BIOE.44

Synthetic Biology Lab

1 April 2010 Lecture / Discussion notes

http://openwetware.org/wiki/Stanford/BIOE44

Today's take aways:

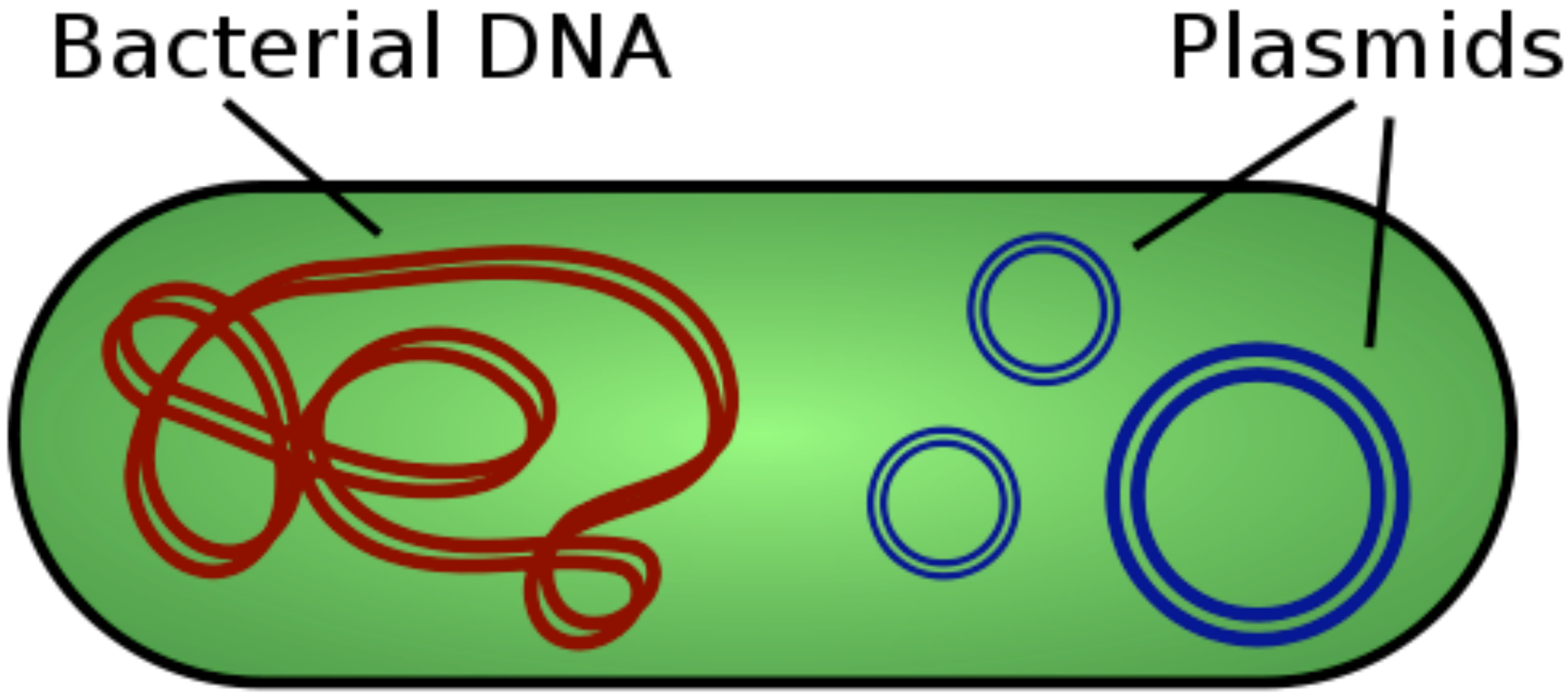
1. What must a plasmid be?

2. Getting at the DNA you want?

3. Cutting DNA with enzymes.

4. Why technical standards matter?

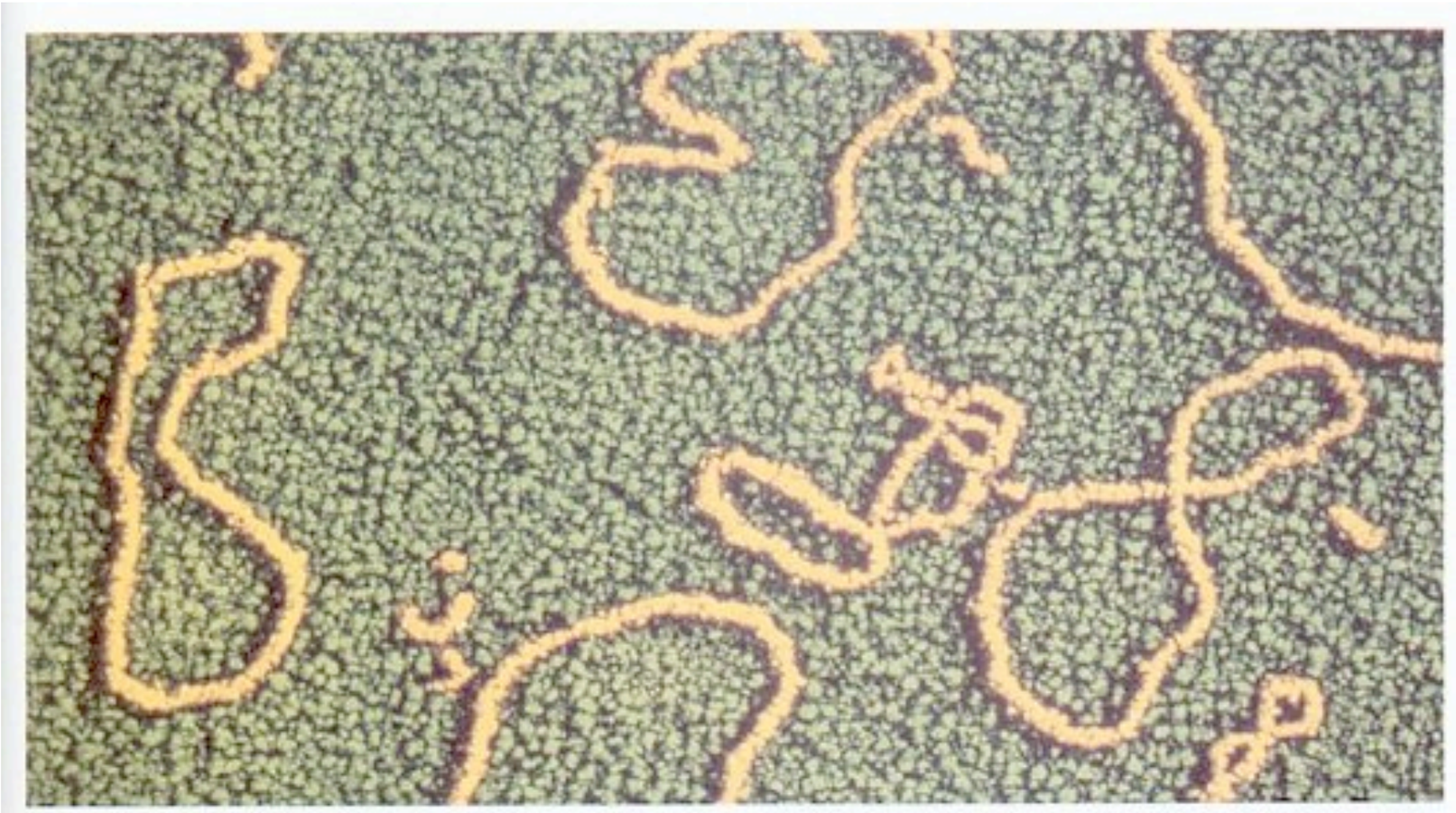
Plasmids, an introduction:



"A **plasmid** is an extra chromosomal DNA molecule separate from the chromosomal DNA"

http://en.wikipedia.org/wiki/Plasmid

Plasmids, an introduction:



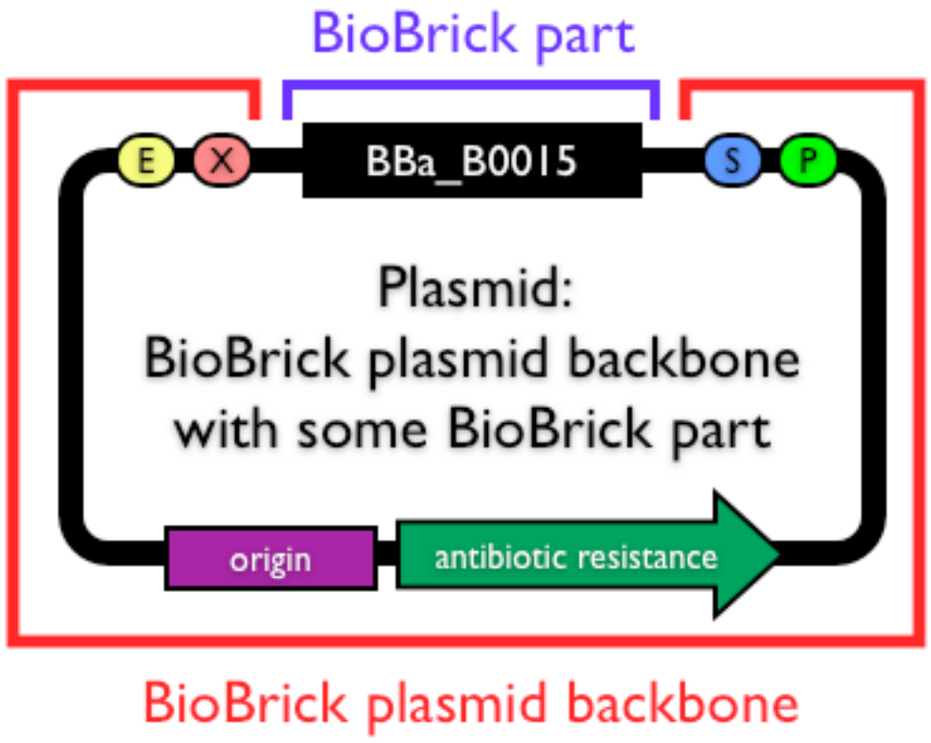
Plasmids are small circles of DNA found naturally in the cells of some organisms. A plasmid can replicate itself as well as any other DNA inserted into it. For this reason, plasmids make excellent cloning vectors—structures that carry DNA from cells of one species into the cells of another.

http://universe-review.ca/I10-71-plasmid.jpg
(but apparently scanned from some textbook)

So, what must a plasmid be?

1. Cells grow and divide; what keeps a plasmid from getting diluted?

2. Genetic material mutates; what keeps a plasmid from being "lost" via evolution?



http://partsregistry.org/Plasmid_backbones

Getting at the DNA you want...

1. How to seperate relevant DNA molecule from all the other stuff inside a cell?

http://www.jensenlab.caltech.edu/coolmovies.html

A brief aside: what type of lab protocol user will you be?

Consumer

Innovator

Volume 7 Number 6 1979 Nucleic Acids Research

A rapid alkaline extraction procedure for screening recombinant plasmid DNA

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Received 3 August 1979

ABSTRACT

A procedure for extracting plasmid DNA from bacterial cells is described. The method is simple enough to permit the analysis by gel electrophoresis of 100 or more clones per day yet yields plasmid DNA which is pure enough to be digestible by restriction enzymes. The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Adequate pH control is accomplished without using a pH meter. Upon neutralization, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant. Large and small plasmid DNAs have been extracted by this method.

PRINCIPLE OF THE ALKALINE EXTRACTION METHOD

Previous workers have shown that there is a narrow range of pH (about 12.0-12.5) within which denaturation of linear DNA but not CCC-DNA occurs and that this property can be used for purifying CCC-DNA (7-10,20,21). We have utilized this approach for developing a rapid extraction method for plasmid DNAs. Plasmid-containing cells are treated with lysozyme to weaken the cell wall and then lysed completely with sodium dodecyl sulfate (SDS) and NaOH. By choosing the ratio of cell suspension to NaOH solution carefully, a reproducible alkaline pH value is obtained without the necessity of monitoring the pH with a meter; further pH control is obtained by including glucose as a pH buffer. Chromosomal DNA, still in a very high molecular weight form, is selectively denatured and when the lysate is neutralized by acidic sodium acetate, the mass of chromosomal DNA renatures and aggregates to form an insoluble network. Simultaneously, the high concentration of sodium acetate causes precipitation of protein-SDS complexes (11,12) and of high molecular weight RNA (13). In this way, most of the three major contaminating macromolecules are co-precipitated and may be removed by a single centrifugation in a bench-top centrifuge. Plasmid DNA (and residual low molecular weight RNA) are recovered from the supernatant by ethanol precipitation. Plasmid DNA may be analyzed by gel electrophoresis either intact in the CCC form or after digestion with a restriction enzyme.

(cont. from previous page)

Modern form...

After harvesting and resuspension, the bacterial cells are lysed in NaOH–SDS (Buffer P2) in the presence of RNase A. SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents. NaOH denatures the chromosomal and plasmid DNAs, as well as proteins. The optimized lysis time allows maximum release of plasmid DNA from the cell without release of cell-wall-bound chromosomal DNA, while minimizing the exposure of the plasmid to denaturing conditions. Long exposure to alkaline conditions may cause the plasmid to become irreversibly denatured. This denatured form of the plasmid runs faster on agarose gels and is resistant to restriction enzyme digestion (see figure "Agarose gel analysis of the plasmid purification procedure").

The lysate is neutralized by the addition of acidic potassium acetate (Buffer P3). The high salt concentration causes KDS* to precipitate, and the denatured proteins, chromosomal DNA, and cellular debris become trapped in salt–detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution. Since any SDS remaining in the lysate will inhibit binding of DNA to QIAGEN Resin, the solution must be thoroughly but gently mixed to ensure complete precipitation of the detergent.

Separation of plasmid from chromosomal DNA is based on coprecipitation of the cellwall- bound chromosomal DNA with the insoluble complexes containing salt, detergent, and protein. Plasmid DNA remains in the clear supernatant. Vigorous treatment during the lysis procedure will shear the bacterial chromosome, leaving free chromosomal DNA fragments in the supernatant. Since chromosomal fragments are chemically indistinguishable from plasmid DNA under the conditions used, the two species will not be separated on QIAGEN Resin and will elute under the same salt conditions. RNase A, which is added at the beginning of the procedure, digests the liberated RNA efficiently during the alkaline lysis. The resulting RNA fragments do not bind to QIAGEN Resin under the salt and pH conditions present in the lysate. The precipitated debris is removed by centrifugation or by use of a QIAfilter Cartridge, producing a cleared lysate for loading onto the QIAGEN-tip. It is important that the lysate is clear at this stage to ensure good flow rates and, ultimately, to obtain protein-free plasmid DNA preparations.

http://www1.qiagen.com/plasmid/KeySteps.aspx

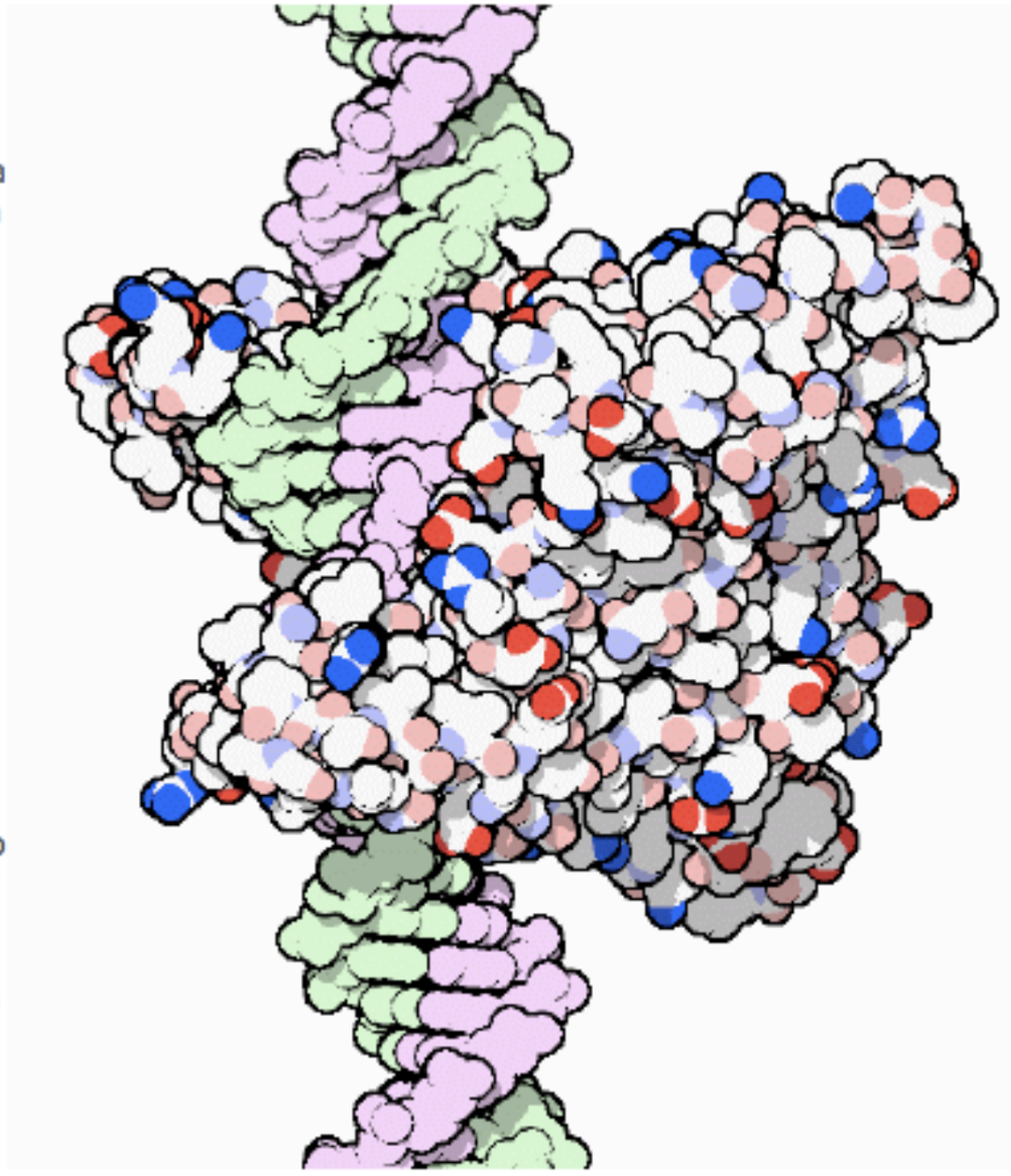
Cutting DNA

Bacteria Fight Back

Bacteria are under constant attack by bacteriophages, like the bacteriophage phiX174 described in an earlier Molecule of the Month. To protect themselves, many types of bacteria have developed a method to chop up any foreign DNA, such as that of an attacking phage. These bacteria build an endonuclease--an enzyme that cuts DNA--which is allowed to circulate in the bacterial cytoplasm, waiting for phage DNA. The endonucleases are termed "restriction enzymes" because they restrict the infection of bacteriophages.

Molecular Scissors

Each type of restriction enzyme seeks out a single DNA sequence and precisely cuts it in one place. For instance, the enzyme shown here, EcoRI, cuts the sequence GAATTC, cutting between the G and the A. Of course, roving endonucleases can be dangerous, so bacteria protect their own DNA by modifying it with methyl groups. These groups are added to adenine or cytosine bases (depending on the particular type of bacteria) in the major groove. The methyl groups block the binding of restriction enzymes, but they do not block the normal reading and replication of the genomic information stored in the DNA. DNA from an attacking bacteriophage will not have these protective methyl groups and will be destroyed. Each particular type of bacteria has a restriction enzyme (or several different ones) that cuts a specific DNA sequence, paired with a methyl-transferase enzyme that protects this same sequence in the bacterial genome.



http://www.pdb.org/pdb/static.do?p=education_discussion/molecule_of_the_month/pdb8_1.html

Cutting DNA

Restriction Enzymes

Sticky Ends

The booming field of biotechnology was made possible by the discovery of restriction enzymes in the early 1950's. With them, DNA may be cut in precise locations. A second enzyme--DNA ligase--may then be used to reassemble the pieces in any desired order. Together, these two enzymes allow researchers to assemble customized genomes. For instance, researchers can create designer bacteria that make insulin or growth hormone or add genes for disease resistance to agricultural plants.

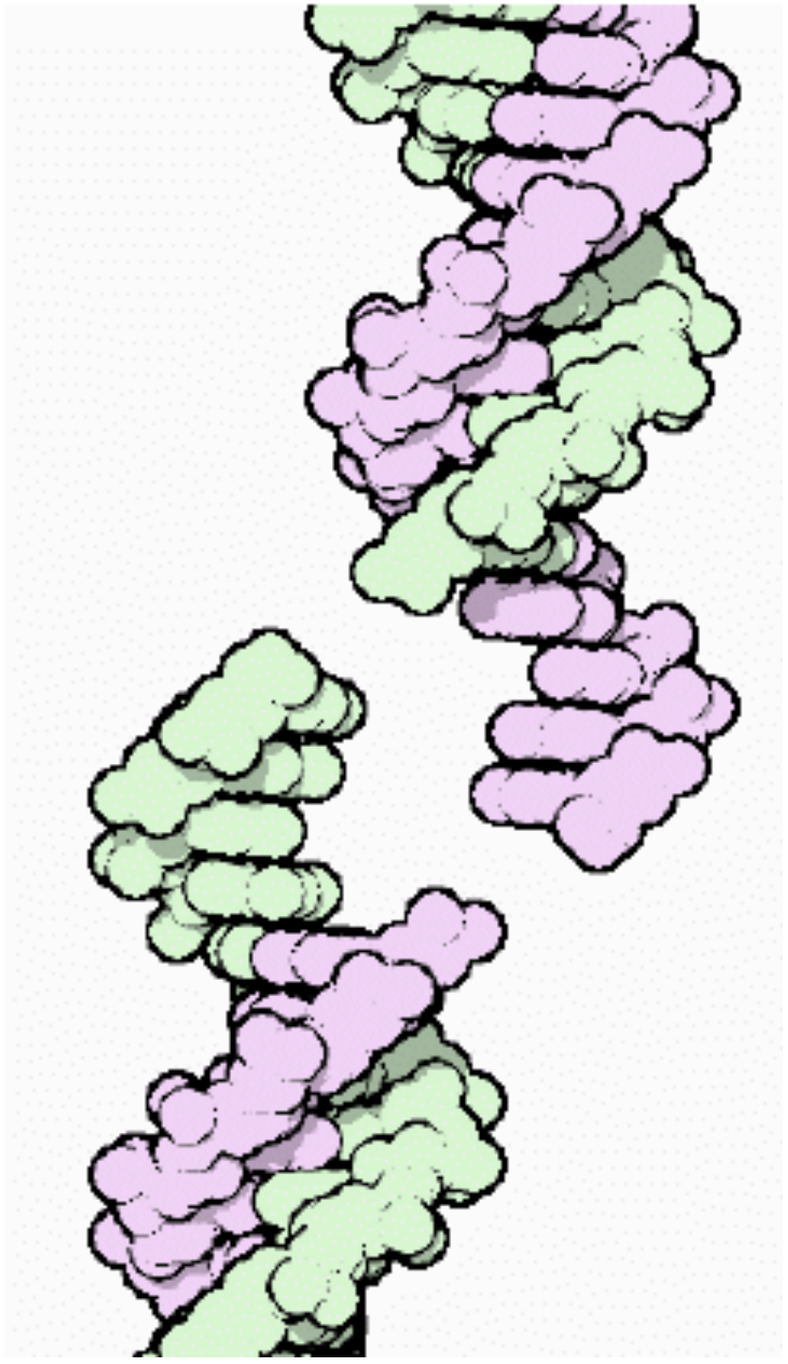
An interesting property of restriction enzymes simplifies this molecular cutting and pasting. Restriction enzymes typically recognize a symmetrical sequence of DNA, such as the site of EcoRI:

```
---GAATTC---
   | | | |
---CTTAAG---
```

Notice that the top strand is the same as the bottom strand, read backwards. When the enzyme cuts the strand between G and A, it leaves overhanging chains:

```
---G    AATTC---
   | |  | |
---CTTAA  G---
```

These are termed "sticky ends" because the base pairs formed between the two overhanging portions will glue the two pieces together, even though the backbone is cut. Sticky ends are an essential part of genetic engineering, allowing researchers to cut out little pieces of DNA and place them in specific places, where the sticky ends match.



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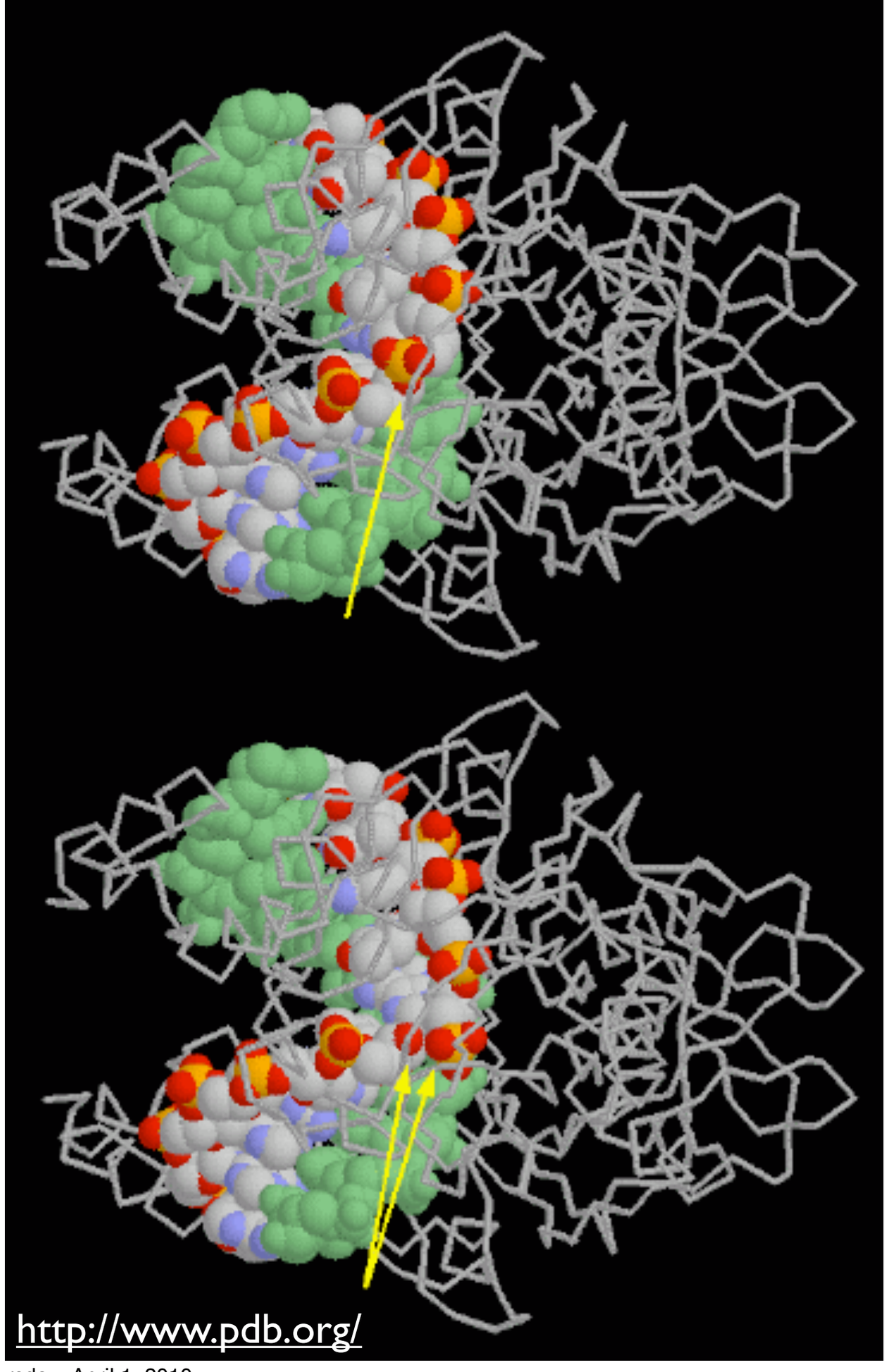
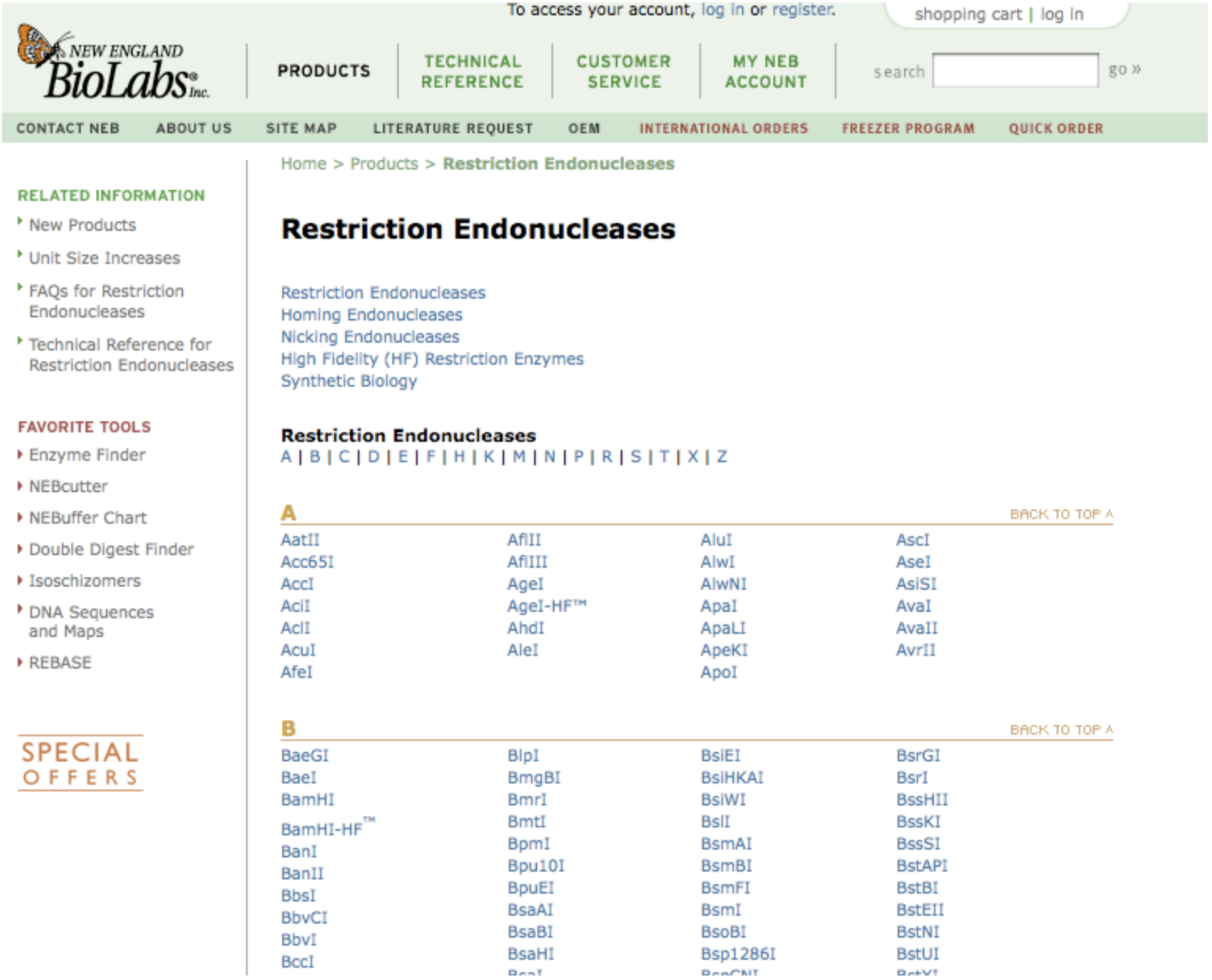


Exploring the Structure

The PDB contains structures for many restriction enzymes. Another example from Escherichia coli--EcoRV--is shown here. The structure at the top, taken from PDB entry 1rva, shows the enzyme bound to a short piece of DNA. The arrow shows the phosphate group that will be cut. The lower illustration, taken from PDB entry 1rvc, shows the structure after the DNA has been cut. A water molecule has been inserted, so there are now two oxygen atoms, close to one another but not bonded together, where there was a single bonded oxygen atom in the intact DNA.

Why standards matter



1973

Construction of biologically functional bacterial plasmids in vitro

Cohen et al., PNAS, 1973

1985

Cloning and expression of the human erthropoietin gene

Lin et al., PNAS,1985

2006

Production of the antimalarial drug precursor artemisinic acid in engineered yeast

Ro et al., Nature, 2006

rt: 2005-11-12577

MATERIALS AND METHODS

E. coli strain W1485 containing the RSF1010 plasmid, which carries resistance to streptomycin and sulfonamide, was obtained from S. Falkow. Other bacterial strains and R factors and procedures for DNA isolation, electron microscopy, and transformation of *E. coli* by plasmid DNA have been described (1, 7, 8). Purification and use of the *Eco*RI restriction endonuclease have been described (5). Plasmid heteroduplex studies were performed as previously described (9, 10). *E. coli* DNA ligase was a gift from P. Modrich and R. L. Lehman and was used as described (11). The detailed procedures for gel electrophoresis of DNA will be described elsewhere (Helling, Goodman, and Boyer, in preparation); in brief, duplex DNA was subjected to electrophoresis in a tube-type apparatus (Hoefer Scientific Instrument) (0.6 × 15-cm gel) at about 20° in 0.7% agarose at 22.5 V with 40 mM Tris–acetate buffer (pH 8.05) containing 20 mM sodium acetate, 2 mM EDTA, and 18 mM sodium chloride. The gels were then soaked in ethidium bromide (5 μg/ml) and the DNA was visualized by fluorescence under long wavelength ultraviolet light ("black light"). The molecular weight of each fragment in the range of 1 to 200 × 10^6 was determined from its

Assembly of Expression Vector for the Epo Gene. For direct expression of the genomic Epo gene, the 4.8-kilobase (kb) *Bst*EII–*Bam*HI fragment of λHE1 (see *Results*), which contains the entire Epo gene, was used. After converting the *Bst*EII site into a *Bam*HI site with a synthetic linker, the fragment was inserted into the unique *Bam*HI site of the expression vector pDSVL (unpublished data), which contains a dihydrofolate reductase (DHFR) minigene from pMg1 (24). The resulting plasmid pDSVL-gHuEPO (Fig. 1*A*) was then used to transfect Chinese hamster ovary (CHO) DHFR$^-$ cells (25) by the calcium phosphate microprecipitate method (26). The transformants were selected by growth in medium lacking hypoxanthine and thymidine. The culture medium used was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine (25).

Plasmid construction. To create plasmid pRS425ADS for expression of *ADS* with the *GAL1* promoter, *ADS* was PCR amplified from pADS[7] using primer pair 9 and 10. (Supplementary Table I). Using these primers the nucleotide sequence 5'-AAAACA-3' was cloned immediately upstream of the start codon of *ADS*. This consensus sequence was used for efficient translation[8,9] of *ADS* and the other galactose-inducible genes used in this study. The amplified product was cleaved with *Spe*I and *Hin*dIII and cloned into *Spe*I and *Hin*dIII digested pRS425GAL1[10].

For integration of an expression cassette for *tHMGR*, plasmid pδ-HMGR was constructed. First *Sac*II restriction sites were introduced into pRS426GAL1[10] at the 5' end of the *GAL1* promoter and 3' end of the *CYC1* terminator. To achieve this, the promoter-multiple cloning site-terminator cassette of pRS426GAL1 was PCR amplified using primer pair 11 and 12. The amplified product was cloned directly into *Pvu*II-

Genetic engineering remains expert driven artwork

ON A SYSTEM OF

SCREW THREADS AND NUTS.

By WILLIAM SELLERS.

[Read before the FRANKLIN INSTITUTE, April 21, 1864.]

The importance of a uniform system of screw threads and nuts is so generally acknowledged by the engineering profession, that it needs no argument to set forth its advantages; and in offering any plan for their acceptance, it remains only to demonstrate its practicability and its superiority over any of the numerous special proportions now used by the different manufacturers. In this country no organized attempt has as yet been made to establish any system, each manufacturer having adopted whatever his judgment may have dictated as the best, or as most convenient for himself; but the importance of the works now in progress, and the extent to which manufacturing has attained, admonish us that so radical a defect should be allowed to exist no longer. The importance of this subject was long ago recognised in England, and the engineers of that country, by mutual agreement, adopted the proportions now in universal use there. Our standard of length being the same as theirs, it would seem desirable that the system which they have adopted should also be employed by us, unless grave objections can be urged against it and a better one substituted. In examining the details of their system, the first in importance appears to be the pitch or the distance from centre to centre of the threads upon each diameter of screw, which is as follows, viz:

FIG. 1

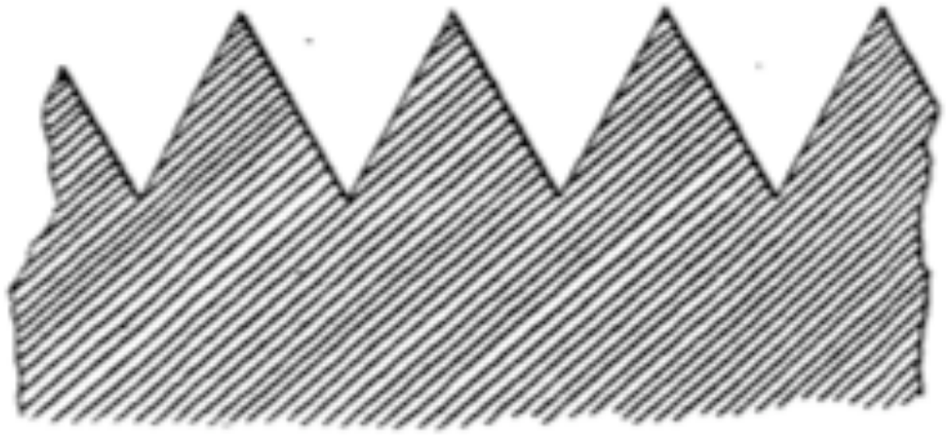


FIG. 2

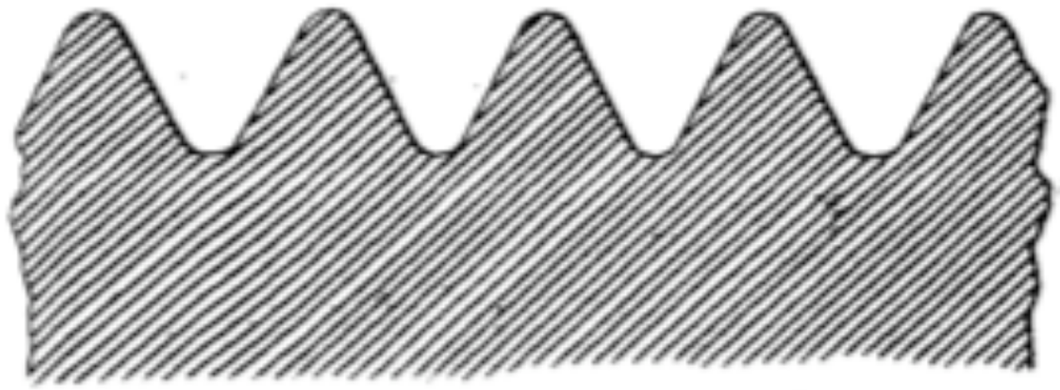
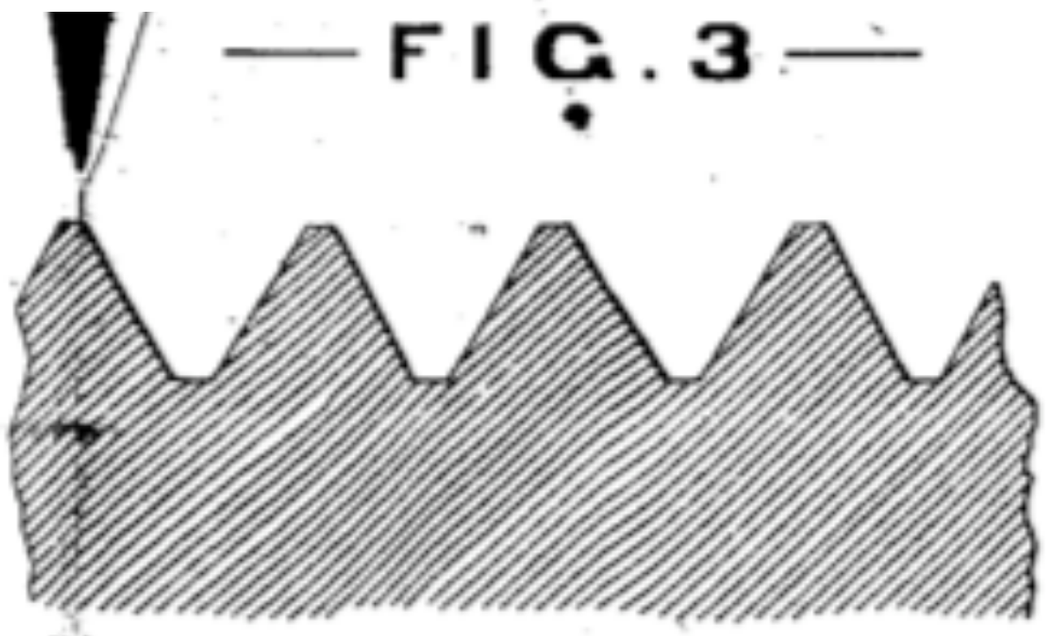


FIG. 3



Why standards matter