BioMan 2013

Montgomery County Community College

Taq polymerase:

An integrated research & production curriculum

Goal

Demonstrate how the *Taq* polymerase core production system can function as a coherent subject for both R &D and production aspects of biotechnology

- Molecular Biology skills
- Fermentation & Protein Purification
- Quality Control

- Can also be used to introduce topics such as bioprospecting, protein structure & function, patents and intellectual property, microbial diversity, etc.

CCBC Program Structure

- Biol 120- Introductory course (mile wide / inch deep) an overview of biotechnology with a focus on developing, producing, and approving protein therapeutics. Basic technique development.
- Bio 253- Molecular biology – a focus on molecular biology techniques starting with nucleic acid isolation (plasmid, genomic), cloning and PCR. Class project is cloning of *Thermus aquaticus pol*I gene. Ends with Tech Transfer!
- Bio 254 – Manufacturing course – focus on production- begins with small process development experiments (media development, time course, etc) progresses through production run (batch and fed batch) of *E. coli* Taq. Ends with labeled tube.

Why *Taq*?

- *Can recreate in the classroom the discovery research, manufacturing, and commercialization pipeline*
  - *Can be adopted to a variety of institutional settings*
- *Can bring in numerous ancillary and related topics:*
  - *Intellectual property*
  - *Bioprospecting*
  - *Microbial diversity and ecology*
  - *Biotechnology product development*
- *Can make a product and compare to commercial preparations!*
- Feel that focus on a single process is a better teaching approach then trying to teach multiple processes (bacterial/yeast/mammalian)

“Taq patents”

Biotechnology is inextricably tied up with Intellectual Property issues. Use of Taq polymerase as model system is a good way to introduce IP (Patent system, licensing issues, how patents are structured, and recent changes to patent system).

- There are three main patents relating to the PCR process and Taq DNA polymerase.
  - US patent 4,683,202 filed in October 1985, listing Dr. Kary Mullis as the inventor, refers to the PCR process for “amplifying nucleic acid sequences.”
  - US patent 4,683,195 filed in February 1986 concerning a “Process for amplifying, detecting, and/or cloning nucleic acid sequences.”
  - US patent US 4,889,818 claims a “Purified thermostable *Thermus aquaticus* DNA polymerase”; the polymerase isolated from *Thermus aquaticus*, andthe same polymerase isolated from a recombinant organism (a bacteria) designed to express the *Thermus aquaticus* DNA polymerase.
- Expired in 2001

Patent title page

Taq and its uses still being patented!

- Rehydratable matrices for dry storage of taq polymerase in a microfluidic device
  US 20120142070 A1 – Published June, 2012
- Polymerase compositions and uses
  WO 2011029866 A2-Published March, 2011
- Detergent free polymerases
  EP 2374874 – Published October, 2011
- Chimeric dna polymerase
  WO 2006010887 A1-Published June, 2008

Global Market for *Taq* polymerase

- Estimate that Global *Taq* market is > $500,000,000 / year
  - Other thermostable polymerases available but *Taq* is still cheapest alternative for routine/diagnostic uses
    - Retail prices range from $0.01-0.10/unit

Life at High Temperature

•High temperatures irreversibly “denature” proteins and nucleic acids.

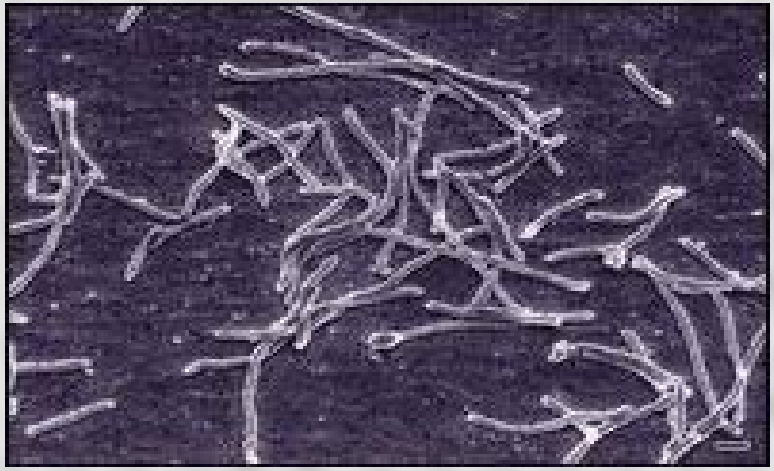
•Postulated that upper limit for life was 73^0C for some photosynthetic Cyanobacteria (Kemper, 1963).

•In 1960’s T.D. Brock started studying hot springs as part of a larger study in microbial ecology (Brock, 1997).

•Mushroom spring near great fountain geyser, Yellowstone national park. Temp 70 C (158 F)

Thermus aquaticus

Image courtesy of David Ward, Montana State University



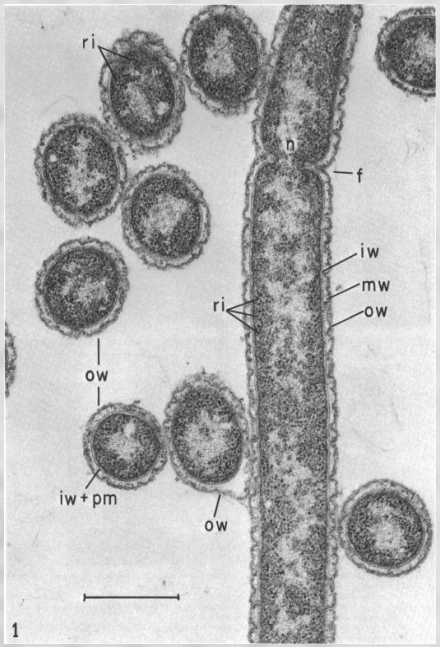
http://en.wikipedia.org/wiki/Thermus_aquaticus

- From Mushroom Spring near Great Fountain Geyser in Yellowstone Park Brock and Freeze isolated *T. aquaticus*
  - T. D. Brock and H. Freeze 1969. *Thermus aquaticus* gen. n. and sp. n., a Nonsporulating Extreme Thermophile *J. Bacteriol.* 98(1): 289–297.
- Thermophiles could also be isolated from hot water heaters, and other “hot” environments.
  - T.D. Brock and K. L. Boylen. 1973. Presence of Thermophilic Bacteria in Laundry and Domestic Hot Water Heaters. *Appl. Microbiology.* 25 (1): 72-76
  - R. Pask-Hughes and R.A.D. Williams 1975. Extremely Thermophilic Gram-negative Bacteria from Hot Tap Water. *J. Gen. Micro.* 88: 321-328

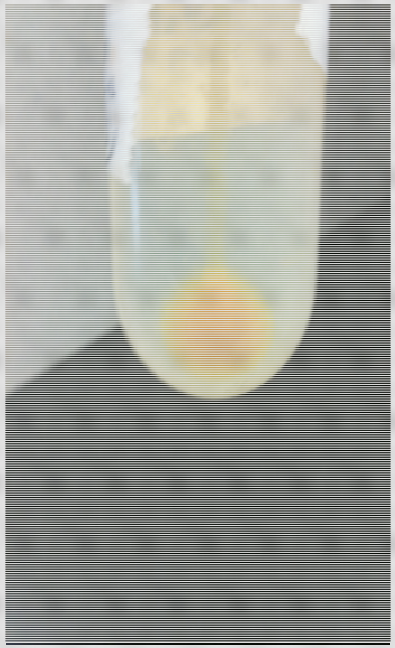
Thermus aquaticus

some characteristics

- Morphology: rod shaped, nonmotile, pigmented
- Gram stain: negative
- Metabolism: obligate aerobe / chemoheterotroph
- Growth media: Castenholz TYE/TT
- Growth Temperature: 70^{o}C
- Doubling Time @ 70^{o}C: 50 min.
- pH optimum: 7.5-7.8



Brock & Edwards, 1970



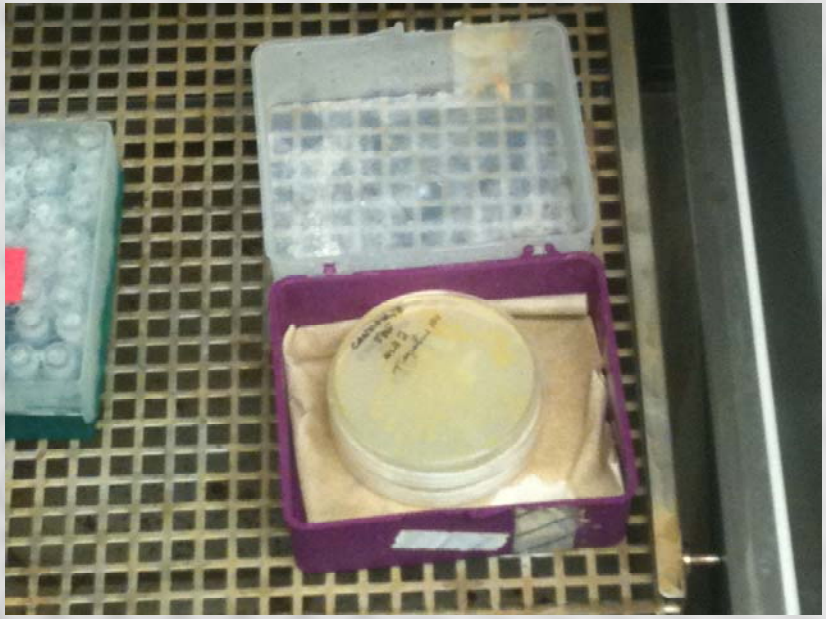
Sources of *Thermus sp.*

- ATCC http://www.atcc.org
  - Strain YT-1 (ATCC # 25104) is source of Taq used in Cetus patents.
  - According to Brock, several isolates were screened for activity by Cetus but YT-1 proved most suitable and served as source for cloning.

- Hot water sources
  - Reported by Brock and Freeze, Brock and Boylen, and later by Pask-Hughes and Williams that *Thermus sp.* Could be isolated from hot tap water and hot water heaters

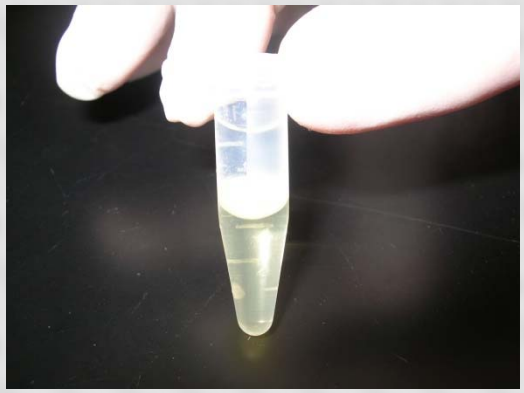
Cultivation of *T. aquaticus*

- Several media formulations:
  - Castenholtz TYE (complicated)
  - TT (simple)
  - USB – Thermophilus vitamin stock
- liquid cultures :
  - No problem with achieving 70^0C with a standard water bath (check specifications)
  - Heavy inoculum from -70^0 (repeated freeze thaw affects viability)
- Solid media
  - More difficult due to drying/condensation on plates
  - Hybridization ovens/platform above hot water bath
  - Higher concentration of agar (3%)

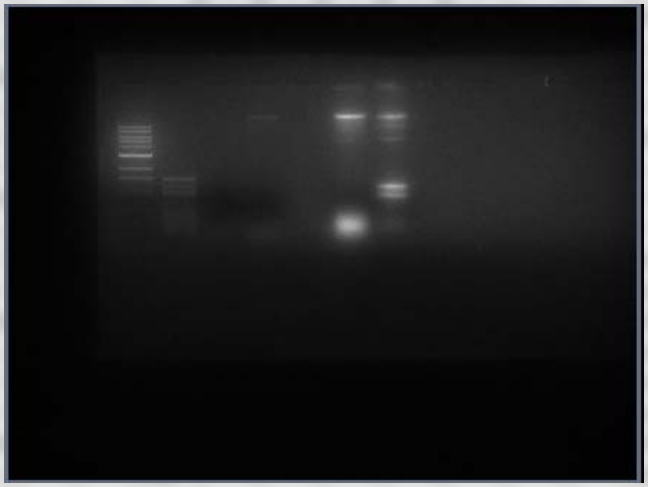


DNA Isolation

- Wizard Preps (Promega) or Phenol/$CHCl_3$ extraction both work well



A B C D



E F

A. 1 Kb ladder
B. 100 bp ladder
C. Wizard Prep (3 mls cells)
D. Homemade prep (3 mls cells)
E. Homemade prep + RNase
F. Homemade prep - RNase

Growth can be problematic

- 2nd generation kits contain lyophilyzed genomic DNA suitable for PCR amplification

- Insert Picture

PCR

- A main focus in our molecular course:
  - What it does, how it can be used, resources

- Goal is that given a particular gene sequence students should be able to design a PCR strategy to clone that gene
  - Primer design
  - Reaction components
  - Reaction conditions

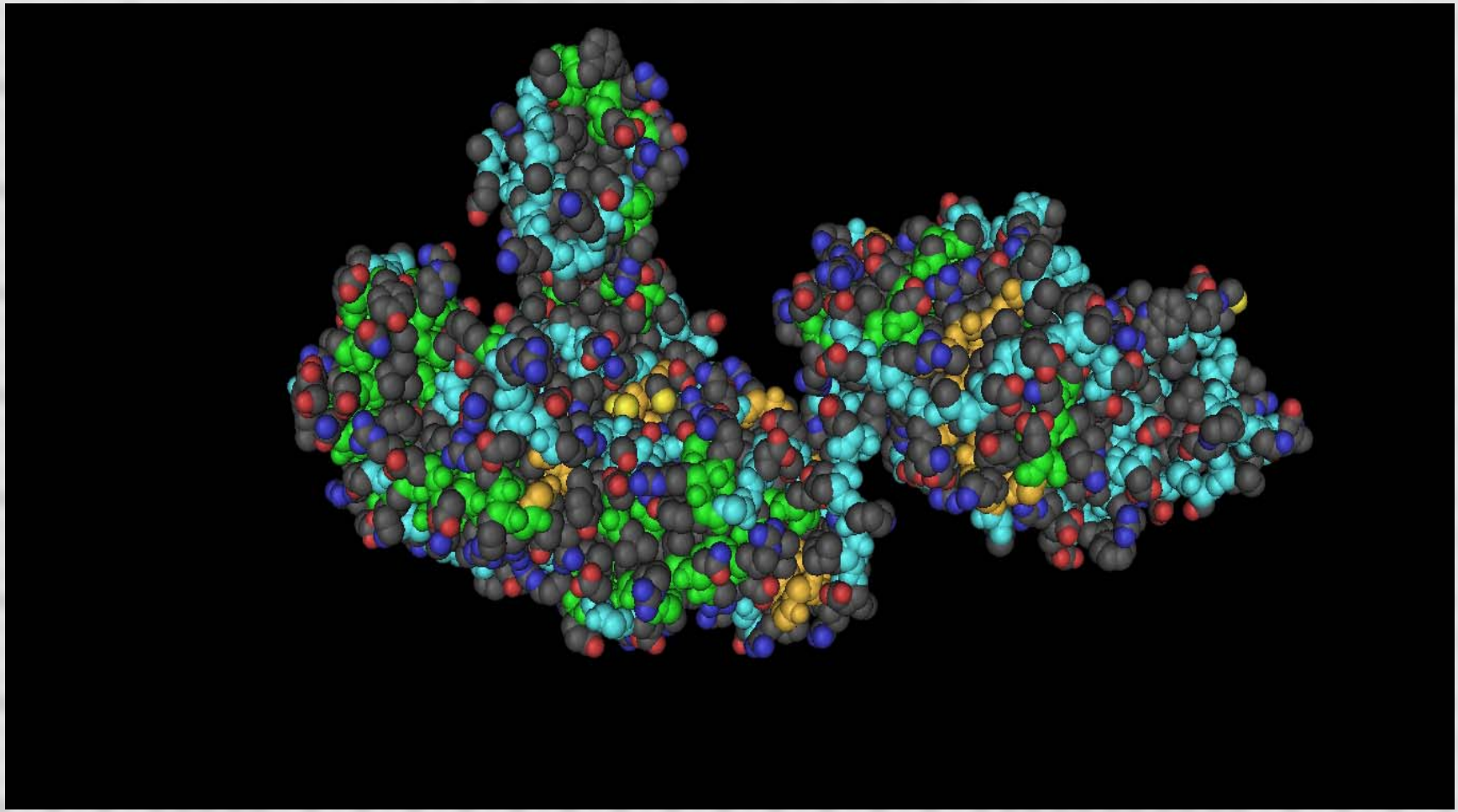
PCR-Objectives

- **Lecture 9: The Polymerase Chain Reaction Part 1: Objectives:**
  - -Be able to describe the components of a typical PCR reaction including reaction concentrations
  - -Be able to calculate the volumes of stock reagents necessary for setting up a PCR reaction
  - -Be able to describe appropriate controls used in PCR

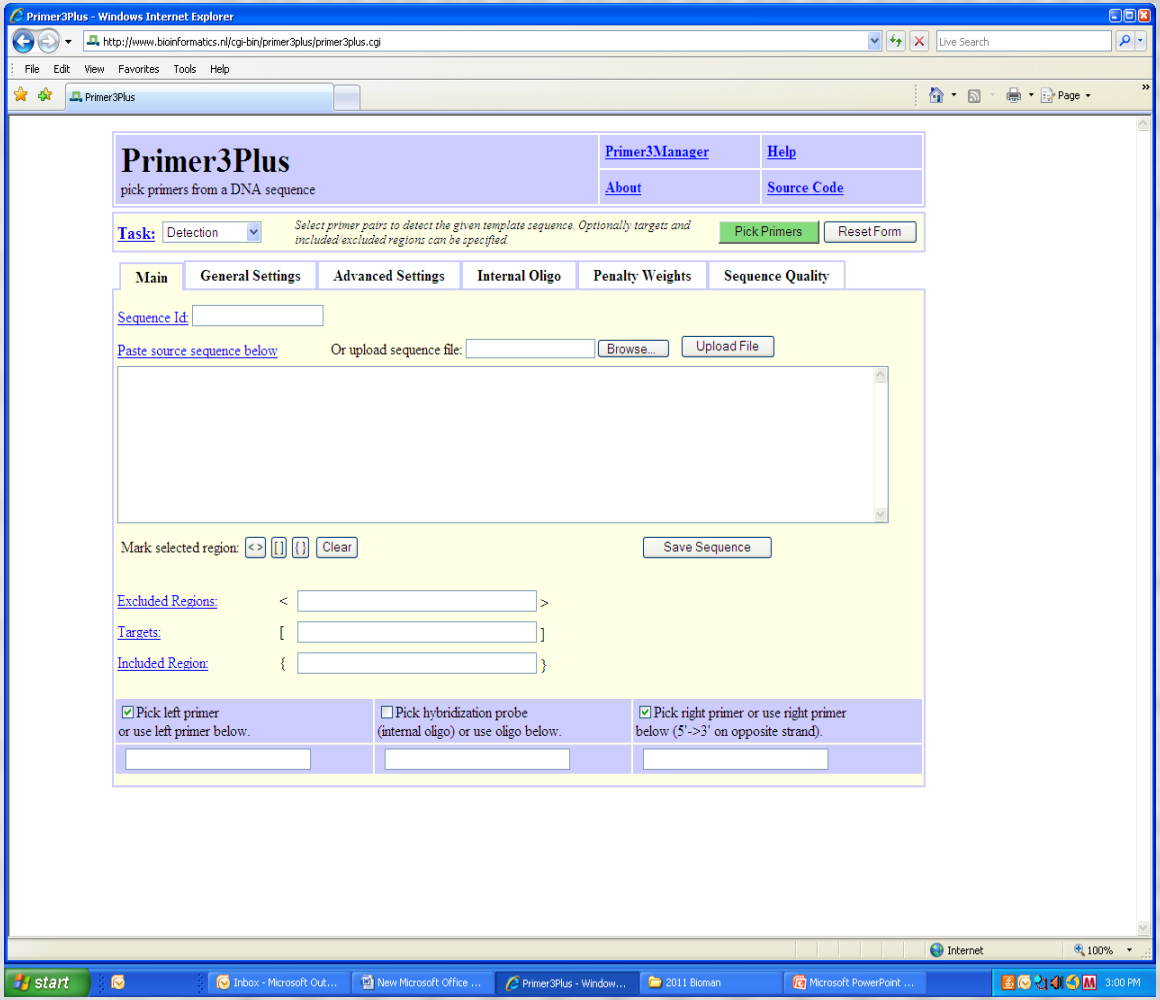
- **Lecture 11: The Polymerase Chain Reaction Part II: Factors that affect primer design - Primer design programs**
  - -Be able to describe criteria for selecting and designing primers for PCR experiments
  - -Correctly calculate the concentration of primers present when reconstituted
  - -Be able to describe different methods for calculating Tm and affects on PCR program
  - -Be able to use software such as Primer 3 to design appropriate PCR primers
  - -Describe the rational for hot start and touchdown PCR

- **Lecture 13: The Polymerase Chain Reaction Part III: Troubleshooting**
  - -Be able to describe common trouble shooting techniques in PCR
  - -Be able to describe the proper use of controls in PCR reactions
  - -Be able to describe and assemble a master mix for PCR
  - -Be able to describe how altering Mg concentration can affect PCR
  - -Be able to describe how DMSO and other enhancing agents are used in PCR

Structure of full length *Taq* polymerase (PDB : 1TAQ)



Primer design



Start students off with basics of primer design, i.e. location, length, Tm, base composition, etc.

Then introduce web based tools for primer design.

Taq polymerase I cloning

- Isolate DNA
- PCR amplify gene
- Digest and ligate into vector
- Analyze clones
- Express protein

PCR assignment

Clone Taq polymerase gene into T7 expression vector

1. Find sequence in Genebank (accession # vs. keywords)
2. Design primers (Primer 3, manual)
3. Do PCR
4. Analyze results !

PCR

- Primers:
  - fwd
    - NNNNNNGGAATTCCATATGCTGCCCCTCTTTGAGCCCAAG
    - *Eco* RI & *Nde* I sites (not underlined)
  - Rev
    - NNNNNNGGAATTCTATCACTCCTTGGCGGAGAGCCAGTC
    - *Eco* RI site (not underlined)

Enzymes

- Initially used enzyme *Pfu* from New England Biolabs – higher fidelity, processivity, and temperature stability then *Taq*
- For lyophilyzed genomic DNA both *Taq* and *Phusion* and *Q5* (maggie) from NEB work well.
  - For class use we now use homegrown Taq and use the original pET-Taq clone (4A4) for protein expression.

Vector- pET-17b

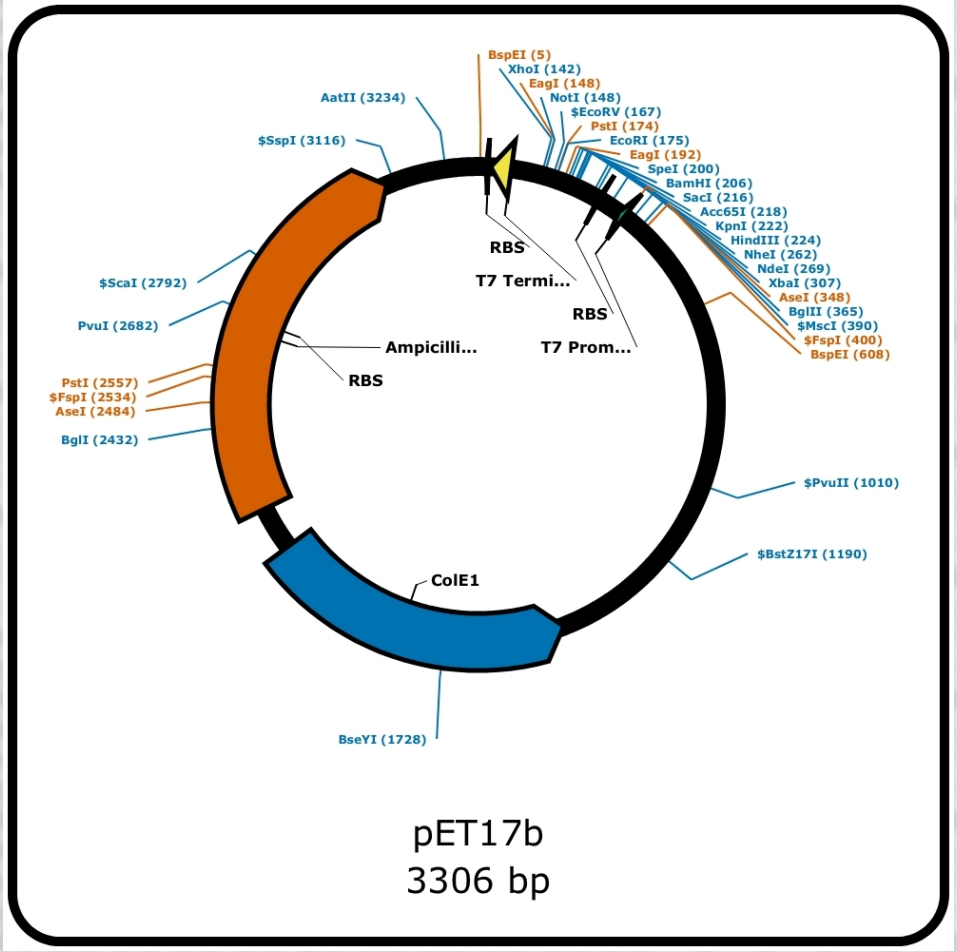
pET vectors developed at Brookhaven national laboratory based on phage T7 gene 10 promoter (Have license to distribute).

T7 RNA polymerase induced by IPTG Requires expression in BL21 (DE3) cells which contain a T7 lysogen

Initial cloning done in JM109 cells then plasmids transferred to BL-21 (DE3) for expression

PlasmaDNA software – available free from:http://research.med.helsinki.fi/plasmadna/

Good program to get students familiar with *in silico* cloning, PCR, ligations, etc.

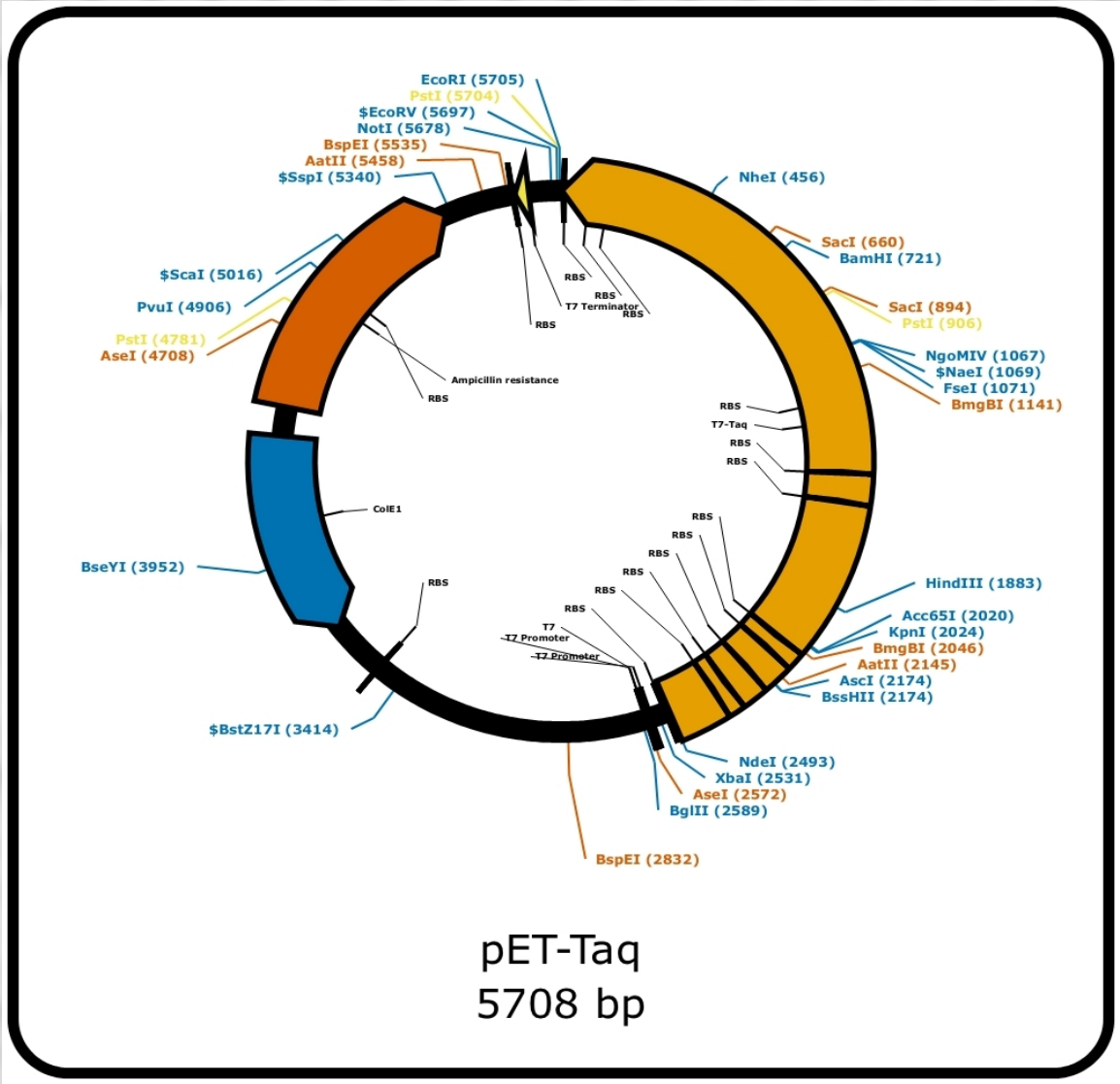


BioBrick® Vectors (NGT)

- BioBrick ® vectors use a standard set of restriction enzymes to allow a “standardized assembly” of genetic constructs.

  - *Eco* RI, *Xba* I (Biobrick ® part) *Spe* I, *Pst* I
    - *Problem with pol* I gene is presence of *Pst*I site.
      - *Did site directed mutagenesis to remove Pst I site and make Biobrick ® compatible*

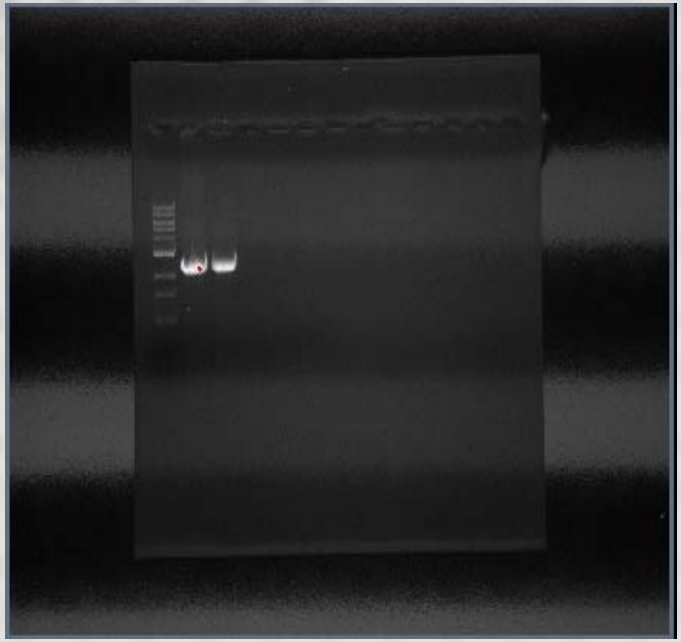
pET-Taq



pET-Taq
5708 bp

PCR

Template (0.6 µg/ µl)	2.5 µl
Fwd Primer (648 µM)	12.0 µl (1:100)
Rev Primer (893 µM)	12.1 µl (1:100)
Buffer (5X)*	10.0 µl
dNTP's (10 mM)	1.0 µl
DMSO (100%)	2.5 µl
Enzyme (*Pfu*)*	0.5 µl
H_2O	9.4 µl
T.V.	50.0 µl



Taq-2 step PCR rxn

1. 98^0 C (30 sec)
2. 98^0 C (15 sec)
3. 72^0 C (1 min)
4. Go to step 2- 29 times
5. 72^0 C (5 min)
6. 4^0 C (forever)

Cloning

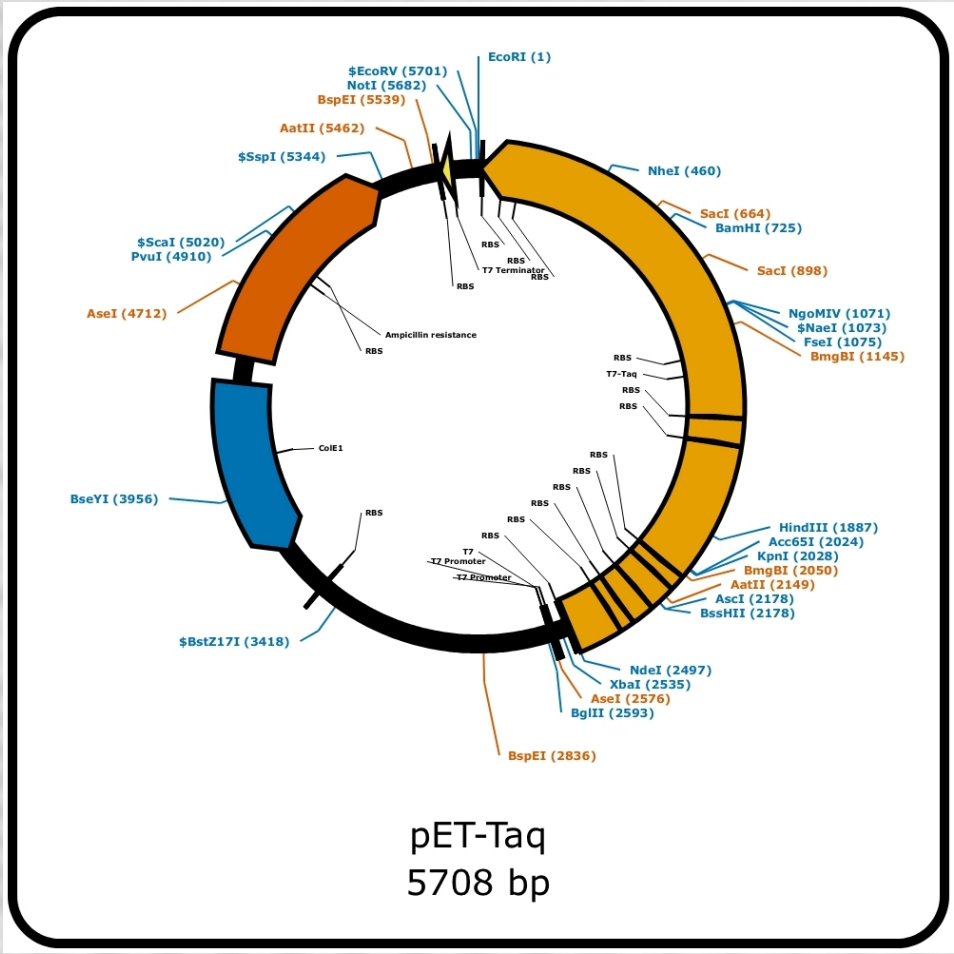
- Isolate fragment with Qiagen Qiaquick gel extraction kit (membrane based method)*
  - See Handout
- Digest with *Nde* I and *EcoI* RI (sequential digestion with *Nde* I first?
- Electrophoresis or PCR cleanup
  - We use electrophoresis and Qiaquick method again
  - Working on onestep digestion/cleanup with *Dpn* I (removes genomic template)
- Ligate into *Nde* I / *Eco* RI digested pET-17b
- Transform
  - Now use homemade cells (TSS) – no need for heat shock & cheaper – student prepared

Cloning

- Analysis of clones
  - Colony PCR (most common problem is too much template)
    - Pick colony-patch small amount (1/4 inch) on plate, remainder suspended in 50 µl H_2O-use 5 µl for template (may have to dilute)
  - Mini-Prep
    - Have used both home-made STET preps (boiling mini-prep) and kits (Qiaquick again!)
    - Equivalent yields but Qiaquick is faster

pET-Taq

1. T7 gene 10 promoter
2. Ampicillin resistance
3. ColE1 origin of replication
4. No T7 “tag” due to use of *Nde* I site.
5. BL21 (DE3) pLysS strain of *E. coli* for expression
6. IPTG inducible



PlasmaDNA program :
http://research.med.helsinki.fi/plasmadna/

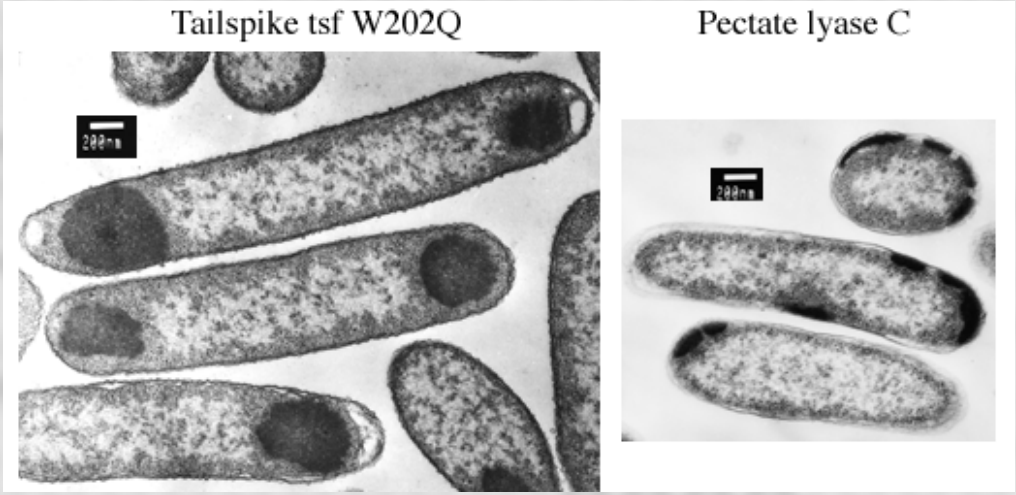
PlasmaDNA: a free, cross-platform plasmid manipulation program for molecular biology laboratories BMC Molecular Biology 2007, 8:77

Small scale expression

- Finish up molecular course with small scale expression, extraction and activity assay (see hand out)
  - Introduces protein techniques
  - Discuss tech-transfer (transfer of R&D products to production)
    - Scale-Up
    - Specifications
    - Quality assays
    - SOP's
    - Master Cell Bank characterization (ICH guidelines)

Small scale expression

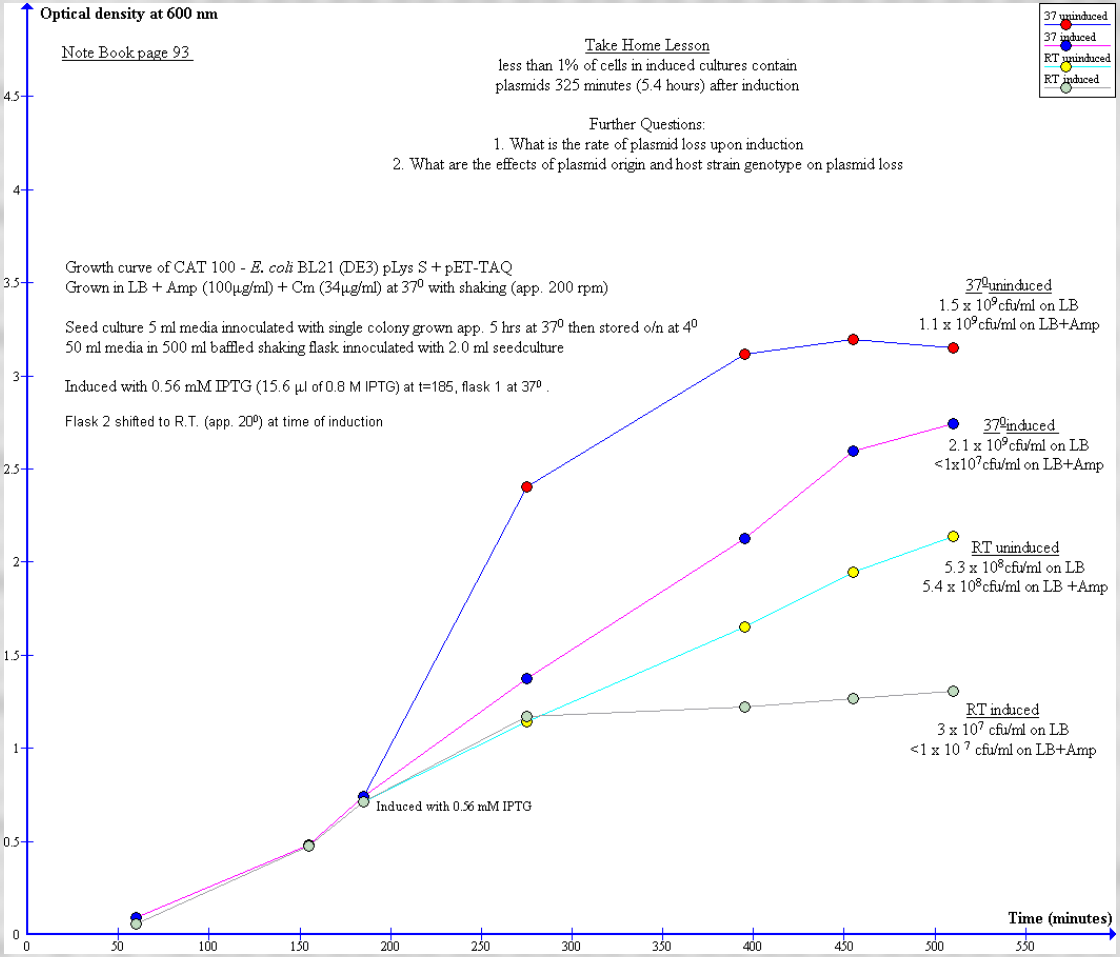
- Induction at 30^0 C / R.T. increases yield of soluble active enzyme
  - Inclusion body formation:
    - Possible to purify enzyme from inclusion bodies but we have not tried-typically not suitable for large scale
  - Similar effect seem with GFP expression using pET



Small scale expression

- Grow cells at 37^0 C till O.D. ~1.0
  - Equilibrate cells at 30^0 C and add IPTG to 0.4 mM
  - Let grow several hours to overnight
    - Significant plasmid loss during expression!
  - Harvest!

Plasmid Loss



Results of experiment performed by biomanufacturing students Fall, 2011

Small scale purification

- Harvest by centrifugation in microfuge
  - 16,000 x *g* for 1 minute
- Cells lysed in Tris, Glucose, EDTA + lysozyme
- KCl, Tween-20, & IPEGAL CA-630 (detergents) added and extract heated to 80^0 C for 30 minutes
- Insoluble fraction centrifuged out at 16,000 x *g* for 10 minutes
- Soluble fraction stored in NaCl, DTT, Triton X-100, and glycerol

Small scale purification

- Relatively crude preparation (DNA contamination) but suitable for many routine PCR amplifications

  - In manufacturing course we use more extensive purification (Ammonium sulfate precipitation, Dnase treatment, DEAE column, ethanol extraction of DNA, size exclusion)

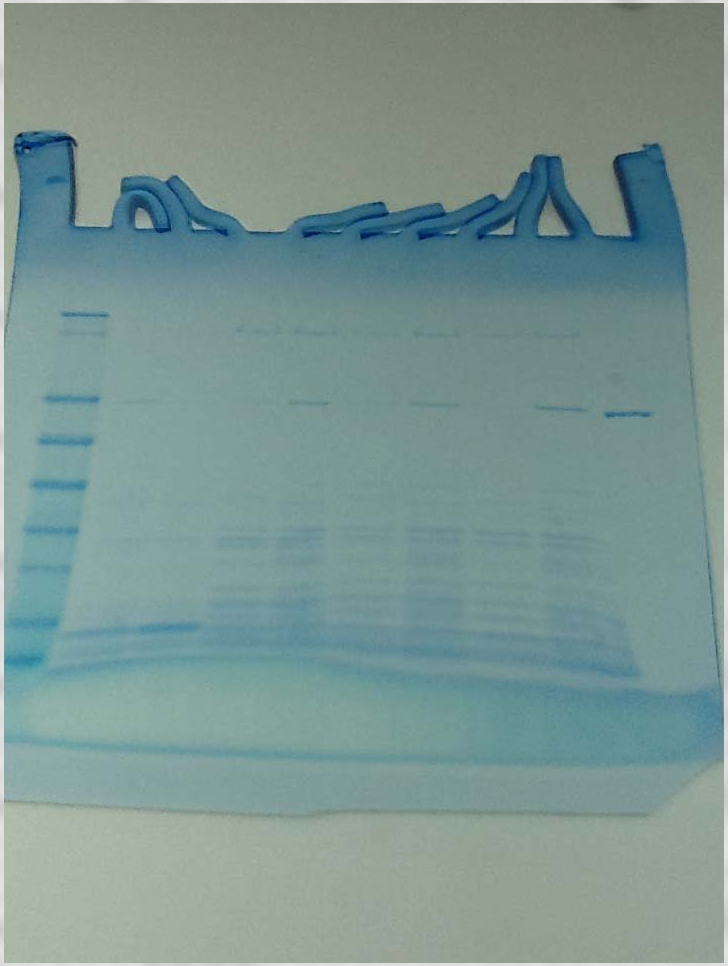
Heat Treatment Denatures & Precipitates Proteins

Samples after 30’ heat treatment at 80^{o}C
And centrifugation for 10’ at max rpm (14K).

Quality Control

- Basic QC
  - SDS-PAGE (protein size, absence of contamination)
  - Agarose electrophoresis (DNA contamination)
  - Western analysis (protein identity)
  - PCR assay (activity)
  - labeled nucleotide incorporation (quantitative activity assay)

SDS-PAGE Gel



4-20% Tris-Glycine gel ran at 125V for 90 minutes. Last Lane is positive control (commercial Taq preparation)

Note contaminating proteins in crude prep.

Bacterial DNA Contamination

0.7% Agarose gel of crude Taq preparations

Manufacturing

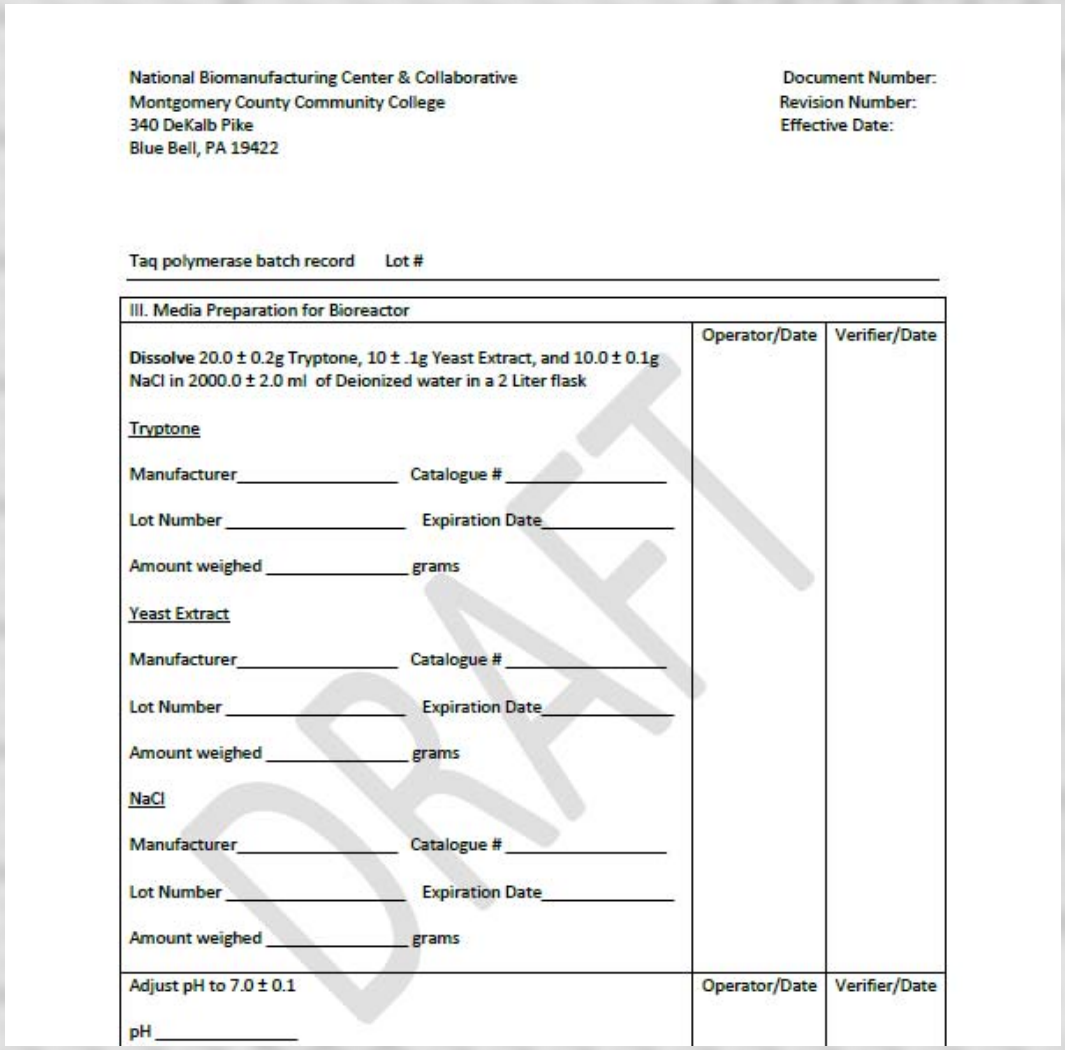
Reproducible production of product that meets predetermined specifications

- What are the specifications for final product
  - Appearance – color, clarity
  - Chemical characteristics- pH, SDS-PAGE, UV absorbance
  - Activity – qualitative, quantitative *
  - Contaminants – DNA, RNA, endo and exo nucleases, endotoxin

Documentation

- Foundation of cGMP's !
  - SOP's
  - Batch Records
  - Quality control assays
  - Labeling!

Batch Records



National Biomanufacturing Center & Collaborative
Montgomery County Community College
340 DeKalb Pike
Blue Bell, PA 19422

Document Number:
Revision Number:
Effective Date:

Taq polymerase batch record Lot #

III. Media Preparation for Bioreactor		
Dissolve 20.0 ± 0.2g Tryptone, 10 ± .1g Yeast Extract, and 10.0 ± 0.1g NaCl in 2000.0 ± 2.0 ml of Deionized water in a 2 Liter flask Tryptone Manufacturer_____ Catalogue # _____ Lot Number _____ Expiration Date_____ Amount weighed _____grams Yeast Extract Manufacturer_____ Catalogue # _____ Lot Number _____ Expiration Date_____ Amount weighed _____grams NaCl Manufacturer_____ Catalogue # _____ Lot Number _____ Expiration Date_____ Amount weighed _____grams	Operator/Date	Verifier/Date
Adjust pH to 7.0 ± 0.1 pH _____	Operator/Date	Verifier/Date

DRAFT

Development of Large Scale Batch Records in progress-anticipated Completion Fall 2013

Scale-Up

- BioFlow 110-115 bench top reactors running biocommand software
- 2 Liter cultures in synthetic media + glycerol
  - In manufacturing course first exercise is small scale media study (LB, minimal, NEB fermentation media)

Large scale purification

- Ammonium sulfate ppt.
- Dnase treatment
- Size exclusion chromatography

Process Development

- Robust, scalable processes
  - Goal is to take what we have learned at small scale and apply it to large scale (economics) production
  - Upstream (i.e. fermentation & expression)
  - Downstream (i.e. purification)

Scale-Up

- Yield from small scale culture –
  - 2 ml/LB+Amp+Cm / 30^0 C / 20 hr induction

    ~ 2,000 units / ml of culture

  Q. What can we get from 2,000 ml culture ?

  Q. What about production loss?

Upstream

Scale Up

Shaker Flask

Bench Top Fermentor

Pilot Scale Fermentor

Production scale Fermentor

Upstream Goal-Maximum cell density

- Physical conditions
  - Temperature control
  - pH control
  - O_2 availability
- Media
  - Simple / complex / antibiotics / cost!
- Feeding strategies
  - Batch, Fed Batch, continuous
- Plasmid stability
  - antibiotic resistance markers

Upstream Goal-Maximum active protein expression

- Alternative Promoters & RBS
- Active vs. inactive protein
- Strain improvement

Downstream

Scale Up

Downstream Goal-Maximum product recovery

Use of scalable technologies – filtration, chromatography

Purification

Avoiding loss during purification-minimize steps, but maintain purity !

- Harvest / Cell lysis
- Heat inactivation
- Concentration with TFF
- DNA extraction
- Ion exchange chromatography
- Size exclusion polishing step
- Final concentration / buffer exchange
- Packaging and labeling

Stability

- Stability studies are a fundamental concept in drug development
  - We haven't done these with Taq, but plan on doing so.
    - Home grown Taq is good for several months when stored at -20
    - Once have quantitative assay down we will see if we can quantify stability

Labeling & Lot release

- In development

Kits

- Taq cloning kit
  - *T. aquaticus* genomic DNA, PCR primers, transformation buffer, *E. coli* strain
- Taq purification kit
  - E. coli strain with pET-Taq plasmid, cell lysis buffer, heat inactivation buffer, storage buffer

Kits

- Taq quality control kit
  - Western Blotting, activity assay, ELISA
- Anti-Taq hybridoma (preliminary development)

Where do I get it!

Kits and Complete curriculum available from BioMan store (when)

References

Literature:

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Web Resources:

- Genebank accession number for sequence J03469
- PlasmDNA software
- Primer 3 – primer design software
- Novagen pET-vector manual