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Quadruplex DNA Structure Characterization by Circular Dichroism

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Abstract

Circular dichroism (CD) arises from the differential absorption of left- and right-handed circularly polarized light, and may be seen for optically active molecules. CD provides useful spectral signatures for biological macromolecules in solution and provides low-resolution structural information about macromolecular conformation. CD is particularly useful for monitoring conformational changes in macromolecules upon environmental perturbations. G-quadruplex structures show unique CD spectral signatures, and CD is an important tool for the characterization of their formation and global structure. This protocol offers step-by-step methods for the determination of reliable and reproducible CD spectra of quadruplex structures, and how to properly normalize these spectra for presentation. CD spectra properly normalized with respect to quadruplex concentration and pathlength are required to facilitate accurate comparison of results among laboratories. The standard operating procedures proposed are recommended to make such comparison accurate and informative.

Keywords

DNA; G-quadruplex; circular dichroism; concentration determination

INTRODUCTION

Circular dichroism (CD) spectroscopy is a powerful tool for the characterization of nucleic acid structure in solution (*UNIT 7.11*; *UNIT 11.5*; Kypr et. al. 2009). While CD cannot provide high-resolution structural information, it is exquisitely sensitive to nucleic acid secondary structure, and is particularly well-suited for monitoring changes in structures resulting from folding, binding and environmental perturbations. DNA secondary structures (A-, B-, Z-form, etc.) have characteristic signature CD spectra. For right-handed, B-DNA, the CD spectra of molecules of different sequences may be calculated using simple nearest-neighbor rules. Higher-order, non-canonical DNA structures (*e.g.*, triplex, quadruplex, i-motif) each display characteristic CD spectra that differ from those of duplex DNA structures and from each other. While CD spectra can, in principle, be calculated using either quantum mechanical theory or a phenomenological approach based on classic electrodynamics, such calculations are not yet quantitatively reliable because of uncertainty

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in the underlying parameters that go into the calculations. Instead, CD spectra are usually used empirically to assess structural changes resulting from changes in temperature, salt concentration, cation type or ligand binding.

Four-stranded G-quadruplex structures are of intense current interest (UNIT 17.2; UNIT 17.5). G-quadruplexes are of biological relevance, for example, by participating in the protection of chromosomes by telomeres, in the control of gene expression, and in recombination. CD is widely used in studies of quadruplex DNA because of the distinctive spectra displayed by different quadruplex structures (Parmasavian et al, 2007; Renciu et. al., 2009; Karsisiotis et al, 2011; Vorlickova et al, 2012; Randazzo et al, 2012). Parallel, tetramolecular quadruplexes, for example, typically show non-conservative CD spectra with a positive peak centered near 260 nm. In contrast, folded unimolecular or bimolecular antiparallel quadruplexes display CD spectra with a positive peak near 295 nm, often with a strong negative peak near 265 nm. Recent calculations show that the exact geometry of the stacking of G-quartets within the quadruplex dictate the shape of at least a portion of the CD spectrum, and these calculations confirm the general empirical assignment of CD spectra to parallel and antiparallel conformations. Comparison of the CD spectrum of a putative quadruplex-forming sequence of unknown structure with the CD spectra of known quadruplex structures (established by NMR) is frequently used to infer the global conformation of the unknown. Such comparisons would be greatly facilitated by the availability of a curated, critically evaluated library of CD spectra of known quadruplex structures.

We encountered difficulties when attempting to build such a curated library of published CD spectra of quadruplex structures and sequences. We found inconsistencies and discrepancies in published spectra for ostensibly the same structure under similar solution conditions. We also found that CD spectra were presented in a wide variety of units, and were often not properly normalized with respect to nucleic acid concentration or pathlength. Descriptions of sample preparation and experimental conditions were often vague or lacking entirely, making it impossible to evaluate the reliability of the data presented, or to reproduce the results of a particular study. These difficulties motivated this protocol, in which we provide recommendations for the proper presentation of CD spectra for G-quadruplexes in particular and nucleic acids in general. Protocols for the determination of nucleic acid CD spectra have appeared previously (*UNIT 7.11*; *UNIT 11.5*) but seem to be widely ignored, necessitating our current plea for standard operating procedures and uniform standards for data presentation.

BASIC PROTOCOL

MEASURE THE CD AND ABSORBANCE SPECTRUM OF A G-QUADRUPLEX

This protocol describes how to obtain reproducible, accurate and reliable CD spectra of G-quadruplexes in order to compare the results between laboratories. In this protocol a properly normalize the CD spectrum with respect to G-quadruplex concentration and path length is performed. Moreover, different methods to present the CD spectra of G-quadruplex (g-factor and second derivative spectra) are described.

Materials

- Jasco J-810 CD spectropolarimeter (Jasco, Easton, MD) equipped with a programmable Peltier thermostatted cell holder and stirrer.
- UV/visible spectrophotometer with temperature-controlled cell holder
- Stoppered quartz cuvette. Depending on DNA strand concentration, 0.01 or 1 cm pathlength
- Folding buffer with composition suitable for ensuring quadruplex formation (See Critical Parameters and Troubleshooting)
- Folded G-quadruplex solution with a total absorbance at 260 nm of 0.8–1.2 in a 1 cm path-length cuvette (see *Support Protocol 1*).

Set up CD spectropolarimeter

- 1 Start up the spectropolarimeter according to the manufacturer's guidelines. A typical procedure consists of:

- 1.1 Purge the spectropolarimeter with nitrogen (15 min) before switching on the light source. The flow rate should be adjusted according to the manufacturer's guidelines to minimize absorbance of oxygen in the wavelength range of interest.

It is necessary to purge the instrument with pre-purified nitrogen gas to remove oxygen from the optical system. Oxygen strongly absorbs in the far UV. In the presence of the strong UV light generated by the Xe lamp, O₂ is converted to ozone that can damage the optics of the instrument.

- 1.2 Turn on the lamp power and wait for 20–30 min until the light source stabilizes.
 - 1.3 Turn on the Peltier thermostatted cell holder or water bath and wait until the desired temperature is reached in the sample compartment.
 - 1.4 Choose appropriate instrument settings to record the CD spectra.

Optimal operating parameters depend on the particular instrument. For our Jasco J-810 instrument, suitable CD spectra are obtained using the following settings: 340–200 nm wavelength range, 1.0 nm step size, 200 nm/min scanning speed, 1.0 nm bandwidth, 2 s integration time, standard sensitivity, with 4 scans averaged.

Record the UV spectrum simultaneously with the CD spectra and monitor the PMT HT voltage. For the Jasco J-810 instrument, CD data collected with PMT HT voltage of >600–800 V are not reliable, and values >800 V are not valid.

- 2 Clean and dry the cuvette.

The typical washing protocol consists of washing the cuvette several times with deionized water and, if necessary, a detergent, followed by thorough rinsing with pure ethanol, and drying under a stream of filtered air.

Measure CD and absorbance spectra

- 3 Record the CD and absorbance spectra of the buffer (the blank) to be subtracted from the experimental spectra.

A small blank CD signal (< 0.5 mdeg) results from birefringence of the cuvette. The blank signal should not vary by more than 5 mdeg across the wavelength range of interest. A high blank CD signal indicates a problem such as high absorbance (resulting in a high PMT voltage) or the presence of chiral impurities in the buffer solution.

See Critical Parameters and Troubleshooting section for the selection of the buffer solution.

- 4 Prepare enough folded G-quadruplex solution with a total absorbance at 260 nm of 0.8–1.2 to fill a 1 cm path-length cuvette for optimal signal-to-noise as described in *Support Protocol 1*.
- 5 Allow the sample to equilibrate for at least 5 min at the desired temperature (usually 20 °C). Record the CD and absorbance spectra between 220–340 nm.

Ensure that optimal instrumental parameters described above are used.

Record the CD spectra using the same clean cuvette positioned in the same orientation that was used to record the reference (blank) spectrum.

Diluting the sample after annealing may alter the distribution or conformation of G-quadruplex species. Therefore, CD and UV spectra should be recorded over time after dilution, especially if a high oligonucleotide concentration was used during annealing. If the G-quadruplex solution is at equilibrium, replicate scans of solutions obviously should overlay each other.

The formation and conformational equilibrium of G-quadruplexes may be sensitive to variations in buffer composition, oligonucleotide strand concentration, temperature, or the presence of organic solvents such as PEG or acetonitrile. For samples that require long equilibration times, especially samples with co-solvents, it may be necessary to store them at 4 °C for 24 hours or more.

Data processing, analysis and software

- 6 Export spectra as ASCII/text files or other data analysis software.

- 7 Subtract the blank (reference) CD and absorbance spectra from the G-quadruplex spectra to obtain the corrected CD and absorbance spectra of the G-quadruplex.
- 8 Normalize the CD spectra of the G-quadruplex solution to the molar concentration of G-quadruplex expressed per strand.

To normalize the CD spectra of G-quadruplex use the formula $\epsilon(M^{-1} \cdot cm^{-1}) = \theta / (32980 * c * l)$ where θ is the CD ellipticity in millidegrees (mdeg), c is the strand concentration in mol/L of G-quadruplex, and l is the path length in cm.

The concentration of G-quadruplex should be estimated using an extinction coefficient determined for the folded quadruplex (see Support protocols 2 and 3), or by precise dilution of a stock solution with an accurately known strand concentration (Support protocol 4). See Table 1 for an example of the effect of extinction coefficient on quadruplex concentration.

As illustrated in Table 1, several methods are suitable for determining the correct molar extinction coefficient of the folded G-quadruplex at 20°C. These include colorimetric phosphate determination (UNIT 7.3), digestion of the oligonucleotide to free bases, alkaline denaturation (see Support protocol 2), or thermal denaturation (see Support protocol 3). Because the extinction coefficients determined by the nearest neighbor method reflect the single stranded state at 20 °C, the DNA concentration DNA determined using this method may be in error by as much as 15%.^[1]

The alkaline denaturation method is a convenient method for the estimation of corrected molar extinction coefficient of the folded G-quadruplex at 20°C (see Support Protocol 2).

- 9 Calculate the g-factor as the ratio of CD spectra (ϵ) and absorbance spectra (ϵ) using the equation 1:

$$g = \frac{A_L - A_R}{A} = \frac{\Delta\epsilon}{\epsilon} \quad \text{Equation 1}$$

- 10 Calculate the second derivative spectra of the normalized G-quadruplex CD spectra if desired.

Data analysis can be performed with the program Origin (OriginLab, Northampton, MA).

A suitable second derivative CD spectrum can be obtained using Savitzky-Golay algorithm with a fourth degree polynomial and 20 point window. The shape of the derivative CD spectra calculated with this algorithm depends on the number of points of the spectrum and the

chosen differentiation parameters such as the order of the polynomial, and the number of points in the window applied. The differentiation parameters may need to optimize for each spectrum. To illustrate the utility of derivative CD spectra, Figure 1 shows the CD spectrum (solid line) and second derivative spectrum (dashed line) of the human telomere G-quadruplex (5'-AGGGTTAGGGTTAGGGTTAGGG-3') under different folding conditions.

SUPPORT PROTOCOL 1

PREPARATION OF A FOLDED G-QUADRUPLEX SOLUTION

The structure of G-quadruplexes in solution and their CD spectra depend on the sequence of the oligonucleotide as well as experimental conditions such as temperature, annealing protocol, buffer composition, and oligonucleotide strand concentration. Therefore, the sample preparation procedure employed to record a CD spectrum of a G-quadruplex should be reported in detail so that other investigators can obtain reproducible CD spectra. This protocol describes how to prepare a reproducible folded G-quadruplex solution.

Materials

- Lyophilized DNA oligonucleotide with standard desalting
- Folding buffer with composition suitable for ensuring quadruplex formation (See Critical Parameters and Troubleshooting)
- Screw cap plastic microcentrifuge tubes
- 1L boiling water bath for annealing DNA samples.

Prepare 1 mM per strand stock solution of the oligonucleotide

- 1 Add a known volume of deionized water (18 M Ω .cm) to the lyophilized DNA to obtain a ~1 mM per strand stock solution of oligonucleotide.

Add 500 μ L of deionized water for 500 nmol of oligonucleotide.

- 2 Incubate the stock solution of oligonucleotide overnight at 4 °C prior to use to achieve complete solubilization.

The solubilization state of the oligonucleotide may influence subsequent quadruplex formation. If the lyophilized DNA is initially dissolved at high concentration in folding buffer, a significant population of aggregated G-quadruplex species may form. The oligonucleotide stock solution (1 mM per strand) is therefore prepared by dissolving the lyophilized DNA in deionized water or non-folding buffer instead of folding buffer.

- 3 Confirm the purity of the synthetic oligonucleotide by HPLC (UNIT 17.7), mass spectrometry (MALDI-TOF; UNIT 10.1 and ESI; UNIT 10.2), and/or denaturing gel electrophoresis (UNIT 10.4).

The purity of the synthetic oligonucleotide should be determined before beginning any experiment. Generally, synthetic, short oligonucleotides (<30 nt) are sufficiently pure for CD studies after standard desalting. Longer oligonucleotides or those with modifications such as fluorescent tags may need additional purification such as PAGE and/or HPLC. (See Critical Parameters and Troubleshooting).

- 4 Determine the concentration using Support Protocol 4.

Folding the oligonucleotide into a G-quadruplex structure: annealing protocol

- 5 Prepare 3 mL the oligonucleotide at 5–10 μ M strand concentration for monomolecular or bi-molecular G-quadruplexes or ~1 mM strand concentration for tetramolecular G-quadruplexes in folding buffer solution.

The rate of bimolecular and tetramolecular quadruplex formation depends strongly on oligonucleotide strand concentration.

Any of the buffer systems described in the Critical Parameters and Troubleshooting may be used for annealing. A monovalent cation (K^+ or Na^+) concentration of 25 mM is usually sufficient for complete G-quadruplex formation, although for novel sequences, the minimum cation concentration for complete folding should be checked by titration.

- 6 Fill a sealed screw cap plastic microcentrifuge tube with enough of the oligonucleotide solution to fill the cuvette being used.

The plastic tube should be suitable for use in a boiling water bath.

- 7 Place the tube in 1 L of boiling water for 20 min. After 20 min, slowly cool the sample to room temperature in the water bath overnight.

When an organic co-solvent is used, first carry out the annealing at 2 $\times$ concentration of the oligonucleotide and 2 $\times$ of the selected folding buffer. After cooling, add an equal volume of the co-solvent to the pre-annealed oligonucleotide. In this way evaporation of the solvent and possible chemical reaction with the oligonucleotide or the buffer are prevented.

Some samples, especially those in the presence of co-solvents, may require long equilibration times in order to reach the thermodynamic equilibrium of the G-quadruplex structure. For samples that require long equilibration times, it may be necessary to store them at 4 $^{\circ}$ C for more than 24 hours. For samples annealed at high oligonucleotide concentration or samples in the presence of a co-solvent, time-dependent CD spectra should be measured to ensure equilibrium.

- 8 Determine the folded G-quadruplex concentration by absorbance spectroscopy (See Support Protocol 4).

Determine the purity of the folded G-quadruplex solution

- 9 Determine the purity of the folded G-quadruplex solution after annealing, especially for samples prone to aggregation or misfolding (See Critical Parameters and Troubleshooting).

SUPPORT PROTOCOL 2

DETERMINATION OF THE MOLAR EXTINCTION COEFFICIENT (ϵ_F) OF A FOLDED G-QUADRUPLEX BY ALKALINE DENATURATION

Extinction coefficients for DNA oligonucleotides are commonly calculated by nearest neighbor methods (Tataurov et. al., 2008) and are usually provided by the manufacturer. It is important to realize that these refer to the *unfolded* oligonucleotide at 20 °C, whereas CD spectra are collected on the *folded* oligonucleotide. Because of hypochromic effects, extinction coefficients for the folded and unfolded forms differ. It is important to use the correct value for concentration determinations. Neglect of this change propagates directly into concentration determination, which in turn propagates into errors in the proper normalization of CD spectra.

Correct molar extinction coefficients for folded quadruplex forms can be obtained by phosphate analysis (as described in Unit 7.3), thermal denaturation or by an alkaline denaturation method. In this protocol, we describe the alkaline method; the thermal denaturation method will be described in *Support Protocol 3*.

In both thermal and alkaline denaturation, the molar extinction coefficient for the folded quadruplex (ϵ_F) is calculated by recording the absorbance spectra of solutions of G-quadruplex under folded and unfolded conditions with identical concentrations. The molar extinction coefficient for the unfolded quadruplex form at 20°C is estimated from nearest neighbor analysis. Since the concentrations of folded and unfolded G-quadruplex are equal, the molar extinction coefficient for the folded quadruplex 20°C (ϵ_F) is calculated by the equation:

$$\epsilon_F = \epsilon_U \frac{A_F}{A_U} \quad \text{Equation 2}$$

where (ϵ_U) is the extinction coefficients for the unfolded quadruplex forms at 20°C, and A_F and A_U are the absorbance value at 260 nm of the folded and unfolded G-quadruplex at 20°C, respectively. In this method, the absorbance at 260 nm of the G-quadruplex solution is measured under both folded conditions (pH 5–8) and denaturing conditions (pH >12). The A_{260} at alkaline pH can be used in conjunction with nearest neighbor extinction coefficients to give an accurate estimate of the DNA concentration.

Materials

- UV/visible spectrophotometer with temperature-controlled cell holder
- 1 cm pathlength stoppered quartz cuvette.

- 1 M NaOH solution
- Folded G-quadruplex solution with a total absorbance at 260 nm of 0.8–1.2 in a 1 cm path-length cuvette (see *Support Protocol 1*).
- Folding buffer with composition suitable for ensuring quadruplex formation (See Critical Parameters and Troubleshooting)

Prepare an unfolded G-quadruplex solution

- 1 Prepare a sufficient volume of unfolded G-quadruplex solution (~ 5 μ M) in 100 mM NaOH.

To prepare this solution use the folded quadruplex solution obtained after the annealing (See *Support Protocol 1*)

For an oligonucleotide with molar extinction coefficient at 260 nm of ~200,000 M⁻¹cm⁻¹ per strand, add 20 μ L of 5 M NaOH into a 980 μ L of a folded G-quadruplex solution (~5 μ M) obtained after the annealing. This solution should give a pH > 12 and an absorbance value ~1.

- 2 Incubate the solution at room temperature for 30 min to obtain the unfolded G-quadruplex solution.

Prepare a folded G-quadruplex solution

- 3 Prepare 1 mL of the folded G-quadruplex solution as in steps 1 to 2 above, but use the corresponding folding buffer.

Use the same volume of folded oligonucleotide solution that it was used above to prepare the unfolded G-quadruplex solution to obtain the same oligonucleotide concentration. This corresponds to adding 20 μ L of the corresponding folding buffer into a 980 μ L of the same folded G-quadruplex solution (~5 μ M) used before to prepare the unfolded G-quadruplex solution.

Determine the absorbance at 260 nm of the unfolded G-quadruplex solution (A_U)

- 4 Fill a clean 1 cm path-length cuvette with 100 mM NaOH and determine a blank spectrum between 220–340 nm at 20 °C.

High absorbance is observed at wavelengths below 240 nm due to absorption by 100 mM NaOH.

- 5 Fill a 1 cm path-length cuvette with the unfolded oligonucleotide pre-equilibrated in 100 mM NaOH.
- 6 Set up the thermostatted cell holder at 20 °C and allow the sample to equilibrate for 5 min.

- 7 Record the absorbance spectrum of the unfolded oligonucleotide between 220–340 nm at 20 °C and determine the value of absorbance at 260 nm of the unfolded G-quadruplex solution (A_U).

A distorted spectrum below 240 nm of the oligonucleotide solution may be observed due to the high absorbance of the 100 mM NaOH.

Determine the absorbance at 260 nm of folded G-quadruplex solution (A_F)

- 8 Record the absorbance spectrum between 220–340 nm at 20 °C of the folded G-quadruplex solution as in steps 4 to 7 above and determine the value of absorbance at 260 nm of the folded G-quadruplex solution (A_F), but use the corresponding folding buffer to record the baseline.

Determine the concentration of unfolded G-quadruplex solution

- 9 Calculate the molar extinction coefficients of the DNA oligonucleotide at 260 nm, ϵ_U (in $M^{-1}cm^{-1}$), by the nearest neighbor method (Tataurov et. al., 2008).

The molar extinction coefficient of the DNA oligonucleotide calculated by the nearest neighbor method is usually provided by the manufacturer and refers to the unfolded oligonucleotide at 20 °C.

It is important to realize that extinction coefficients for the folded and unfolded forms at 20 °C differ because of hypochromic effects.

- 10 Determine the concentration (C) of the unfolded oligonucleotide in 100 mM NaOH solution by using the value of absorbance at 260 nm (A_U) and the molar extinction coefficients (in $M^{-1} cm^{-1}$ per strand) calculated using the nearest-neighbor method (ϵ_U).

$$C = \frac{A_{U \text{ at } 260 \text{ nm, pH}=12}}{\epsilon_{\text{nearest-neighbor}}^{260 \text{ nm}} * l} \quad \text{Equation 3}$$

where C is the molar concentration of the unfold oligonucleotide, A_U is the absorbance at 260 nm of the oligonucleotide in 100 mM NaOH solution at 20°C where the oligonucleotide is completely unfolded, ϵ_U is the molar extinction coefficient at 260 nm calculated using the nearest-neighbor method, and l is the pathlength in cm.

Calculate the value of the molar extinction coefficient (ϵ_F) of the folded G-quadruplex

- 11 Calculate the molar extinction coefficient for the folded quadruplex at 20 °C (ϵ_F) by using the equation:

$$\epsilon_{\text{folded G-quadruplex}}^{260 \text{ nm}} = \frac{A_{F \text{ at } 260 \text{ nm}}}{C * l} \quad \text{Equation 4}$$

where C is the molar concentration of the unfolded oligonucleotide per strand in 100 mM NaOH solution ($\text{pH} > 12$), A_F is the 260 nm absorbance at 20 °C of the annealed oligonucleotide, and l is the pathlength in cm.

SUPPORT PROTOCOL 3

DETERMINATION OF THE MOLAR EXTINCTION COEFFICIENT (ϵ_F) OF A FOLDED G-QUADRUPLEX BY THERMAL DENATURATION METHOD

As emphasized above, the extinction coefficients for unfolded and folded G-quadruplexes differ. Extinction coefficients for DNA oligonucleotides calculated by nearest neighbor methods (Tataurov et. al., 2008) and usually provided by the manufacture are valid only for unfolded DNA samples at 20 °C. Since CD spectra are collected on the *folded* oligonucleotide it is necessary to extrapolate the A_{260} values measured at high-temperatures to 20 °C for the most precise estimate of concentration. This protocol describes how to obtain the correct molar extinction coefficients for folded quadruplex forms at 20 °C by a thermal denaturation method.

Materials

- UV/visible spectrophotometer with temperature-controlled cell holder
 - 1 cm pathlength stoppered quartz cuvette.
 - Folded G-quadruplex solution with an A_{260} of 0.8–1.2 in a 1 cm path-length cuvette (see *Support Protocol 1*).
 - Folding buffer with composition suitable for ensuring quadruplex formation (See Critical Parameters and Troubleshooting).
1. Prepare a suitable volume of the folded quadruplex solution ($\sim 5 \mu\text{M}$) with an A_{260} of 0.4–1 in a 1 cm pathlength cuvette. See *Support Protocol 1*.
 2. Perform a UV melting experiment of the folded quadruplex solution following the protocol described in UNIT 17.1.

For accurate determination, use only 1 cm cuvettes since a precise determination of the pathlength can be achieved.

3. Plot the blank-corrected absorbance profile at 260 nm as a function of temperature.

If the solvent is water, it is not necessary to determine a blank spectrum at high temperature because the change of the absorbance with temperature for the deionized water is negligible. However, if a buffer is used, a blank spectrum should be determined at all temperatures used in the extrapolation.

4. Determine the upper and lower baselines of the melting profile at 260 nm as described in UNIT 17.1.

The upper and lower baselines allow determining the values of the absorbance at different temperatures for the completely folded and unfolded G-quadruplex forms, respectively.

These baselines are determined as straight lines using a few values at low and high temperature. However, the upper baseline may be difficult to obtain due to the high thermal stability of the G-quadruplex if the concentration of monovalent cations (Na^+ or K^+) is high in the folding buffer.

If poor baselines are obtained at 260 nm an accurate value of the molar extinction coefficient (ϵ_F) for the folded G-quadruplex at 20 °C may be not obtained by this method.

5. Determine the 260 nm absorbance value of the unfolded G-quadruplex at 20 °C by extrapolation of the upper baseline.
6. Estimate the concentration of unfolded oligonucleotide by using the A260 extrapolated to 20 °C in conjunction with the molar extinction coefficients (in $\text{M}^{-1} \text{cm}^{-1}$ per strand) calculated using the nearest-neighbor method.

From the Beer-Lambert law, $C=A_\lambda/(\epsilon\lambda *l)$ where C is the concentration of the unfolded oligonucleotide, A_λ is the extrapolated A260 of the denatured oligonucleotide, ϵ is the molar extinction coefficient at 260 nm calculated using nearest-neighbor method, and l is the pathlength in cm.

7. Determine the 260 nm absorbance value of the folded G-quadruplex at 20 °C.
8. Calculate the molar extinction coefficient for the folded quadruplex at 20 °C (ϵ_F) by using the equation:

$$\epsilon_{\text{folded G-quadruplex}}^{260 \text{ nm}} = \frac{A_{F \text{ at } 260 \text{ nm}}}{c * l} \quad \text{Equation 5}$$

where C is the molar concentration of the unfolded oligonucleotide determine above, A_F is the 260 nm absorbance at 20°C of the folded oligonucleotide, and l is the pathlength.

This method can be used to calculate the concentration of the stock solution.

SUPPORT PROTOCOL 4

DETERMINATION OF FOLDED G-QUADRUPLEX CONCENTRATION BY ABSORBANCE SPECTROSCOPY

This protocol describes how to measure the concentration of an oligonucleotide in solution by absorbance spectroscopy using the corresponding molar extinction coefficient determined by several methods (See Support Protocol 2, 3, and Table 1).

Materials

- UV/visible spectrophotometer with temperature-controlled cell holder
- 1 cm path length stoppered quartz cuvette.
- Folding buffer with composition suitable for ensuring quadruplex formation (See Critical Parameters and Troubleshooting)
- Folded G-quadruplex solution with a total absorbance at 260 nm of 0.8–1.2 in a 1 cm path-length cuvette (see *Support Protocol 1*).

1. Calculate the extinction coefficients for the DNA oligonucleotide.

As illustrated in table 1, several methods are suitable for determining the correct molar extinction coefficient of the folded G-quadruplex at 20°C.

2. Turn on the spectrophotometer and allow the lamp to warm up for 30 min.

The oligonucleotide concentration can be determined directly if the absorbance spectrum of the G-quadruplex is recorded simultaneously to the CD spectra.

3. Set up the thermostatted cell holder or water bath to at 20 °C.

4. Fill a clean 1 cm path-length cuvette with folding buffer and determine a blank spectrum between 220 – 340 nm at 20 °C.

For accurate determination, use only 1 cm cuvettes since a precise determination of the pathlength can be achieved.

Some organic solvents can interfere with determination of the G-quadruplex concentration by UV absorbance. Accurate volumetric addition of the co-solvent after annealing allows calculation of the exact concentration of the G-quadruplex (and the co-solvent concentration).

5. Record the absorbance spectrum of the folded oligonucleotide between 220–340 nm at 20 °C and determine the value of absorbance at 260 nm (A_F).

For accurate determination of the concentration the absorbance of the sample at 260 nm should be between 0.4–1.2 in a 1 cm path-length cuvette

For a oligonucleotide with molar extinction coefficient at 260 nm of ~200,000 M⁻¹ cm⁻¹ per strand, prepare a folded G-quadruplex solution (~5 μM) (See Support Protocol 1). This solution should give an absorbance value ~1.

If the absorbance of the DNA sample at 350 nm is not close to zero (where DNA does not absorb), light-scattering due to aggregates or precipitates may be present. If so, the sample should be filtered or centrifuged to remove particulate matter and the spectrum recorded again. If the light-scattering is still present after the filtration, a further

purification of the oligonucleotide by one of the methods mentioned above should be carried out.

6. Estimate the concentration of folded G-quadruplex by using the A₂₆₀ at 20 °C in conjunction with the molar extinction coefficients (in M⁻¹ cm⁻¹ per strand) calculated for the folded form at 20 °C.

From the Beer-Lambert law,

$$C = \frac{A_{F \text{ at } 260 \text{ nm}}}{\epsilon_{F \text{ at } 20^\circ \text{C}}^{260 \text{ nm}} * l} \quad \text{Equation 6}$$

where C is the concentration of the folded oligonucleotide, A_F is the 260 nm absorbance at 20°C of the folded G-quadruplex, ε_F is the molar extinction coefficients (in M⁻¹ cm⁻¹ per strand) calculated for the folded form at 20 °C, and l is the pathlength in cm.

COMMENTARY

Background Information

Circular dichroism is defined as the difference in absorbance (A) of left and right circularly polarized light:

$$CD = \Delta A = A_L - A_R \quad \text{Equation 7}$$

When normalized for nucleic acid concentration (C) and pathlength (l), CD can be cast into the difference in molar extinction coefficients for absorbance of left and right circularly polarized light, and then is expressed as the CD version of the Beer-Lambert law:

$$\Delta \epsilon = \epsilon_L - \epsilon_R = \frac{\Delta A}{C \cdot l} (M^{-1} \cdot \text{cm}^{-1}) \quad \text{Equation 8}$$

Most CD instruments measure the difference in absorbance but display spectra as ellipticity (θ) in units millidegrees. Molar CD and molar ellipticity ([θ]) are related by the expression:

$$[\theta] = 3298 \Delta \epsilon \quad \text{Equation 9}$$

The use of ellipticity is antiquated, and dates from when CD was calculated in terms of the change of polarization of linear polarized light into elliptically polarized light when it passed through the sample. Modern instruments measure the true differential absorbance of circularly polarized light, A, so there is no justification for the continued use of the obsolete term “ellipticity”.

Recommendation: CD spectra of quadruplexes should be presented as molar circular dichroism, ϵ , in units of $M^{-1}cm^{-1}$. Presentation of CD spectra of quadruplexes as raw data in degrees or millidegrees is unacceptable. Data must be normalized with respect to DNA concentration and pathlength.

This recommendation is made to facilitate comparison of the amplitudes of CD spectra. Without normalization, the shape of the CD spectra may be adequately represented, but the amplitude of the circular dichroism cannot be easily evaluated. One problem that could result is an inability to detect partially unfolded structures. Partially folded quadruplexes may exhibit CD spectra with similar shapes of fully folded forms, but with reduced absolute spectral amplitudes.

A neglected, but important, property in CD measurements is the g-factor, which is the ratio of a sample's CD and absorbance signals:

$$g = \frac{A_L - A_R}{A} = \frac{\Delta\epsilon}{\epsilon} \quad \text{Equation 10}$$

Note that the g-factor is a dimensionless parameter that expresses the strength of CD relative to absorbance. Modern CD instruments generally measure both CD and absorbance, and save and present both spectra as separate data channels. The g-factor can be easily calculated as the ratio of these data channels. One significant advantage of the g-factor is that is an intensive property, and is independent of concentration and pathlength. A plot of g as a function of wavelength provides a signature spectrum for a sample that does not require an independent determination of the sample concentration.

Viglasky and coworkers (Viglasky et al, 2011) have shown that the first derivative of a CD spectrum is a powerful tool for the evaluation of any G-quadruplex, and could be more unambiguous than a direct analysis of original spectra. We agree, and illustrate here that the second derivative has additional value, in particular by revealing more subtle transitions within the major bands of the CD spectrum.

In order to properly normalize CD spectra, accurate DNA concentrations must be known *for the folded form*. Accurate concentrations are most easily determined for nucleic acids by absorbance measurements if an accurate molar extinction coefficient is available. From the Beer-Lambert law,

$$C = \frac{A_\lambda}{\epsilon_\lambda \cdot l} (\text{mol/L}) \quad \text{Equation 11}$$

where C is the concentration, A_λ is the absorbance at wavelength λ , ϵ is the molar extinction coefficient at the same wavelength, and l is the pathlength in cm. The problem is to get a reliable value for ϵ *for the folded form*, a task that is straightforward though somewhat tedious.

What to use as the basis for expressing concentration needs consideration. Historically the concentration of isolated genomic DNA or RNA was expressed in moles of phosphate, nucleotides or (for duplex forms) base pairs. This was because the exact molecular weight of the isolated nucleic acids polymers was not known exactly or the DNA sample was heterogeneous with respect to length, so the monomeric unit of the polymer was selected as a concentration reference. For most quadruplex studies, synthetic oligonucleotides of precisely defined length and molecular weight are used, so it makes sense to define concentration in terms of molar concentration of strands, a practice we recommend. Whatever basis is chosen for the concentration scale, it must be reported clearly.

Critical Parameters and Troubleshooting

The G-quadruplex structures present in solution depend on the sequence of the oligonucleotide as well as experimental conditions such as temperature, annealing protocol, buffer composition, and oligonucleotide strand concentration. Therefore, the sample preparation procedure employed to record a CD spectrum of a G-quadruplex should be reported in detail so that other investigators can obtain reproducible CD spectra. Since experimental and instrumental parameters (including the accuracy of the cuvette pathlength, oligonucleotide concentration, measurement parameters and instrument calibration) also affect the magnitude and the quality of the CD signal of G-quadruplexes. It is important to specify all conditions under which spectra are recorded.

The protocol outlined below provides guidelines to ensure reproducibility, accuracy, quality and comparability of CD spectra of G-quadruplexes in particular and nucleic acids in general. Some important points to be considered to obtain reproducible and high quality CD spectra of G-quadruplexes are:

1. Preparation and composition of buffers

1.1 Folding buffer composition: The nature and concentration of cations present in the buffer, especially monovalent cations Na^+ and K^+ , significantly influence G-quadruplex structure and stability. Therefore, the *exact* final buffer composition used to record the CD spectra should be reported. For example, the chemical composition and final concentrations of all components of the buffer solution, should be given (e.g. BPES (6 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1 mM Na_2EDTA , 185 mM NaCl , pH=7.0)).

Special attention should be taken in describing the cation composition and concentration when ethylenediaminetetraacetic acid (EDTA) is used since di-, tri, tetra sodium or potassium salts are available. If titrations with different ions are to be performed, the exact final ionic composition should be reported. Several annealing buffers can be used to form G-quadruplex structures. Tetrabutylammonium phosphate, Tris, borate, phosphate and cacodylate are generally acceptable annealing buffers used in CD spectroscopy since these buffers have low absorbance and thus minimize interference at short wavelengths. In addition, since annealing is carried out at high temperatures, one should be aware of the strong temperature-dependence of the pK_a of Tris-containing buffers ($\text{pK}_a/ T = -0.028$). Thus, a Tris buffer prepared to have a pH of 7.4 at 25 °C will have a pH of 9.4 at 95 °C. In addition, certain solvent/buffer systems, typically solutions that contain high concentrations

of chloride or organic solvents, have high absorbance in the far UV (see the paper by Grimsly and Pace (2003) for a list of buffer components that interfere with far UV CD). A short pathlength cell should be used to minimize the absorbance due to the buffer.

1.2. pH of the buffer solution: Between pH 5 and 11, the pH of the buffer does not appreciably influence the CD spectra of G-quadruplexes. However, one should avoid adjusting the pH of the buffer with NaOH or KOH by direct addition to the sample, since it may then be difficult to know accurately the total cation concentration. Buffers should be prepared by using the appropriate conjugate acid-base pairs to give the desired final pH so that the exact concentration of Na or K ions is accurately known.

1.3. Organic co-solvents: Addition of organic co-solvents, (for example acetonitrile or polyethylene glycols) to the buffer has been used to induce conformational changes in G-quadruplexes. When a solvent is used, the absorption properties should be checked since some organic solvents absorb in the UV region.

The co-solvent should be added after annealing the oligonucleotide to avoid evaporation of the organic component, reaction of the solvent with the oligonucleotide, and/or hydrolysis of the solvent. The quadruplex structures present in the presence of co-solvents may need a longer time to reach thermodynamic equilibrium. Therefore, the duplicate spectra should be recorded over a period of time to ensure equilibrium.

For preparation of buffers in the presence of a viscous material such as polyethylene glycols (PEGs) one should weigh the additive instead of pipetting from a concentrated stock solution in order to know the exact composition of the mixture. In addition, the low conductivity of some organic co-solvents could cause problems with the reproducible pH determination of the sample. Therefore, the pH of the buffer should be determined and specified before adding the co-solvent.

2. Purity of the oligonucleotide sample—The purity of desalted oligonucleotide generated by solid-phase synthesis (usually greater than $\approx 95\%$ for oligonucleotides < 30 bases), is usually sufficient for CD spectroscopy. Standard desalting purification removes salts but not truncated oligonucleotides from the completed synthesis products.

To eliminate undesired deletion and/or truncation products, purification by HPLC, (Unit 17.7) SEC (Unit 17.3) or PAGE is recommended for oligonucleotides > 40 bases in length and for oligonucleotides with complex modifications such as fluorescent labelling.

To rule out the presence of aggregates and misfolded structures, the purity of the folded sample should be checked by a technique such as non-denaturing gel electrophoresis, analytical ultracentrifugation (AUC) or size exclusion chromatography (SEC). If the folded sample is not homogeneous as assessed by one of these methods, preparative PAGE, SEC or reversed-phase chromatography should be used to remove undesired folded or unfolded contaminating structures (Unit 17.3, Unit 17.7).

3. Experimental and instrumental parameters to record CD spectra—A number of parameters influence the magnitude of the CD signal, including cell pathlength,

oligonucleotide concentration, scan rate, spectral bandwidth, signal averaging and instrumental calibration. Since conventional circular dichroism instruments are routinely calibrated by the manufacturer and cell pathlengths are usually accurately known, the major potential source of error comes from uncertainty oligonucleotide concentration. Below we list critical factors that influence the magnitude of the CD signal.

3.1. Accuracy of CD measurements: CD instruments can be calibrated by recording the CD spectrum of camphor sulfonic acid or ammonium camphor sulfonate at 290 nm by protocols described in the manufacturer's literature.

3.2. Accuracy of cell pathlength: Cuvettes with pathlengths between 0.1–1 cm are usually accurately reported by the manufacturers. However, the pathlength reported for cuvettes with short pathlengths (<0.1 cm) may be erroneous, notably for cells consisting of quartz plates pressed together by a spring-loaded holder. The pathlength of these demountable cells can be influenced by the volume of liquid loaded, by thermal expansion of the liquid within the cell, and by the pressure applied by the cell holder to the two quartz plates. Obviously, any error in the pathlength leads to error in the magnitude of the CD signal of the G-quadruplex. Thus, the pathlength of cuvettes shorter than 0.1 cm should be determined. Accurate measurement of the cell pathlength can be achieved by using the interference fringe method for cuvettes with a pathlength shorter than ~20 μm or by using the absorbance of potassium chromate standard solutions (Miles et al, 2003; Miles et al, 2005).

3.3. Sample concentration and linearity of detector response: At high concentrations quadruplexes may form aggregates or higher order structures. This can distort the shape and magnitude of the CD spectrum as a result of light scattering artefacts as well as from structure-dependent changes in the CD spectrum. Thus, for concentrated DNA samples, the absence of aggregates should be confirmed by analytical ultracentrifugation and/or size exclusion chromatography. To obtain a reliable CD spectrum of the monomeric species, aggregates should be removed by centrifugation, size exclusion chromatography or filtration.

Highly absorbing samples (e.g. those with absorbance $>\sim 1.0$ resulting from a high concentration of oligonucleotide or co-solvents) lead to noisy and unreliable CD spectra because of the relatively small fraction of light reaching the photomultiplier detector (PMT). In these cases, it is important that the voltage applied to the photomultiplier remain within the manufacturer's specified limits of linearity of response in the wavelength region of interest. Depending on the particular instrument being used, the PMT voltage should be 500 V in the wavelength region of the CD spectrum. The theoretically maximum signal/noise ratio for CD spectrum is obtained when the absorbance of the sample is 0.87. If the photomultiplier voltage exceeds 500 V in standard 1 cm cell, the sample should be diluted or a short pathlength cuvette should be used to avoid instrumental artefacts due to high PMT voltage.

4. Concentration of the oligonucleotide for annealing—The annealing protocol may affect the formation and population of G-quadruplex structures present in solution. For multi-strand quadruplexes, the rate of bi- and tetra-molecular formation depends strongly on oligonucleotide strand concentration. Generally, higher amounts of monomeric G-

quadruplexes and lower amounts of higher-order G-quadruplexes are obtained when annealing is carried out at low concentration (μM).

4.1 Mono- and bimolecular structures: either high ($\sim 1\text{mM}$) or low ($<10\text{ }\mu\text{M}$) strand concentrations can be used for formation of monomolecular quadruplexes. If a high strand concentration is used during annealing, a significant amount of higher-order (aggregated) G-quadruplex structures that persist after dilution of the sample could be formed. A higher yield of monomeric G-quadruplexes is often realized when a low concentration ($\sim 10\text{ }\mu\text{M}$) of the oligonucleotide is annealed.

4.2 Tetramolecular structures: To ensure maximum formation of tetramolecular quadruplexes, a high strand concentration ($\sim 1\text{mM}$) of the oligonucleotide should be used. Tetramolecular G-quadruplexes form very slowly, if at all, if the concentration of oligonucleotide is lower than $\sim 100\text{ }\mu\text{M}$ during the annealing (Mergny et al, 2005). Dilution after annealing may also alter the distribution of G-quadruplex species. Therefore, to ensure thermodynamic equilibrium, serial spectra over time should be recorded after dilution of the sample if high strand concentrations were used during annealing. If time-dependent spectral changes are observed, it is useful to check the macromolecular homogeneity of the folded G-quadruplex solution by a method such as PAGE, SEC or AUC.

5. Reporting results—CD spectra provide valuable signatures for assigning G-quadruplex conformations. To accurately compare results between laboratories, properly normalized spectra that have been obtained using standard operating procedures are needed. Published spectra should clearly and fully describe sample preparation and how the CD spectra were recorded. How concentrations were determined and molar extinction coefficients used should be clearly stated. CD spectra should be presented as a molar circular dichroism, normalized with respect to sample concentration and cell pathlength. The exact solution conditions (temperature, pH, buffer composition) should be described for each spectrum.

Anticipated Results

Table 1 illustrates the apparent extinction coefficients obtained using different methods for the folded quadruplex formed from the human telomere sequence 5'-AGGG(TTAGGG)₃-3'. Note that there is about 15% hypochromicity at 260 nm upon folding. This is the magnitude of the potential error that would be propagated into values of ϵ by use of calculated nearest neighbor extinction coefficients for the single-stranded form.

Figure 1 shows absorbance and CD spectra for the folded human telomere quadruplex under different solution conditions that favor particular conformations. While there is little difference among the absorbance spectra of the different conformational forms (figure 1B), their CD spectra (Figure 1A) provide distinctive signatures. In sodium solution an antiparallel “basket” form is adopted that shows a characteristic spectrum with a minimum near 260 nm and a maximum near 295 nm. In potassium solution, a “hybrid” conformation forms with a characteristic spectrum with a minimum near 235 nm and a maximum near 290 nm. In the presence of potassium and cosolutes (polyethylene glycol or acetonitrile) a

parallel “propeller” conformation is adopted with a minimum near 240 nm and a maximum near 255 nm. In addition to the shapes of the spectra in Figure 1A, the amplitudes provide additional distinctive and important differences between the conformations. Figure 1C shows the g-factor calculated for the different conformations from the absorbance and CD spectra in panels A and B of Figure 1. The g-factor is of interest and utility for at least two reasons. First, the g-factor spectra provide a distinctive signature for each conformational form, and can be obtained from primary CD and absorbance spectra directly without knowledge of the exact quadruplex concentration. Second, the g-factor shows the rotational power across the spectrum. This helps in interpretation of CD spectra. For example, the CD spectrum of the parallel form shows the largest magnitude in molar circular dichroism at 260 nm. The g-factor at this wavelength shows that this high magnitude results from the high molar extinction at that wavelength rather than any extraordinary rotational strength from the structural features of the parallel quadruplex. Indeed, the rotational strength seems to be maximal for all conformations in the vicinity of 300 nm where absorbance is low but circular dichroism is high.

Figure 2 shows the second derivative spectra calculated for the human telomere conformations. These spectra provide a signature for different conformations. The utility of derivative spectra is that they reveal more subtle features of the broad, overlapping transitions in the primary CD spectra. Derivative spectra of all orders can be useful in assigning structural topology and, possibly, can reveal complexity due to sample heterogeneity (Viglasky et al, 2011).

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Figure 1.

Spectral features of human telomer quadruplex conformations. A. Circular dichroism for the antiparallel "basket" form in sodium (blue), the antiparallel "hybrid" form in potassium (red) and the parallel "propeller" form in 50% acetonitrile (black). B. Absorbance spectra for the same samples with the same color coding. C. Calculated g-factor as a function of wavelength obtained using the data in panels A and B. Spectra were recorded at 20 °C in BPS buffer (6 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 15 mM NaCl, pH=7.1; blue curves), BPK

buffer (6 mM K_2HPO_4 , 4 mM KH_2PO_4 , 15 mM KCl, pH=7.1; red curves) or BPK + 50% (v/v) acetonitrile (black curves).

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Figure 2. CD spectra (solid lines) and their second derivatives (dashed lines) for the human telomere “hybrid” form (A), parallel form (B) and the antiparallel “basket” form (C). Note that the scale of the ordinate axes for the second derivative curves have been reversed to facilitate comparison to the CD spectra. Solution conditions for the different conformations are identical to those given in Figure 1.

Table 1

Values for the apparent molar extinction coefficient at 260 nm for a human telomeric G-quadruplex sequence determined by various methods. Values refer to strand concentration in a buffer containing 6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM Na₂EDTA, and 185 mM NaCl, pH 7.0, 20°C.

Method	ϵ , M ⁻¹ cm ⁻¹	Comments
Calculated, Nearest Neighbor ^a	228,500	Unfolded, single-strand form
Colorimetric Phosphate ^b	196,480 ± 5,680	Folded
Colorimetric Phosphate	200,310 ± 1,000	Folded; independent determination
Absorbance, thermal denaturation	194,600	Folded
Absorbance, enzymatic digestion	201,180	Folded
Absorbance, alkaline denaturation	199,700	Folded
Average of all methods	198,450 ± 2,790	Folded; %H ^c = 15.1

^a[1] R. Buscaglia, R. D. Gray and J. B. Chaires, *Biopolymers* **2013**, *99*, 1006–1018.

^c%H is the per cent hyperchromism at 260 nm