Polymerases and polymerase chain reaction (PCR)

Dr. Nick Coleman

Polymerases and PCR

Learning outcomes. Be able to…

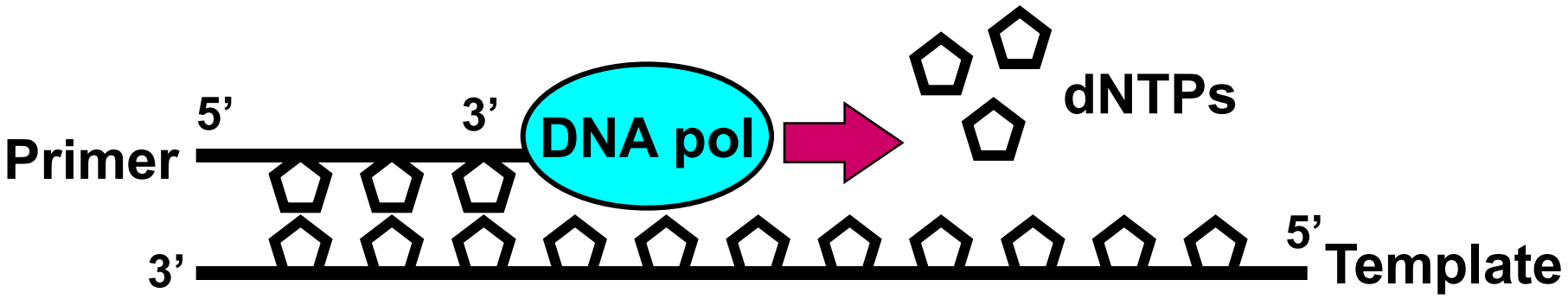
- **Define the functions of the following DNA polymerases and describe their relative advantages / disadvantages, and their useful applications in microbiology:**
  - **Taq**
  - **Pfu**
  - **Reverse transcriptase**
  - **Phi29**
- **Explain in detail how PCR works, and describe the major factors that influence the success of PCR**
- **Interpret and troubleshoot PCR experiments**

DNA polymerases

- All organisms depend on DNA polymerases for replication and repair of DNA
- Most are DNA-directed (copy from DNA template) but some are RNA-directed (copy from RNA template)

General properties of DNA polymerases:

- Require single stranded DNA or RNA template
- Require an RNA or DNA primer to begin reaction
- Require deoxynucleoside triphosphates (dNTPs)
- Only polymerize in 5'→3' direction



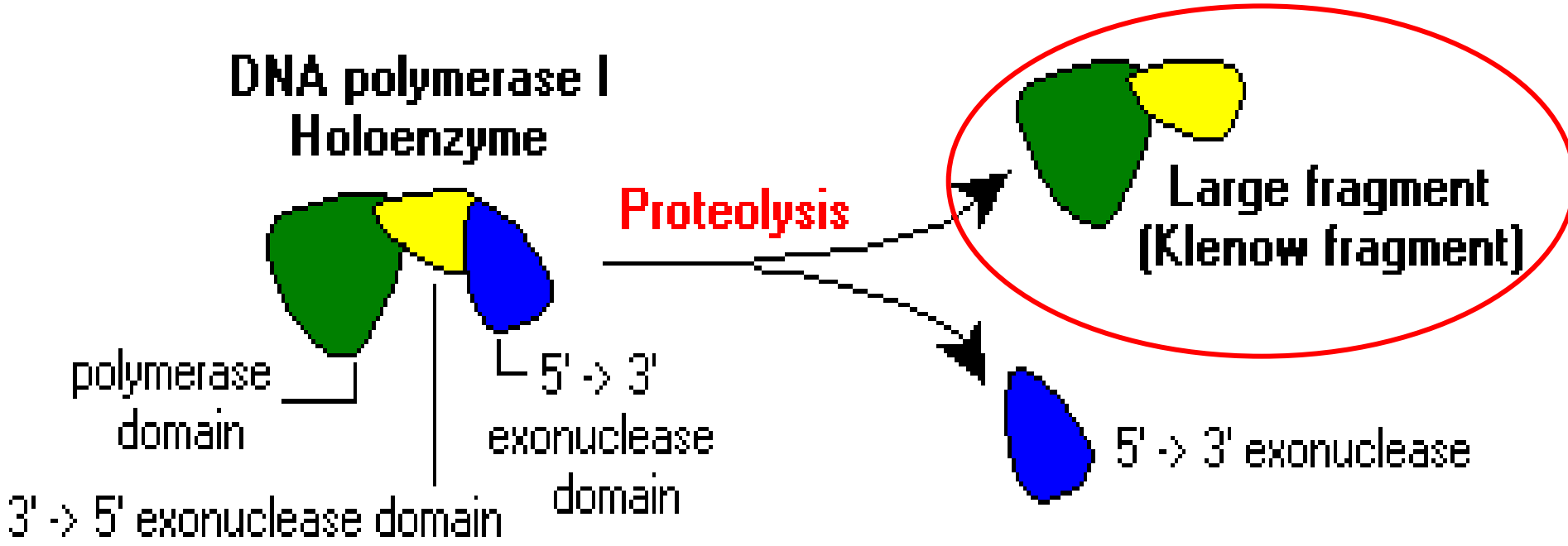
DNA polymerases

Properties relevant to applications in mol.micro.

- **Error rate:** DNA replication is a critical cellular function, error rate is low (10^{-4} to 10^{-6} per base), but not zero!
- **Extension rate:** speed of polymerization; ranges from ~20 bases/sec (DNA pol I) to 1000 bases/sec (DNA pol III)
- **Processivity:** distance travelled before 'falling off', ranges from ~100 (DNA pol I) to ~500,000 bases (DNA pol III)
- **Thermostability:** for use in PCR, polymerase must have thermostability and a high optimum temp. for activity

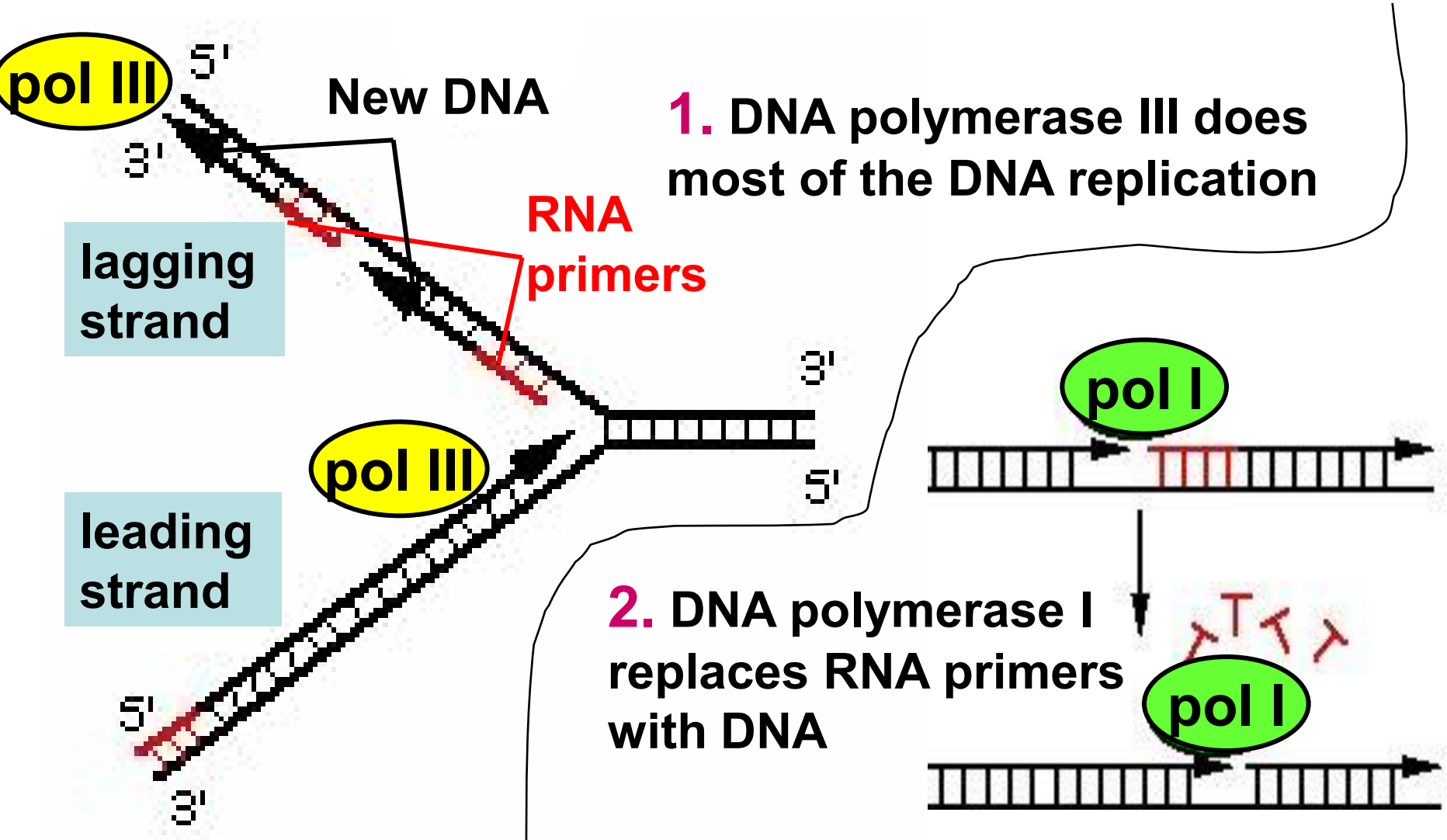
DNA polymerase I

- **Different domains of DNA pol I:**
  - **DNA-directed DNA polymerase: copies DNA 5'→3', makes double-stranded DNA from single-stranded DNA**
  - **5'→3' exonuclease: removes upstream RNA primers**
  - **3'→5' exonuclease: proofreading of new DNA strand**



DNA polymerase I

- Specifically useful for completing DNA synthesis on '<u>lagging strand</u>' during DNA replication

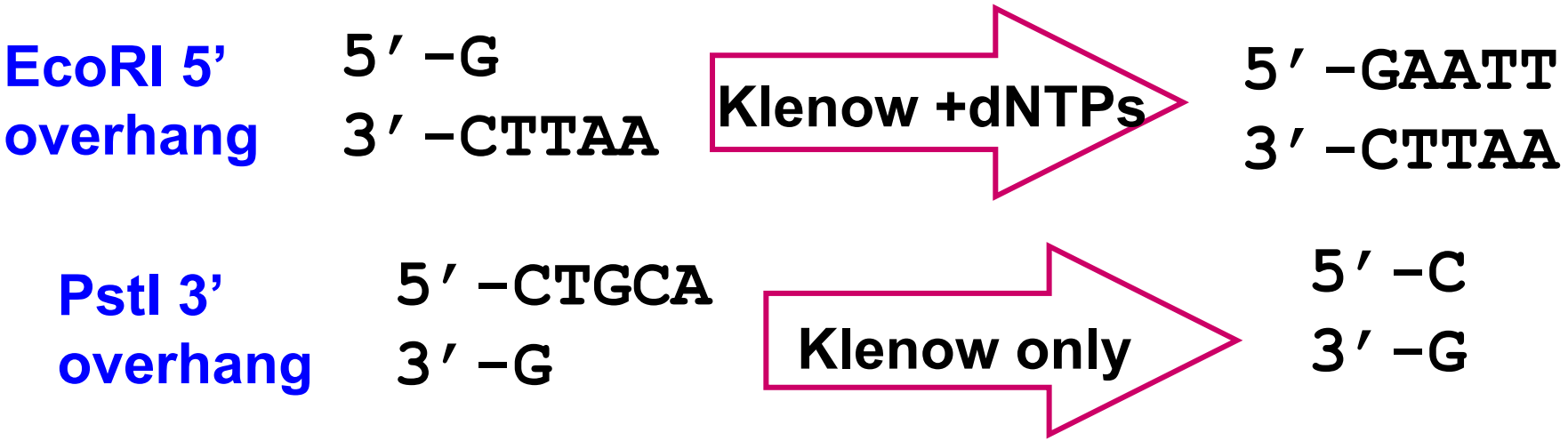


DNA polymerase I

- Klenow is a fragment of *E.coli* DNA polymerase I, produced by proteolytic treatment with subtilisin

How is Klenow used?

- Blunting sticky ends produced in restriction digests
  - 5' overhang: filled in by polymerase, needs dNTPs
  - 3' overhang: removed by exonuclease, don't add dNTPs



- Blunting allows more versatile use of restriction enzymes

Taq polymerase

- "Taq polymerase" is the DNA polymerase I of the hyperthermophilic bacterium *Thermus aquaticus*
- Properties and function similar to DNA pol. I of *E.coli*, but lacks 3'-5' exonuclease (proofreading function), and has high temperature optimum for activity (70-80°C)
- Taq is the crucial component that allowed commercial development of the polymerase chain reaction (PCR)

Hot spring in Yellowstone National Park, USA, source of *T.aquaticus* and PCR

Importance of environmental microbiology to biotechnology

Pfu polymerase

- **"Pfu polymerase" is the DNA polymerase I of the hyperthermophilic archaeon *Pyrococcus furiosus***

Domain ARCHAEA

Phylum *Euryarchaeota*

Class *Thermococci*

Strictly anaerobic motile cocci.

Temperature optimum of 100ºC.

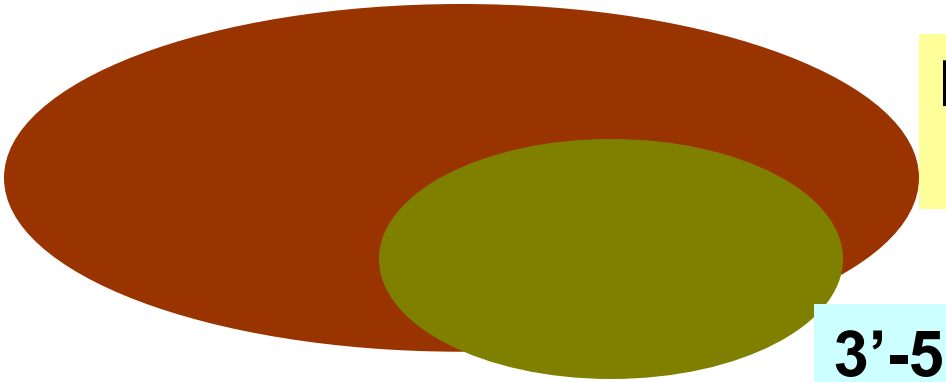
Fermentative heterotrophs.

Hydrothermal vent habitat.

Genome sequenced (1.9 Mb)

Pfu polymerase

- **Monomeric, soluble, 90 Kda protein**



Polymerase domain

3'-5' exonuclease proofreading domain

Advantages:

- **Error rate lower than *Taq* polymerase** ★
- **Thermostability higher than *Taq***

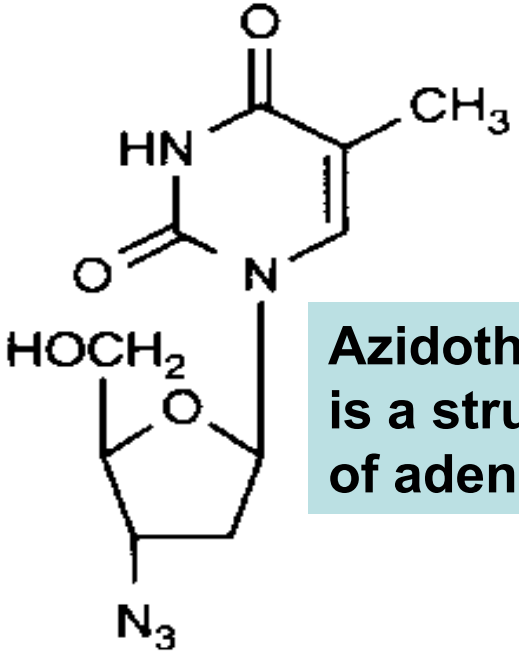
Disdvantages:

- **Extension speed is slower than *Taq***
- **3'-5' exonuclease can 'eat' primers**

Reverse transcriptase (RT)

- RNA-directed DNA polymerase – an exception to the 'central dogma' of molecular biology (DNA→RNA→protein)
- Derived from retroviruses, eg. Moloney murine leukemia virus: RNA genome → DNA → integrates into host genome
- Medical significance: Several AIDS medications (eg. AZT) are specific inhibitors of RT enzyme in HIV

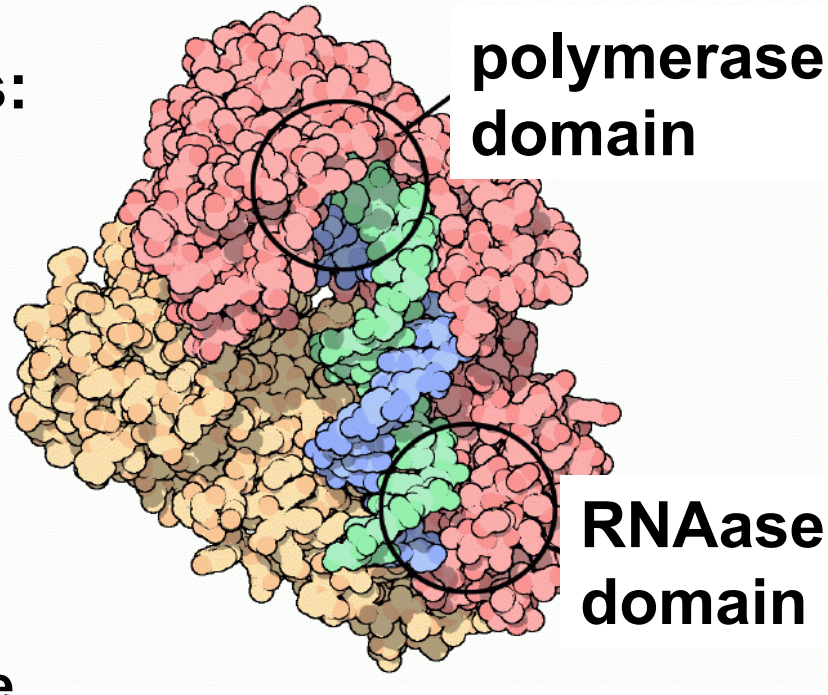
Model of HIV surface

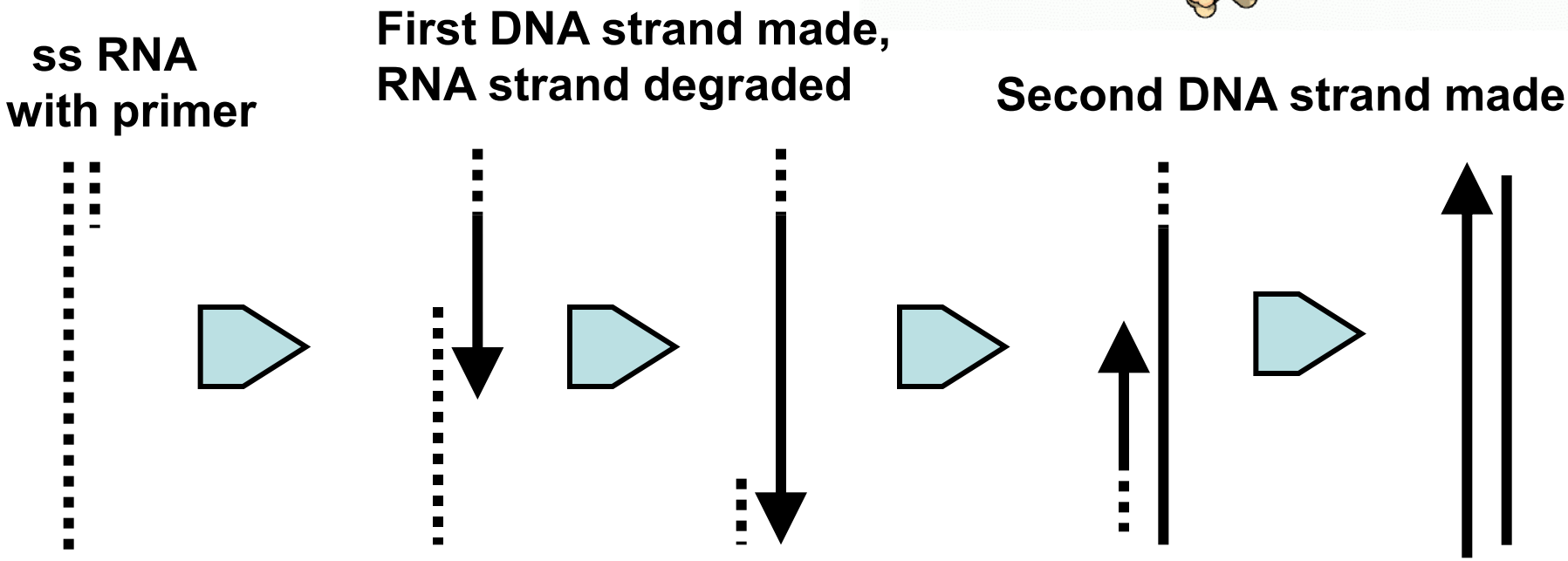


Azidothymidine (AZT) is a structural analog of adenine base in DNA

Reverse transcriptase (RT)

- Similarities with other DNA pols:
  - –make DNA in 5'→3' direction
  - –need primer, dNTPs, Mg^{2+}
- Differences to other DNA pols:
  - – Use RNA or DNA template
  - – contain RNAase H domain

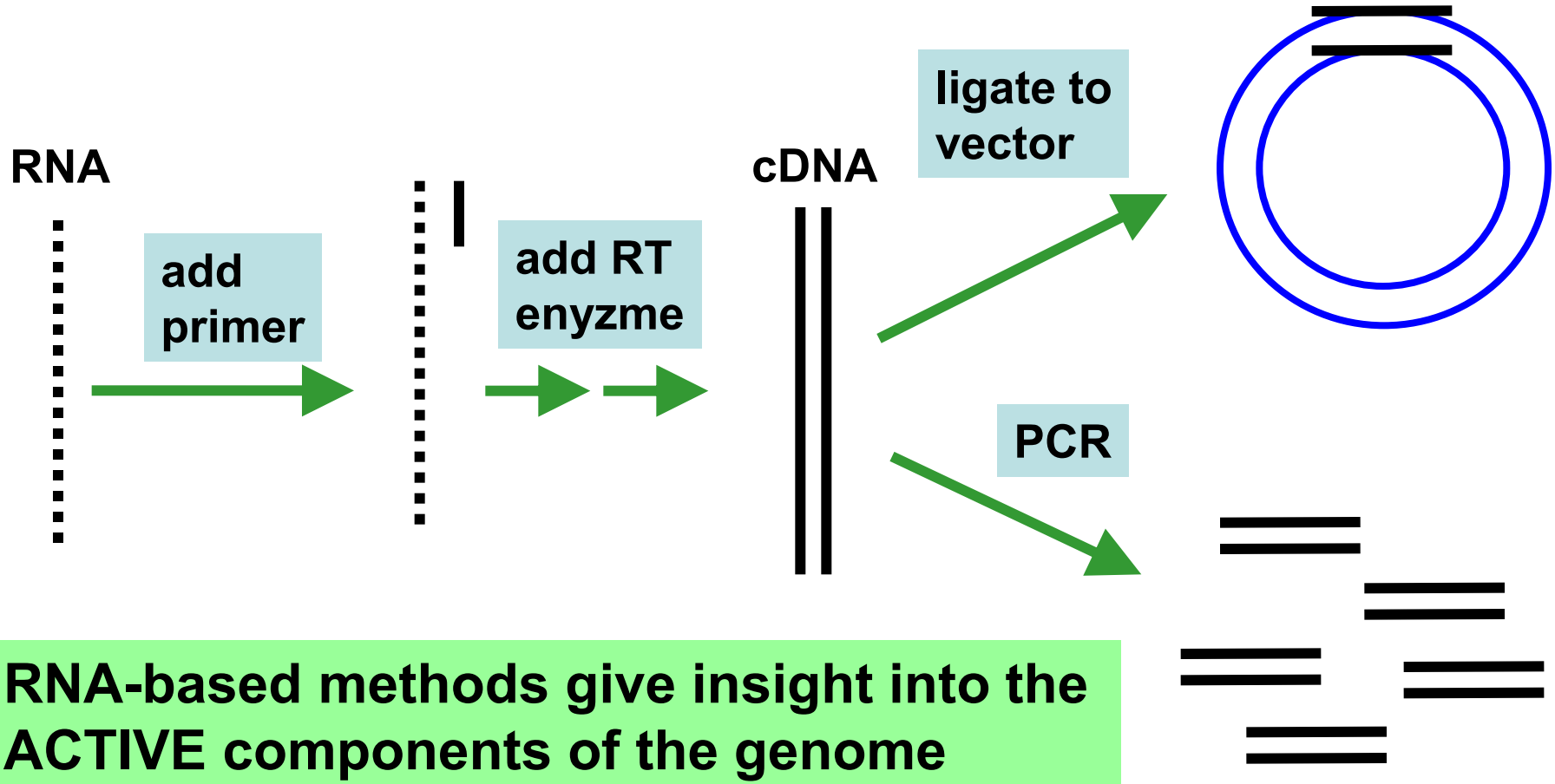




Reverse transcriptase (RT)

Applications

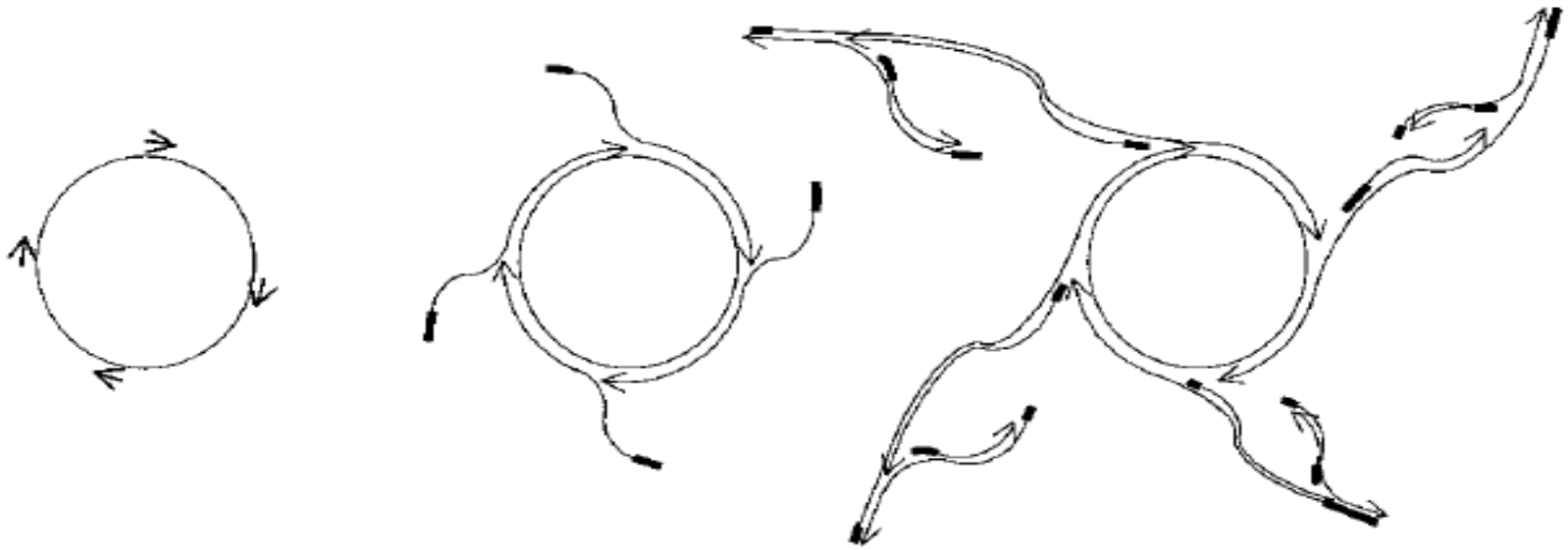
- Complementary DNA (cDNA) made from RNA is useful for identifying genes that are EXPRESSED
- Approaches: either cDNA library or RT-PCR



RNA-based methods give insight into the ACTIVE components of the genome

phi29 polymerase

- Replication enyzme of *Bacillus* phage phi29
- Distant homolog of DNA pol I of bacteria / archaea
- Extremely processive enzyme – does not detach easily from template strand; can replicate up to 70 kb of DNA
- Strand-displacing activity; rolling circle amplification



Dean et al, 2001. Genome Res.

phi29 polymerase

Applications: "Whole Genome Amplification"

- **Unbiased amplification of genomic DNA from very small amounts of starting template**
- **NOT a type of PCR ! Only one primer is used (usually NNNNNN), reaction is isothermal (no thermocycling)**
- **Extremely useful for :**
  - **Forensic trace samples**
  - **Unculturable bacteria**

OPEN ACCESS Freely available online

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PLoS one

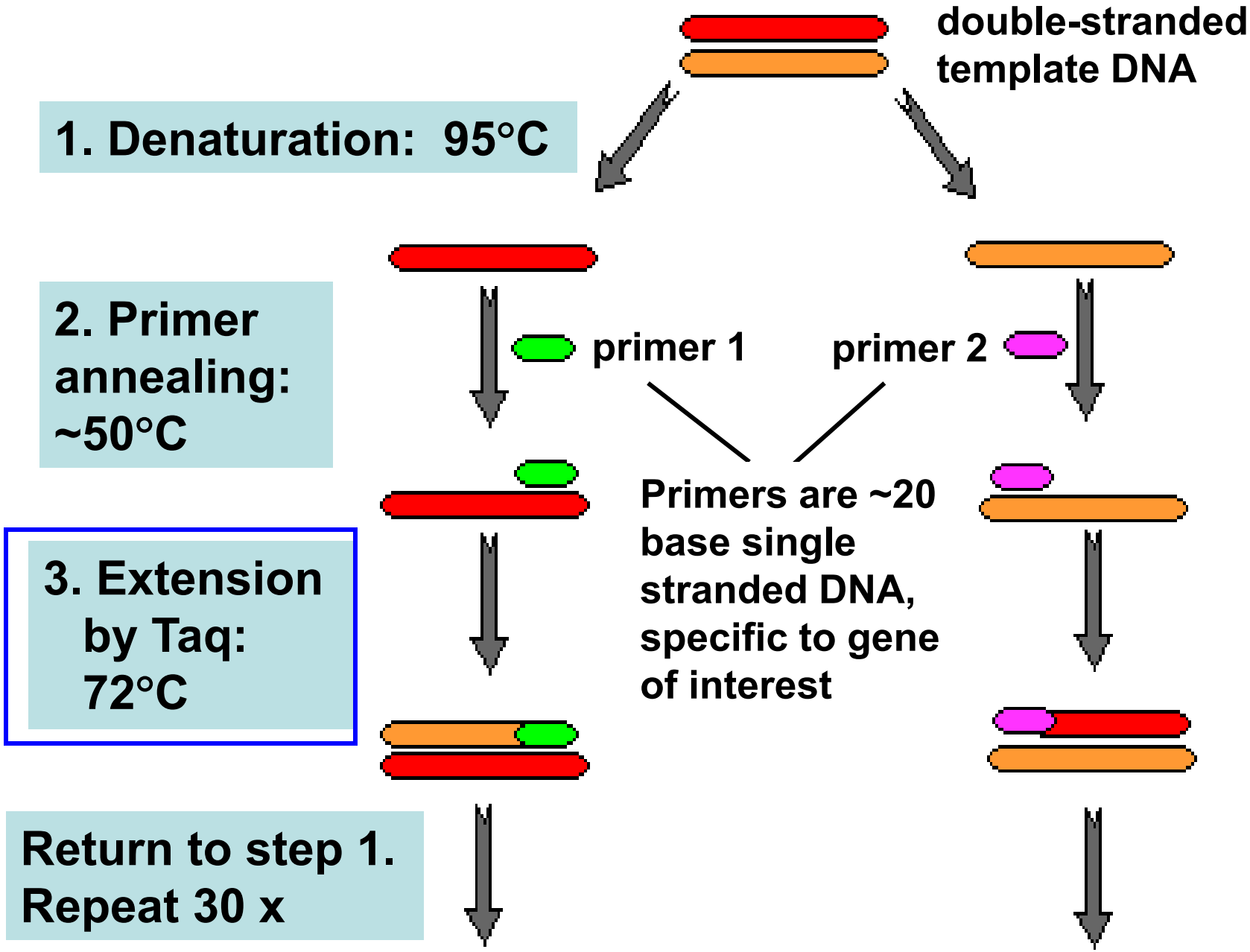
One Bacterial Cell, One Complete Genome

Tanja Woyke[1], Damon Tighe[1], Konstantinos Mavromatis[1], Alicia Clum[1], Alex Copeland[1], Wendy Schackwitz[1], Alla Lapidus[1], Dongying Wu[1], John P. McCutcheon[2], Bradon R. McDonald[2], Nancy A. Moran[2], James Bristow[1], Jan-Fang Cheng[1]*

1 Department of Energy Joint Genome Institute, Walnut Creek, California, United States of America, 2 Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, United States of America

Pipette Tip Personalities

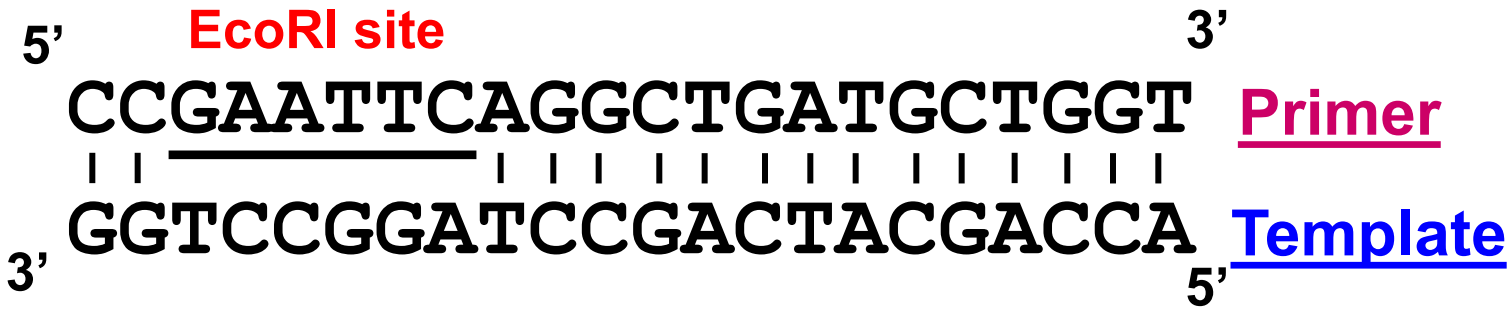
How does PCR work?



Significance of PCR

- Allows specific and sensitive amplification of a target gene, even from complex samples and/or small amounts of DNA

- VERY useful for cloning genes
  - easily make large amounts of a specific DNA
  - can include restriction sites at 5' end of primers, which are incorporated into PCR product



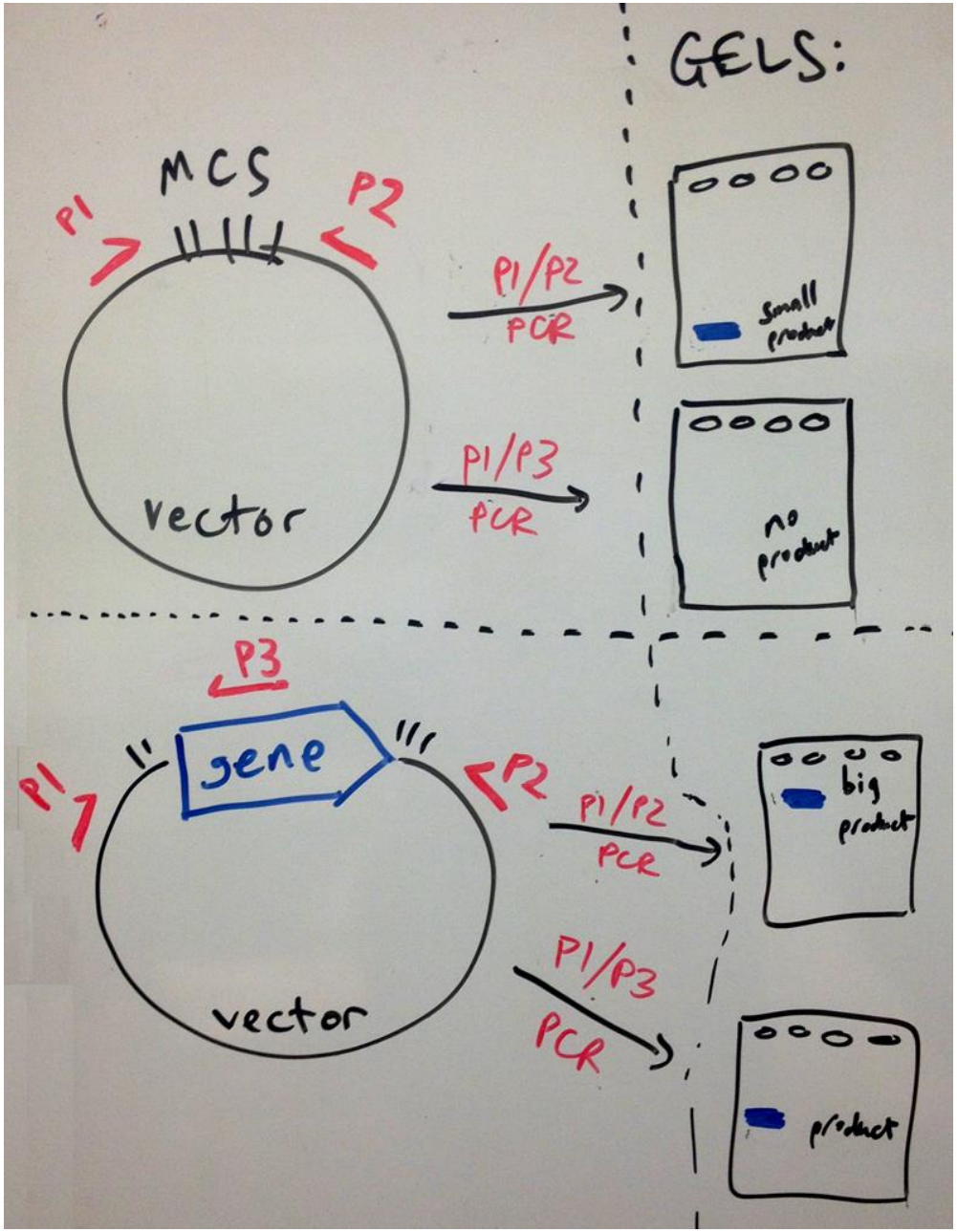
PCR tolerates mismatches at 5' end of primer, but not at 3' end because Taq binds at 3' end

Significance of PCR

- **PCR is invaluable for finding positive clones in libraries**

- **Different combinations of primers can be used → info. on presence and size of insert DNA**

- **Many clones can be screened – much less work than doing plasmid extractions + restriction digests**

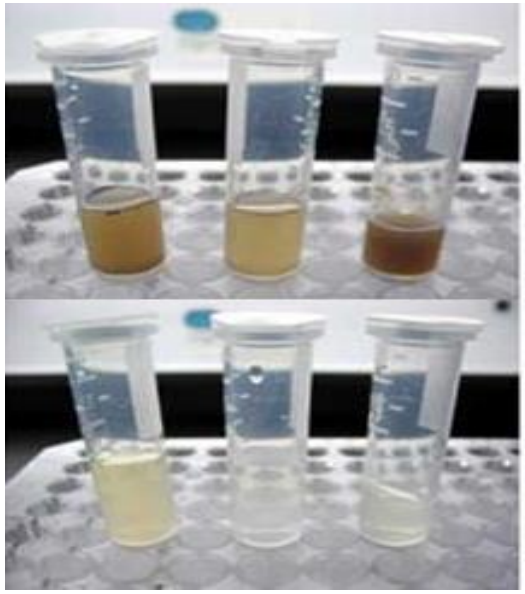


Troubleshooting PCR. 1. No product

Inhibitors in template?

- **PCR-inhibitory chemicals are common in many types of samples, and may co-extract with DNA.**

eg. Soil : humic acids
Blood : haemoglobin
Faeces: bilirubin
Bone: calcium



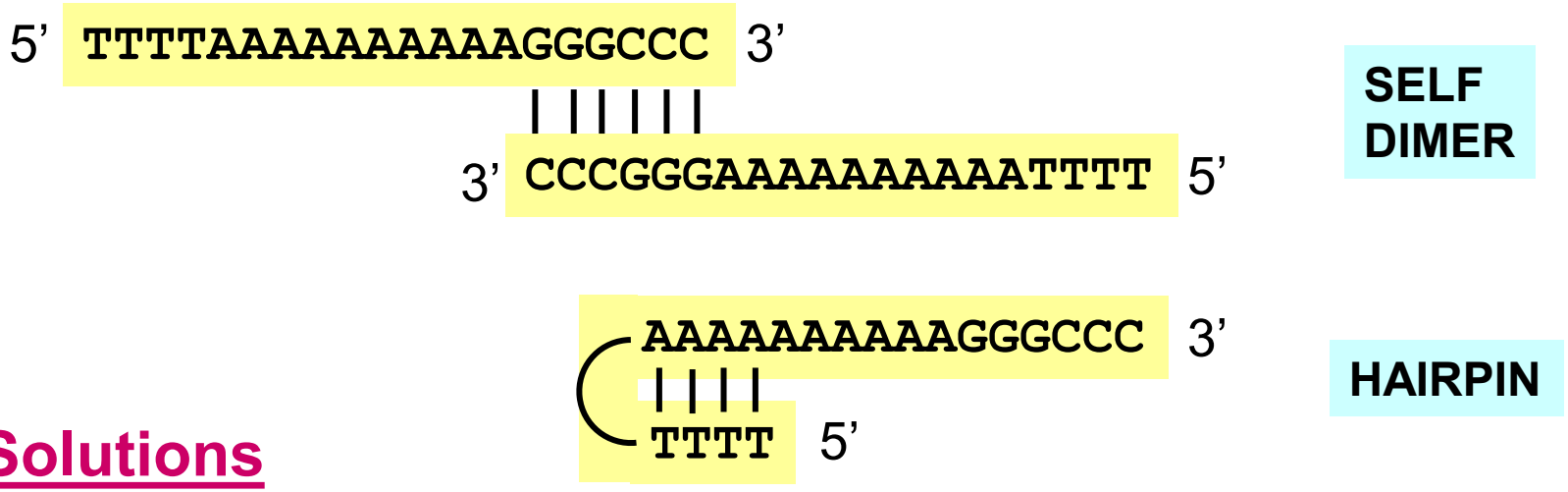
Solutions

- **Test different dilutions of template DNA**
- **More-rigorous / different DNA purification method**
- **PCR additives eg. bovine serum albumin (BSA)**

Troubleshooting PCR. 1. No product

Primer not binding to the template ?

- **Primers can form self-dimers / heterodimers / hairpins → these structures don't bind well to the template DNA**
- **Multiple primer-binding sites may be present in the template, which compete with the intended site**



Solutions

- **Good primer design**
- **Check uniqueness of primers by BLAST search**

Troubleshooting PCR. 1. No product

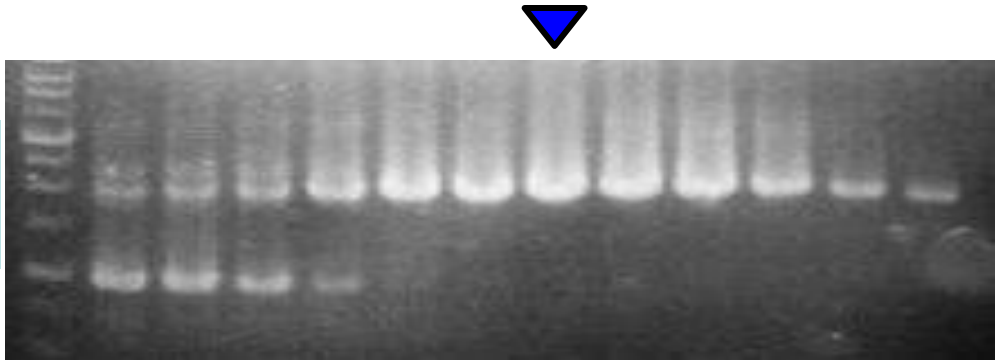
PCR conditions wrong ?

- Annealing temperature? Extension time? No. of cycles?

Bad reagent ?

- Repeated freeze-thawing damages reagents
- Contamination with nucleases

Annealing temp. gradient reveals optimum Tm



Solutions

- Use gradient to optimise annealing temp.
- Single-use aliquots of reagents
- Systematic troubleshooting and use of controls
- Excellent aseptic technique

Troubleshooting PCR. 2. Too many products

→ **Primers are binding non-specifically**

Solutions

- **Redesign primers**
- **Increase annealing temp.**
- **Lower the Mg^{2+} conc.**
- **Lower template DNA conc.**
- **Fewer PCR cycles**

Design of PCR primers - factors to consider

- where do forwards and reverse primers bind?

- what is the size of the expected product?

- do the primers make self- or pair dimers?

- do the primers make hairpins?

- what are the predicted melting temperatures (Tm)?

- do the primers bind in unexpected locations?

- do any bases need to be changed? (eg. adding a restriction site, introducing a mutation in a gene)

PCR Protocol

Picture credits

http://www.nps.gov/yell/nature/thermophiles/biopro.html
http://www.rrsjamescook.com/Oceanography
http://i.zpravy.cz/05/111/Incl/VVRec358_hiv.jpg
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