Probing Nucleic Acid Structure with Nickel- and Cobalt-Based Reagents

Use and application of nickel and cobalt reagents are described for characterizing the solvent exposure of guanine residues within DNA and RNA. These reagents promote guanine oxidation in the presence of a peracid such as monopersulfate, and the extent of reaction indicates the steric and electronic environment surrounding the N7 and aromatic face of this residue. Together with the complementary reagents described elsewhere in this chapter, the secondary and tertiary solution structure of polynucleotides may be deduced.

Very low concentrations of the metal reagents are sufficient to promote guanine oxidation under near physiological conditions (see Basic Protocol). Nucleic acid recognition and oxidation does not perturb target structure or result in direct strand scission. Therefore, secondary procedures are necessary to identify sites of reaction. For polynucleotides of <200 to 300 residues, guanine oxidation is most conveniently detected by its induction of strand fragmentation upon treatment with piperidine (DNA; see Support Protocol) or aniline acetate (RNA; see *UNIT 6.1*). For larger polynucleotides, guanine oxidation is detected by its characteristic termination of primer extension (*UNIT 6.1*). Although the nickel reagent demonstrates the greatest specificity, the cobalt reagent maintains its selectivity at high temperatures and salt concentrations that are not compatible with the nickel reagent.

NICKEL- AND COBALT-DEPENDENT OXIDATION OF NUCLEIC ACIDS

BASIC PROTOCOL

This protocol describes a method for selectively oxidizing guanine residues (RNA or DNA) that do not stack within a double-helical structure. After excess oxidant is quenched and removed, the modified guanines are detected by procedures common to nucleic acid sequencing. These include either direct strand scission by subsequent treatment with piperidine (DNA) or aniline acetate (RNA) or termination of primer extension. The resulting polynucleotides are then separated and identified by denaturing gel electrophoresis.

CAUTION: Perchlorate is often used as a counter ion to the nickel complexes. Perchlorate salts containing organic materials are potentially explosive and should be handled carefully and in small quantities.

Materials

1 µg/µL carrier DNA or RNA
100 mM potassium phosphate buffer, pH 7 (*APPENDIX 2A*)
1 M NaCl in water
RNA or DNA sample: 30,000 cpm end-labeled (*UNIT 6.1 or UNIT 6.3*) or 1 pmol unlabeled
60 µM $[NiCR](PF_6)_2$ solution (see recipe and Fig. 6.4.1) *or* 60 µM $CoCl_2$ in water
0.6 mg/mL OXONE solution (see recipe)
20 mM HEPES/100 mM EDTA, pH 7, in water
NaOAc/EDTA/Tris solution (see recipe)
1 mM EDTA, pH 8 (*APPENDIX 2A*)

Microdialyzer
Lyophilizer

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Additional reagents and equipment for phenol/chloroform extraction and ethanol precipitation (*APPENDIX 2A* and, e.g., *CPMB UNIT 2.1A*), piperidine treatment (see Support Protocol), aniline acetate treatment (*UNIT 6.1*), PAGE (e.g., *APPENDIX 3B* or *CPMB UNIT 7.6*), partial alkaline hydrolysis and T1 nuclease digestion (*UNIT 6.1*), and primer extension (*UNIT 6.1*)

Oxidize nucleic acid

1. Combine the following in a microcentrifuge tube:

   2 µL 100 mM potassium phosphate buffer

   2 µL 1 M NaCl

   6 µL 1 µg/µL carrier RNA or DNA.

   Other buffers may also be used (see Critical Parameters).

2. Add 30,000 cpm end-labeled RNA or DNA, or 1 pmol unlabeled RNA or DNA (for primer extension), and sufficient deionized water to obtain a total volume of 18 µL.

   If the sample is <250 residues in length, it should be 5′ or 3′ end labeled with ^{32}P, permitting detection of the modified sites after chemical cleavage and electrophoretic separation. Longer samples should not be end labeled; these will be analyzed via primer extension. CPMB provides additional labeling methods, see e.g., CPMB UNITS 3.5, 3.6 & 3.10.

3. Initiate reaction by adding the following (total 20 µL/tube), vortexing, and incubating for 30 min at room temperature:

   1 µL 0.6 mg/mL OXONE solution

   1 µL 60 µM $[NiCR](PF_6)_2$ or $CoCl_2$

   This amount of OXONE results in a final concentration of 0.1 mM potassium peroxymonosulfate ($KHSO_5$) in the reaction.

   The extent of target modification can be controlled by adjusting a number of parameters (see Critical Parameters).

Stop reaction and purify sample

4a. *For subsequent piperidine treatment:* Stop reaction by adding 2 µL of 20 mM HEPES/100 mM EDTA and vortex. Dialyze oxidized samples in a microdialyzer against 1 mM EDTA, pH 8 (twice) and deionized water (once) to remove the inorganic salts. Lyophilize the dialysate.

   The resulting material may be analyzed immediately or stored at −20°C for >1 week.

4b. *For subsequent primer extension:* Stop reaction by adding 180 µL NaOAc/EDTA/Tris solution and vortexing. Extract with phenol/chloroform (see *APPENDIX 2A* and *CPMB UNIT 2.1A*). Precipitate DNA by adding 3 vol ethanol, incubating 60 min at −20°C, and microcentrifuging 10 min at 4°C. Remove supernatant, rinse with ethanol, microcentrifuge, and remove supernatant. Dry the pellet. (Also see *CPMB UNIT 2.1A*.)

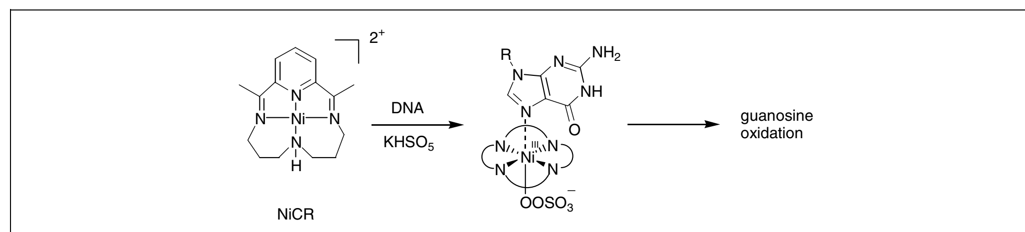


Figure 6.4.1 The mechanistic basis for specific oxidation of guanine.

Dialysis can be omitted for large RNA and for large DNA that is easily precipitated; however, precipitation alone is often not sufficient to purify RNA/DNA for subsequent analysis. The resulting material may be analyzed immediately or stored at −20°C for >1 week.

Identify sites of oxidation

5a. *Chemical cleavage:* For polynucleotides of <250 residues, assess modification sites by their characteristic strand scission after treatment with piperidine (for DNA; see Support Protocol; see *CPMB UNIT 7.5*) or aniline acetate (for RNA; Peattie and Gilbert, 1980; see DMS modification and cleavage of RNA, steps 8 and 9, in *UNIT 6.1*). Determine the ultimate profile of strand fragmentation by denaturing polyacrylamide gel electrophoresis (PAGE; e.g., *APPENDIX 3B* or *CPMB UNIT 7.6*). For RNA samples, subject aliquots of labeled RNA to partial alkaline hydrolysis and T1 nuclease digestion to use as sizing standards on the gel (*UNIT 6.1*).

5b. *Termination of primer extension:* For polynucleotides >250 residues, assess modified sites by determining their ability to terminate extension of a radiolabeled primer. Use this procedure for both DNA and RNA (Woodson et al., 1993; see *UNIT 6.1*).

DNA STRAND SCISSION BY PIPERIDINE TREATMENT

SUPPORT PROTOCOL

DNA samples ≤250 nucleotides in length that were modified at exposed guanines using the nickel and cobalt reagents described in Basic Protocol 1 can be analyzed by treatment with piperidine. Piperidine causes strand scission at the modified guanine residues. The resulting DNA fragments can then be analyzed by denaturing polyacrylamide gel electrophoresis.

Materials

Modified DNA sample pellet (see Basic Protocol)
0.2 M piperidine, freshly prepared in sterile water

1.5-mL screw-cap microcentrifuge tubes
90°C water bath
Speedvac evaporator

1. Resuspend modified DNA sample pellets in 20 µL of 0.2 M piperidine.

2. Transfer samples to 1.5-mL screw-cap microcentrifuge tubes, tightly cap the tubes, and incubate 30 min at 90°C.

   During this reaction, the tubes must be tightly sealed to prevent piperidine loss.

3. Microcentrifuge tubes briefly to collect any condensate from the sides of the tubes. Transfer the tubes to a Speedvac evaporator and evaporate the piperidine to dryness.

4. Resuspend dried samples in 30 µL sterile water, transfer to new tubes, and dry in a Speedvac evaporator.

5. Repeat step 4 using 30 µL sterile water.

   Failure to completely remove piperidine will result in smearing of bands on the sequencing gel. The samples are now ready for gel fractionation by PAGE (e.g., APPENDIX 3B or CPMB UNIT 7.6).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX.***

NaOAc/EDTA/Tris solution

0.3 M NaOAc
10 mM EDTA
10 mM Tris·Cl, pH 7.6 (*APPENDIX 2A*)
0.5% (w/v) SDS
Store up to 1 month at 4°C

[NiCR](PF_6)$_2$ solution, 60 μM

This complex is prepared according to Karn and Busch (1969) except that $NaPF_6$ is used in place of $NaClO_4$ and $HClO_4$ (see caution below). [NiCR](PF_6)$_2$ is also available from the authors' laboratories upon written request. A concentrated aqueous solution of [NiCR](PF_6)$_2$ (0.3 mM; 0.36 mg/mL) is stable for several months when stored at 4°C and protected from ambient light. Aliquots may be diluted to a working stock solution of 60 μM as needed.

[Ni(II)cyclam](ClO_4)$_2$ (see recipe) may be used as an alternative to [NiCR](PF_6)$_2$. However, Ni(cyclam) is less efficient than [NiCR](PF_6)$_2$.

CAUTION: *Perchlorate is often used as a counter ion to the nickel complexes. Perchlorate salts containing organic materials are potentially explosive and should be handled carefully and in small quantities.*

[Ni(II)cyclam](ClO_4)$_2$

Dissolve 0.110 g 1,4,8,11-tetraazacyclotetradecane (cyclam; 0.55 mmol) in 10 mL ethanol and heat to 60°C. In a separate container, dissolve 0.200 g (0.55 mmol) $Ni(ClO_4)_2 \cdot 6H_2O$ in 5 mL ethanol, and add in a dropwise fashion to the warm cyclam solution. Stir the resulting orange suspension at 60°C for an additional 10 min and then cool to room temperature. Collect the yellow product by vacuum filtration and wash with ice-cold ethanol. Use in the same fashion as [NiCR](PF_6)$_2$.

Ni(II)cyclam is easily prepared from commercially available materials (Martin et al., 1977) with a recovered yield of 0.243g (96%).

CAUTION: *Perchlorate is often used as a counter ion to the nickel complexes. Perchlorate salts containing organic materials are potentially explosive and should be handled carefully and in small quantities.*

OXONE solution, 0.6 mg/mL

Dissolve 6 mg $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$ (OXONE; Sigma, Aldrich) in 10 mL deionized water (final 2 mM $KHSO_5$). Prepare fresh each day.

The suggested concentration refers to $KHSO_5$ only.

COMMENTARY

Background Information

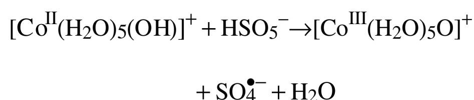
Chemical and enzymatic probes are essential for characterizing nucleic acid structure when nuclear magnetic resonance and crystallography are not appropriate or possible (Ehresmann et al., 1987; Knapp, 1989; Nielsen, 1990). The variable extent of modification induced by each reagent indicates the relative accessibility of its target site. Information on multiple sites may in turn be used to predict the three-dimensional structure of a polynucleotide. Although a limited analysis may provide some conformational data, most reliable structures are derived from the results of a broad series of experiments using a variety of probes that examine each functional region of each residue. Such an effort is necessary because the accessibility of one region of a nucleotide will not necessarily predict the accessibility of another region. For example, exposure of the phosphoribose backbone and N7 of guanine in the *Tetrahymena* group I intron

RNA were found to be independent (Chen et al., 1993).

The use of multiple probes to characterize a particular site is also often recommended to compensate for the inherent limitations of each reagent (Zheng et al., 1998). The solvent accessibility of guanine N7 has typically been examined by its alkylation with dimethyl sulfate (Ehresmann et al., 1987; Nielsen, 1990; *UNIT 6.1*). Failure to react suggests that a polynucleotide tertiary structure blocks access to this site. In contrast, variation in secondary structure does not significantly affect this reaction. Diethylpyrocarbonate (DEPC; *UNIT 6.1*) is more sensitive to base stacking within helical polynucleotides, but it is also much more efficient at adenine N7 than guanine N7 modification (Conway and Wikens, 1989). The structural environment of guanine N7 in RNA may also be investigated with RNase T1 (*UNIT 6.1*), but this enzyme requires access to the N1, O6, and N7 positions of guanine for activity (Heinemann and Saenger, 1985) and may disrupt local structure during substrate turnover (Zheng et al., 1998).

The nickel reagent appears to bind directly to guanine N7 and deliver its oxidizing equivalent specifically to this guanine (Fig. 6.4.1). The intermediate size of the nickel complex provides for greater selectivity than dimethyl sulfate, and provides access to more target sites than is possible for a macromolecule such as RNase T1. Nickel-dependent oxidation is selective for guanine residues that are not held within canonical A or B helices (Chen et al., 1992, 1993). Modification occurs at extrahelical and single-stranded guanines in addition to base-paired guanines at a helical terminus or junction (Chen et al., 1992, 1993). Since this probe does not affect the structure of its target, its relative ability to oxidize guanine has been a very strong indicator of both static and dynamic conformations of DNA and RNA (Chen et al., 1993; Shih et al., 1998).

Cobalt provides a complementary probe for guanine structure by generating a diffusible rather than metal-bound oxidant. Simple inorganic salts of cobalt, in contrast to nickel, are sufficient to catalyze formation of the sulfate radical from $KHSO_5$ (Muller et al., 1996).



$$[Co^{II}(H_2O)_5(OH)]^+ + HSO_5^- \rightarrow [Co^{III}(H_2O)_5O]^+ + SO_4^{\bullet -} + H_2O$$

This radical exhibits an inherent specificity for guanine relative to the other bases and the phosphoribose backbone. More importantly, guanine residues with highly accessible aromatic faces are most rapidly oxidized. The profiles of cobalt- and nickel-induced modification are often similar (Muller et al., 1996; Zheng et al., 1998), but will differ significantly when the environments surrounding the N7 and aromatic face of guanine are not equivalent (e.g., $tRNA^{Phe}$; Muller et al., 1996). Overall, the conformational selectivity of cobalt is less than that of nickel, as expected for the smaller size of the cobalt- versus nickel-dependent oxidants. Comparative investigations based on these two probes are also useful for determining the influence of electrostatic potential on modification. The anionic sulfate radical generated by cobalt and the cationic radical-nickel complex may respond to surface charge in the opposite manner.

Critical Parameters

The oxidation protocol described in this unit may be used as a starting point or guide for characterizing a variety of polynucleotides under different conditions. Typically, nucleic acid structure should be examined under conditions optimized for the nucleic acid's natural function rather than for chemical modification. Variations in reaction and quenching conditions are described in the cited literature and other studies based on these reagents.

Buffers and temperature. Contamination of the nucleic acid may inhibit the oxidative process. Buffers such as phosphate, Tris, and cacodylate are compatible with the nickel- and cobalt-dependent reactions. However, the Good buffers (for example, MOPS, MES, HEPES), EDTA, and thiols quench the reaction. Borate also inhibits the guanine-specific reaction and seems instead to promote variable modification of all polynucleotides. In addition, the nickel reagent is inhibited by high concentrations of salt (>1 M NaCl or $NaClO_4$) and appears to decompose in the presence of $KHSO_5$ at temperatures above 35°C. Although the cobalt reagent is also inhibited by NaCl, it remains active in the presence of 4 M $NaClO_4$ and at temperatures above 80°C. Both reagents tolerate the presence of $MgCl_2$, a common counterion required to form native RNA structure. Finally, bromide salts should be avoided as $KHSO_5$ will oxidize bromide, which in turn leads to modification of C residues (Ross and Burrows, 1996).

Extent of reaction. Oxidation should be limited to approximately 10% of the initial target nucleic acid in order to minimize secondary reactions of the oxidized products. The extent

of target modification is best controlled by increasing or decreasing the concentration of $KHSO_5$, although the concentration of nickel and cobalt reagents as well as incubation time and temperature may also be varied. A common cause for a lack of reaction is the use of a $KHSO_5$ solution that is not prepared fresh daily or the use of a metal solution that has been stored for an excessive time (typically >3 months). Old reagents may also induce abnormal reactions of uridine residues in RNA. If primer extension is used to detect sites of oxidation, phenol/chloroform extraction of the polynucleotide is generally required. This procedure likely removes and quenches materials that might otherwise inhibit polymerase activity.

Anticipated Results

The nickel complexes most readily oxidize guanine residues with highly accessible N7 positions, and the extent of reaction is indicative of the relative accessibility and electrostatic environment surrounding this position. In A- and B-helical structures of DNA and RNA, guanine N7 resides on the surface of the major groove, but is protected from reaction with nickel. Guanine residues are similarly unreactive when stacked within a duplex structure and paired in a noncanonical manner (Schmidt et al., 1995; Zheng et al., 1998). In contrast, guanine residues that are base paired at a helical junction or terminus are subject to nickel-dependent oxidation. The N7 of guanine within a Z helix is also exposed and demonstrates predictably high reactivity (Burrows et al., 1995). Guanines exhibiting the greatest reactivity are commonly those in highly disordered loops or single-stranded regions, and all guanines within such regions are oxidized with an equally high efficiency. When multiple structures are formed by a target nucleic acid, modification reflects the relative accessibility of each guanine weighted by the fractional concentration of its alternative conformations (Shih et al., 1998).

The cobalt reagent generates equivalent data on guanine, except the extent of reaction is more dependent on the exposure of its aromatic surface than its N7 position (Muller et al., 1996). The specificity for unstacked guanine residues is also lower for cobalt- than nickel-dependent oxidation. In the absence of an exposed target, both reagents will begin to oxidize guanine residues within a duplex in accord with their ionization potential. Therefore, under these conditions, the 5′ guanine of a GG sequence will react preferentially (Sugiyama and Saito, 1996; Burrows and Muller, 1998). Only one anomalous result has yet been detected with NiCR, and this, too, may provide useful structural data. Uridine residues are subject to direct strand scission if they are on the 3′ side of an adjacent uridine that forms a wobble base pair with guanine (5′-UU paired to 3′-GA; Hickerson et al., 1998).

Time Considerations

Probing the structure of nucleic acids with nickel and cobalt reagents can be accomplished very rapidly and performed in tandem with other typical modification reactions. Samples can be left overnight to dialyze if this method is used for purification. Product characterization by strand scission (using piperidine or aniline acetate) or primer extension, followed by gel electrophoresis, can be completed on the second day.

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A summary of initial mechanistic studies.

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