

Understanding and measuring variations in DNA sample quality

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Product types:

Chromosome-specific arrays, Custom arrays

Reliable measurement of DNA concentration and purity is important for many applications in molecular biology, especially array comparative genomic hybridisation (aCGH) where accurate determination of DNA concentration is critical. Impurities in DNA can lead to inaccurate measurement of DNA concentration and could potentially inhibit subsequent labelling reactions. This article provides recommendations for measuring DNA quality for use in aCGH, such as OGT's CytoSure arrays, based on our experience of running thousands of samples per week in our high-throughput genomic services laboratory. In addition, detailed information is provided on the theory and practice behind measuring DNA purity and concentration.

Overview

DNA concentration can be assessed using four different methods: absorbance (optical density), agarose gel electrophoresis, fluorescent DNA-binding dyes and a luciferase-pyrophosphorylation-coupled quantitation system. The two most common methods of measuring DNA purity and concentration are absorbance (measured using a spectrophotometer) and agarose gel analysis.

At Oxford Gene Technology (OGT), we ensure all DNA samples run in our high-throughput laboratory are of suitable purity for accurate, reliable and reproducible results. We typically measure DNA purity and concentration using a NanoDrop™ spectrophotometer. A260/280 and A260/230 values greater than 1.8 are typically suitable for analysis (see section "Absorbance" for more information on Absorbance ratios). Lower A260/280 values may indicate protein contamination. At OGT, we use QIAGEN DNeasy® Kits, incorporating the optional proteinase K digestion step, to re-purify DNA samples with low A260/280 ratios. Lower A260/230 values indicate contamination with salts or some solvents (e.g. phenol). We typically re-purify these samples by ethanol precipitation, resuspending the DNA in TE buffer.

Accurate dilution of DNA samples for microarray analysis is very important as differences in DNA input between sample and references can adversely affect data quality. At OGT, to ensure accurate measurement of DNA concentration prior to dilution, all samples are warmed to 37°C for at least 30 minutes and are then carefully mixed before running on a spectrophotometer. This ensures that the DNA is completely in solution. Careful mixing using a pipette is essential to prevent DNA shearing. We also undertake a two-step dilution to ensure greater accuracy.

Example 2-step dilution suitable for all array formats:

1. Warm and mix DNA samples (as described above)
2. Measure sample concentration using a spectrophotometer
3. Dilute samples to 100 ng/μl
4. Measure sample concentration to check samples are at expected concentration
5. Dilute samples from step 3 to 50 ng/μl based on DNA concentration from step 4
6. Measure sample concentration to ensure high accuracy, repeat dilution as required

The DNA amount and concentration for use on arrays depends on both the application and the array format (see Table 1).

Application	OGT/Agilent Array format			
	8x60k	4x180k	2x400k	1x1M
	8x15k	4x44k	2x105k	1x244k
aCGH (prokaryotic)	250–500 ng gDNA	1 µg (range 1–3 µg) gDNA		2–3 µg (range 1–3 µg) gDNA
Enzymatic aCGH (eukaryotic)	200–500 ng gDNA	1 µg (range 0.5–3 µg) gDNA		2–3 µg (range 0.5–3 µg) gDNA
Enzymatic aCGH (eukaryotic) from amplified DNA	2–3 µg gDNA			
Non-enzymatic aCGH (incl. FFPE DNA, eukaryotic)	250 ng gDNA	500 ng gDNA	1 µg gDNA	1.5 µg gDNA or 2 µg FFPE† DNA
ChIP on chip (prokaryotic)		200–400 ng IP* DNA		
ChIP on chip (eukaryotic)	2µg IP DNA			

Table 1: Amount of DNA required for various microarray applications using OGT/Agilent arrays.

*IP (Immunoprecipitated DNA). †FFPE (Formalin-fixed, paraffin-embedded).

The range values indicate the upper and lower limits of DNA quantity we would like to receive.

Absorbance

Nucleic acids absorb ultraviolet (UV) light due to the heterocyclic rings of the nucleotides; the sugar-phosphate backbone does not contribute to absorption. The wavelength of maximum absorption for both DNA and RNA is 260nm ($\lambda_{\text{max}} = 260\text{nm}$) with a characteristic value for each base.

Base	pH	λ_{max} nm
Adenine	1	262.5
	7	260.5

	12	269
Cytosine	1	276
	7	267
	14	282
Guanine	1	276
	7	276
	11	274
Thymine	4	264
	7	264
	12	291

Table 2: Spectral data for nucleotides at different pH.

The absorption properties of DNA can be used for detection, quantification and assessment of purity. Although the λ_{max} is constant, the extinction coefficient of nucleic acids depends on their environment. The absorbance of isolated nucleotides is greater than that of RNA and single-stranded DNA (ssDNA) which is in turn greater than that of double-stranded DNA (dsDNA). With the absorption of dsDNA being approximately 40% less than would be exhibited by a mixture of free nucleotides of the same concentration. This difference is due to the structural properties of the nucleic acid and is called the hypochromic effect. It results from hydrophobic and dipole-dipole interactions between the electron systems of the individual bases made possible by their stacking in the parallel array of the double helix. In ssDNA the bases have a tendency to stack on top of one another but this is maximised in dsDNA. It is these interactions and stacking arrangement that stabilise the helical structure of DNA and also RNA rather than hydrogen bonding which determines the specificity of base pairing.

Before measuring absorbance, it is important to make sure that the DNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes.

Effect of Acid

Under acidic conditions or at extremely high temperatures nucleic acids are completely hydrolysed. At a lower pH, for example pH 3–4 the most easily hydrolysed bonds are broken. These are the glycosylic bonds which attach purine bases to the ribose ring.

Effect of alkali

If the pH increases above pH 7–8 the tautomeric state of the bases is affected. This is when some of the hydrogen atoms associated within a base change their location producing a tautomer. For example, an amino group ($-\text{NH}_2$) can tautomerize to an imino form ($=\text{NH}$). Likewise a keto group ($-\text{C}=\text{O}$) can tautomerize to an enol form ($=\text{C}-\text{OH}$). These tautomers can form non-standard base pairs that fit into the double helix and can cause the introduction of mutations during DNA replication. They also change the specific hydrogen bonding between base pairs and destabilise the structure of dsDNA. This change in structure results in a change of absorption at 260nm as shown in Table 2.

Effects of solvents

Absorption of nucleic acids depends on the solvent used to dissolve them. Reports in the literature have compared

solubilising DNA in water with a low salt buffer (e.g., Tris or TE buffer). It was found that a low salt buffer gave very reproducible readings whereas readings in water could give up to 14% variation. This is most likely due to differences in the pH of the water caused by the solvation of CO₂ from air. The amount of CO₂ that is dissolved in the DNA/water solution is dependent on and varies with the amount of agitation used to re-suspend the DNA. As DNA can be difficult to solubilise in water thorough mixing may be needed. A₂₆₀/A₂₈₀ ratios measured in water were also shown to be variable and ratios obtained were typically less than 1.8. As the protein content in these examples was less than 1% these ratios were misleading. The A₂₆₀/A₂₈₀ ratios measured in low salt buffer were completely reproducible.

Quantification of DNA

The absorbance at 260nm is used to calculate the concentration of nucleic acids. At a concentration of 1 µg/ml and a 1 cm path length* dsDNA has A₂₆₀ = 50. The absorbance value is also dependent on the amount of secondary structure in the DNA due to hypochromicity.

To ensure the concentration reading is accurate, the absorbance reading should be within the linear range of the spectrophotometer. The Lambert-Beer law relates the absorption of light to the properties of the material through which the light is travelling. The law states that there is a logarithmic dependence between the transmission of light through a substance and the product of the absorption coefficient of the substance and the distance the light travels through the material (i.e. the path length). The law tends to break down at very high concentrations, especially if the material is highly scattering. So for concentrated solutions the absorbance value and therefore the concentration can be inaccurate. It is often useful to prepare and measure a series of dilutions to check not only the concentration but the accuracy of the dilutions as well. Inaccurate dilutions can occur if the DNA is not homogeneously re-suspended. For reliable spectrophotometric DNA quantification A₂₆₀ readings should lie between 0.1 and 1.0.

To improve the accuracy of DNA concentration determination allowance should be made for any impurities in the solution. This can be estimated by adjusting the A₂₆₀ measurement for turbidity which is measured at an absorbance of A₃₂₀. The equation below can be used:

$$\text{Concentration (}\mu\text{g/ml)} = (\text{A}_{260} \text{ reading} - \text{A}_{320} \text{ reading}) \times \text{dilution factor} \times 50\mu\text{g/ml}$$

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

$$\text{DNA Yield (}\mu\text{g)} = \text{DNA Concentration} \times \text{Total Sample Volume (ml)}$$

* Path length defines the distance that light (UV) travels through a sample in an analytical cell.

Purity of DNA

A₂₆₀/A₂₈₀ ratio to measure Protein Contamination

This procedure was first described to measure protein purity in the presence of nucleic acids. However it is now commonly used to assess protein contamination of DNA. It is important to note that the A₂₆₀/A₂₈₀ ratio is only an indication of purity rather than a precise answer. Pure DNA preparations have an A₂₆₀/A₂₈₀ ratio of greater than or equal to 1.8.

When the A₂₆₀/A₂₈₀ ratio is determined for a range of different DNA/protein mixtures it has been shown that the ratio is relatively insensitive to the addition of protein to pure nucleic acid. However the utility of the ratio becomes apparent when DNA is prepared from tissue or blood. These samples have a protein content that greatly exceeds nucleic acid on a weight basis and so in these cases the approximate purity of dsDNA preparations and contamination by protein may be estimated by determination of the ratio of absorbance at 260nm and 280nm.

Several factors can influence the accuracy of the A₂₆₀/A₂₈₀ ratio. For example, readings from very dilute samples

will have very little difference between the absorbance at 260 and 280nm leading to inaccurate ratios. The type(s) of protein present will also have an effect. Absorbance in the UV range by proteins is primarily the result of aromatic ring structures. Proteins are composed of 22 different amino acids of which only three contain aromatic side chains. Thus the amino acid sequence of proteins would be expected to influence the ability of a protein to absorb light at 280 nm. For example bovine serum albumin (BSA) has an extinction coefficient value of 0.7 for a 1 mg/ml solution at 280nm, while streptavidin has an extinction coefficient of 3.4, absorbing almost five times as much light at 280nm at the same concentration.

It is also important to note the phenol and other contaminants can also absorb at 280 nm and can affect the ratio calculation. Phenol absorbs with a peak at 270nm and has an A260/A280 ratio of 2. Nucleic acid preparations uncontaminated by phenol should have an A260/A280 ratio of around 1.8. As well as affecting the ratio calculations, contamination by phenol can significantly contribute to overestimation of DNA concentration and inhibit downstream reactions.

The pH of the solution can also affect the A260/A280 ratio, with acidic solutions having a lower ratio of up to 0.2–0.3 and alkaline solutions having an increased ratio by a similar amount.

Due to their different absorption spectra, the nucleotide composition of the bases present in DNA will have different A260/A280 ratios.

Nucleotide	A260/A280 ratio
Adenine	4.50
Cytosine	1.51
Guanine	1.15
Thymine	1.47

Table 3: A260/A280 ratios for nucleotides.

Therefore the ratio will be approximately equal to the weighted average of the A260/A280 ratios estimated for each nucleotide if measured independently, which explains why the accepted ratio of 1.8 for pure DNA is an approximation.

The ratio can be calculated after subtracting the non-nucleic acid absorbance at A320.

$$\text{DNA Purity (A260/A280)} = (\text{A260 reading} - \text{A320 reading}) \div (\text{A280 reading} - \text{A320 reading})$$

RNA Contamination

Pure RNA has an A260/A280 ratio of 2.0, therefore if a DNA sample has an A260/A280 ratio of greater than 1.8 this could suggest RNA contamination. However, due to the similarity in absorption profiles of RNA and DNA, probably the most accurate way to determine RNA contamination is to run the sample on an agarose gel where the RNA will clearly be seen migrating ahead of the DNA. RNA can easily be removed by adding RNase A during purification.

A260/A230 ratio

This ratio is used as a secondary measure of nucleic acid purity. The A260/A230 ratio values for pure samples are often higher than the respective A260/A280 ratio values. Strong absorbance around 230nm can indicate that organic compounds or chaotropic salts are present in the purified DNA. A ratio of 260nm to 230nm can help evaluate the level of salt carryover in the purified DNA. The lower the ratio, the greater the amount of salt present. As a guideline, the A260/A230 ratio should be greater than 1.5, ideally close to 1.8. Urea, EDTA, carbohydrates and phenolate ions

all have absorbance near 230nm. The TRIzol[®] reagent which can be used for DNA extraction, although more commonly used for RNA preparation is a phenolic solution which absorbs in the UV spectrum at 230 nm and around 270nm. Guanidine HCL which is used in the extraction of DNA will absorb at 230nm while guanidine isothiocyanate, used for RNA isolations will absorb at 260nm.

As discussed previously, a reading at 320nm will indicate if there is turbidity in the solution, another indication of possible contamination. Therefore, taking a spectrum of readings from 230nm to 320nm is most informative.

Gel Electrophoresis

Agarose gel electrophoresis of the purified DNA compliments the data from absorbance readings. Concentration can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard. For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the solution concentration is 50 ng/µl (100 ng divided by 2 µl). Standards used for quantitation should be labelled in the same way and be the same size as the sample DNA being analyzed. In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR Green is required. This method is useful for cases where concentration is too low to accurately assess with spectrophotometry and in cases where contaminants absorbing at 260nm make accurate quantitation by that method impossible.

However the most common reason for running a gel is to assess DNA quality. On a 1 to 1.5% agarose gel, intact genomic DNA should appear as a compact, high-molecular-weight band with no low-molecular-weight smears. Degraded DNA results in biased labelling.

Conclusion

High quality, intact pure DNA is required for many applications, particularly aCGH. Care must be taken to ensure reliable and reproducible results. Using poor quality DNA or DNA of unknown concentration can lead to problems during analysis leading to inaccurate results. If sample quality checks are not done properly it is still sometimes possible to identify DNA quality issues after the labelling reactions however this is not guaranteed. It is likely that problems with sample quality will only be identified after running the microarray, at which point significant investment in time and money will have been made.

Before making any measurements it is important to make sure that the DNA is completely in solution by pipetting up and down. If needed, incubate at 37°C for 30 minutes. More accurate measurements are typically obtained when the DNA is suspended in a salt-based (e.g., Tris) buffer with neutral pH. Tris buffer also has the added benefit of inhibiting nucleases thereby preventing DNA degradation during storage.

For more information on how to detect sample quality issues on your microarray, please read our QC metric article.

About Oxford Gene Technology (OGT)

OGT provides high-quality products and services for accurate disease analysis. Our CytoSure range of aCGH products provide the complete solution for clinical genetics research. A wide range of arrays are available for research into cytogenetics, molecular genetics, cancer and pre-implantation genetic screening (PGS). In addition, we offer sensitive DNA labelling kits, automation solutions and class-leading analysis software.

OGT's Genefficiency Genomic Services are designed to lead the researcher all the way from project conception to high-quality results. Our Genefficiency[™] high-throughput array service provides researchers with the choice of standard or custom arrays to achieve maximum resolution over either the whole genome or particular regions of interest. The Genefficiency[™] NGS Service provides offers a tailored approach to next generation sequencing and includes, exome sequencing, custom panel design and sequencing plus RNA-Seq. All projects are accompanied by an interactive analysis report that allows rapid access to meaningful results without the need for bioinformatics support.

Contact us for more information about OGT's products and services.

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