# Large-scale DNA Sequencing

## Current Topics in Genome Analysis
## October 28, 1997

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# Large-scale Sequencing

I Current technology

- Sanger method
- Fluorescent sequencing chemistry: dye-primer and dye-terminator
- Four-color fluorescent detection

II Classical sequencing strategies

- Directed
  - Primer "walking"
  - Controlled nested deletions
  - Transposon insertions
- Random

III Shotgun sequencing

- Laboratory techniques
- Poisson distribution
- Directed vs. random strategies

IV EST sequencing

V Bacterial genomes

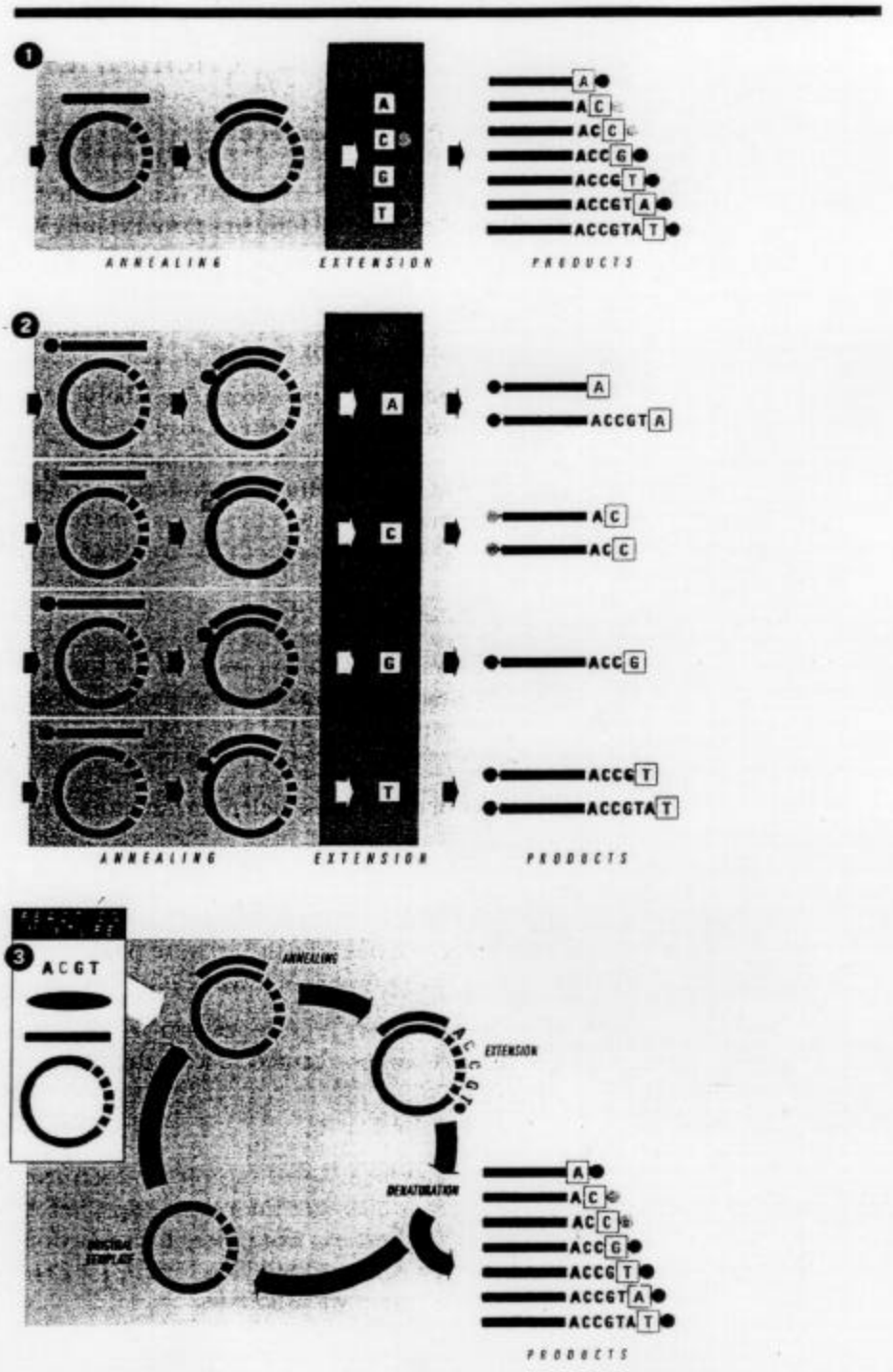
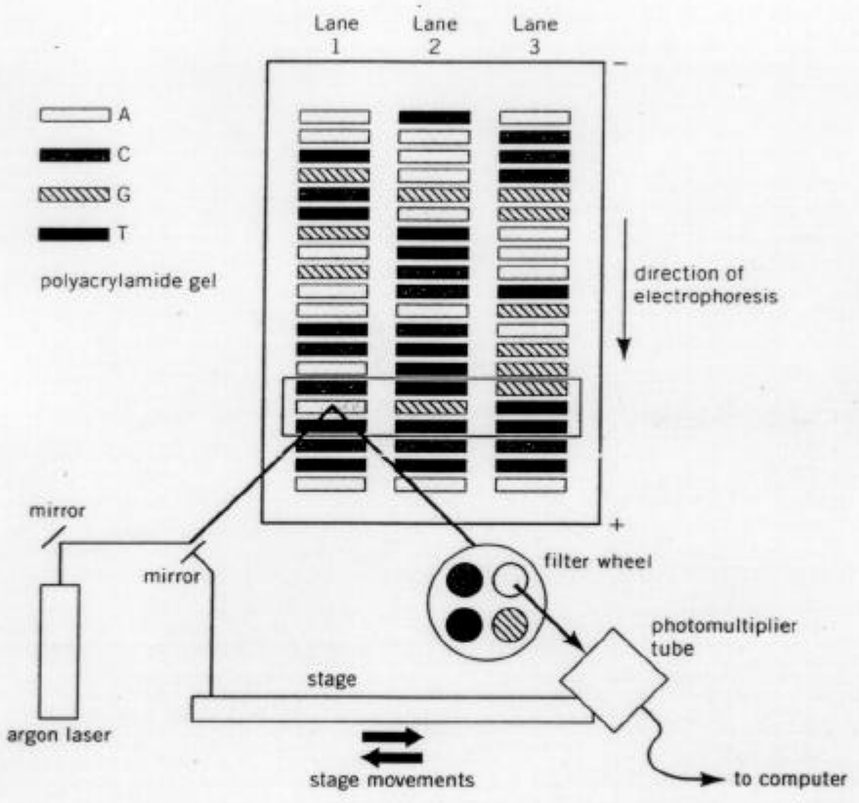



**Figure 1-1. (1) Dye terminator labeling, (2) Dye primer labeling, (3) Cycle sequencing**

# Four Color Fluorescent Sequencing



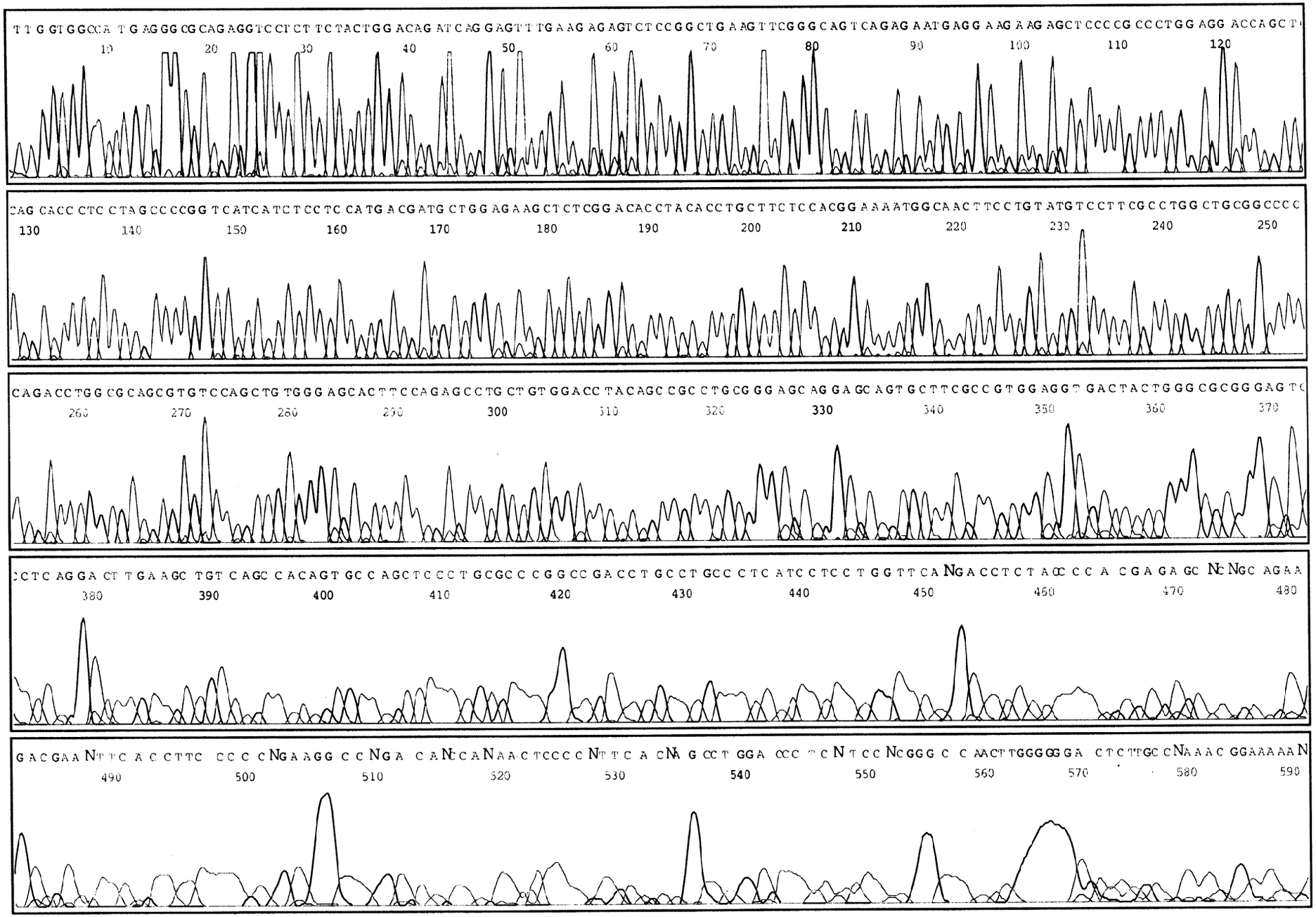
DYE TERMINATOR

ABI PRISM™

Model 373 31
Version 3.0
ABI50 31
Version 3.0 Lane 5

Signal G:212 A:580 T:219 C:225
DT6%Ac{A Set-AnyPrimer}
94061942S
Points 944 to 7164 Base 1: 944


Mon, Oct 20, 1997 10:32 PM
Sat, Feb 1, 1997 12:41 PM
Spacing: 10.79{10.79}



DYE PRIMER

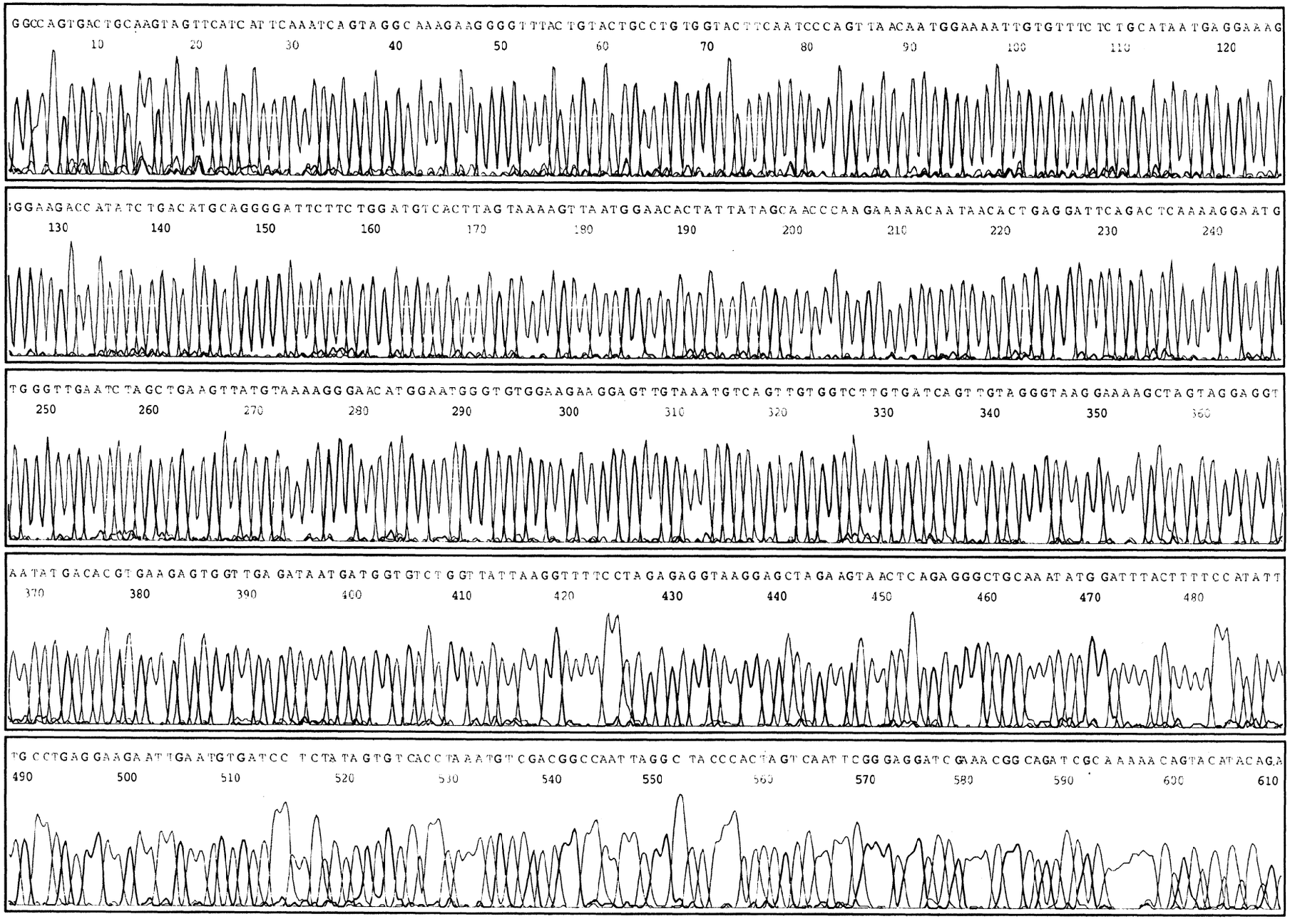
ABI PRISM™

Model 377
Version 3.0
ABI200
Version 2.1.1

c58t01d09.s1
c58t01d09.s1
Lane 21

Signal C:392 A:367 G:272 T:365
ET{-40m13}960208
ET-Matrix960720
Points 1121 to 8008 Base 1: 1121

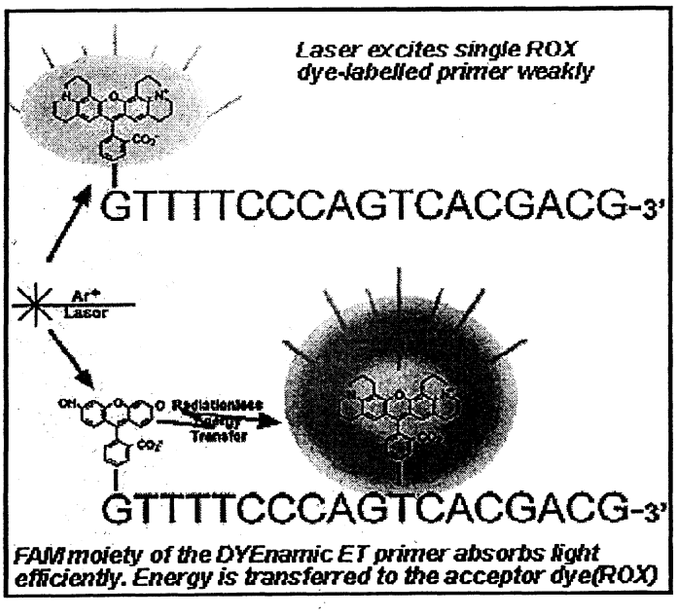

Tue, Jul 30, 1996 9:36 AM
Mon, Jul 29, 1996 12:51 PM
Spacing: 10.30{ 0}



# Energy Transfer Technology

(Ju et al., 1996; Lee et al., 1997)

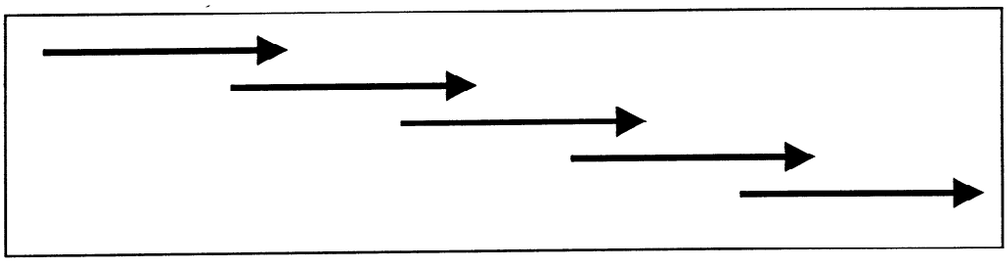
- Less DNA needed in sequencing reaction
- Fewer PCR cycles allowable
- Longer read lengths
- Direct loading onto sequencing gels possible (primers only)



# Classical large-scale sequencing strategies

## 1. Directed or Ordered Approaches

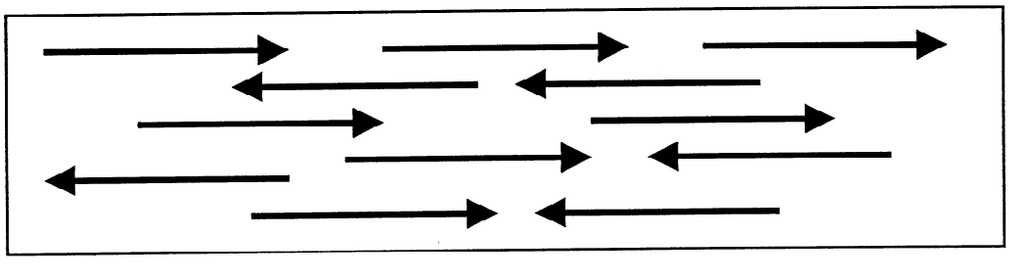
- Generate ordered clones
- Minimal redundancy sequencing



- Primer walking
- Controlled nested deletions
  (Bal31 digestion, ExoIII/S1 digestion, ssDNA deletions)
- Transposon insertion
- Restriction fragment subcloning

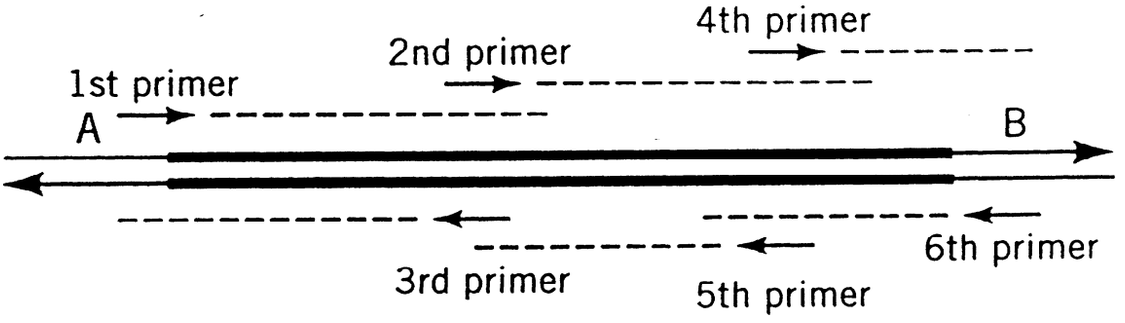
## 2. Random Approaches

- High redundancy sequencing
- Gap closure



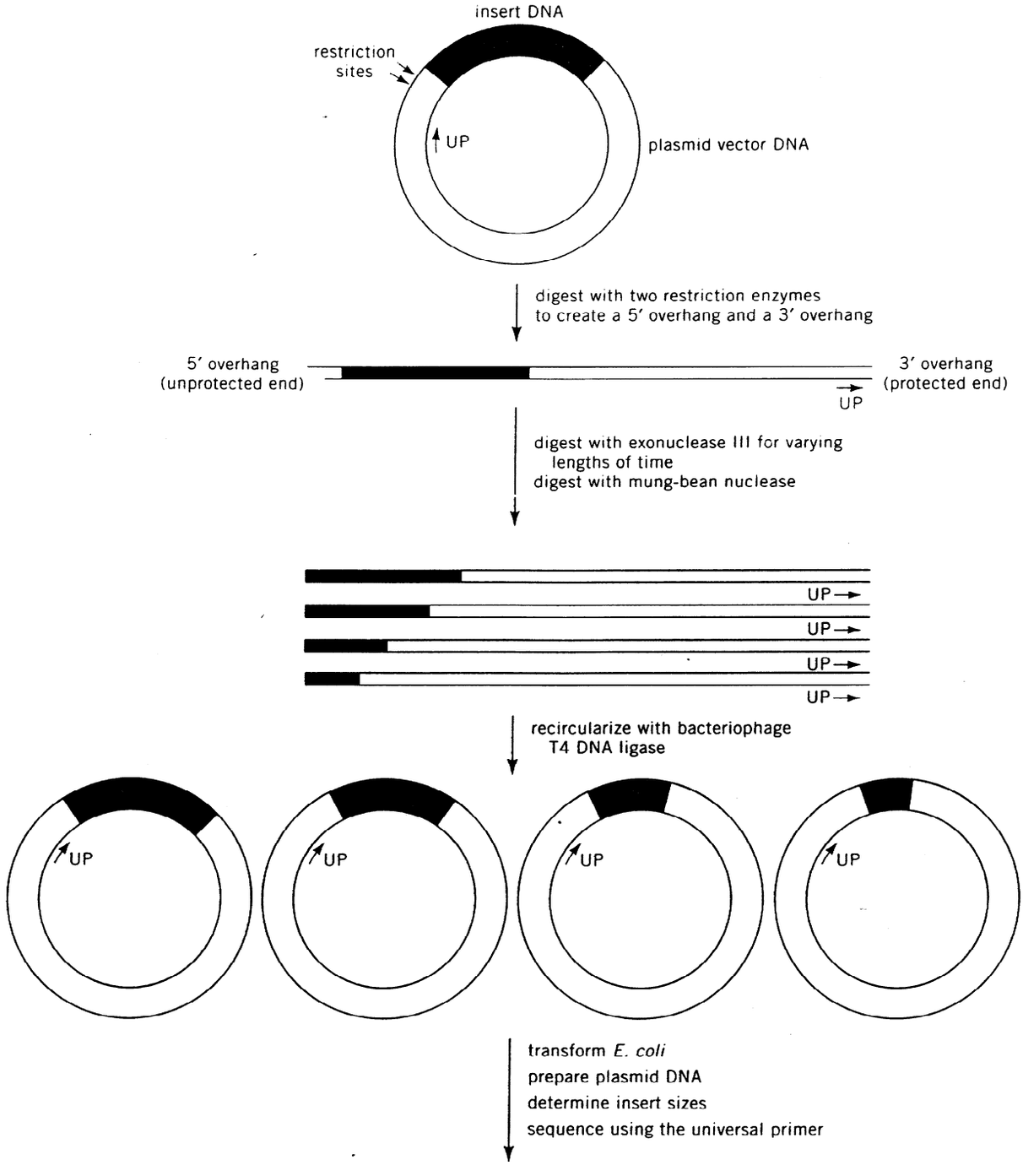
- "Shotgun"

# Primer-directed Strategy

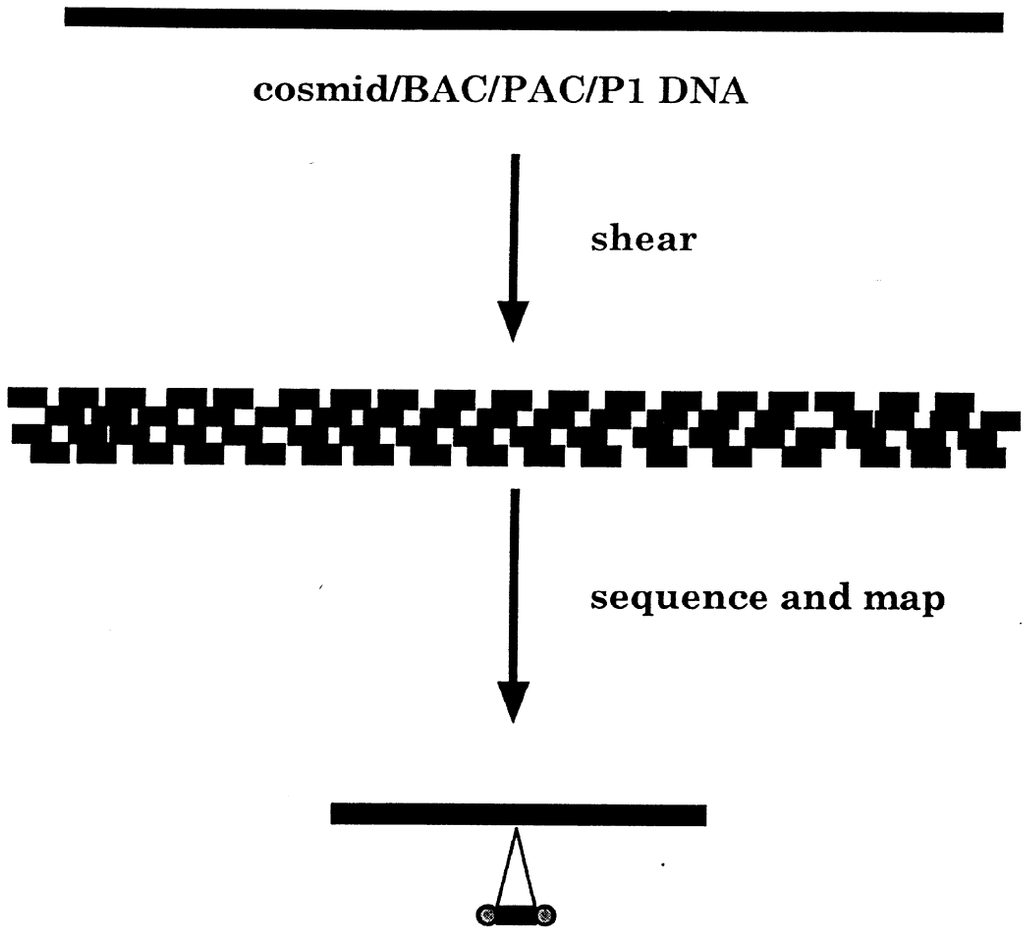



*Note*: 1st and 6th primers anneal to universal (i.e., vector) sequences

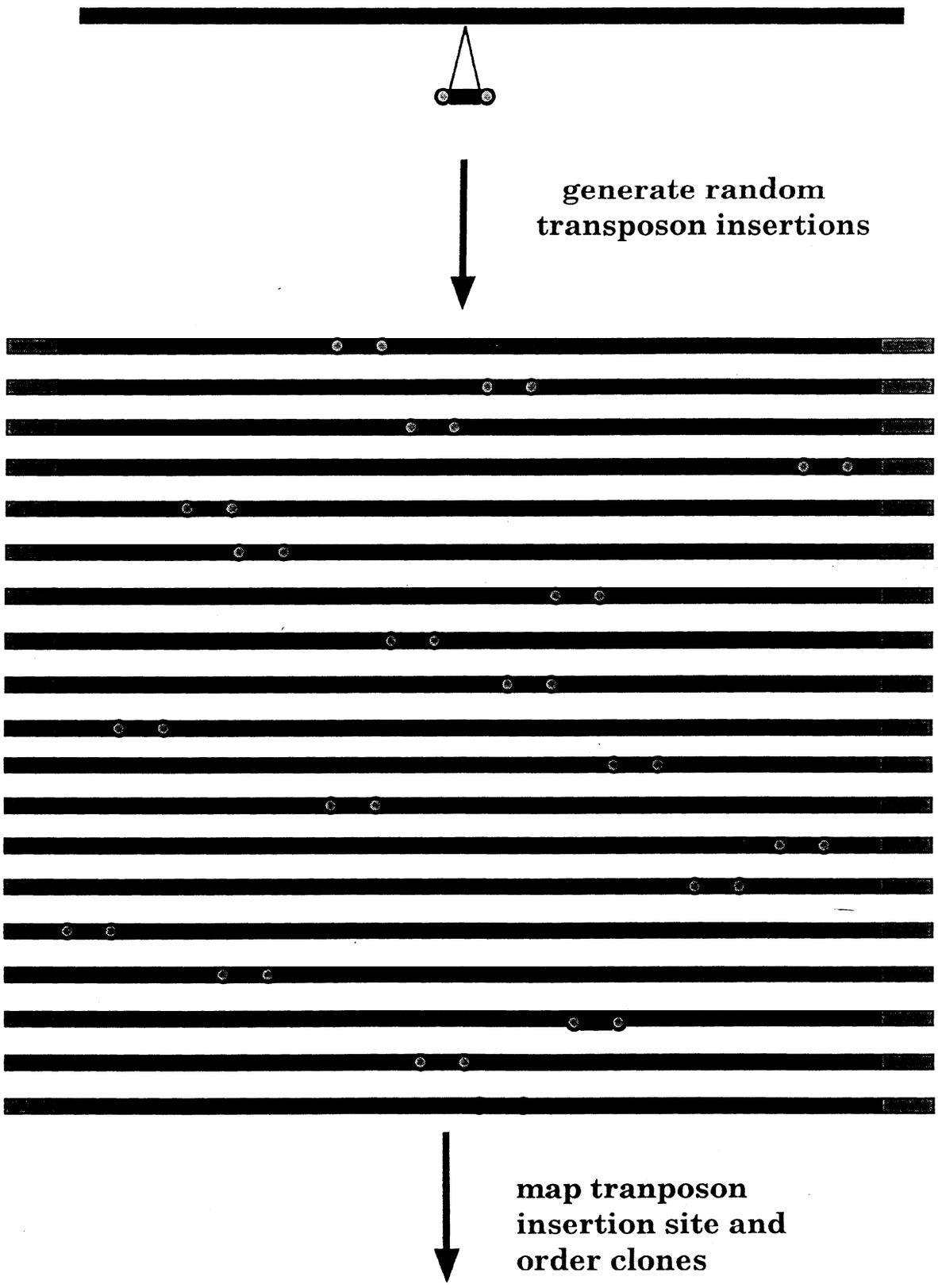
# Nested Deletions



# Sequencing from ordered transposon insertions into mapped random subclones



# Ordering transposon insertions



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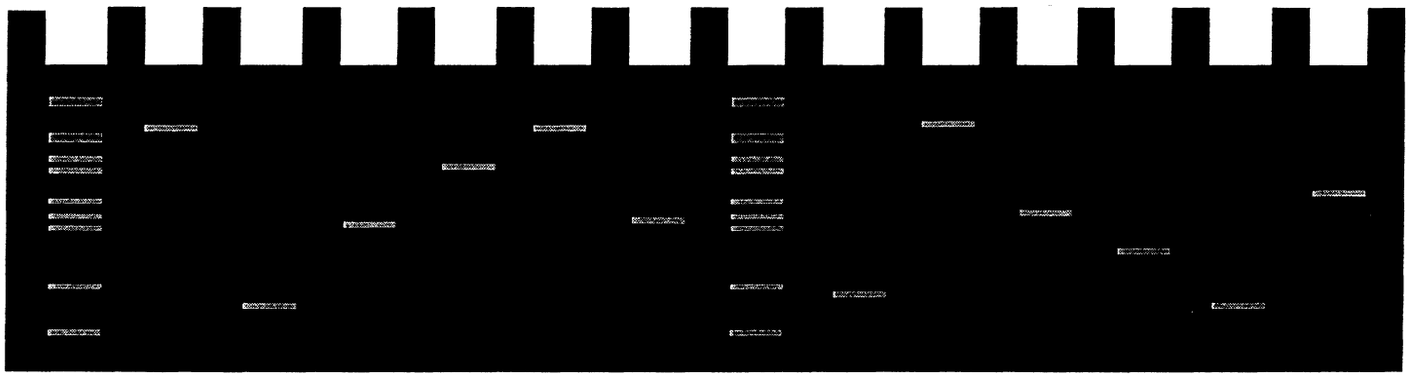
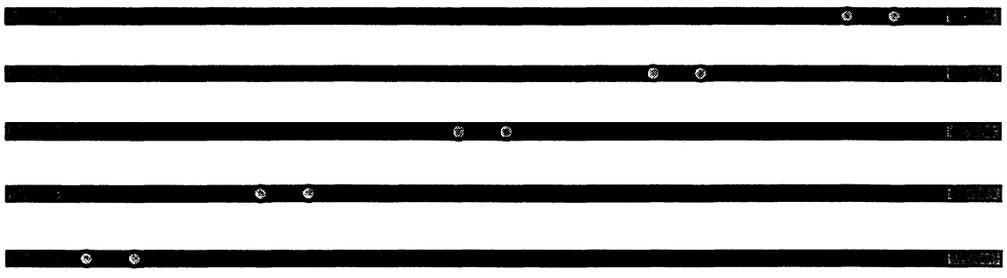
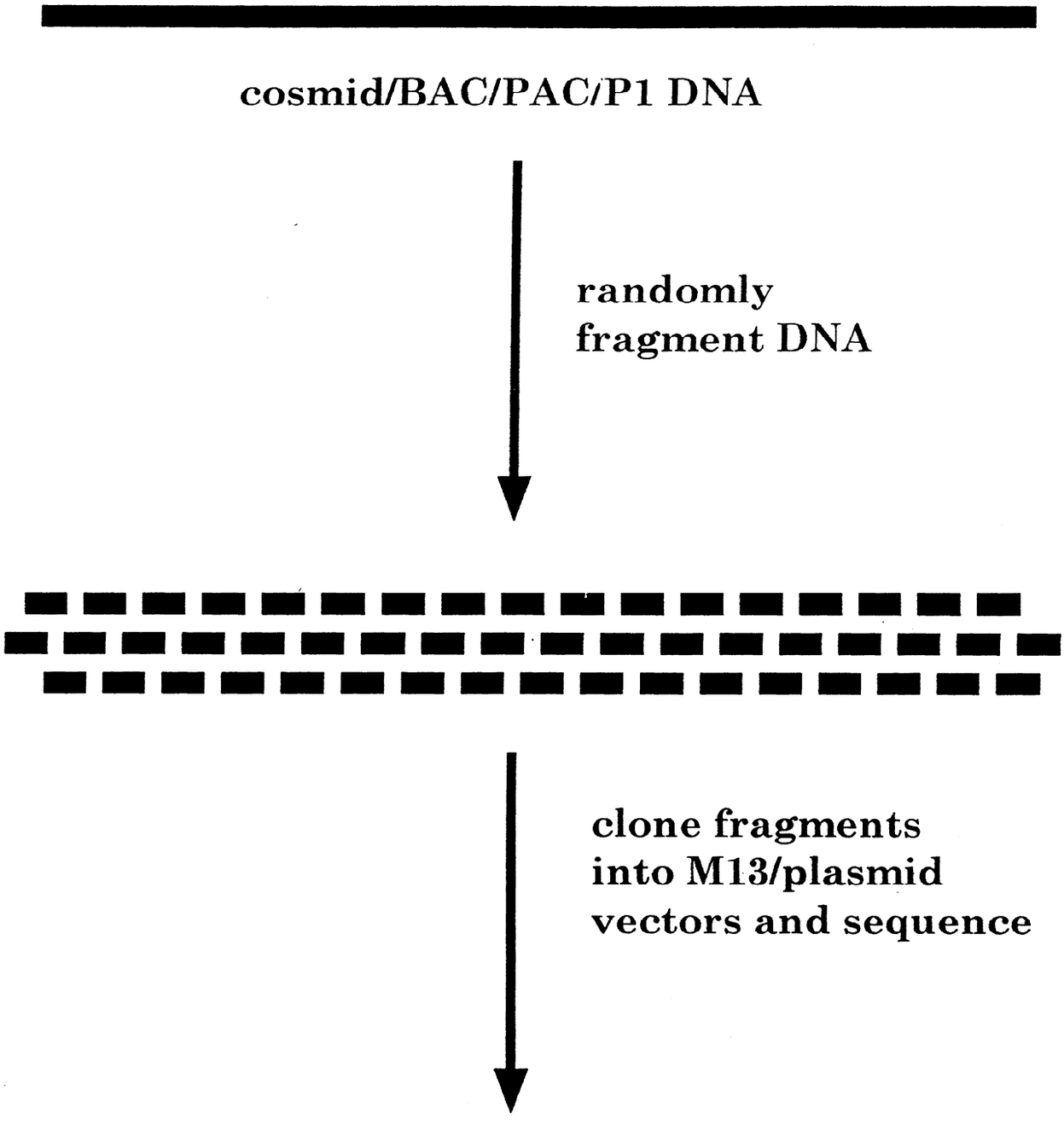


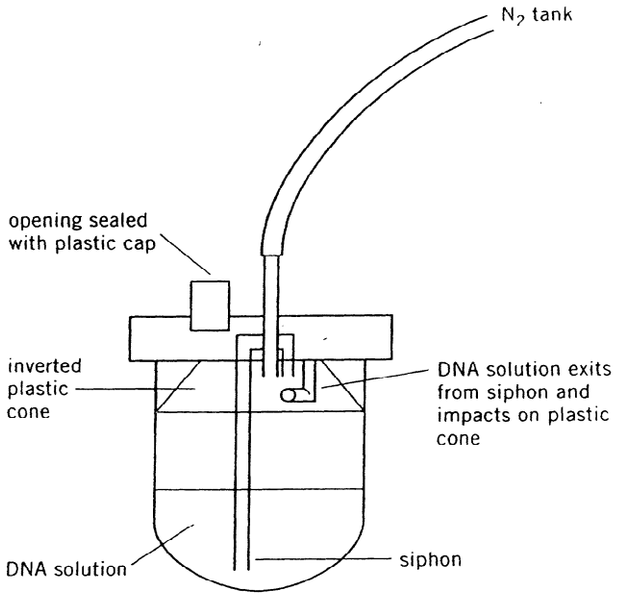


# Random Shotgun Sequencing with Directed Gap Closure

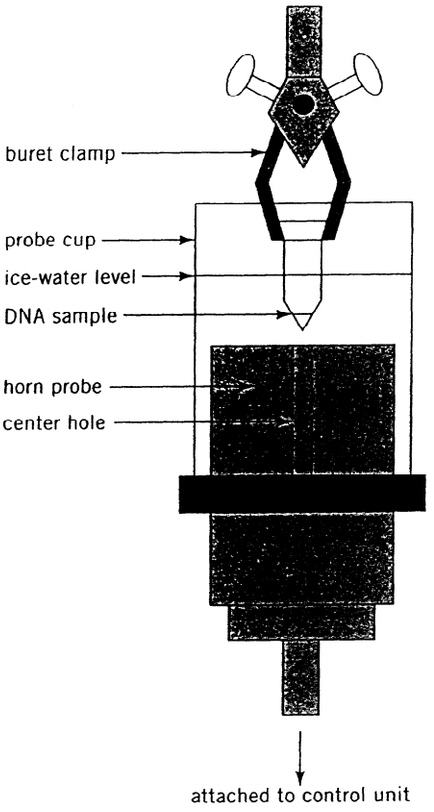


# Popular DNA Shearing Strategies

- **Sonication**
  Physical shearing with high frequency sound waves
- **Nebulization**
  Physical shearing with high pressure
- **French Press Shearing**
  Physical shearing with high pressure
- **Partial Digestion**
  Enzymatic, e.g. Sau3AI, DnaseI/$Mn^{++}$

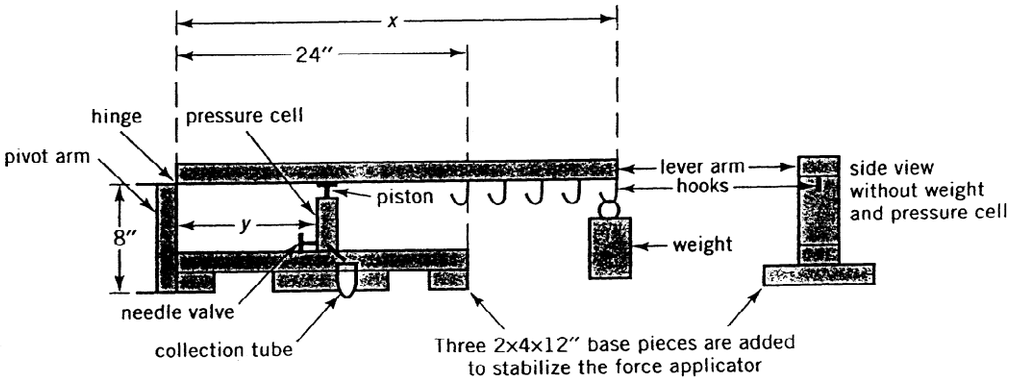



## Nebulizer

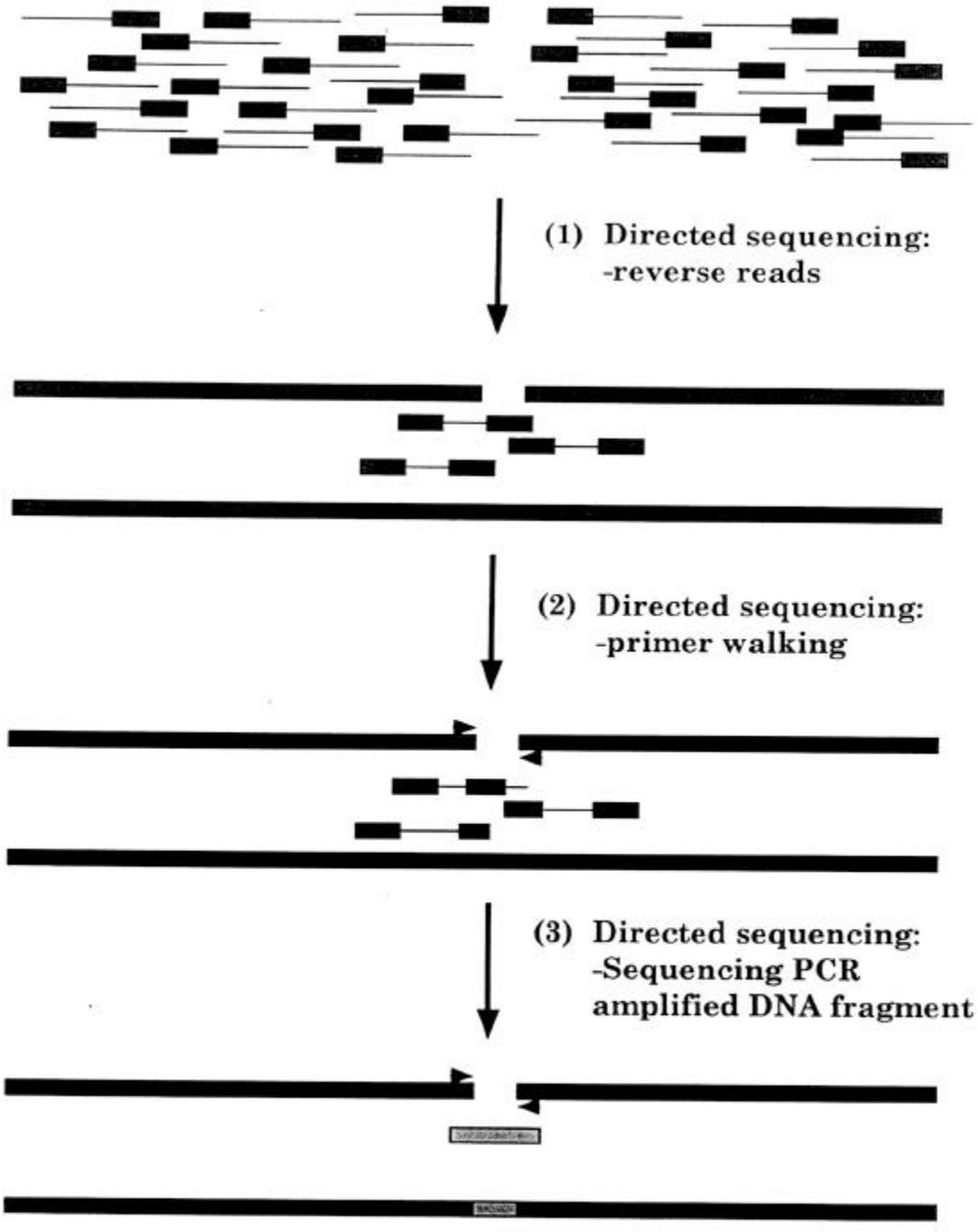



## Cup Horn Sonicator

## French Press



# Random Shotgun Sequencing with Directed Gap Closure



# Sequence Assembly Software

**DNA Star**
**Sequencher (Gene Codes)**
**Assembler (PE/ABI)**
**Gelassemble (GCG)**
**XBAP/XGAP (Staden)**
**Phrap (Green)**

# Poisson calculations

**The sequencing strategy for the shotgun approach follows the Lander and Waterman application of the Poisson distribution (Lander and Waterman, 1988)**

**The probability a base is not sequenced is given by:** $P_0=e^{-c}$

**Where:**

- **c = fold sequence coverage (c=LN/G),**
- **LN = # bases sequenced, i.e. L = average sequencing read length and N = # reads**
- **G = target sequence length**
- **e = 2.718 (e=2.718281828459)**

| Fold Coverage | $P_0=e^{-c}$ | % not sequenced | % sequenced |
|---|---|---|---|
| 1 | 0.37 | 37% | 63% |
| 2 | 0.135 | 13.5% | 87.5% |
| 3 | 0.05 | 5% | 95% |
| 4 | 0.018 | 1.8% | 98.2% |
| 5 | 0.0067 | 0.6% | 99.4% |
| 6 | 0.0025 | 0.25% | 99.75% |
| 7 | 0.0009 | 0.09% | 99.91% |
| 8 | 0.0003 | 0.03% | 99.97 |
| 9 | 0.0001 | 0.01% | 99.99% |
| 10 | 0.000045 | 0.005% | 99.995% |

% sequenced is independent of read length when fold coverage is considered

# Total Gap Length

**Total Gap Length (bp) = $Ge^{-c}$**

**Where:**

- **c = fold coverage**
- **G = target sequence length**
- **$e^{-c} = P_0$**

| Genome size = | 50 kb | 150 kb | 300 kb | 2 Mb | 4 Mb |
|---|---|---|---|---|---|
| Fold coverage | $Ge^{-c}$ | $Ge^{-c}$ | $Ge^{-c}$ | $Ge^{-c}$ | $Ge^{-c}$ |
| 1 | 18,500 | 55,500 | 111,000 | 740,000 | 1,480,000 |
| 2 | 6,750 | 20,250 | 40,500 | 270,000 | 540,000 |
| 3 | 2,500 | 7,500 | 15,000 | 100,000 | 200,000 |
| 4 | 900 | 2,700 | 5,400 | 36,000 | 72,000 |
| 5 | 335 | 1,005 | 2,010 | 13,400 | 26,800 |
| 6 | 125 | 375 | 750 | 5,000 | 10,000 |
| 7 | 45 | 135 | 270 | 1,800 | 3,600 |
| 8 | 15 | 45 | 90 | 600 | 1,200 |
| 9 | 5 | 15 | 30 | 200 | 400 |
| 10 | 2 | 6 | 12 | 90 | 180 |

# Total Number of Gaps

**Total number of gaps = $Ne^{-c}$**

**Where:**

- **N = Gc/L = number of reads for x-fold coverage**

  **G = Target sequence length**
  **c = Fold Coverage**
  **L = Average sequencing read length**

- **$e^{-c} = P_0$**

## 50 kb Target Clone:

| Read length | 400 | | | 500 | | | 600 | | |
|---|---|---|---|---|---|---|---|---|---|
| Fold Cov. | N | $e^{-c}$ | #Gaps $=Ne^{-c}$ | N | $e^{-c}$ | #Gaps $=Ne^{-c}$ | N | $e^{-c}$ | #Gaps $=Ne^{-c}$ |
| 1 | 125 | 0.37 | 46 | 100 | 0.37 | 37 | 84 | 0.37 | 31 |
| 2 | 250 | 0.135 | 34 | 200 | 0.135 | 27 | 168 | 0.135 | 23 |
| 3 | 375 | 0.05 | 19 | 300 | 0.05 | 15 | 242 | 0.05 | 12 |
| 4 | 500 | 0.018 | 9 | 400 | 0.018 | 7 | 326 | 0.018 | 6 |
| 5 | 625 | 0.0067 | 4 | 500 | 0.0067 | 3 | 410 | 0.0067 | 3 |
| 6 | 750 | 0.0025 | 2 | 600 | 0.0025 | 2 | 500 | 0.0025 | 1 |
| 7 | 875 | 0.0009 | 1 | 700 | 0.0009 | 1 | 583 | 0.0009 | 1 |
| 8 | 1000 | 0.0003 | 0 | 800 | 0.0003 | 0 | 667 | 0.0003 | 0 |
| 9 | 1125 | 0.0001 | 0 | 900 | 0.0001 | 0 | 750 | 0.0001 | 0 |
| 10 | 1250 | 0.000045 | 0 | 1000 | 0.000045 | 0 | 833 | 0.000045 | 0 |

**The values for each fold coverage for a 50kb cosmid (G=50,000) with average read length of 500 bases are:**

| Fold coverage | Total bases sequenced | $e^{-c}$ | Total gap length_in bases $=Ge^{-c}$ | Number of Gaps = $Ne^{-c}$ | Gap Length/# gaps = # bases per gap | % complete |
|---|---|---|---|---|---|---|
| 1 | 50000 | 0.37 | 18,500 | 37 | 500 | 63 |
| 2 | 100000 | 0.135 | 6,750 | 27 | 250 | 87.5 |
| 3 | 150000 | 0.05 | 2,500 | 15 | 167 | 95 |
| 4 | 200000 | 0.018 | 900 | 7 | 129 | 98.2 |
| 5 | 250000 | 0.0067 | 335 | 3 | 112 | 99.4 |
| 6 | 300000 | 0.0025 | 125 | 2 | 63 | 99.75 |
| 7 | 350000 | 0.0009 | 45 | 1 | 45 | 99.91 |
| 8 | 400000 | 0.0003 | 15 | 1 | 15 | 99.97 |
| 9 | 450000 | 0.0001 | 5 | 1 | 5 | 99.99 |
| 10 | 500000 | 0.000045 | 2 | 1 | 2 | 99.995 |

**The values for each fold coverage for a 150kb BAC (G=150,000) with average read length of 500 bases are:**

| Fold coverage | Total bases sequenced | $e^{-c}$ | Total gap length_in bases $=Ge^{-c}$ | Number of Gaps = $Ne^{-c}$ | Gap Length/# gaps = # bases per gap | % complete |
|---|---|---|---|---|---|---|
| 1 | 150000 | 0.37 | 55,500 | 111 | 500 | 63 |
| 2 | 300000 | 0.135 | 20,250 | 81 | 250 | 87.5 |
| 3 | 450000 | 0.05 | 7,500 | 45 | 167 | 95 |
| 4 | 600000 | 0.018 | 2,700 | 22 | 123 | 98.2 |
| 5 | 750000 | 0.0067 | 1,005 | 10 | 101 | 99.4 |
| 6 | 900000 | 0.0025 | 375 | 5 | 75 | 99.75 |
| 7 | 1050000 | 0.0009 | 135 | 2 | 68 | 99.91 |
| 8 | 1200000 | 0.0003 | 45 | 1 | 45 | 99.97 |
| 9 | 1350000 | 0.0001 | 15 | 1 | 15 | 99.99 |
| 10 | 1500000 | 0.000045 | 6 | 1 | 6 | 99.995 |

For more calculations, see http://www.genome.ou.edu/poisson_calc.html

## Directed Approaches

+ Reduction of sequencing redundancy (to 2-3X)
+ Better organization of raw sequence data
+ Ability to sequence highly repetitive regions

- Manual work requires highly skilled personnel
- Labor-intensive procedures difficult to automate
- Works only from 5-10 kb each time
- Primer walking expensive and slow

## Random Approaches

+ Rapid accumulation of data
+ Over-determination of each nucleotide minimizes errors in the sequence
+ More convenient to automate
+ Currently works up to 50-250 kb

- Large redundancy (5-10X) increases cost
- Gap-filling ("finishing") is tedious and slow
- Progress of a project is more difficult to control
- Highly repetitive regions difficult to handle

# Goals

**Maximize efficiency and accuracy**

**Minimize cost**

# Method of choice:

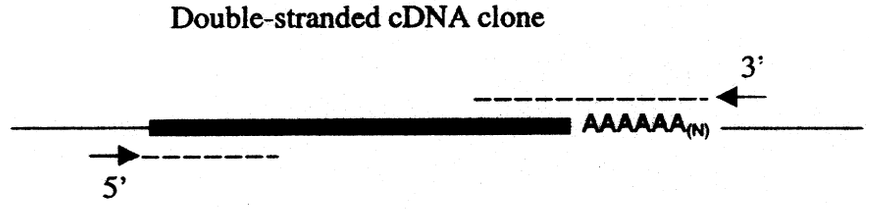
**Random sequencing** **98-99%**

**followed by**

**Directed sequencing** **1-2%**

# Expressed Sequence Tags (ESTs)

- **Single-pass partial sequencing of random cDNA clones**




- Sample or "tag" sequence from both 5' and 3' ends of normalized, tissue-specific cDNA libraries
- 3' ESTs more likely to be unique among gene family members
- 5' ESTs more likely to yield homology information indicative of gene function

# Usefulness of ESTs

- Rapid means of gene discovery for those searching for human disease genes
- The study of temporal and spatial expression patterns
- Construction of a genome-wide STS based transcript map

NCBI dbEST BLAST Entrez

# dbEST release 101097

## Summary by Organism - October 10, 1997

## Number of public entries: 1,266,975

| | | |
|---|---|---|
| Homo sapiens (human) | 826,089 | |
| Mus musculus + domesticus (mouse) | 234,814 | |
| Caenorhabditis elegans (nematode) | 72,521 | |
| Arabidopsis thaliana (thale cress) | 32,964 | |
| Oryza sativa (rice) | 20,708 | |
| Drosophila melanogaster (fruit fly) | 16,859 | |
| Brugia malayi (parasitic nematode) | 10,721 | |
| Toxoplasma gondii | | 10,671 |
| Rattus sp. (rat) | 6,703 | |
| Schistosoma mansoni (blood fluke) | 3,394 | |
| Danio rerio (zebrafish) | 3,145 | |
| Saccharomyces cerevisiae (baker's yeast) | 3,042 | |
| Plasmodium falciparum (malaria parasite) | 2,872 | |
| Trypanosoma brucei rhodesiense | 2,626 | |
| Caenorhabditis briggsae | 2,424 | |
| Zea mays (maize) | 1,757 | |
| Onchocerca volvulus | 1,616 | |
| Brassica napus (oilseed rape) | 1,427 | |
| Sus scrofa (pig) | 1,323 | |
| Brassica campestris (field mustard) | 965 | |
| Dictyostelium discoideum | 795 | |
| Ricinus communis (castor bean) | 750 | |
| Pristionchus pacificus | 703 | |
| Pyrococcus furiosus (hyperthermophilic archaeon) | 577 | |
| Trypanosoma cruzi | 575 | |
| Cryptosporidium parvum | 567 | |
| Paralichthys olivaceus | | 550 |
| Leishmania major | | 535 |
| Schistosoma japonicum (blood fluke) | 455 | |
| Citrus unshiu | 426 | |
| Emericella nidulans | 272 | |
| Pinus taeda (loblolly pine) | 256 | |
| Capra hircus (goat) | 245 | |
| Saccharum sp. | 245 | |
| Strongylocentrotus purpuratus (purple urchin) | 242 | |
| Gallus gallus (chicken) | 216 | |
| Oryctolagus cuniculus (rabbit) | 207 | |
| Gracilaria gracilis | 200 | |
| Boophilus microplus (southern cattle tick) | 143 | |
| Trypanosoma brucei brucei | 143 | |
| Avena sativa (oat) | 125 | |
| Wuchereria bancrofti | 120 | |
| Biomphalaria glabrata | | 111 |
| Canis familiaris (dog) | 107 | |
| Entamoeba histolytica | 106 | |
| Glycine max (soybean) | 97 | |
| Sorghum bicolor (sorghum) | 91 | |
| Antheraea yamamai | 90 | |

# Other Strategies for EST/cDNA Sequencing

## Directed Methods

- Nested deletions
- End sequencing and primer walking
- Sequencing via ordered transposon insertions

## Random Methods

- Random shearing of clone, or PCR product of clone
- Random shearing of multiple cDNA clones ligated together

REVIEW

# Big Time for Small Genomes

Eugene V. Koonin[1]

National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland 20894

## Completed microbial genomes

| Link | Genome | Strain | Domain | Size (Mb) | Institution | Funding | Publication |
|---|---|---|---|---|---|---|---|
| | ***Haemophilus influenzae* Rd** | KW20 | **B** | 1.83 | **TIGR** | **TIGR** | Fleischmann *et. al.*, *Science* **269**:496-512 (1995) |
| | ***Mycoplasma genitalium*** | G-37 | **B** | 0.58 | **TIGR** | **DOE** | Fraser *et. al.*, *Science* **270**:397-403 (1995) |
| | ***Methanococcus jannaschii*** | | **A** | 1.66 | **TIGR** | **DOE** | Bult *et. al.*, *Science* **273**:1058-1073 (1996) |
| | ***Synechocystis* sp.** | PCC 6803 | **B** | 3.57 | **Kazusa DNA Research Inst.** | | Kaneko *et. al.*, *DNA Res.* **3**: 109-136 (1996) |
| | ***Mycoplasma pneumoniae*** | | **B** | 0.81 | **Univ. of Heidelberg** | | Himmelreich et. al., *Nuc. Acid Res.* **24**:4420-4449 (1996) |
| | ***Saccharomyces cerevisiae*** | | **E** | 13 | **European & North American consortium** | | Goffeau et. al., *Nature* **387** (Suppl.) 5-105 (1997) |
| | ***Helicobacter pylori*** | 26695 | **B** | 1.66 | **TIGR** | **TIGR** | Tomb *et. al.*, *Nature* **388**:539-547 (1997) |
| | ***Escherichia coli*** | K-12 | **B** | 4.60 | **University of Wisconsin** | **NHGRI** | Blattner *et. al.*, *Science* **277**: (1997) |
| | ***Bacillus subtilis*** | | **B** | 4.20 | **European & Japanese Consortium** | | manuscript submitted |
| | ***Archaeoglobus fulgidus*** | VC-16, DSM4304 | **A** | 2.20 | **TIGR** | **DOE** | manuscript submitted |
| | ***Borrelia burgdorferi*** | | **B** | 1.30 | **TIGR** | **Mathers Foundation** | manuscript in preparation |

# Microbial genomes in progress

| Genome | Strain | Domain | Size (Mb) | Institution | Funding | Anticipated Publication |
|---|---|---|---|---|---|---|
| *Actinobacillus actinomycetemcomitans* | | B | 2.2 | University of Oklahoma | NIH / NIDR | |
| *Aquifex aeolicus* | | B | 1.50 | Recombinant BioCatalysis Inc. | | |
| *Caulobacter crescentus* | | B | 3.80 | TIGR | | |
| *Chlamydia trachomatis* | serovar D (D/UW-3/Cx) | B | 1.05 | UC Berkeley & Stanford | NIAID | |
| *Clostridium acetobutylicum* | ATCC 824 | B | 4.1 | Genome Therapeutics | DOE | |
| *Deinococcus radiodurans* | R1 | B | 3.00 | TIGR | DOE | 1997 |
| *Enterococcus faecalis* | | B | 3.00 | TIGR | NIAID | 1999 |
| *Halobacterium salinarium* | | A | 4.0 | Max-Planck-Institute for Biochemistry | | |
| *Legionella pneumophila* | | B | 4.10 | TIGR | | |
| *Methanobacterium thermoautotrophicum* | delta H | A | 1.75 | Genome Therapeutics & Ohio State Univ. | DOE | |
| *Mycobacterium avium* | | B | 4.70 | TIGR | NIH | 2000 |
| *Mycobacterium tuberculosis* | CSU#93 (clinical isolate) | B | 4.40 | TIGR | NIAID | 1998 |
| *Mycobacterium tuberculosis* | H37Rv (lab strain) | B | 4.40 | Sanger Centre | Wellcome Trust | |
| *Neisseria gonorrhoeae* | | B | 2.20 | University of Oklahoma | NIAID | |
| *Neisseria meningitidis* | | B | 2.30 | TIGR / Oxford University | TIGR | |
| *Plasmodium falciparum* **Chr1** (clone 3D7) | | E | 0.8 | Sanger Centre | Wellcome Trust | |
| *Plasmodium falciparum* **Chr2** (clone 3D7) | | E | 1.00 | TIGR / NMRI | NIAID | 1997 |
| *Plasmodium falciparum* **Chr3** (clone 3D7) | | E | 1.20 | Sanger Centre | Wellcome Trust | |
| *Plasmodium falciparum* **Chr4** (clone 3D7) | | E | 1.5 | Sanger Centre | Wellcome Trust | |
| *Plasmodium falciparum* **Chr9** (clone 3D7) | | E | 1.80 | TIGR / NMRI | | |
| *Plasmodium falciparum* **Chr10** (clone 3D7) | | E | 2.10 | TIGR / NMRI | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Plasmodium falciparum* **Chr12** (clone 3D7) | | **E** | 2.4 | Stanford University | Burroughs Wellcome Fund | |
| *Plasmodium falciparum* **Chr14** (clone 3D7) | | **E** | 3.4 | **TIGR / NMRI** | Burroughs Wellcome Fund | |
| ***Porphyromonas gingivalis*** | | **B** | 2.20 | **TIGR / Forsyth Dental Center** | **NIDR** | |
| *Pseudomonas aeruginosa* | PAO1 | **B** | 5.90 | **International collaboration** | | |
| ***Pyrobaculum aerophilum*** | | **A** | 2.3 | **Caltech / UCLA / Recombinant BioCatalysis Inc.** | **ONR / DOE** | |
| ***Pyrococcus furiosus*** | | **A** | 2.10 | **Center of Marine Biotechnology / Univ. Utah** | **DOE** | |
| ***Pyrococcus shinkaj*** | | **A** | 2.00 | **MITI / University of Tokyo** | | |
| ***Rickettsia prowazekii*** | | **B** | 1.10 | **University of Uppsala** | **SSF / NFR** | |
| ***Salmonella typhimurium*** | | **B** | 4.50 | **TIGR** | | |
| ***Shewanella putrefaciens*** | MR-1 | **B** | 4.50 | **TIGR** | **DOE** | |
| ***Streptococcus pneumoniae*** | | **B** | 2.20 | **TIGR** | **TIGR** | 1997 |
| *Streptococcus pyogenes* | | **B** | 1.98 | **University of Oklahoma** | **NIAID** | |
| *Sulfolobus solfataricus* | | **A** | 3.05 | **Canadian & European Consortium** | | |
| *Thermotoga maritima* | MSB8 | **B** | 1.80 | **TIGR** | **DOE** | 1998 |
| ***Treponema denticola*** | | **B** | 3.00 | **TIGR / Univ. Texas** | | |
| *Treponema pallidum* | | **B** | 1.05 | **TIGR / Univ. Texas** | **NIAID** | 1997 |
| ***Thermoplasma acidophilum*** | | **A** | 1.7 | **Max-Planck-Institute for Biochemistry** | | |
| ***Ureaplasma urealyticum*** | serovar 3 | **B** | 0.75 | **U. Alabama / PE-ABI** | **PE-ABI / NIH / UAB** | 1997 |
| *Vibrio cholerae* | serotype O1, Biotype El Tor, strain N16961 | **B** | 2.50 | **TIGR** | **NIAID** | 1998 |

**KEY**

| **Domain** | **A: Archaea** | **B: Eubacteria** | **E: Eucaryote** |
|---|---|---|---|

Comments and questions to: Anthony R. Kerlavage

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# URLs

Links to almost ALL Genome related sites:

http://www-ls.lanl.gov/HGhotlist.html

Bacterial genomics database:

http://www.tigr.org/