$ difference of 1 would have a two-fold difference in starting template amount.

- Can be used to determine relative template levels in test samples vs. controls

This is only accurate if amplification efficiency is 100%.

- Assay validation should include standard curves to calculate efficiency
- If amplification efficiency is less than 100%, then calculations can be adjusted to correct for the decreased efficiency


Relative Quantitation Compares the \( C_q \) of the Experimental Sample to the \( C_q \) of a Control Sample.

Gene of Interest: LIN28

Reference Gene: GAPDH

\[
\Delta C_q = (C_q \text{ experimental sample}) - (C_q \text{ control sample})
\]

\[
\Delta \Delta C_q = (\Delta C_q \text{ gene of interest}) - (\Delta C_q \text{ reference gene})
\]
RT-qPCR is the Detection of RNA Transcripts Using a Combination of Reverse Transcription & qPCR

RT-qPCR: Reverse Transcription followed by qPCR amplification.

Conversion of a specific RNA molecule into cDNA followed by DNA-based PCR amplification.

RT-qPCR is a powerful method of gene expression analysis.
• Determine the presence or absence of a transcript
• Quantitate mRNA expression levels (transcript abundance)
RT-qPCR is a Highly Quantitative Measure of mRNA Levels

Advantages

• Highly quantitative measurements
• Limited multiplexing is possible (probe-based systems only)
• Wide dynamic range

Disadvantages

• Limited number of target mRNAs assessed per reaction

Key Challenges

• Wide variation in mRNA expression levels per sample
• RT-qPCR enzymes may be sensitive to inhibitors in starting RNA sample – e.g.:
  • RT and/or Taq DNA Pol in 1-step RT-qPCR systems
  • RT in 1\textsuperscript{st} step of 2-step RT-qPCR experiments
**Better Sensitivity Increases Your Ability to Analyze Weakly Expressed Genes**

- **GoTaq® 2-Step RT-qPCR System**
  - Combination of GoScript® RT Kit and GoTaq® qPCR Master Mix

- **GoTaq® 1-Step RT-qPCR System**
  - Single tube rxn for both RT and qPCR
  - Built on strength of GoScript® RT and GoTaq® qPCR Master Mix
  - Sensitive, reproducible detection of low abundance transcripts in a single tube

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**Improved Sensitivity**

- **GoTaq® 2-Step RT-qPCR System**
  - Brighter Fluorescence
  - Earlier Cq's

**EXPRESS Two-Step SYBR® GreenER™ Universal**

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Promega Corporation
Template Purity Is Key to Amplification as Inhibitors May Be Present in Starting Samples

• Environmental samples can be challenging with qPCR analysis
  o Food samples, fecal samples, soil, wood, plant material, textiles
  o Inhibitors from these samples may be present in “purified” nucleic acid

• Inhibitors can also be introduced during nucleic acid purification
  o Part of purification protocol/kit
  o Chaotropes, alcohols, chelators, detergents, solvents

Some qPCR master mixes will perform better in the presence of inhibitors
Higher Tolerance to Inhibitors Allows for Amplification of RNA from Difficult Samples

GoTaq® qPCR Master Mix  
Power SYBR® Green Master Mix

Customer data courtesy of Andrei C., University of Louisiana Lafayette

Amplification of target RNA in difficult samples with high levels of contaminants
Template Integrity is Important as Fragmented Samples May Affect qPCR Results

Some sample types are more likely to be degraded

- Samples exposed to nucleases, heat and UV light
- FFPE samples: cross-linking introduced by the fixation and embedding process results in nucleic acids that are characteristically partially degraded after extraction
- When designing downstream amplification assays, best results will be achieved when targeting regions of 200 nucleotides or less

Options for assessing template integrity, include:

- Gel analysis
- Agilent Bioanalyzer (Agilent Technologies)
- Fragment Analyzer (Advanced Analytical)
DNA Fragmentation Directly Affects Amplifiability

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ave. BP</th>
<th>Amplification Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>100bp</td>
<td>NO amp, NO amp</td>
</tr>
<tr>
<td>Sample 2</td>
<td>500bp</td>
<td>+ amp (2/3), NO amp</td>
</tr>
<tr>
<td>Sample 3</td>
<td>1500bp</td>
<td>+ amp (3/3), + amp (2/3)</td>
</tr>
</tbody>
</table>
Amplifiability of the Sample Can Affect Quantitation

- Important to note that fragmentation occurs as part of fixation
- Highly fragmented gDNA is less amplifiable, as the peak is about 100bp
- The difference in area under the curve >300bp is the advantage of Chem A over Chem B
Advantages of Real-Time PCR Quantitation Include High Sensitivity and Wide Dynamic Range

✓ Amplification and detection occur together
✓ Highly sensitive
✓ Wide dynamic range
✓ High throughput capability
✓ Multiplex capability
✓ Requires minimal sample
Disadvantages of Real-Time PCR Quantitation Include Expensive Equipment and Sensitivity to Inhibitors

- Requires specialized instrumentation

- Higher cost compared to UV absorbance and fluorescent dye-based methods

- Sensitivity to inhibitors
Several Different Metrics Can Be Used to Predict the Likelihood of Success in Downstream Assays

- **Quantitation by:**
  - Absorbance
  - Fluorescent dye
  - Amplification (qPCR)

- **Purity by:**
  - Absorbance ratio

- **Factors that impact quality:**
  - Purification chemistry contaminants
  - Sample specific inhibitors
  - Fragmentation

All are used to predict likelihood of success in downstream assays
Quantitation of DNA by Absorbance can be Overestimated Due to Contaminants

**gDNA Extraction from Matched Lung Tissue FFPE Slides**

**Chemistry A**

- No peak at 230nm

**Chemistry B**

- Large peak at 230nm

Absorbance is unreliable for Chemistry B – is qPCR a better choice?
Absorbance at 260nm May Not Be an Accurate Measure of Amplifiable Yield

Large difference in quantitation

- Absorbance at 260nm may not be an accurate measure of amplifiable yield
- Absorbance and amplifiability may correlate, but several other factors play a role
One Sample of DNA Isolated from Saliva and Quantitated by the Three Methods Shows Different Results

Absorbance shows all nucleic acid present: dsDNA, ssDNA and RNA

Fluorescent-dye quantitation measures only dsDNA present

qPCR quantitates human DNA present
Summary

✓ Sample quantitation is a key step in DNA and RNA analysis workflows

✓ Key challenges of quantitation include accuracy, sensitivity, and specificity
  • NanoDrop® is fast but lacks specificity and may overestimate
  • Fluorescent dye-based methods are highly sensitive and can be specific
  • Real-time PCR is highly sensitive over a wide range, but costs may be prohibitive

✓ Products are available that overcome the key challenges associated with quantitation
  • Fast, sensitive and inexpensive quantitation systems
    ➢ QuantiFluor® dsDNA, RNA and ssDNA Systems
  • qPCR ad RT-qPCR enzymes & kits that are resistant to inhibitors
    ➢ GoScript® RT & GoTaq® enzymes & qPCR/RT-qPCR kits
  • Isolation of pure genomic DNA and RNA
    ➢ ReliaPrep™ and Maxwell® 16 DNA and RNA purification systems
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