Reconstitution of duplex circular DNA chromosomes by simple re-annealing of the separated, complementary single strands

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NOTE: A concise protocol, in the form of an Excel spreadsheet, accompanies this document. Virtually all the entries in the spreadsheet are self-adjusting formulae, so that if certain key quantities, concentrations or percentages change, most of the other values will self-adjust. (Most – not necessarily all!)

Detailed Experimental Protocol

Summary

This protocol may seem long and complicated, but it's conceptually simple:

(1) You'll nick the "**top**" strand of an aliquot of pBR322 plasmid DNA with the "nicking enzyme" N**t**.BbvCI, then digest the nicked strand with Exonuclease III. This leaves only the "bottom" strand.

(2) You'll nick the "**bottom**" strand of another aliquot of pBR322 DNA with the "sister" enzyme N**b**.BbvCI, then digest that nicked strand with Exonuclease III. This leaves only the "top" strand.

(3)You'll mix the purified "top" and "bottom" strands together, and re-anneal them under conditions known to regenerate Form I duplex DNA. (The phrase "conditions known to regenerate Form I" is key here; these conditions are completely *non*-intuitive, and are only known through the study and understanding of years of work by other investigators).

(4) You'll analyze the re-constituted duplex by agarose gel electrophoresis at various concentrations of ethidium bromide, and show that it co-migrates with control DNA at any and all ethidium bromide concentrations.

(6) You'll alkali-denature the experimentally-reconstituted DNA, and show that it still co-migrates with alkali-denatured control DNA, at various key salt concentrations.

(7) You'll *re*nature some of the alkali-denatured experimental DNA, and show that its electrophoretic properties have reverted to those of the native chromosome.

(8) You'll verify the strandedness of your single- and double-stranded DNA by demonstrating that the single-stranded DNA only is susceptible to Mung Bean nuclease.

Estimated time: If you are experienced with these DNA laboratory procedures, and all your materials and supplies have been obtained and prepared, you can do the actual experiment in as little as 1-2 days.

WARNING!

Circular DNA re-annealing is a tricky business, fraught with pitfalls. The DNA concentrations, pHs, ionic strengths and temperatures given here were determined by years of pain and suffering, by dedicated scientists. Therefore, these instructions must be followed *exactly*. If you "cut corners", or take what you think are "shortcuts", you will almost surely fail.

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Materials

NOTE: If your daily job involves plasmid molecular biology, you'll have almost all of the following chemicals and instruments already, and you won't have to spend more than a few hundred dollars, mostly for enzymes. If you *don't* do this sort of work routinely, the cost will go up, depending upon how many of the following items you lack.

Note that the vendors referenced below are not the only sources of these materials. Any competent vendor will suffice, except that whatever plasmid/viral DNA you elect to use must have the proper cleavage sites for the nicking enzymes you have selected:

Enzymes & DNA:

(List last updated July, 2018)

1. **DNA from the plasmid "pBR322":**
NOTE: Although the DNA of any plasmid can be used for this experiment, I am suggesting this one, because it has the proper cleavage sites for the readily-available nicking enzymes below.
(Size: 4361 bp (4.3 kb), MW 2.67×10^6

Life Technologies (formerly ThermoFisher, formerly Fermentas)**:**
100 μg total, supplied at a concentration of 0.5 μg/μl
Stored in 10 mM Tris-HCl, 1 mM EDTA, pH 7.6.
Cat. #: SD0041. Cost: $126.
URL: https://www.lifetechnologies.com/order/catalog/product/SD0041
You need to double this order, *i.e.,* **200 μg.** Cost $236.

Alternative source: **New England Biolabs**:
250 μg @ 1000 μg/ml
Stored in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
Cat. # N3033L, price $289.
https://www.neb.com/products/n3033-pbr322-vector

Allegedly 90% pure. Store at –20º.

2. **Strand-specific, site-specific endonucleases (= "nicking enzymes"):**
New England Biolabs "top" and "bottom" enzymes N**t**.BbvCI and N**b**.BbvCI, which cleave the "top" and "bottom" strands of the plasmid pBR322 respectively. There is exactly *one* cleavage site for each enzyme on its respective strand. Supplied with "CutSmart" buffer (warning: to get 100% enzyme activity, you must pay strict attention to buffer salt concentrations). Cost: 1000 u, $69 each.
"Top" enzyme (*i.e.,* N**t**.BbvCI), : Cat #: R0632S
URL: https://www.neb.com/products/r0632-ntbbvci

"Bottom" enzyme (*i.e.*, N**b**.BbvCI),: Cat #: R0631S
URL: https://www.neb.com/products/r0631-nbbbvci

3. **Exonuclease III** (which digests single-stranded DNA from a free end, but has little or no effect on intact, covalently-closed circular DNA); New England Biolabs. Concentration: 100,000 u/ml = 100 u/µl. Supplied with 10X reaction buffer (NEBuffer 1*).
Cat. # M0206S. Cost: **5000 u**, $60.
URL: https://www.neb.com/products/m0206-exonuclease-iii-e-coli

*NOTE: Like the nicking enzymes, Exo iii is finicky about the buffer salt concentration. NEBuffer 1 has no sodium at all. In other NEBuffers, the activity ranges from 25%- 75%:

4. **Mung Bean nuclease**, New England Biolabs, to establish single-strandedness.
Cat #: M0250S. Cost: 1500 u, $64.
URL: https://www.neb.com/products/m0250-mung-bean-nuclease

NOTE: You will need additional 10X Mung Bean nuclease reaction buffer, for dialyzing DNA at the end of the experiment. This product, as a separate order, has been discontinued. You may therefore have to make the extra 1X buffer yourself.

1X Buffer Components:
30 mM NaCl
50 mM sodium acetate
1 mM ZnSO4
pH 5.0@25°C

Materials for agarose gel electrophoresis:

Electrophoresis apparatus (Edvotek)
M12 Complete Electrophoresis Package, Cat. #: 502/504, cost $199.
URL: http://www.edvotek.com/M12

Power source:
Edvotek "DuoSource 150 Power Supply" (75/150v).
Cat. #509, cost $179.
URL: http://www.edvotek.com/509.html

Gel Scoop, Sigma-Aldrich (MSMINIDUO UV gel scoop), 7 cm
Cat# EP1112, $33.10. URL:
http://www.sigmaaldrich.com/catalog/product/sigma/ep1112?lang=en&region=US
OR
Gel Scoop, Sigma-Aldrich (MSCHOICETRIO UV gel scoop), 15 cm

Cat# EP1213, $46.60. URL:
http://www.sigmaaldrich.com/catalog/product/sigma/ep1213?lang=en&region=US&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-1

Agarose
UltraSpec-Agarose™ 100 g Cat.#: 605-100g
Cost (100 g): $149
URL: http://www.edvotek.com/605-100g_4

Ethidium bromide
Fisher Scientific
URL: (Click here)
There are a multitude of different items, starting at about $70.

Rubber gloves

6X loading dye
Life Technologies (formerly ThermoFisher, formerly Fermentas):
Loading dye (glycerol) (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA)
Cat. #: R0611. Cost, 5 ml, $40.25.
URL: https://www.lifetechnologies.com/order/catalog/product/R0611

UV transilluminator
Midrange UV Transilluminator 7 x 14 cm 558 (NO CAMERA!)
Edvotek 558, cost: $579
http://www.edvotek.com/558.html
If you use this, you must photograph the gels with your own camera.

EdvoFoto™ Digital GelCam (camera)
Edvotek 551, cost: $649
http://www.edvotek.com/551

BOTH OF THE ABOVE COMBINED (i.e., transilluminator + camera):
UV Digital Photodocumentation System
Edvotek 555, cost $1,149.
http://www.edvotek.com/555

Miscellaneous chemicals:

Sodium Phosphate Dibasic Heptahydrate ($Na_2HPO_4 \cdot 7\ H_2O$)
Fisher, Cat. #: S373-500
500g, $125.22
URL: (Click here)
pH of a 5% solution about 9.0

Sodium phosphate, tribasic dodecahydrate ($Na_3PO_4 \cdot 12\ H_2O$)
Fisher, Cat. #: AC42440-5000, Acros Organics, No.:424405000.
500g, $84.00.
URL: (Click here)
pH of a 5% solution about 12.0

(NOTE: POTASSIUM PHOSPHATE CAN BE USED IN PLACE OF SODIUM PHOSPHATE, AND IN FACT HAS SOME MINOR ADVANTAGES — BUT IT COSTS A LOT MORE).

Deionized, nuclease-free purified water. Buy it or make your own.

Sodium chloride, Fisher, S271-1, 1 kg, $83.45
URL: (Click here)
EDTA, nuclease-free "for molecular biology"
Cat #: AC32720-5000, 500g, $104.10.
URL: (Click here)
(NOT NECESSARY UNLESS YOU MAKE YOUR OWN TAE).

Sodium hydroxide pellets, for pH adjustment.
Fisher ACS Reagent Grade, 1kg, Cat. # RDCS0530100, $91.40.
URL: (Click here)

Hydrochloric acid, 6N, for pH adjustment. "EMD Millipore" (?)
Fisher, Cat.#: MHX0603M6, 1L, $62.05 (cannot be purchased online):
URL: (Click here)

OR

Fisher, Cat.#: SA56-1, 1L, $119.54. "Fisher Certified"
URL: (Click here)

Phenol, chloroform, isoamyl alcohol. If purchased separately:

Phenol, Fisher, Cat.# A931I-500, 500 ml, $198.49. (Cannot buy online)
https://www.fishersci.com/shop/products/phenol-liquid-certified-fisher-chemical-4/a931i500

Chloroform, Fisher, 1L. Cat.#: BP1145-1, 1L, $204.27.
Application: This solvent is used in Phenol/Chloroform extractions to remove proteins from DNA or RNA samples.
URL: (Click here)

Isoamyl Alcohol (Clear, Colorless/Certified), "Fisher Chemical"
Cat.#: A393-500, 500 ml, $174.77.
URL: (Click here)

If purchased as a pre-mixed combination:

Chloroform/Isoamyl alcohol, 24:1, Fisher. Cat.#: AC327155000
500 ml, $288.00.
URL: (Click here)

Phenol/Chloroform/isoamyl alcohol 25:24:1, Fisher
Cat.#(s)(?)AC327115000; ACROS Organics; No.:327115000
500 ml, $246.00.
URL: (Click here)

Thermo Scientific™ 3 M Sodium Acetate Solution, pH 5.2
"Precipitate DNA and RNA with this 0.22µm membrane-filtered, sterile, nuclease-free solution of sodium acetate."
5x1mL, $20.50.
URL: (Click here)

Sodium acetate, Fisher. (This is a the solid—better buy).
"Used to facilitate ethanol precipitation of DNA".
Cat.# BP333-500, 500g, $82.22.
URL: (Click here)

Zinc Sulfate Heptahydrate (Crystalline/Certified ACS), Fisher Chemical
Cat.# Z68-500, 500g, $166.41.
Formula: $O_4SZn \cdot 7\ H_2O$; MW 287.58.
URL: (Click here)
NOTE: Look for a stock solution. Fisher has one, but gives no purity information. There ought to be a cheaper product than this, which is a lot of money for something that's going to be used to make a 1 mM solution!

Ethanol, Absolute (200 Proof), Molecular Biology Grade, Fisher BioReagents™
Cat.# BP2818500, 500 ml, $74.88.
URL: (Click here)

TAE electrophoresis buffer (Edvotek, sold as 50x concentrate):
Electrophoresis Buffer 50x TAE, Cat.#: 607, qs for 5L, $30
Tris base 1M
Sodium acetate 0.3 M
EDTA 50 mM
(Final concentration after dilution: 20mM Tris, 6 mM acetate, 1mM EDTA)
http://www.edvotek.com/607

NOTE: At the end of this experiment, you'll have to perform electrophoresis at a higher salt concentration, possibly as high as 1M. Prepare those gels with this TAE buffer, modified by addition of solid NaCl to the desired ionic strength.

Miscellaneous:

Spectrophotometer (UNICO® S1200 Visible Spectrophotometer). Edvotek, $1,549.
http://www.edvotek.com/567

Temperature-controlled water bath.
http://www.edvotek.com/539
1.8 L capacity, $379.

UPTO:

Micropipetters:

Edvotek® Variable Micropipet 0.5 - 10 µl
Cat#: 589, ~~$179~~ $149 ("sale price")
http://www.edvotek.com/589?category=1808

Edvotek® Variable Micropipet 5 - 50 µl
Cat#: 590, ~~$179~~ $149 ("sale price")
http://www.edvotek.com/590?category=1808

Edvotek® Variable Micropipet 20 - 200 µl
Cat#: 591-1, ~~$179~~ $149 ("sale price")
http://www.edvotek.com/591-1?category=1808

Tips (for example):
Yellow Micropipet Tips (1-200 µl) Bag of 1000 tips
Cat#: 636-B, $45.00 (4.5¢ ea)
http://www.edvotek.com/636-B?category=4438

Fine Tip Micropipet Tips (1-200 µl) 1 rack of 204
Cat #:638, $25 (13¢ ea)
http://www.edvotek.com/638?category=4438

Ultra Micropipet Tips (0.5-10 µl) 2 racks of 96 each
Cat#: 635, $15 (8¢ ea)
http://www.edvotek.com/635?category=4438

Centrifuge

http://www.edvotek.com/533
Mezzo™ Microcentrifuge, Cat.# 533, $599
Speed is variable from 0 to 12,500 rpm (9,100 x g maximum)
Includes a 12-place rotor for 1.5 ml to 2.0 ml tubes (adapters available for smaller tubes).

Vortex

Tornado™ Vortexer, Cat.#: 5023, $279.
http://www.edvotek.com/5023

Plastic storage/reaction tubes

i.e., Microtest Tubes 630
500 tubes, 1.5 ml, $29 (6¢ ea)
http://www.edvotek.com/630

pH meter (starts at $54, the sky's the limit)

Test tube rack ($18)

Source of ice

Dialysis

Dialysis Buttons (Hampton Research, http://hamptonresearch.com/)
The buttons themselves: http://hamptonresearch.com/product_detail.aspx?cid=10&sid=63&pid=111
"Sampler"; 5 of each size, range 5-350 µl, #40.
Cat. # HR3-336: , $108.

Button Applicator: http://hamptonresearch.com/product_detail.aspx?cid=10&sid=63&pid=583
Cat. # HR4-348, $58.70.

Dialysis membrane discs, pre-cut, sold in packs of 50, with MW cutoff 3,500→14,000.
http://hamptonresearch.com/product_detail.aspx?cid=10&sid=63&pid=112
Cat. # HR3-338, HR3-344 or HR3-346. $251.00 each (!)

WARNING! Dialysis buttons are notoriously difficult to use. Practice with water before attempting to use the buttons and applicator on your hard-earned DNA.

Dialysis tubing. Thermo Scientific ("Pierce Protein Biology Products").
http://www.piercenet.com/product/snakeskin-dialysis-tubing-7k-mwco
7K MWCO, 22 mm, 35 ft (we don't need all this), Cat# 68700, $164.00.

Experimental protocol

Part 1

Prepare reagents

1. "3X Strider/Warner" re-annealing buffer: In the first part of this experiment, we are going to adjust the pH of our DNA to 10.75, the pH proven by the work of Strider, Warner and associates[1] to be a re-annealing optimum at 70°, 1M NaCl. We shall adjust the pH exactly as they did, employing 1 volume of pH-adjusting buffer mixed with 2 volumes of DNA.

This is the buffer composition:

3.0 M NaCl, 0.3 M sodium phosphate, 9 mM EDTA

After addition of one volume of this to 2 volumes DNA, we will have 1X Strider buffer in the reaction mixture, *i.e.*,

1.0 M NaCl, 0.1 M sodium phosphate, 3 mM EDTA

Make two batches of the 3X buffer; one with sodium phosphate dibasic ($Na_2HPO_4 \cdot 7 H_2O$), and the other with sodium phosphate tribasic dodecahydrate ($Na_3PO_4 \cdot 12 H_2O$).

NOTE: Make sure you take the water content of your phosphate crystals into account when you calculate your molarities. Phosphates are sold as anhydrous, monohydrates, dehydrates, heptahydrates and dodecahydrates, each with a different molecular weight.

Mix the dibasic and tribasic buffer solutions together under the pH meter to pH 10.75.

This is the "3X Strider/Warner" buffer which will be used for the re-annealing part of the experiment.

One final step: Prepare, in advance, a liter (or at least a few hundred ml) of the 1X buffer now. This will be used as a dialysis buffer at the end of the experiment.

Store these Strider buffers at room temperature (they precipitate in the cold).

2. Alkali denaturation: Prepare a 1.5 M solution of NaOH (FW 40). For 100 ml:

(1.5 x 40)/10 = **6 g NaOH in 100 ml water**

Prepare also a neutralizing solution of 1.5 N HCl from 6 N HCl. For 100 ml:

(1.5 x 100)/6 = **25 cc HCl plus 75 cc water**

3. Purchase or mix chloroform and isoamyl alcohol together to give a 24:1 mix, to be used in purifying DNA. Alternatively, purchase 24:1 pre-mixed chloroform/isoamyl alcohol, and/or 25:24:1 pre-mixed phenol/chloroform/isoamyl alcohol, from Fisher or some other source.

4. Make a 50-fold dilution of the 50x TAE buffer concentrate, using deionized, nuclease-free water for the dilution. This is 1X electrophoresis buffer.

The 1X buffer contains very little salt. At the end of the experiment, however, you will need *four* additional batches of this buffer, with salt concentrations adjusted to 0.03M, 0.1M , 0.3M and 1.0 M NaCl respectively. The easiest way to do this is probably to make a concentrated NaCl stock solution, and to use that to create the others. The stock solution must be made up in TAE:

5 M NaCl TAE stock solution: 292.2 g NaCl in 1 liter of TAE (not water)

These are the salt-enhanced TAE solutions you'll need for the alkali-denaturation work:

0.03 M = 6 cc stock sol'n, 994 cc TAE (or 0.6 cc stock, 99.4 cc TAE)
0.1 M = 20 cc stock sol'n, 980 cc TAE (or 2.0 cc stock, 98.0 cc TAE)
0.3 M = 60 cc stock sol'n, 940 cc TAE (or 6.0 cc stock, 94.0 cc TAE)
1.0 M = 200 cc stock sol'n, 800 cc TAE (or 20.0 cc stock, 80.0 cc TAE)

An easier way to do this, especially if you need more "on the fly", is to add the stock solution to either 100 cc or 1000 cc of TAE, so you only really have to measure (by pipette) the following volumes of 5 M NaCl stock solution into a full 100-ml or 1-liter bottle of 1X TAE:

Final concentration	**To 100 ml bottle add:**	**To 1 liter bottle add:**
0.03 M	0.6 cc	60.4 cc
0.1 M	2.0 cc	20.4 cc
0.3 M	6.4 cc	63.8 cc
1.0 M	25 cc	250 cc

(Obviously you cannot do this unless the bottle is accurately graduated at the 100 ml or 1 liter fill-line, and there yet remains room at the top for the additional NaCl solution).

The literature on high-salt agarose is scant, and it would be advisable to make a 1.0 M NaCl agarose gel up front, to make sure it gels properly. Here's the recipe for ordinary agarose, at the commonly-used concentration of 0.8%. Note that if ethidium bromide is to be added, it's done at the end, not the beginning**:**

1. To prepare 50 ml of an 0.8% agarose solution, measure 0.4 g agarose into a glass beaker or flask and add 50 ml 1X TAE (using the salt-free or salt-enhanced TAE, as called for by the instructions).

2. Microwave or stir on a hot plate until agarose is dissolved and solution is clear.
3. Allow solution to cool to about 55°C before pouring. It's at this point that ethidium bromide is added (EtBr cannot be added to hot agarose). If you add EtBr you must *reduce* the volume of TBE/TAE in step 1 accordingly.

5. Prepare 3M sodium acetate solution. Don't forget that the exact weight of sodium acetate will depend upon whether you use the anhydrous form or the trihydrate.

6. Take some of your 100% ethanol and prepare a 75% ethanol stock solution for use in washing DNA pellets.

Part 2

I. Confirm the purity of the pBR322 plasmid

NOTE: Vendors claim that their DNA is "pure". Don't believe it! First of all, it may be excessively nicked. Secondly, it is usual for commercially-purchased plasmids to have histone-like proteins adherent to them as sold. These will change the superhelical winding, and hence the electrophoretic mobility. I encountered both of these problems when working with another plasmid, pUB. To my astonishment, and unpleasant surprise, the electrophoretic mobility of this supposedly "pure" plasmid DNA changed significantly when I merely dialyzed it overnight, in the cold, against a simple phosphate buffer at pH 10.75! Who would have predicted that? (I certainly didn't).

SINCE THE INTERPRETATION OF OUR EXPERIMENTAL RESULTS DEPENDS UPON PROVING THAT CERTAIN PRODUCTS CO-MIGRATE IN AGAROSE GELS, THESE "SLIGHT" CHANGES IN ELECTROPHORETIC MOBILITY WILL DESTROY THE ENTIRE EXPERIMENT.

If this experiment is done with the pBR322 plasmid, it will be absolutely necessary to deal intelligently with the problem of whether your supply of plasmid DNA has residual histone-like proteins. The only safe way to do this is to subject the control DNA to EVERY STEP OF THE PROTOCOL BELOW. DO NOT OMIT ANYTHING! (Except, of course, addition of the nucleases which constitute the experiment itself).

That means that the control DNA will be subjected to many "unnecessary" PCIA (phenol-chloroform-isoamyl alcohol) extractions and EtOH (ethanol) precipitations, as well as numerous heatings, coolings, vortexings and centrifugations. There is simply no way to know with certainty what any of these steps will do to the electrophoretic mobility of the experimental DNA, and unless the control DNA is treated exactly alike (except for addition of enzymes), you're asking for trouble.

Therefore, the de-proteinization method we shall use is as follows:

De-proteinization method for control DNA:

In this method you simply subject your control DNA to EVERY STEP of the experimental protocol, EXCEPT, of course, the addition of nucleases.

If you do this, then it doesn't matter what trace proteins are bound to the original DNA you purchased, because (*presumably!)* the experimental and control DNA will have the same bound protein content at each step of the experiment.

Electrophoresis of control DNA (original duplex pBR322)

Before proceeding with the actual experiment, the pBR322 plasmid should be subjected to electrophoresis, to test the DNA and the electrophoresis equipment. According to the vendors, at least 90% of it should migrate as a single band. If so, you're OK. If, on the other hand, it's extensively nicked, giving rise to more than one prominent band, you're in trouble.

We shall assume that the DNA is supplied at a concentration of 0.5μg/μl (*i.e.*, 500 μg/ml). One μl therefore contains the appropriate amount of DNA for agarose gel electrophoresis.

If the DNA comes at a different concentration, you must either dilute it accordingly, or else adjust all volumes for the entire experiment, which shouldn't be terribly difficult, since there is an accompanying Excel spreadsheet, virtually all of whose entries are formulae based upon the initial DNA amount/concentration.

Prepare an agarose slab (with ethidium bromide, 0.5 μg/ml). Mix together, and introduce (to one or more wells):

1.0 μl	DNA (*i.e.*, 0.5 μg)
14.0 μl	TAE buffer
3.0 μl	loading dye
TOTAL	18.0 μl

Run the electrophoresis at 70v (typically, anywhere from 40-90 minutes). If the material is 90% pure, as per the manufacturer's claim, there should only be one important band.

If there are 2 or more prominent bands, then the Form I DNA has been nicked and degraded. This can be the result of physical handling, freezing and thawing, or a multitude of other causes. Regardless of the cause, in that case either new DNA must be obtained, or else the old DNA must be purified by using Exonuclease III to digest all nicked strands, then Mung Bean nuclease to digest the remaining intact single-stranded circles. The DNA must then be carefully de-proteinized as described below. Obviously

this is extra work, and I have not written a separate detailed protocol for it, because I would far rather start with good DNA.

II. Create single-stranded circular DNA from the "bottom" and "top" strands of the pBR322 chromosome

The object of this step is to introduce a single nick in the pBR322 "top" strand, using the enzyme N**t**.BbvCI (the "**t**" stands for "top"), and to digest that strand with Exonuclease III, which only attacks DNA from a cut end.

ASIDES:

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1. GENERAL NOTE: In all steps not calling for specific temperatures, you should (almost) always keep your DNA on ice. The enzymes we shall be using are never 100% removed, and no nuclease is 100% specific for the type of DNA it preferentially attacks. If DNA is left standing for prolonged periods at room temperature, it may be significantly degraded by residual nuclease activity in the solution.

2. Nt.BbvCI unit Definition: One unit is defined as the amount of enzyme required to convert 1 µg of supercoiled plasmid DNA to open circular form in 1 hour at 37°C in a total reaction volume of 50 µl. Since the enzyme activity can be affected by a variety of factors, we shall add twice the theoretically-required amount.

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1. Turn on your 37º water bath. Get your ice ready, and make sure your 100% and 75% EtOH are on ice. Prepare three reaction mixtures as follows:

EXPERIMENTAL #1 (to create pure "bottom" strand SS DNA):

Substance	**Volume**
Plasmid pBR322 DNA (60 µg @0.5µg/µl)	120 µl
NEB "CutSmart" buffer	40 µl
N**t**.BbvCI (120u @ 10 u/µl)	12 µl (do not add to control DNA!)
Water, nuclease-free	228 µl

Total:	400 µl

(OVER)→

EXPERIMENTAL #2 (to create pure "top" strand SS DNA):

Substance	Volume
Plasmid pBR322 DNA (60 µg @0.5µg/µl)	120 µl
NEB "CutSmart" buffer	40 µl
Nb.BbvCI (120u @ 10 u/µl)	12 µl (do not add to control DNA!)
Water, nuclease-free	228 µl

Total:	400 µl

CONTROL:

Substance	Volume
Plasmid pBR322 DNA (60 µg @0.5µg/µl)	120 µl
NEB "SmartCut" buffer	40 µl
No enzyme!	0 µl
Water, nuclease-free	240 µl

Total:	400 µl

2. Vortex the tubes and spin in a microcentrifuge for 3-5 sec.

3. Incubate at 37°C for 1 hour.

4. Add ½ volume of phenol (*i.e.*, 200 µl) and ½ volume of chloroform/isoamyl alcohol (24:1) (200 µl). (Alternative, if available: Add 1 volume of 25:24:1 PCIA). Do this for both experimental and control DNA. Vortex for 10 sec and centrifuge at maximum speed for 5 minutes.

NOTE: If there's too much salt in the DNA (almost inconceivable in this experiment), the layers might invert. In this unlikely event, we are advised that the protein layer can be identified by virtue of its having a yellow color.

5. Transfer the upper aqueous phase to a fresh tube, and ***measure it*** **(most writers recommend a 1000 µl pipette for the transfer; some recommend instead the removal and discarding of the bottom layer, whereupon the top layer can simply be decanted).** All the quantitative calculations, in the accompanying spreadsheet, arbitrarily presume that 85% of the aqueous layer will be recovered in each extraction, and 85% of the DNA will be recovered after EtOH precipitation. If the measured volume of extracted aqueous layer is either more or less than 85%, you'll have to adjust the spreadsheet accordingly.

After recording the extracted volume, add 1 volume (~400 µl) of chloroform/isoamyl alcohol (24:1). Vortex and centrifuge for 5 minutes.

6. Repeat step 5 twice more.

7. Transfer the upper aqueous phase to a fresh tube. Add 1/10 volume (*i.e.*, ~40 µl) of 3M sodium acetate and 2.5 volumes (*i.e.*, ~1000 µl) of ice-cold, 100% ethanol. Mix and incubate at -20° (-4º F) for 1 hour.

NOTE: Before the following centrifugation, we are advised to place the hinge of the tube lid on the side that the DNA is expected to pellet to, which will indicate which wall the pellet is on, in the event that it's hard to see.

8. Centrifuge at maximum speed for 10 minutes.

(This is entirely adequate for DNA at this concentration, 150 µg/ml. Lower concentrations might require more time. For example, at 10 µg/ml, the centrifugation time must be extended to 30 minutes. See <ethanol precipitation.doc> in this directory. Ideally, the expected yield, when the pellet is re-suspended, could exceed 90%).

9. Pour off the supernatant. Carefully wash the pellet with 200 µl of 75% ice-cold ethanol. (CAUTION! IT IS SAID THAT THE PELLET IS ***NOT*** FIRMLY ATTACHED AFTER 75% ETOH WASH, AND THAT IT CAN BE ACCIDENTALLY POURED OUT!). (Some authors recommend washing twice, with centrifugation after each wash to re-pellet the DNA).

Dry the pellet (Methods: (1) Hair dryer x 3 min., (2) invert tube on clean paper towel, tap gently, air-dry 10 min., (3) use a pipette to remove visible drops of EtOH, check dying progress by smell.) Do not over-dry, which is said to promote denaturation.

10. Dissolve DNA in 50 µl of water, nuclease-free (see below for calculation). (Wikipedia says "Finally, the pellet is air-dried and the DNA is resuspended in water or other desired buffer"). BUT SHOULD WE BE ULTRA-CAREFUL AND USE EXO III BUFFER INSTEAD OF WATER? (Probably not).

If necessary, store at –20º C (-4º F).

11. Treat with Exonuclease III by adding the following components to a reaction tube (have your 37º and 70º water baths ready):

~~~~~~

Aside - Exonuclease iii: how much?

One unit is defined as the amount of enzyme required to produce 1 nmol of acid-soluble total nucleotide in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X NEBuffer 1. See <Exonuclease iii-2012.doc> in this directory.
~~~~~~

I've seen 6 different estimates of the amount of enzyme necessary, which estimates, when extrapolated to our DNA amount (a total of 27 μg/experiment, of which only half will be digested, i.e., the nicked strand only), give answers ranging from 34 units to 1350 units!

Two of these estimates, one of which was from the current NEB web site, actually agree, giving an answer of about 300 units. I think that we should err in putting in too much enzyme, but not overwhelmingly too much, because there is an issue of non-specific enzyme degradation of intact SS circles. Therefore, let us use 600 units per reaction mixture; which may be twice as much as needed.

~~~~~~

EXPERIMENTAL #1
(Digest "top" strand; to create pure "bottom" strand):

Substance	**Volume**
DNA from Step 10 (25-40 μg)	50 μl
10X Exonuclease III reaction buffer	20 μl
Exonuclease III (600u @ 100u/μl)	6 μl
Water, nuclease-free	124 μl

	Total: 200 μl

EXPERIMENTAL #2
(Digest "bottom" strand; to create pure "top" strand):

Substance	**Volume**
DNA from Step 10 (25-40 μg)	50 μl
10X Exonuclease III reaction buffer	20 μl
Exonuclease III (600u @ 100u/μl)	6 μl
Water, nuclease-free	124 μl

	Total: 200 μl

CONTROL:

Substance	**Volume**
CONTROL DNA from step 10	50 μl
10X Exonuclease III reaction buffer	20 μl
Don't add Exonuclease III	0 μl
Water, nuclease-free	130 μl

	Total: 200 μl
~~~~~~

12. Mix and incubate at 30°C for 10 minutes. Stop the reaction by heating at 70°C for 10 minutes. (You may need a second water bath in this step).

13. Extract the reaction mixture with phenol/chloroform as described in Steps 4-6 above.

(IMPORTANT VOLUME NOTE: The protocol you are following reduces the volume to 200 µl because the exonuclease iii digests half the DNA. But some authors caution against attempting PCIA extraction with such a small volume. You CAN add another 200 µl of TAE to each reaction mixture, after the enzyme treatment and just before the PCIA extraction, the only issue being the concentration of DNA for EtOH precipitation. If you add the TAE, the exonuclease iii-digested DNA concentration will be reduced to about 33 µg/µl. At this concentration, the EtOH-precipitated DNA should spin down in 10 minutes, but this conclusion is based upon data for 40 µg/µl, so maybe you should spin down the precipitated DNA for 15 minutes, or even 20 minutes.)

Then Precipitate the DNA as described in steps 7-9 (taking care to adjust volumes according to any changes you make in this protocol). Then dissolve the DNA in **20** µl of purified water.

RESULT: According to current estimates (see spreadsheet), the two experimental solutions should, at this point, each contain about 6 µg of a pure population of single-stranded circular DNA molecules exclusively from the pBR322 "bottom" and "top" strands respectively, at a concentration of 0.3 µg/µl.

III. Reannealing of a mixture of single-stranded "top" and "bottom" strands according to the reannealing protocols of Robert Warner/William Strider

The idea is to mix together the separated "top" and "bottom" single-stranded DNA (at a final concentration of 33 µg/ml), add ½ volume of the 3-fold concentrated "Strider" buffer for adjustment of the pH (10.75 in this experiment), then to re-anneal the DNA at 70°.

Before proceeding, let us review the amounts and concentrations of single-stranded circular DNA from Step 13. Remember that after digesting with Exonuclease III, only half the DNA remains, the other half having been eaten up by the enzyme:

"Bottom" strand SS DNA
6 μg DNA in 20 μl water
i.e., DNA concentration = 0.3 μg/μl

"Top" strand SS DNA
6 μg DNA in 20 μl water
i.e., DNA concentration = 0.3 μg/μl

TOTAL:
12 μg DNA in 40 μl water
i.e., DNA concentration = 0.3 μg/μl

PROTOCOL FOR RE-ANNEALING

This protocol uses 5/6 of the total volume of DNA created in Step 13 of part II, *i.e.*, 5 μg of "bottom" strand DNA and 5 μg of "top" DNA, totaling 10 μg DNA. That will be sufficient for the twelve electrophoresis runs which are planned. Unfortunately, this leaves only 1 μg of each SS species for control experiments, which is enough, but only if nothing goes wrong.

(If an electron microscope is available to confirm the single-strandedness of our DNA, we may not have enough SS material remaining to take advantage of it. In that case we shall have to adjust the entire protocol, to squeeze out another microgram for EM.)

1. If you have not already done so, prepare the 3X Strider/Warner re-annealing buffer, pH 10.75 (Part I, Step 1).

2. Mix together 5 μg each of single-stranded DNA from the "bottom" and "top" strands (Part II, Step 13), and Strider/Warner buffer, in the proportions given below. The final buffer composition of this DNA solution will be very close to 1 M NaCl, 0.1 M phosphate, 3 mM EDTA, pH 10.75. (Since this is exactly the method used by Strider for DNA re-annealing, any small deviations in "actual" pH or ionic strength should not prevent us from getting the same results he did)**:**

EXPERIMENT:

17 μl DNA (*i.e.,* 5 μg) from "bottom" strand
17 μl DNA (*i.e.,* 5 μg) from "top" strand
100 μl 3X Strider/Warner buffer, pH 10.75
167 μl H_2O

Total: 300 μl (final DNA concentration 33 μg/μl)

CONTROL:

17 μl CONTROL DNA (*i.e.*, 10 μg of DNA from Step 13 above)
100 μl 3X Strider/Warner buffer, pH 10.75
183 μl H_2O

Total: 300 μl (final DNA concentration 33 μg/μl)

3. Place the experimental and control tubes in a 70° water bath, for 20 minutes. Cool on ice.

IV. Gel electrophoresis

"Ethidium bromide fingerprint"
To rule out "Form V"

As explained in our Experimental Overview, Stettler *et al* (1979) have persuaded the world that re-annealing of complementary SS circular DNA yields a bizarre and novel form they dubbed "Form V", whose properties differ markedly from Form I. The primary purpose of the following step is to rule out any possibility that our re-annealed DS DNA is "Form V", by showing that its physical properties are, in fact, indistinguishable from those of Form I.

This we shall accomplish by electrophoresis at differing concentrations of EtBr, then by an alkali denaturation-renaturation experiment.

METHOD

Prepare 6 agarose gels for electrophoresis. Within each gel, each well shall contain 0.5 μg of DNA. Ethidium bromide (EtBr) shall be incorporated directly into each gel except the first.

You will run six gels, mainly differing only in the EtBr concentration.

The first gel, however, will differ in *two* ways: (1) It will have no EtBr, wherefore it will have to be stained, and (2) it will have both single-stranded "bottom" DNA and single-stranded "top" DNA control lanes.

Gels 2-6 will have only re-annealed [presumably] double-stranded experimental DNA, sham-reannealed control DNA, and "original" DNA, that is, DNA "from the factory" which has not been touched. Single-stranded DNA control lanes are not included in gels 2-6, mainly because we simply won't have enough single-stranded DNA to use in all 6 gels:

First agarose gel (No EtBr - gel must be stained)

	Lane 1	**Lane 2**	**Lane 3**	**Lane 4**	**Lane 5**
Type of DNA:	Expl	Control	ss-Bottom	ss-Top	Original
Volume of DNA:	15.2	15.2	1.7	1.7	1
Loading dye:	3	3	3	3	3
TAE:	0	0	13.0	13	14
TOTAL:	18.2	18.2	17.7	17.7	18.0

Second agarose gel (EtBr 0.1 μg/ml in agarose)

	Lane 1	**Lane 2**	**Lane 3**
Type of DNA:	Expl	Control	Original
Volume of DNA:	15.2	15.2	1
Loading dye:	3	3	3
TAE:	0	0	14
TOTAL:	18.2	18.2	18

THE THIRD, FOURTH, FIFTH AND SIXTH GELS ALL HAVE EXACTLY THE SAME WELL COUNT AND CONTENT AS THE SECOND, DIFFERING ONLY IN THE ETHIDIUM BROMIDE CONCENTRATION:

Third agarose gel: (EtBr 0.2 μg/ml in agarose)
Fourth agarose gel: (EtBr 0.4 μg/ml in agarose)
Fifth agarose gel: (EtBr 0.6 μg/ml in agarose)
Sixth agarose gel: (EtBr 1.0 μg/ml in agarose)

EXPECTED RESULT: This is the "ethidium bromide fingerprint" mentioned in the Experimental Overview. The EtBr concentrations in the gels have been chosen quite deliberately, to maximize the probability that all phases of the EtBr titration curve will be represented:

- With no EtBr (gel #1) the experimental and control DNA should be maximally supertwisted in the interwound, right-handed sense, and should migrate relatively rapidly in the electric field.
- The 2nd and 3rd gels (0.1 and 0.2 μg EtBr, respectively) are in the range of EtBr concentration within which Form I unwinds, eventually to the point of complete relaxation, so we should see a progressive decrease in electrophoretic mobility relative to gel #1.

- The 4th, 5th and 6th gels (0.4, 0.6 & 1.0 μg/ml of EtBr, respectively) are in the range of EtBr concentration within which the Form I becomes supertwisted in the opposite sense (*i.e.*, interwound left-handed), increasing in electrophoretic mobility until the rapid mobility of Gel #1 is again seen.

If both experimental and control DNA co-migrate in all six gels, it will be difficult to argue against the proposition that the re-annealed and sham-treated control DNA have the same structure.

FINAL EXPERIMENTS

Alkali denaturation-renaturation
Mung Bean nuclease control

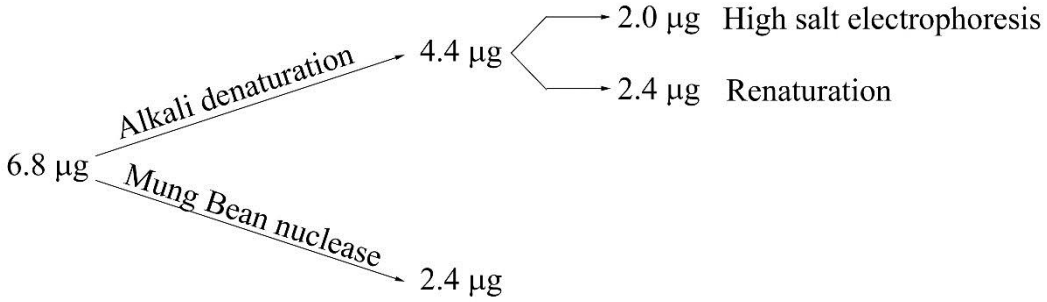
Introduction

DNA remaining:

	Exp'l	Cont'l
Amount:	6.8 μg	6.8 μg
Volume:	209 μl	209 μl
Conc.:	0.033 μg/μl	0.033 μg/μl

There is very little DNA remaining, but much work still remains to do. The 6.8 μg of DNA will be divided into two portions, one for alkali denaturation, and the other for Mung Bean nuclease digestion:

The following flow chart is applicable to both the experimental and control DNA. Note that the amounts of DNA in the chart are not literal, but merely the results of formula cell auto-calculations in the accompanying spreadsheet. The actual amounts will be somewhat different, but hopefully not too far from the values shown here:



The upper branch indicates that we shall subject 4.4 μg of our DNA (that is, 4.4 μg each of experimental and control DNA) to alkali denaturation at pH 13.4. Of this DNA, 2.0 μg will be immediately analyzed by gel electrophoresis in agarose gels containing four

different concentrations of salt (see below for further explanation). The other 2.4 μg of denatured DNA will be *renatured* by dialysis against the same pH 10.75 Strider buffer used earlier, followed by incubation under the same conditions used in the earlier renaturation experiment, *i.e.*, 30 minutes at 70º.

The lower branch of the chart indicates that we shall subject 2.4 μg of DNA to digestion by Mung Bean nuclease, which is specific for single-stranded DNA. The purpose is to prove that our re-annealed DNA, prepared from separated single strands, has now had its native duplex structure restored, and is no longer single-stranded.

V. Alkali denaturation - renaturation

A. Denaturation

Assuming that this experiment goes as I predict, which is virtually certain (since it's been done before – see https://NotAHelix.net), I still expect the "establishment" to resist accepting the obvious, namely that DNA is non-helical in living chromosomes. To some extent, this stubbornness is desirable, because the subject matter is of the utmost importance to the future of molecular biology, and the questions we are raising here must therefore be answered with the utmost certainty.

The following step, however, will dispel the slightest lingering doubt about the non-helicity of the native plasmid chromosome. Circular DNA, when subjected to alkali denaturation above pH 13 (yielding the so-called "Form IV"), nearly *triples* in density! [1] Neutralization reduces the density, but even at pH 7, alkali-denatured circular DNA remains over twice the density of its non-denatured cousin. This increase in density will, under the right circumstances, also result in a parallel increase in electrophoretic mobility.

According to "traditional" Watson-Crick theory, above pH 13 there is no earthly reason for our re-annealed duplex DNA to do anything other than to instantly fall apart into its component single strands, which should move relatively slowly on agarose gels. If, on the other hand, we see a doubling or tripling of the electrophoretic mobility of our experimental DNA at pHs above 13, then the identity of the re-constituted and control DNA is confirmed beyond any shadow of a reasonable doubt:

PROTOCOL (For the acid-base calculations, see <pH-calc_2012.doc>):

1. If you haven't already done so, make stock solutions, 100 ml each, of 1.5 M NaOH and 1.5 N HCl. YOU ***MUST*** CHECK THE EFFECT OF YOUR NaOH AND HCl STOCK SOLUTIONS ON A BLANK SPECIMEN OF STRIDER BUFFER, pH 10.75, ***BEFORE*** USING THEM ON YOUR DNA!

The following proportions are precisely those you will use in the actual experiment, as long as the volumes in the accompanying Excel spreadsheet have not changed. Mix together:

Strider buffer, pH 10.75:	**10 ml**
1.5 M NaOH:	**632 µl**

Check the pH. If it's clearly and unequivocally over 13.0, you're all right. If not, then your DNA will not denature, wherefore you must increase the volume of added NaOH in this test until the necessary pH increase has been obtained.

Remember that if you increase the volume of NaOH here, you must also increase it proportionately in the actual experiment.

After you've established that the added NaOH has brought the pH to a value >13, add an equivalent amount of HCl:

1.5 N HCl:	**632 µl**

Check the pH. The purpose of this step is merely to reduce the high $[OH]^-$ concentration, so that later, when you actually do this to your experimental DNA, hydroxide ions do not migrate to the neighboring control DNA well, causing conformational changes or denaturation. Since that seems unlikely to happen, *this step is probably unnecessary*, but it's unlikely to cause any harm, so I am including it.

There is no exact target pH for this step. Most likely it will restore the solution to about pH 10.75, but if it over- or under-shoots a bit, it's not a problem.

2. Assuming that you have not altered the above volumes of NaOH and HCl, mix, with good stirring:

Experimental DNA:	**136.6 µl**
1.5 M NaOH:	**8.6 µl**

and

Control DNA:	**136.6 µl**
1.5 M NaOH:	**8.6 µl**

(The fractions of a µl are copied literally from spreadsheet formula cells – obviously you can't accurately measure small fractions of a single µl – just do the best you can).

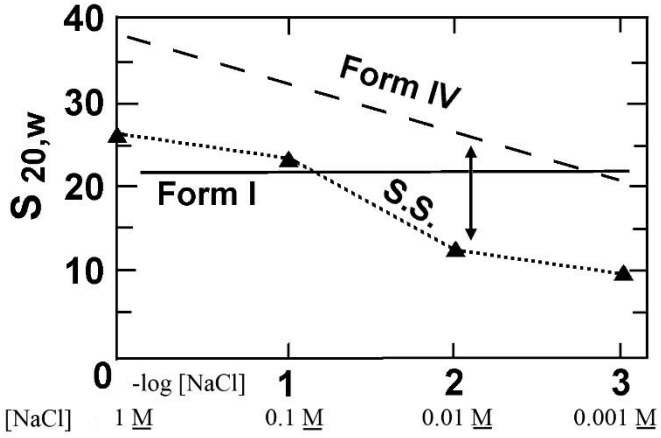
This should instantly denature all the DNA.

Now, slowly and with even better stirring (since there's more risk of hydrolyzing DNA with acid than with base) add:

1.5 N HCl: **8.6 µl**

...to each reaction mixture, which will neutralize most of the previously-added NaOH.

3. Electrophoresis: You will need modified TAE buffers containing high salt concentration. We shall employ ***four*** different salt concentrations, namely 0.03 M, 0.1 M, 0.3 M, and 1.0 M NaCl. Here's why: The typical agarose gel contains only a small amount of salt, usually about 0.01M NaCl (see the double-headed arrow in the diagram below[2,3]), but at that salt concentration, Forms I & IV have about the same compactness, as measured by CsCl density gradient centrifugation. If, however, the salt concentration of the agarose is increased to 0.1M, the ***s*** value of Form IV (and presumably the electrophoretic mobility) will increase 50% *over* that of Form I. At 1M, it will increase 100%. In contrast, Form I is not significantly affected by changes in salt concentration:



Dependence of the sedimentation coefficient (*s*) of DNA on the salt concentration. Note that at the salt concentration of the typical agarose gel (double-headed arrow) Form IV and Form I have about the same *s* value. It is known that, at that salt concentration, they also have about the same electrophoretic mobility. At a salt concentration of 0.1 M, however, the *s* value of Form IV increases about 50%, but that of Form I doesn't change at all. Finally, at 1.0 M NaCl, the *s* value of Form IV is nearly twice as high as that of Form I! This is virtually certain to be reflected in an equally enormous change in the electrophoretic mobility of Form IV in 1.0 M salt. (Data adapted from: Pouwels, P.H., C.M. Knijnenburg, J. Van Rotterdam, J.A. Cohen and H.S. Jansz (1968). Structure of the replicative form of bacteriophage ϕX174. VI. Studies on Alkali-denatured Double-stranded ϕX DNA. J. Mol. Biol. 32, 169-182. doi:10.1016/0022-2836(68)90002-8).

The salt concentrations we shall use, namely 0.03 M, 0.1 M, 0.3 M, and 1.0 M NaCl, represent 4 equidistant points on the logarithmic graph shown above, and should give a linearly-increasing rate of electrophoretic migration on agarose.

I confess that I have never had the opportunity to prepare and use agarose gels with high salt concentrations. On the subject of high-salt agarose gel electrophoresis, the few references I have been able to unearth warn of odd-shaped bands, but none of these references say that the gels can't be run.

The DNA may now be immediately subjected to gel electrophoresis in four agarose gels, each one containing one of the four above-referenced NaCl concentrations. MAKE SURE YOU ALSO EMPLOY THE HIGH-SALT BUFFERS IN THE ELECTROPHORESIS TANK.

PROTOCOL

Prepare four agarose gels, with the above-referenced NaCl concentration.

	Lane 1	**Lane 2**	**Lane 3**
Type of DNA:	Expl	Control	Original
Volume of DNA (μl):	17.2	17.2	1
Loading dye:	3	3	3
TAE:	0	0	16
TOTAL:	20.2	20.2	20.0

The exp'l and control DNA is at a slightly higher volume than the last time it was used, because of the slight dilutional effect of the added NaOH and HCl. The "original" DNA is still at its "factory" concentration of 0.5 μg/μl.

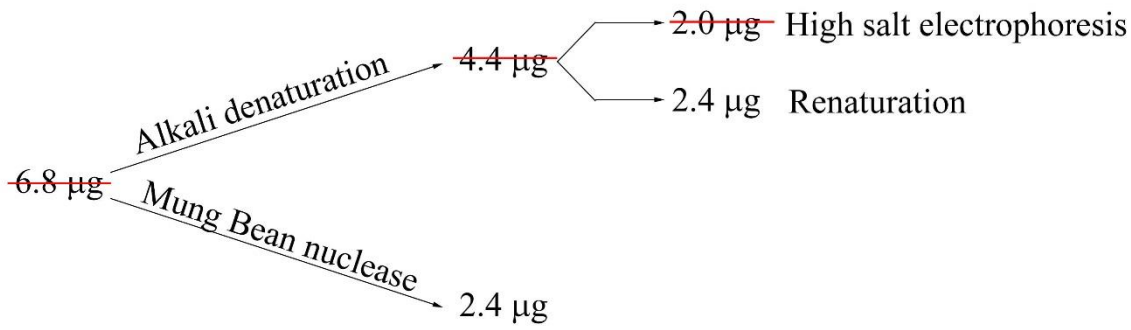
WARNING #1: Don't forget that this denatured DNA is expected to migrate much faster than control DNA. Watch your gels carefully, or the denatured bands of DNA will run off the bottom of the gel before you see them!

WARNING #2: Don't forget that denatured DNA binds much less ethidium bromide than duplex DNA. The bands of denatured DNA will therefore be very faint. But, with 0.5 μg of DNA in each well, they should be clearly visible.

EXPECTED RESULT: The "original" DNA should migrate as always, having never been denatured. The experimental and control DNA should migrate anywhere from 25% to 100% *faster,* depending upon the NaCl concentration.

B. Renaturation

The four high-salt electrophoresis runs will use up about 2.0 μg of the original 4.4 μg of DNA which you denatured. This experiment is almost over. Here's the DNA remaining:



There are, before you, 2.4 μg (in 84 μl volume) each of denatured experimental and sham-treated control DNA remaining. They need to be re-adjusted to the original Strider buffer, pH 10.75, for re-annealing. Unfortunately you cannot merely add 3X Strider buffer, as you did before, because there is an unknown amount of hydroxide ion present. You must dialyze this out. Therefore:

1. Retrieve your 1 liter of pre-prepared 1X Strider buffer (*i.e.*, 1 M NaCl, 0.1 M phosphate, 3 mM EDTA, pH 10.75). Put these DNA samples into 100 μl dialysis buttons, *carefully* apply the dialysis membrane, and dialyze them against this buffer.

CAUTION! DIALYSIS BUTTONS ARE NOTORIOUSLY DIFFICULT TO USE Practice using the applicator with tap water until you're sure you've mastered the art of applying the dialysis membrane to the button, otherwise all your DNA will surely wind up on the floor, and you'll wind up in the university infirmary getting a large dose of intravenous tranquilizers.

NOTE: 1X Strider buffer may precipitate in the refrigerator. Therefore, carry out this dialysis in an insulated cooler (styrofoam or whatever) with artificial ice, in which the measured temperature is 12°-15° C. Use several changes of buffer, to be certain of the final pH, which is critical.

2. Remove the DNA from the dialysis buttons. Place immediately into a 70° water bath for 20 minutes. Perform electrophoresis:

	Lane 1	**Lane 2**	**Lane 3**
Type of DNA:	Expl	Control	Original*
Volume of DNA:*	17.2	17.2	1
Loading dye:	3	3	3
TAE:	0	0	16

TOTAL: 20.2 20.2 20.0

***Totally untouched pBR322 DNA; still @0.5µg/µl**

EXPECTED OUTCOME: All three should run as Form I. The experimental and control DNA should run in parallel. The original pBR322 control, having not been de-proteinized, should run a little faster.

COMMENT: The rapid electrophoretic migration of the denatured experimental DNA (presuming we see it!) rules out any possibility that it might be separated single strands. The restoration of the much slower native electrophoretic mobility here, after re-annealing, provides virtually incontrovertible evidence that DNA, in this plasmid, has a topologically non-helical structure.

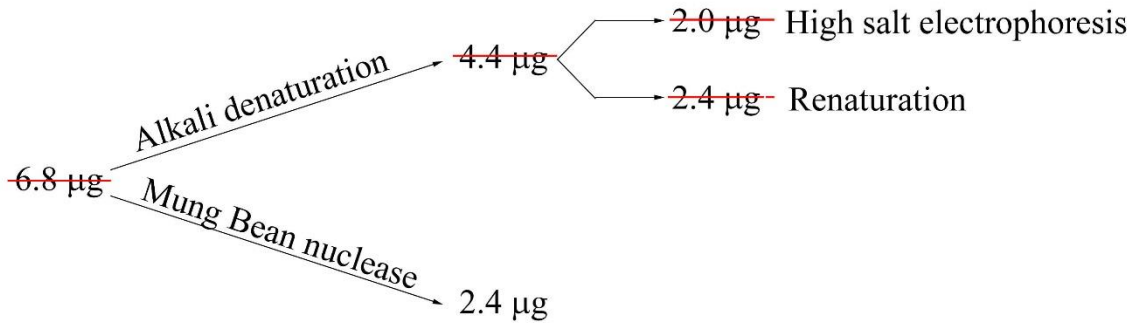
NOTE: If all has gone well, there should be about 2 µg each of re-annealed and sham-reannealed DNA remaining at this point. There is, at present, no use for it. Put it in the freezer.

VI. Mung Bean nuclease control experiment to prove that the DNA in Part II, Step 13 is really single-stranded

Mung Bean nuclease digests single-stranded DNA, but has little effect on double-stranded DNA. It will be highly-desirable to prove that the DNA we have created in Part II.13 is really single-stranded, and that the re-annealed DNA of Part III.3 is really double-stranded. Toward this end, we shall treat the "top" and "bottom" strand single-stranded DNA, the experimentally re-annealed DNA, the sham-treated control DNA, and the untouched native pBR322 with Mung Bean nuclease.

Hopefully, the control DNA and the re-annealed DNA will be unaffected, but the top and bottom strand single-stranded DNA will be totally degraded.

1. Retrieve the 2.4 µg of DNA which you put aside for the Mung Bean nuclease control experiment:



This DNA is still in the Strider re-annealing buffer (*i.e.*, 1 M NaCl, 0.1 M phosphate, 3 mM EDTA, pH 10.75), which must now be removed, because Mung Bean nuclease is very fastidious about its environment. In particular, this nuclease requires zinc, but it "hates" a lot of NaCl. The only easy way to adjust the buffer composition is dialysis.

2. *Carefully* place the DNA (both exp'l and control, 2.4 µg of each, in 73 µl of solution) in 100 µl dialysis buttons, and dialyze against 1X Mung Bean nuclease buffer.

The nuclease is sold by New England Biolabs with a 10X buffer, but it's not a large volume. They used to sell additional 10X buffer as a separate product, in 6 ml batches (*i.e.*, an amount sufficient to make 6 x 10 = 60 ml of *1X* buffer). Unfortunately, this product was discontinued, so you'll have to make your own. Make 100 ml, as follows:

1X Buffer Components:

30 mM NaCl	0.175 g/100 ml
50 mM sodium acetate*	0.292 g/100 ml*
1 mM $ZnSO_4$ **:	0.029 g/100 ml**
pH 5.0@25°C	

*** Assuming that the NaAc is *anhydrous.***

****Assuming the heptahydrate, FW 287.58. There are $ZnSO_4$ solutions for sale, but I cannot, so far, find purity data for them. The protocol above is for solid $ZnSO_4$. This is probably better done by making a stock solution, and making a suitable dilution thereof.**

Once you've made your 1X Buffer, divide it into at least two 50cc batches. Dialyze the DNA against these two changes of buffer.

3. Remove the DNA from the dialysis buttons, and set up the following reaction mixtures. Each contains 5u of Mung Bean nuclease in a total volume of just over 15 µl of solution. Each solution is either all, or nearly all 1X Mung Bean nuclease buffer:

MUNG BEAN NUCLEASE REACTION MIXTURES:

DNA type:	Original pBR322	Exp'l reannealed	Sham-treated control	SS Bottom*	SS Top*
DNA amount (µg):	0.5	0.5	0.5	0.5	0.5
DNA source conc. (µg/µl):	0.5	0.033	0.033	0.3	0.3
DNA added volume (µl):	1	15	15	1.7	1.7
MB nuclease, 5u, vol (µl):*	0.5	0.5	0.5	0.5	0.5
1X Mung Bean buffer (µl):*	14.0	0	0	13.3	13.3
TOTAL volume, rxn mixture:	**15.5**	**15.5**	**15.5**	**15.5**	**15.5**

***If you can't measure 0.5 µl, dilute enzyme with 1 vol of 1X Mung Bean buffer and use 1 µl.**
***Note this is 1X buffer, *not* 10X buffer. Note also that none is put into the exp'l and control DNA samples. That's because that DNA was dialyzed against 1X Mung Bean buffer, and no more is needed.**
***Both SS species were dissolved in 20 µl of water, so there should not be any buffer issues here.**

4. Incubate at 30ºC for 30 minutes. Add 3 µl of loading dye to each reaction mixture, and subject the entirety of each reaction mixture to electrophoresis. The EtBr may be incorporated into the gel at 0.5 µg/ml, or the gels may, alternatively, be stained with EtBr later.

ELECTROPHORESIS

	Original pBR322	Exp'l reannealed	Sham-treated control	SS Bottom*	SS Top*
DNA (µl) (approximate):	15	15	15	15	15
Loading dye:	3	3	3	3	3
TOTAL:	**18**	**18**	**18**	**18**	**18**

Expected result: Lanes 3 & 4 should have NO bands of DNA, since Mung Bean Nuclease should totally degrade the single-stranded DNA in the lanes. Lanes 2 & 5 should be totally unaffected by the nuclease, and should co-migrate with the untreated control DNA in Lane 1.

References

1. For a complete discussion of the denaturation and re-annealing of circular DNA, and for the print references pertaining to that subject, see https://NotAHelix.net. Start the PowerPoint slide show entitled *The Double Non-Helix, Part I, "The Science and History of Topologically Non-Linked ('TN') DNA".* From the menu (slide 5), select the topic *"Form IV, structure and properties"*.

2. *Ibid.* From the menu (slide 5), select the topic *"Critical evaluation of Stettler et al (work of Charles Weissmann)".* This section starts at slide 298. If you merely wish to see the data on the effect of salt concentration on the ***s*** value of circular DNA, you can skip most of this and go directly to slides 331-334.

3. Figure re-drawn from Pouwels PH *et al*, JMB 32:169-182 (1968).