



Site-directed hydroxy radical mapping of nucleosome positions *in vitro* (PROT21)



Andrew Flaus, Chris Stockdale, Elisa Garcia-Wilson, Maik Engholm, Helder Ferreira & Tom Owen-Hughes
National University of Ireland Galway
Distillery Road
Galway, Ireland

Email feedback to:

andrew.flaus@nuigalway.ie

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Introduction

The dynamics of chromatin structure is becoming an area of increasing interest. Both thermal energy and ATP-dependent chromatin remodelling enzymes can alter nucleosome structure and positioning. In addition, modifications to both the DNA and histones and changes in their composition can influence the rate of these changes. The ability to precisely determine the position of a nucleosome *in vitro* is extremely useful when studying the biochemical behaviour of chromatin and its modifying enzymes.

This protocol gives details of a method for mapping the position of nucleosomes *in vitro* by attaching an EDTA-derived reagent to a specific site on the histone octamer which then catalyses local DNA cleavage. The sites of cleavage reveal the position of the nucleosome at base pair resolution (Flaus *et al.*, 1996; Flaus and Richmond, 1999a; Flaus and Richmond, 1999b; Bruno *et al.*, 2004). Nucleosome positions and movements on DNA fragments of some 500 bp have been mapped (Flaus and Owen-Hughes, 2003).

Background information

Most methods for determining the translational position of nucleosomes such as Restriction Enzyme accessibility, Micrococcal Nuclease, DNaseI, Exonuclease III or solution iron/EDTA are footprinting assays based on the fact that DNA assembled within a nucleosome is more resistant to cleavage than free DNA. In ideal cases with strongly positioned nucleosomes these methods can yield high resolution mapping of nucleosome positions. However, nucleosomal DNA is typically less tightly bound at the edges of the nucleosome, assays can require conditions which also encourage nucleosome mobility, and footprints may be difficult to interpret for mixed populations of positions.

In the early 1990s several investigators developed methods based on attaching chemical reagents which altered DNA binding proteins to become site-specific nucleases (Flaus *et al.*, 1996; Chen and Signman, 1987; Ebright *et al.*, 1992; Sluka *et al.*, 1987; Hayes, 1996). The most popular reagents are based on EDTA-derivatives capable of chelating iron and take advantage of the fact that a chelator will localise the Fenton reaction. This reaction involves donation of an electron from iron II to hydrogen peroxide, yielding iron III, a hydroxyl ion and a highly reactive hydroxyl radical ([Figure 2](#)). The hydroxyl radical is capable of reacting with DNA (or protein (Rana and Meares, 1991) or RNA (Wang and Cech, 1992) to yield a break in the backbone at the C1' and C4' carbons of the deoxyribose ring (Hertzberg and Dervan, 1984). The advantage of this approach is that it uses a diffusible species which does not require or select for a tightly defined arrangement of the protein-DNA interaction, unlike chemical crosslinking methods.

If the reaction is also supplied with a reducing agent (e.g. ascorbic acid or DTT), the iron III product can be recycled to iron II so

that the iron/chelator complex acts as a catalytic centre (Figure 2). Generating multiple hydroxyl radicals per molecule is necessary because the probability of any one causing DNA chain scission is low. This is due to stereochemical preferences of the radical reaction with nucleotides and their high reactivity with many other bonds in the vicinity, and results in a short effective range of around 7-16 angstroms from the reaction centre for cleavages (Ebright *et al.*, 1992). This sensitivity of reactivity means that the degree of cleavage at different sites should not be compared without appropriate controls.

The iron-EDTA reaction centre is localized to a specific site on the protein but near the DNA by tethering the EDTA molecule. The most convenient method to do this is by attachment at a cysteine residue, because covalent links can be specifically formed with the cysteine thiol and there is only a single cysteine (H3 Cys110) in the four *Xenopus laevis* core histones. The equivalent residue is an alanine in yeast and the alanine can be mutated to cysteine without any known biochemical effect.

A popular reagent for site directed mapping is BABE (bromoacetamidobenzyl EDTA (Meares *et al.*, 1984)). However, the predominant side reaction of common thiol-active functional groups such as bromoacetamide and maleimide is with lysine. There are a very large number of accessible lysines in the histone octamer in addition to the unique desired cysteine site, and we have observed significant levels of cross reaction using such reagents even under optimized conditions. To avoid this cross-reaction, we instead tether the EDTA via a disulphide bond which is highly selective for the target cysteine. The core EDTA molecule is therefore extended on one arm by reaction with cysteamine to introduce a terminal thiol. This thiol is 'activated' as an unstable disulphide bond with 2-thiopyridine which readily undergoes thiol-disulphide exchange with the histone cysteine sidechain (Gilbert, 1995). The only disadvantage of this is that the reagent tether is disrupted by the presence of reducing agents such as DTT and beta-mercaptoethanol.

The low rate of radical attack on DNA and the kinetic limitations of supplying the sequence of reactants to the tethered iron-EDTA centre mean that the reaction occurs on the order of tens of minutes. Presumably because the reagent tethering itself is also attacked by the hydroxyl radicals, DNA scission does not proceed to completion. Typically 5-20% of the nucleosomes will be mapped. The advantage of the high specificity of the radical attack on DNA is that it results in scission of the DNA backbone at a single site, yielding true single base pair resolution mapping of the nucleosome position.

The site of reagent tethering was chosen as H4 serine 47, close to the centre of the nucleosome (Flaus *et al.*, 1996; Luger *et al.*, 1997). For this site, cleavage occurs at a single strong nucleotide on one DNA strand and two secondary weaker nucleotides on the opposite strand (Figure 1). Because of its dyad symmetry, there are two reagents symmetrically attached per complete nucleosome. This means the single strong cut is generated on a strand by one reagent site whilst the two secondary cuts are generated by the other (Figure 1). The secondary cuts are 7 and 8 nucleotides downstream of the strong cut. The symmetry axis of the nucleosome can be mapped to the midpoint of these cleavages, 2 nucleotides downstream of the site of the strong cut (Flaus *et al.*, 1996). The symmetry axis defines the centre of the nucleosome and is therefore a convenient measure of the nucleosome position. Interestingly, because this axis passes through a nucleotide, it demonstrates that an idealized nucleosome has 147bp of DNA as subsequently confirmed by high resolution nucleosome structures (Luger *et al.*, 1997). This structure could nevertheless be in a dynamic state (Negri *et al.*, 2001; Richmond and Davey, 2003).

Related links

1. Fenton reaction
<http://www.iupac.org/goldbook/FT06786.pdf>,
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=8138191
2. Mapping of a linker histone using a similar method
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=8810896
3. Site-specific mapping with FeBABE
<http://www.biologicalprocedures.com/bpo/arts/1/49/m49.htm>

Procedure

Preparation of Derivatised Octamer

1. Refold and purify histone octamer containing histones H2A wild type, H2B wild type, H3 Cys110Ala and H4 Ser47Cys following standard methods (Dyer *et al.*, 2004) (see [note 1](#));
2. If necessary, re-reduce histone octamers by incubating in 10 mM DTT for 30 minutes (see [note 2](#) and [comment 2](#));
3. Dialyse histone octamer three times 2 hours or more against at least 100 times volume of 5mM sodium cacodylate pH 6.0, 2 M NaCl buffer (see [note 3](#));
4. Carefully measure the absorbance at 276 nm of the dialysed histone octamer. Calculate the concentration (see [note 4](#) and [comment 3](#));
5. Carefully weigh out a small amount of reagent into an Eppendorf tube. Dissolve in 100mM Tris-Cl pH 7.5 to a final concentration of 10 mM. Gentle agitation may be necessary (see [note 5](#));
6. Take desired amount of histone octamer for reagent attachment. Add 0.05 volumes of 1 M TrisCl pH 7.5. Add 50-100 fold molar excess of freshly dissolved 10 mM reagent (see [note 6](#)). Allow to react overnight at room temperature (see [comment 1](#)). Reagent reaction can be monitored by SDS PAGE or triton urea acetic acid gels (Flaus *et al.*, 1996);
7. Calculate new concentration of histone octamer allowing for dilution caused by adding Tris buffer and reagent (see [note 7](#));

8. Dialyse histone octamer three or four times 3 hours or more against at least 100 times volume of 20mM Tris-Cl pH7.5, 2M NaCl buffer (see [note 8](#)). Store the reagent-tethered octamer at 0-4°C, where it is typically stable for at least a month.

Preparation of Labelled DNA

1. We routinely prepare DNA fragments for mono- and di-nucleosomes made by preparative PCR and ion exchange purification (Bruno *et al.*, 2004). Alternatively, fragments can be isolated by restriction digestion of plasmids after large scale plasmid preparations (see [note 9](#));
2. DNA fragments should be strand-specifically labelled, for example with a fluorophore dye or radioisotope (see [note 10](#)).

Nucleosome Reconstitution

1. It is necessary to minimize metal ions at each stage from nucleosome reconstitution onwards. This is the major cause of high background and low DNA scission efficiency (see [note 11](#) and [comment 4](#));
2. Reconstitutions can be carried out by the standard dialysis methods for 2 hours against buffers of 20mM Tris-Cl pH 7.5, 1mM EDTA with sequentially 0.85M, 0.65M, 0.5M NaCl or KCl and then two steps with only 50mM Tris-Cl pH 7.5 (ie no NaCl, KCl or EDTA) (see [note 12](#)).

Mapping Reaction

1. A typical sample for mapping has a total volume of 10-20µl and contains 0.2-0.5µM reconstituted nucleosome, 50mM Tris-Cl pH 7.5 and appropriate monovalent salt. After desired experimental manipulation, place sample on ice (see [note 13](#));
2. Freshly prepare reagent solution by degassing 3 small sidearm flasks, each containing 50ml water. Add 37.5mg of ferrous ammonium sulphate, 200mg of ascorbic acid and 333µl of hydrogen peroxide to separate flasks. Swirl under vacuum to dissolve/mix (see [note 14](#) and [comment 5](#));
3. Dilute 20µl 1mM degassed ferrous ammonium sulphate in 980µl water. Add 1µl to each mapping reaction sample. Remove 1µl from each mapping reaction for a native gel. Incubate 5-15 minutes (see [note 15](#));
4. Mix 400µl degassed ascorbic acid solution with 100µl 1M Tris-Cl pH 7.5. For a 10µl sample, add 5µl buffered ascorbate to the sample to be mapped (see [note 16](#), [comment 7](#) and [comment 8](#));
5. For a 10µl sample, add 5µl 0.2% v/v hydrogen peroxide;
6. Incubate sample for 30-90 minutes on ice;
7. Terminate the reaction by adding one reaction volume of phenol/chloroform/isoamyl alcohol (ie 20µl for a 10µl original sample volume). Mix then spin 1 minute to separate phases (see [comment 6](#));
8. Add upper aqueous phase to a fresh tube containing 2µl 3M sodium acetate pH 5.2. Add 3 volumes of absolute ethanol. Spin 15 minutes to precipitate. Remove the supernatant. Spin a further 5 minutes and remove residual liquid. Resuspend pellet in 10µl formamide loading dye solution (see [note 17](#)).

Readout and Analysis

1. Incubate samples 3-5 minutes at 90°C to denature. Load 4µl per sample on a standard high resolution denaturing gel ('sequencing gel') and run as appropriate (see [note 18](#) and [note 19](#));
2. Image the gel as appropriate (see [note 20](#));
3. Nucleosome positions can be easily diagnosed as a strong cut with weaker cuts 7 and 8 nucleotides downstream (i.e. further up gel for 5' labelled DNA). The actual position of the dyad axis is 2 nucleotides downstream of the main strong cut (see [note 21](#) and [figure 1](#)).

Materials & Reagents

Originally the chelating reagent was manually synthesized with a 2-nitrophenyl activating moiety. However, an equally effective identical EDTA reagent activated instead with 2-pyridine is available commercially from Toronto Research Chemicals (TRC Inc, Toronto, Canada) as N-[S-(2-Pyridylthio)cysteaminy]ethylenediamine-N,N,N',N'-tetraacetic acid ([product P996250](#)).

Any high purity grade, low trace metal chemicals should be sufficient. We source ascorbic acid (cat 95209), hydrogen peroxide (cat 95321), sodium chloride (cat 71378) or potassium chloride (cat 60129), and hydrochloric acid (cat 84415) from Fluka because explicit specifications for trace metal contaminants are provided. We have successfully used ferrous ammonium sulphate from both Fluka (cat 09719) and Aldrich (cat 20,350-5).

Phenol:chloroform:isoamyl alcohol at 25:24:1 is prepared by standard methods. Formamide loading dyes comprise deionised formamide with 10mM EDTA. 0.1g of bromophenol blue and xylene cyanol can be added for visualization but these dyes can mask fluorimager readout. We store the loading dye at -20°C in small aliquots.

We use Millipore Amicon Ultra 5000 MWCO and Centricon YM30 concentrators. Multivalent metal ions can be removed by BioRad Chelex 100 (200-400 mesh) resin. Sequencing gel solutions are SequaFlowgel 6 and 8 (cat H18049 and H18051) from Flowgen UK. Millipore Stericup filter devices are convenient for filtration.

Xenopus laevis

H2A wild type, H2B wild type, H3 Cys110Ala and H4 Ser47Cys as pET3 recombinant expression plasmids are available from [Prof Tim Richmond](#), [Dr Karolin Luger](#) or myself.

Authors Notes

1. For subsequent steps it is convenient to prepare approximately 10-50nmol (1-5mg) of octamer. Histone octamer should be concentrated to approximately 20-30 μ M. Concentrations below this will mean octamers can become inconveniently dilute for reconstitutions, and at concentrations above this making accurate spectrophotometric measurements become complicated;
2. For example, if beta-mercaptoethanol has been used in the histone octamer preparation a proportion of H4 cysteine 47 thiols will typically be in a disulphide bond with the beta-mercaptoethanol and hence unavailable for reaction (see [comment 2](#));
3. Efficient dialysis is necessary to remove all reducing agents, 2M NaCl is necessary to maintain the histone octamer. A slightly acidic buffer is used because this stabilizes the free thiol form of the cysteine. Cacodylate is used for historical reasons but is an arsenic-based compound so requires appropriate precautions. Any buffer with pKa around pH 6 that does not chelate metal could be a suitable alternative (e.g. 'good' buffers (Good and Izawa, 1972));
4. A 1mg/ml solution of histone octamer has an absorbance of 1.0 at 276 nm. The molecular weight of the histone octamer is approximately 100 kDa (see [comment 3](#));
5. It is convenient to weigh approximately 0.5mg to maintain accuracy but minimise wastage. The reagent molecular weight is 461Da, so a typical solution would require dissolving 0.5mg in 108 μ l of 100mM Tris-Cl pH 7.5. The reagent is very hygroscopic and somewhat labile to oxidation. Store the reagent sealed in a dry environment and minimize exposure to air and humidity. It has limited but sufficient solubility in a neutral pH buffer;
6. A typical example reaction mixture comprises 400 μ l of 25 μ M histone octamer, 20 μ l of 1M Tris-Cl pH 7.5 and 100 μ l of 10mM reagent solution. Adding Tris buffer alters the pH so that the disulphide bond between the reagent and histone is favoured. Avoid diluting the histone octamer by more than 25% as this will reduce the salt concentration maintaining the histone octamer complex significantly. We have not tested how quickly the reaction occurs so shorter incubations may be possible;
7. For the example above, the final concentration is 19.2 μ M histone octamer. The pyridine ring of the reagent absorbs strongly compared to histones so trace remnants make it difficult to accurately determine the histone octamer concentration spectrophotometrically after reaction, even if extensively dialysed;
8. This is to remove excess reagent and 2-nitrophenol activating group. Efficient dialysis is necessary because they may not pass through membrane as rapidly as small ions.
9. Care should be taken to minimize nicking and premature terminations because this can introduce background into mapping. For fluorophore labelling of PCR-prepared DNA fragments, we use Cy3 or Cy5 labelled oligonucleotide primers from standard commercial sources. It is possible to detect both strands in the same reaction by labelling with different dyes. Cy5 is preferred because of its better signal to noise ratio in gels;
10. For fluorophore labelling of PCR-prepared DNA fragments, we use Cy3 or Cy5 labelled oligonucleotide primers from standard commercial sources. It is possible to detect both strands in the same reaction by labelling with different dyes. Cy5 is preferred because of its better signal to noise ratio in gels. For radioactive labelling of PCR-prepared DNA fragments, we combine a primer for the strand to be labelled with a free 5' hydroxyl together with a primer for the other strand with a covalently linked 5' blocking moiety such as a dye or biotin. After preparative PCR and purification of the fragments, we transfer gamma 32P onto the free 5' hydroxyl terminus using a standard polynucleotide kinase reaction and Sephacryl S200 spun column cleanup;
11. Reagents should have the lowest possible metal ion contaminant specifications (see materials section for recommended products). Only molecular biology grade purified water should be used. Containers made of plastic should be preferred over glass where possible. Stock 4M NaCl or 3M KCl solution used to prepare dialysis buffers should be mixed with a few grams of Chelex resin to remove metal ions then filtered in a plastic apparatus to remove the resin. Where practical, dialysis apparatus, stock bottles and flasks for degassing should be acid washed by soaking in 0.1M HCl overnight and extensive washing with purified water. Finally they should be re-equilibrated to neutrality by washing with 10mM Tris-Cl pH 7.5 to ensure trace acids will not affect subsequent buffer pHs (see [comment 4](#));
12. Samples should be mixed in a total volume of 25-50 μ l with final concentrations of 2M NaCl, 1-4 μ M reagent-tethered histone octamer, and equimolar DNA to histone octamer. Where necessary, the molar ratio of DNA to octamer can be optimized to minimize free DNA. Nucleosomes tend to be unstable when stored at concentrations below 1-2 μ M. Although we use a custom dialysis apparatus (Flaus and Richmond, 1999b), commercial alternatives are available, such as the Pierce Microdialyzer System, Spectrum MicroDialyzer devices or Spectrum Micro DispoDialyzers;
13. For mapping with fluorescent detection, at least 4pmol of nucleosome are required due to the lower detection limits achievable. Concentrations of NaCl up to 400 μ M and MgCl₂ up to at least 1mM can be tolerated. Mg²⁺ binds many orders of magnitude more weakly to EDTA than Fe²⁺. Residual amounts of detergents and glycerol from enzyme buffers can be tolerated, but the bulk of reducing agents such as DTT and beta-mercaptoethanol need to be removed before an enzyme is added to a reagent-tethered nucleosome sample. Experimental treatment of the nucleosome samples can be carried out at any condition where the nucleosome is stable (ie up to 55°C). The mapping reaction itself is typically carried out on ice to minimize nucleosome dynamics during the hydroxyl radical cleavage incubation. We have had poor results with some types of tubes which appear to inhibit the hydroxyl radical reaction. Eppendorf brand 0.5ml Safelock tubes work well and are convenient for the subsequent phenol/chloroform extraction;

14. The flasks therefore contain respectively 2mM ferrous ammonium sulphate, 24mM ascorbic acid and 0.2% v/v hydrogen peroxide;
15. Typically we add the 1µl sample to 10µl 5% sucrose and load all on a pre-run 160x200x1mm, 5% (1:40) acrylamide, 0.2xTBE native gel equilibrated at 4°C. The gel is both pre-run and run at 300V for 180-240 minutes. When the sample contains larger amounts of nucleosome it may be necessary to increase the amount of ferrous ammonium sulphate added to ensure all tethered chelators are loaded;
16. For larger samples add proportionately more ascorbate and hydrogen peroxide (i.e. half of original sample volume). The final concentrations in our typical reaction sample are 0.1-0.4µM nucleosome, 75mM Tris-Cl pH 7.5, 2µM ammonium ferrous sulphate, 4.8mM ascorbate, 0.05% hydrogen peroxide and monovalent salt as required (see [comment 7](#)). We find ascorbate to be a major limiting factor in the reaction. However, adding too much will affect the pH and ionic strength of the sample. The mapping reaction is optimal at pH 7.0-7.5 (see [comment 8](#));
17. Incubating ethanol precipitations at low temperature is unnecessary. Adding calf thymus DNA or tRNA may be useful to improve the yield of small DNA fragments. Air or vacuum drying ethanol precipitate is unnecessary. Brief vortexing and sucking up/down with pipette is sufficient;
18. We use 6% and 8% commercial premixed gel solutions according to the manufacturers instructions for optimal observation of fragments in the ranges approximately 75-300 bp and 25-200 bp respectively. We use BioRad SequiGen gel equipment with a BioRad 3000 power supply with temperature control set to maintain a constant 50°C gel temperature. Gels are pre-run for 45-60 minutes to bring them up to temperature and loaded immediately, by taking samples directly from 90°C hot block. Run times vary from 100-200 minutes as appropriate. Some DNA samples do not denature readily and may require elevated urea concentrations or formamide added to the gel;
19. For radioactively labelled samples, Maxim-Gilbert G-specific reaction products ('G tracks') generate chemically equivalent products to the hydroxyl radical cleavage and are ideal size marker ladders. For fluorescent dyes, restriction digest products provide useful markers because the G-track reaction damages the fluorophores. To achieve base pair resolution it is essential to use an identical sequence as a marker. It is also possible to have suitably fluorescently labelled mapping reaction products analysed by submitting it to a standard sequencing facility (Yindeeoungyeon and Schell, 2000).
20. Allow gel to cool significantly before separating plates to reduce problems of gel not remaining attached to a plate. If gels are fixed prior to drying, ensure gel does not come away from plate during soaking step as this will stretch it and reduce resolution. For reading radioactively labelled samples, expose to phosphorimager plate or x-ray film (e.g. BioMax MS with screens). Note that x-ray film typically gives a non-linear response. For reading fluorescently labelled gels, do not fix because exposure to low pH damages Cy3 and Cy5 dyes. We image gels directly on the glass plate using a Fuji FLA5100 imager with standard Cy3 and Cy5 filter sets;
21. The presence of the diagnostic pair of strong and weak cuts in the denaturing gel, and agreement of the mapped positions with the migration of bands in the native gel is an important confirmation of the significance of the mapping results.

Reviewer Comments

Reviewed by: [Jeff Hayes](#), University of Rochester Medical Centre, Rochester, US.

1. The extent of available cysteines reacted with the reagent can be determined by briefly reacting a portion of the sample with an excess of fluorescein-maleimide or 14°C-N-ethylmaleimide (NEM) for 10-15 minutes, followed by direct fluorography or autoradiography (the latter may require soaking the gel in an enhancer). Note that the maleimides must be added in excess of the disulfide reagent.
2. Note that the proportion of beta-mercaptol-protein disulfides is likely to be very small as long as the concentration of β-MeOH is kept high and thus this statement may be a bit misleading. However, DTT is definitely the reducing reagent of choice given its greater reducing power. Andrew Flaus replies: "Although this is true according to the textbook theory, our [note 2](#) is based on our direct experience and analytical observations - presumably there is extra stabilisation of disulphides due to the local environment. We have found the DTT pre-treatment to be important for effective reagent attachment."
3. For less concentrated preparations of core histones (about 0.1mg/ml), the absorbance at 230nm can be used to measure concentrations (~4.2 O.D. per 1mg/ml). However in this case be careful not to use buffers that absorb in the near UV (i.e. Tris).
4. Note, it is especially difficult to completely remove iron from the high salt solutions used for dialysis. One solution we found was to include high quality highly polymerized DNA (calf thymus or salmon sperm) in the dialysis buffers to ensure that metal ions from the solution do not accumulate on the sample DNA. For this purpose we used 50-100ng/ml calf thymus DNA in the dialysis buffers.
5. One can also bubble N₂ through the solutions to remove O₂.
6. In some cases, it may be desirable to separate cleaved native complexes on nucleoprotein gels after the reaction. In such cases, the cleavage can be terminated by addition of EDTA and glycerol to 5. Note that free EDTA binds the metal several orders of magnitude better than the tethered chelator and glycerol is an effective radical scavenger.
7. Note that Tris buffers >~20mM are fairly good radical scavengers. In some cases, utilizing buffers that do not scavenge such as phosphate may increase cleavage signals. On the other hand having such high Tris concentrations may actually help keep the cleavages localized.
8. Fresh sodium ascorbate can be used in this step to help avoid the acidification problem. Still care must be taken to not overcome the buffering capacity of the solution.

Figures

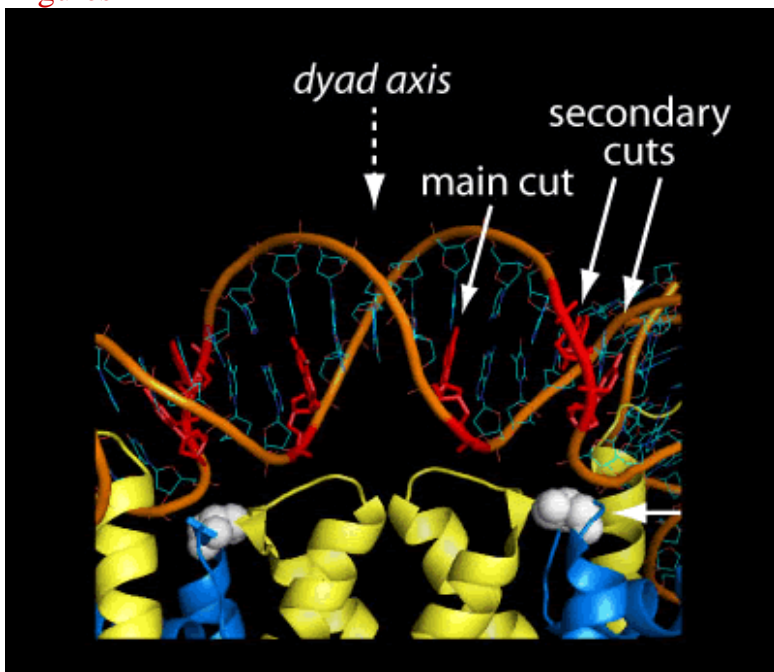


Figure 1

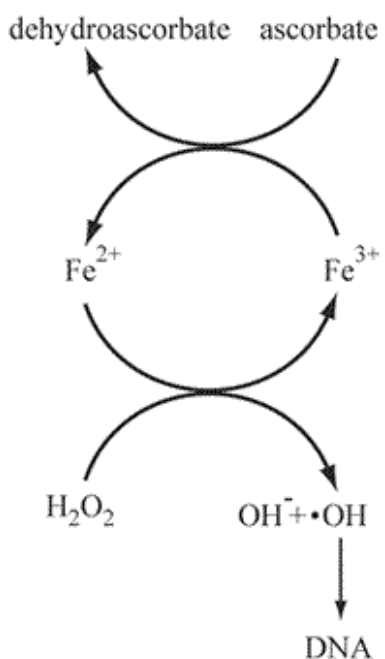


Figure 2

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