

Use of Chromophoric Ligands to Visually Screen Co-Crystals of Putative Protein-Nucleic Acid Complexes

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ABSTRACT

Distinguishing between crystals of protein-nucleic acid complexes and those containing protein alone is a common problem in structural studies of protein-nucleic acid interactions. Currently, there are several methods available for detecting nucleic acid in crystals, including gel electrophoresis, SYBR Gold fluorescence dye staining, and methyl violet staining. However, they require either that the crystals be sacrificed or access to a fluorescence microscope. In this protocol, we describe an approach that allows direct visualization of either the presence or absence of oligonucleotides in crystals grown from solutions containing both protein and nucleic acid—labeling with the Cy5 dye. In addition to offering the advantage of being able to distinguish between crystals of complex and protein alone with the naked eye or a light microscope, crystals of covalently Cy5-labeled DNA can be directly used for X-ray diffraction data collection. *Curr. Protoc. Nucleic Acid Chem.* 46:7.15.1-7.15.8. © 2011 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit describes a method for establishing the presence of oligo-2'-deoxynucleotides in crystals of putative protein-DNA complexes by covalent attachment of water-soluble fluorescent dyes to either the 5'- or the 3'-terminus of an oligonucleotide (UNITS 4.2, 4.3, 4.5, & 4.9) or at internal sites.

The Cy5 and Cy3 compounds (Fig. 7.15.1; Ernst et al., 1989) belong to the cyanine dye family and are blue (excitation 650 nm/emission 670 nm) and red (excitation 550 nm/emission 570 nm), respectively (Jackson ImmunoResearch Laboratories; <http://www.jacksonimmuno.com/technical/f-cy3-5.asp>). Oligonucleotide-Cy5 and -Cy3 conjugates are commercially available, i.e., from Integrated DNA Technologies (<http://www.idtdna.com/catalog/Modifications/Dyes.aspx>). Cy5- or Cy3-modified oligonucleotides are purified (UNIT 10.3) by PAGE (UNIT 10.4) or HPLC (UNIT 10.5) and, following desalting (UNIT 10.7), are subjected to crystallization experiments (UNITS 7.6 & 7.13) with protein similar to complexes between native protein and DNA (Hollis, 2007). Once crystals appear, it is straightforward to establish by naked eye, or using a standard light microscope, whether they are colored or not (Georgescu et al., 2008). Colorless crystals either contain protein alone or are salt. Coloring of crystals most likely indicates that they contain both protein and DNA, although the conjugated oligonucleotide may occasionally crystallize by itself.

This unit provides procedures for the crystallization of Cy5- or Cy3-labeled oligo-2'-deoxynucleotides, and for identifying crystals that contain protein-DNA complexes.

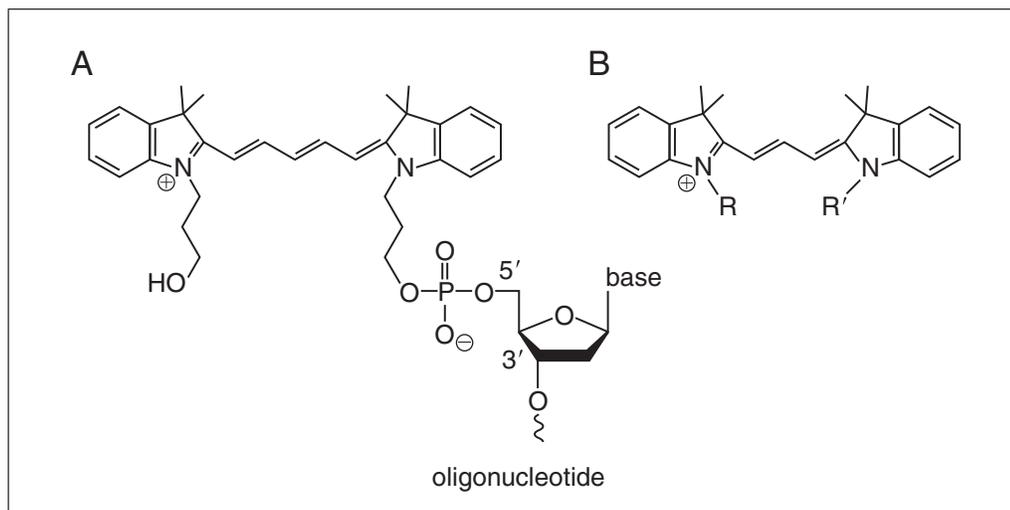


Figure 7.15.1 Chemical structures of the (A) Cy5 and (B) Cy3 dyes. Panel A depicts a conjugate between Cy5 and the 5'-terminal residue of an oligo-2'-deoxynucleotide via a phosphodiester moiety.

NOTE: Although colored crystals most likely contain the protein-nucleic acid complex, it remains possible that the nucleic acid portion is not well ordered in the crystal, thus preventing a meaningful analysis of the protein-DNA interactions. Furthermore, to prevent extension of the primer strand by the polymerase at the 3'-end, the catalytic Mg^{2+} ion can be replaced by Ca^{2+} in the crystallization experiments. Other measures to inhibit the enzyme in the crystallization drops include the use of a 2',3'-dideoxynucleotide at the 3'-end of the primer [for example (Silverstein et al., 2010)] and/or nonhydrolyzable 2'-deoxynucleotide triphosphate analogs (Batra et al., 2006).

BASIC PROTOCOL

CO-CRYSTALLIZATION OF A DUPLEX COMPOSED OF 5'-CY5-LABELED DNA PRIMER AND UNLABELED DNA TEMPLATE WITH DNA POLYMERASE η FROM *SACCHAROMYCES CEREVISIAE* (*scPOL* η)

The following procedure outlines the mixing and annealing of color-labeled oligonucleotides and DNA polymerase to form the complex and its crystallization by the hanging drop vapor diffusion technique. However, the solid-phase synthesis of the Cy5-labeled DNA primer strand is not described in detail because the modified oligonucleotide is commercially available and was used as provided by the manufacturer. Similarly, we refer the experimenter to published procedures regarding plasmid preparation and over-expression of the *scPol* η enzyme in *E. coli*, as well as purification. The described approaches apply to crystallizations of DNA/protein complexes in general.

Materials

- Purified and desalted 5'-Cy5-labeled primer strand (5'-Cy5-GTGGTCAAG-3'; Integrated DNA Technologies; <http://www.idtdna.com/Home/Home.aspx>)
- Purified and desalted native DNA template strand 5'-CTTCTTGACCAC-3' (IDT)
- scPol* η enzyme, catalytic core, residues 1-513 (*scPol* η_{1-513}) (Trincao et al., 2001; Alt et al., 2007)
- Ice
- Magnesium chloride (Fisher Scientific)
- 2'-Deoxyadenosine-5'-[(α,β)-methylene]triphosphate (dAMPcPP; Jena Bioscience)
- PEG Suite crystallization screen (Qiagen) containing reservoir solution

Microcentrifuge tubes
Various pipetman pipets and tips (Gilson)
Benchtop centrifuge
Heating block to anneal the primer and template strands
Vortex mixer
Siliconized glass circle cover slides (Hampton Research)
24-well plastic tray (Hampton Research)
Light microscope
Nylon loops for harvesting and flash-freezing crystals (Hampton Research)
Liquid nitrogen

1. Using a microcentrifuge tube, dissolve the Cy5-labeled primer and template strands in double distilled H₂O to a final concentration of 0.5 mM (duplex).
2. Anneal the primer-template duplex by heating the solution to 80°C for 10 min in a heating block, followed by slowly cooling to room temperature in the heating block. Centrifuge the tube containing the cooled solution in a benchtop centrifuge.
3. Mix *scPol* η_{1-513} and DNA in a 1:1.25 molar ratio using a vortex mixer. Incubate the mixture on ice for 1 hr, followed by incubation at room temperature for 10 min after addition of MgCl₂ (5 mM) and dAMPcPP (1 mM). Centrifuge the tube using a benchtop centrifuge.
4. Set up crystallization droplets on siliconized glass circle cover slides by mixing 1 μ L of annealed protein-DNA complex with 1 μ L of reservoir solution (PEG Suite crystallization screen) and equilibrate hanging drops against a 0.7 mL reservoir (see *UNIT 7.13*, Fig. 7.13.16).
5. Store the 24-well plate in an incubator at 18°C.
6. Crystals will appear after a period of \sim 12 hr, but may take longer.
7. Check individual droplets for colored crystals using a standard light microscope.

Crystallization plates should be checked daily.

Crystals were obtained from the following condition: 0.2 M magnesium formate and 20% PEG 3350.

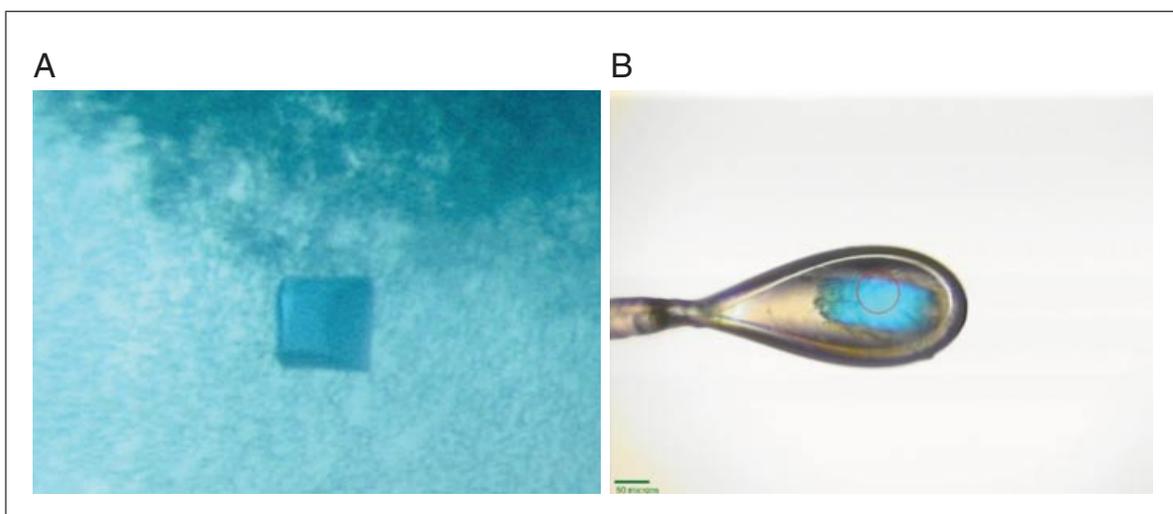


Figure 7.15.2 (A) Micrograph of a crystal of the complex between *scPol* η and a DNA primer-template duplex with a 5'-Cy5-labeled primer strand. (B) A color-labeled crystal of the complex inside a cryoloop, ready for data collection. The scale bar at the bottom left represents 50 μ m. For the color version of this figure go to <http://www.currentprotocols.com/protocol/nc0715>.

7.15.3

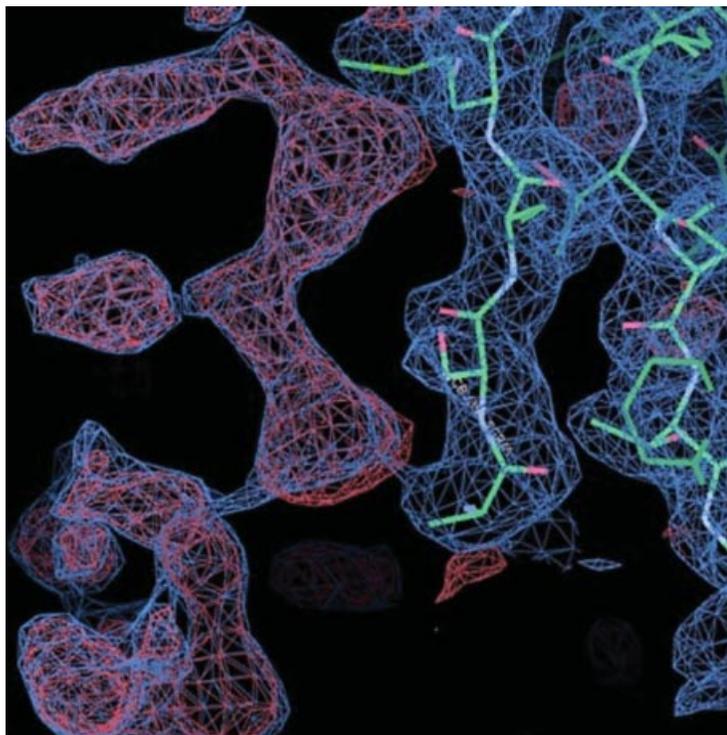


Figure 7.15.3 Electron density maps for the complex between *scPol* η_{1-513} and a DNA duplex containing the 5'-Cy5-labeled primer are consistent with partially ordered DNA. Superimposed Fourier (2*F_o*-*F_c*) sum (blue) and (*F_o*-*F_c*) difference electron density (red) maps drawn at the 1σ level reveal only a tetranucleotide fragment (left) that maps to the central part of the DNA template strand. Virtually no density existed in the region presumably occupied by the primer strand or at the active site (not shown). Conversely, the protein portion is well ordered and fully surrounded by sum electron density. Carbon, nitrogen, and oxygen atoms of *scPol* η are colored in green, blue, and red, respectively. Diffraction data of high completeness with a resolution of 1.92 Å were collected on the LS-CAT 21-ID-F beamline, Advanced Photon Source, Argonne National Laboratory (Argonne, Illinois). The maps were computed at a stage of the refinement with values for R-work and R-free of 27.1% and 30.2%, respectively. For the color version of this figure go to <http://www.currentprotocols.com/protocol/nc0715>.

- Mount colored crystals in nylon loops and freeze in liquid nitrogen for X-ray diffraction data collection and structure determination (UNIT 7.13) (Figs. 7.15.2 and 7.15.3).

COMMENTARY

Background Information

Many basic biological processes, such as replication, recombination, transcription, and translation involve both nucleic acids and proteins. Structural biology approaches provide insight into the mechanisms of these processes and are indispensable for elucidating structure-function relationships. X-ray crystallography is a powerful tool to study protein-nucleic acid interactions at the atomic level and is essential for a detailed understanding of protein function and the mechanisms underlying a myriad of protein and RNA enzyme-catalyzed chemical reactions. The foundation for all successful X-ray structure determinations is the

ability to produce diffraction-quality crystals. Unfortunately, crystallization has remained a trial and error approach; typically, hundreds of conditions need to be screened for each new protein or macromolecular complex under investigation, with no guarantee of success. Crystallization of complexes involving two or more components (proteins or nucleic acids or both) constitutes an even bigger challenge because of the need to establish that crystals indeed contain the complex (Hollis, 2007).

Crystals grown from solutions containing both nucleic acid and protein may contain either the complex or one of the components alone, or may simply be salt. The ability to

reliably distinguish between crystals of complex and protein or nucleic acid crystals is crucial, as exclusion of undesirable specimens at the structure determination or refinement stages can be very time consuming. However, differentiating between crystals of the complex and those containing solely the DNA/RNA or the protein is not straightforward. This is because of the inability to visually distinguish between such crystals. Similarly, unit cell constants and/or crystallographic space group symmetries often do not allow any firm conclusions either.

Currently, there are several ways to separate protein crystals from salt crystals. For example, methylene blue dye and Izit (Hampton Research) are helpful because the dye is expected to only stain protein crystals. Nucleic acid crystals can often be identified by their strong birefringence. For protein-nucleic acid complexes, protein detection is fairly easy by methylene blue dye or Izit. To identify DNA in protein-nucleic acid complexes, a traditional method, such as mobility shift assays, can be used (Sam et al., 2006; Loukachevitch and Egli, 2007). To achieve this, a few crystals are harvested from droplets, carefully washed and then dissolved prior to electrophoresis. Nucleic acid in native gels can be stained by ethidium bromide. However, if the nucleic acid concentration is low in the complex crystals, it may be hard to detect its presence. Furthermore, this method requires multiple steps and crystals need to be destroyed, which is less suitable for the purpose of rapid screening.

Detection of nucleic acids in putative crystals of complexes can also be achieved by methyl violet dye (Wilkosz et al., 1995). This is a direct method to recognize DNA in a crystal. Crystals containing DNA turn violet in the presence of the dye applied at the appropriate concentration. However, the method needs to be practiced first on crystals of known composition, and the concentration of the dye has to be adjusted carefully for accurate results (Wilkosz et al., 1995). SYBR Gold can be used to discriminate nucleic acid in crystals too. Molecular Probes SYBR Gold is the most sensitive fluorescent stain available for detecting double- or single-stranded DNA or RNA, and is more sensitive than ethidium bromide. It has been widely used for DNA/RNA gel staining. Moreover, it was reported that DNA in protein-DNA complexes is detectable by SYBR Gold. The method allows the identification of nucleic acid in crystals even when it only constitutes 3% of the crystalline materials (Kettenberger and Cramer, 2006). Whether

crystals contain DNA or not can then be detected with the help of a fluorescence microscope. To do so, crystals are soaked in harvest solution to completely remove nonspecifically bound nucleic acid. Compared with mobility shift assays, this method is simpler and more direct, but it requires access to a fluorescence microscope.

A visible dye would certainly facilitate detection of nucleic acids in crystals. Thus, Cy5 and Cy3 are reactive water-soluble fluorescent dyes that belong to the cyanine dye family (Fig. 7.15.1) and can be attached to primary or secondary amines and hydroxyl groups and therefore incorporated into nucleic acids or proteins at selected locations. Cy5 is popular for antibody labeling and has been used with nucleic acids in applications, such as comparative genomic hybridization, gene chips, and RNA localization (Blower et al., 2007). The use of Cy5 dye has recently also been reported in the co-crystallization of a sliding clamp processivity factor with DNA (Georgescu et al., 2008). This method is simple and offers the advantage of direct visualization in crystallization experiments.

Our protocol describes the use of Cy5-labeled DNA to detect its presence in putative protein-DNA complex crystals. We find that Cy5 labeling is very efficient for characterizing the makeup of crystals grown from solutions containing protein-nucleic acid complexes.

Critical Parameters

Although one may initially attempt to grow crystals of a complex containing native DNA, screening of oligonucleotides of various sequences and lengths could be directly performed with dye-labeled material. Thus, the appearance of colored crystals will provide an indication that sequence and length of the DNA may be optimal and that the crystals are of the complex. However, not all crystals are of diffraction-quality and crystallization conditions may have to be further optimized to establish crystals suitable for X-ray data collection. Occasionally, colored crystals may turn out to contain only nucleic acid. However, the volume of the unit cell and/or the space group symmetry might then hint at the absence of the complex.

For the crystallization experiments with DNA polymerase-DNA complexes described here, we relied on modified primer strands with the dye attached to the 5'-end. Clearly, the dye should not be attached at the 3'-end of the primer or at certain internal sites, i.e.,

at phosphate moieties, as this could compromise the formation of the complex with the polymerase and potentially crystals. This is because a dye attached at the 3'-end of the primer would likely interfere with binding of the incoming 2'-deoxynucleoside triphosphate. Similarly, a dye attached to the phosphate group in the center of either the primer or the template strands could drastically reduce the stability of the complex between primer-template duplex and polymerase as the bulky Cy5 or Cy3 moieties might clash with protein side chains. On the other hand, we would expect attachment of the dyes at either the 5'- or 3'-terminal ends of the template strand not to interfere with complex formation and crystallization.

It is possible that crystals of the native protein-DNA complex and the complex containing colored-labeled DNA will not crystallize under the same conditions. It is then advisable to either modify the initially established crystallization conditions slightly or to screen entirely new conditions. If crystals can only be obtained for the complex with labeled DNA, the structure can be determined and refined without the need to identify crystallization conditions for the native complex.

In cases where crystallization experiments remain unsuccessful with complexes containing Cy5- or Cy3-labeled oligonucleotides, it may be advisable to turn to alternative dyes. For example, we have also tested *scPol* η complexes in which the 5'-end of the DNA

primer was modified with the TAMN dye (Fig. 7.15.4). DNA and RNA oligonucleotides modified with this dye are also available from IDT Inc.

Troubleshooting

Although the approach described here for distinguishing between crystals of complex and protein alone is direct and efficient, it has some limitations. The dye can only indicate that the nucleic acid is in the crystal, but it does not provide any information as to whether DNA is stably and specifically bound to the protein or not. Currently, the only way to establish whether the crystal structure can yield useful insights into the protein-DNA interactions is to actually determine the structure of the complex.

In the case of the *scPol* η enzyme described here, polymerase molecules interact with each other to form dimers in crystals of the apo-form, whereby the pairing mode hampers formation of specific interactions between polymerase and DNA primer-template duplex. In other words, packing in crystals of the polymerase alone and of the polymerase-DNA complex is dictated by protein-protein interactions that prevent proper binding of the DNA. Although the DNA is clearly present in the crystal (Fig. 7.15.2), crystals of both protein alone and of the complex exhibit virtually identical unit cell constants and are of the same space group (data not shown). The electron density maps visualized after several rounds of refinement indicate that the primer-template

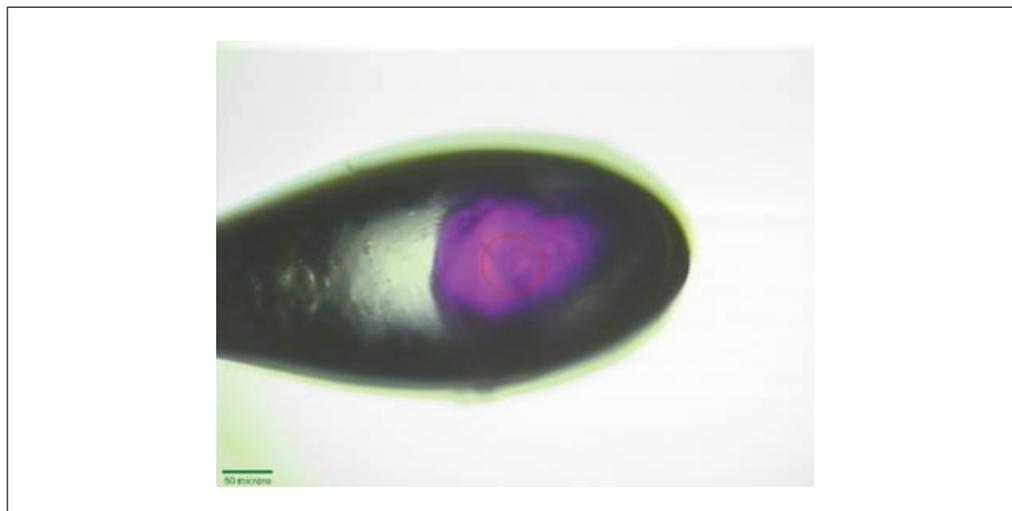


Figure 7.15.4 Cryoloop with a crystal of the complex between *scPol* η and a DNA primer-template duplex with a 5'-TAMN-labeled primer strand. The purple tint of the crystal indicates presence of both protein and DNA. The scale bar at the bottom left represents 50 μm . For the color version of this figure go to <http://www.currentprotocols.com/protocol/nc0715>.

duplex is disordered, with only a few nucleotides of the template strand adopting a defined conformation (Fig. 7.15.3). This problem is not related to the location or nature of the dye, and we grew crystals of the complex with native DNA with a range of lengths and sequences and with various modifications in the template strand, i.e., an abasic site or containing adducted bases, including 8-oxodG, O⁶-methyl-dG, 1, N²- ϵ -etheno-dG, or a TT dimer. In every case, the calculated electron density maps were consistent with disordered DNA. It was recently shown that the tyranny of the protein lattice could be overcome by introducing double mutations in *scPol* η_{1-513} , resulting in the formation of a new crystal form by the complex and well-ordered DNA (Silverstein et al., 2010).

The problem of partial occupancy of DNA in crystals of protein-DNA complexes, even in cases where such crystals diffract X-rays to medium or high resolution, may be quite common. For example, the interactions between RRM domains in crystals of FUSE-binding protein-interacting repressor (FIR) complexed with single-stranded DNA differed only minimally from those in crystals of the apo-form. Thus, the movement of one protein subunit relative to the other in the presence of the DNA amounted to just one degree relative to the orientation in the DNA-free crystal (opening the dimer slightly toward the DNA), and only a few nucleotides were subsequently visible in electron density maps (Crichlow et al., 2008; G. Crichlow, pers. comm.).

Anticipated Results

In this unit, we showed that a cyanine dye-labeled nucleic acid provides a facile way to distinguish nucleic acid-protein complex crystals from protein crystals. Cy5 labeling is simple, efficient, and cost effective. Cy5 is covalently linked to the nucleic acid sequence, which eliminates the need for staining. The dye was linked to the 5'-terminus of the DNA primer strand and did not seem to interfere with protein binding. Crystals with either the labeled or the native DNA duplex could both be obtained from the same crystallization conditions, and labeling did not change the crystal packing of the complexes.

Compared with gel electrophoresis or dyes requiring the use of a fluorescence microscope, Cy5 labeling is easier to handle and the crystals containing the complex can be distinguished from those containing the protein alone by naked eye or with a standard light microscope. The approach also eliminates the possibility of

false positives or negatives by incorrect concentration of the dye, as is potentially the case with SYBR Gold staining. Color labeling of DNA eliminates the need for a fluorescence microscope or relatively large amounts of material from dissolved crystals for conducting gel electrophoretic assays. Crystals containing DNA with covalently attached Cy5 dye can be directly used for X-ray diffraction data collection.

In summary, Cy5 labeling of nucleic acids is a direct and simple way to ascertain the presence of a protein-nucleic acid complex in crystals. While it provides a clear indication for the presence of nucleic acid in crystals, the approach provides no information regarding the degree of order of the DNA or RNA in a crystal or its diffraction limits.

Time Considerations

Cy5- or Cy3-labeled oligonucleotides, both DNA and RNA, are commercially available, thus precluding the need for in-house synthesis. The use of covalently modified oligonucleotides saves time compared to indirect staining or gel electrophoretic assays and requires fewer crystals. Color-labeled oligonucleotides may not crystallize under the conditions established with native complexes, thus requiring one to identify new crystallization conditions via screening. As well, crystals of complex with labeled DNA may appear earlier or later compared to the native complex.

Acknowledgments

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