What can molecular biology do for you?

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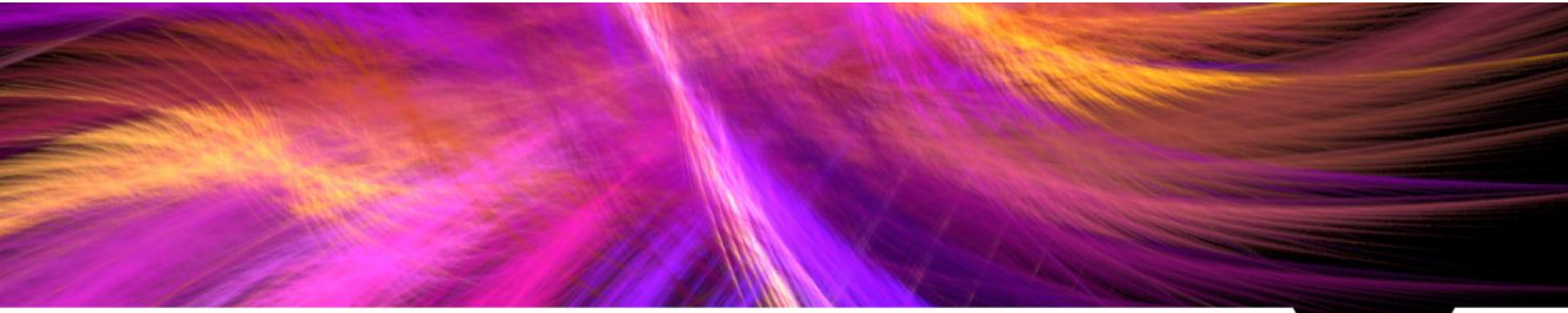
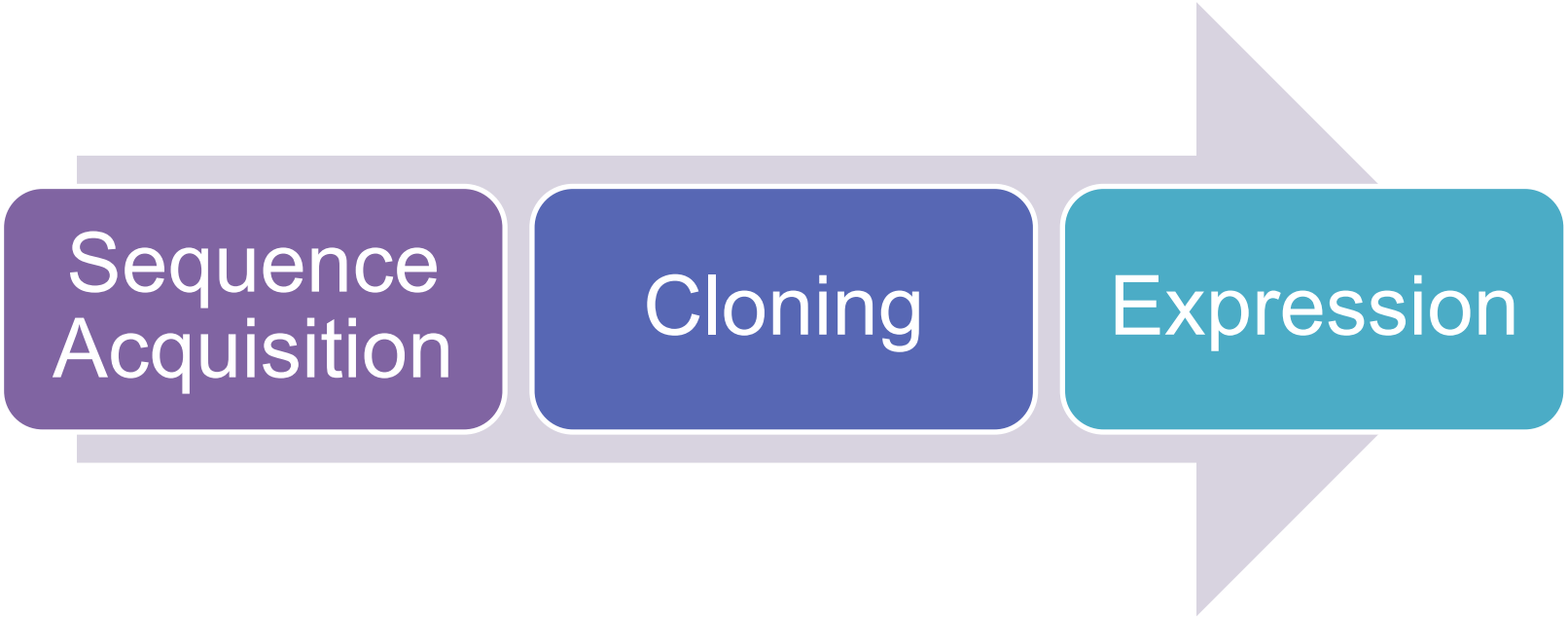


Midlands Young Researcher Meeting on Nanomaterials, 17-12-15
University of Warwick

WARWICK

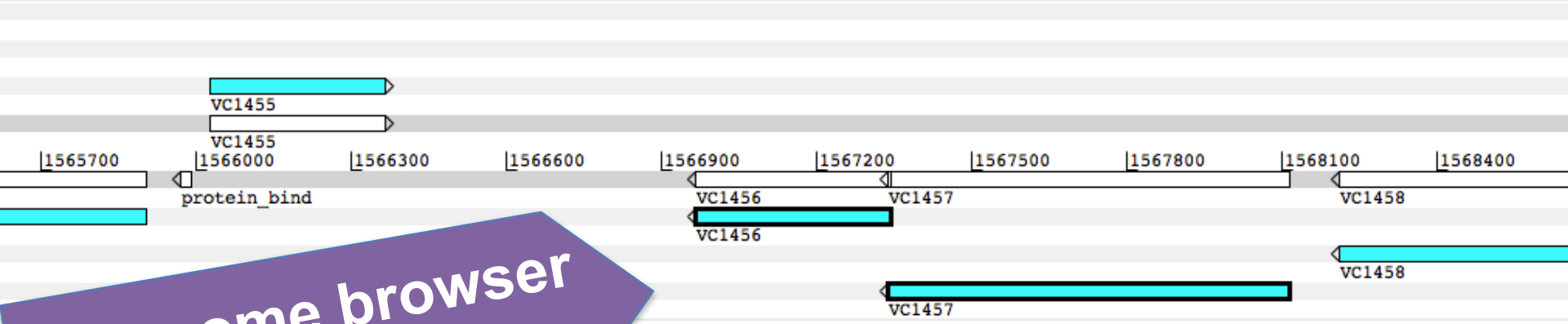
Talk Outline

- Classic molecular biology workflow
- Alternative techniques
- Why it might be useful to Gibson lab work



Sequence I: Identify Gene of Interest

Step 1: Find your gene sequence of interest, e.g. via:



A **Genome browser** (e.g. Artemis)

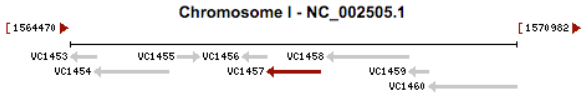
Summary

Gene symbol	VC1457
Gene description	cholera enterotoxin subunit A
Locus tag	VC1457
Gene type	protein coding
RefSeq status	REVIEWED
Organism	Vibrio cholerae O1 biovar El Tor str. N16961 (strain: N16961, serotype: O1, biotype: El Tor, old-name: Vibrio cholerae O1 biovar eltor str. N16961)
Lineage	Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio

Genomic context

n: chromosome: I

ence: Chromosome: I; NC_002505.1 (1567338..1568114, complement)



Genomic regions, transcripts, and products

Genomic Sequence: NC_002505.1

Go to reference sequence details

Go to nucleotide: Graphics FASTA GenBank

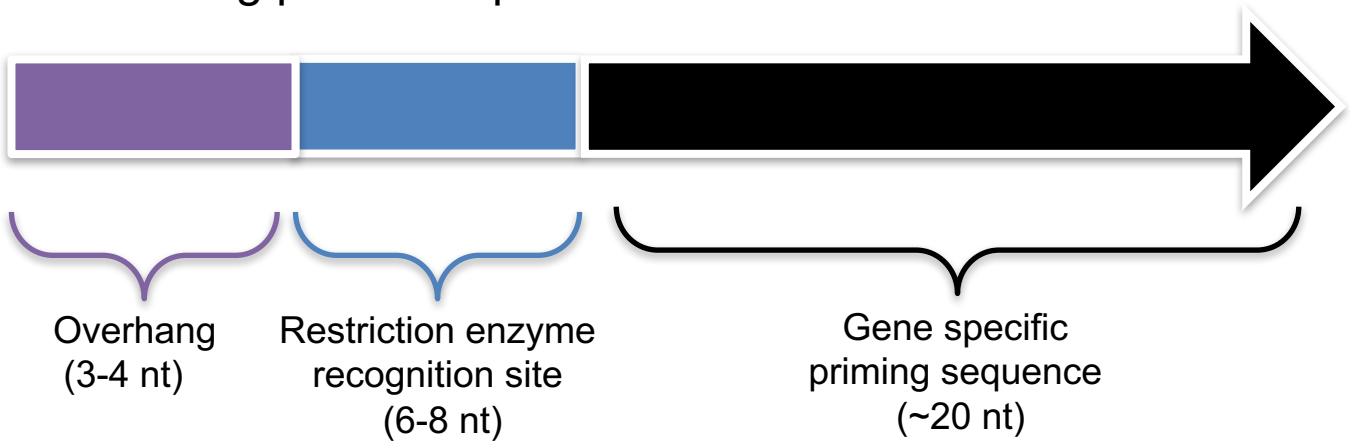
NC_002505.1: 1.6M..1.6M (1.0Kbp) C | Find: | Tools | Tracks

Web resources (NCBI's BLAST results shown here)

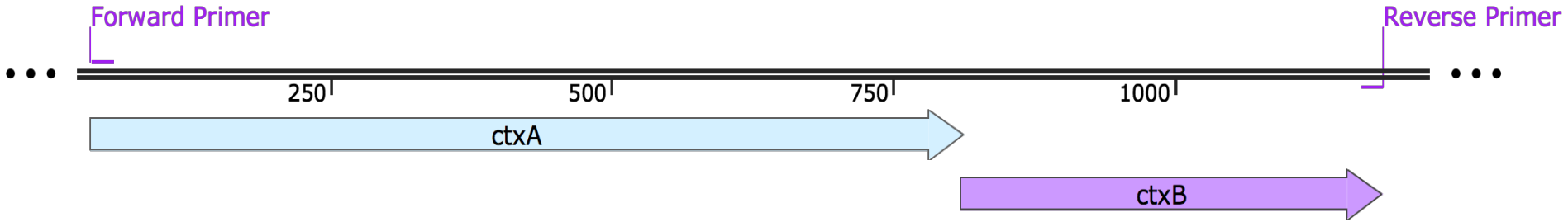
Sequence II: Designing Primers

Step 2: pull out the relevant sequence and **design primers**

Basic 'layout' of a cloning primer sequence:

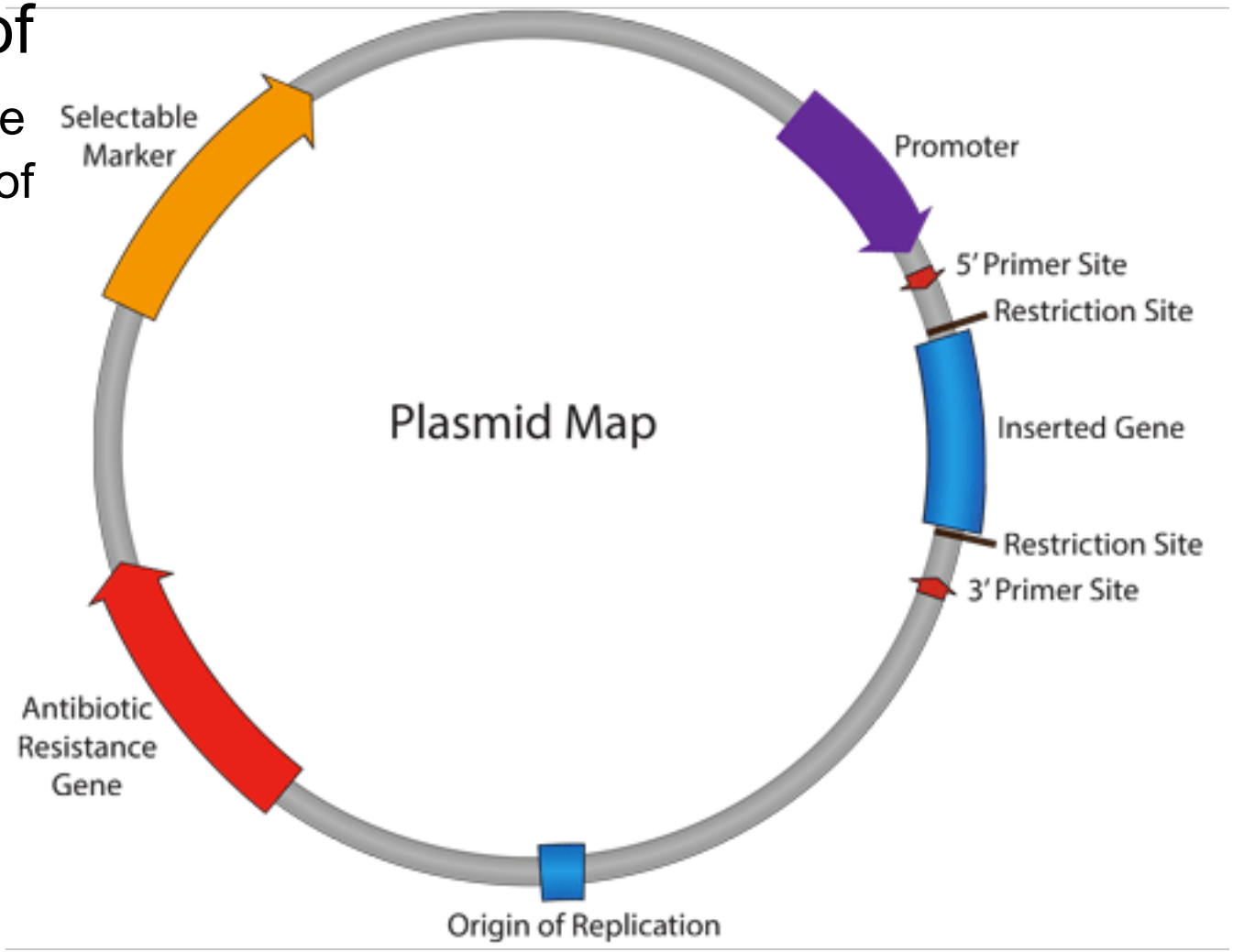


2 primers **(a 'Forward' and 'Reverse') flank the genes**, binding to each strand:



Sequence III: Choosing a Vector

Basic structure of a plasmid (but there are literally thousands of different versions)



Sequence III: Choosing a Vector

Examples of **common vectors/vector types**

Vector	Application
pBAD30	Protein expression inducible with arabinose
pET29	Tagging expression vector, can incorporate Strep-Tag and/or C-terminal HIS-tag
pSGFP2-C1	Plasmid for expression of GFP fusion proteins in mammalian systems.
pBluescript	Expression vector with both antibiotic and colour (“Blue/White” Screening) selection
pWEB	A ‘cosmid’ for cloning of larger sequences and packaging them in to phages

Cloning I: PCR of Insert Sequence

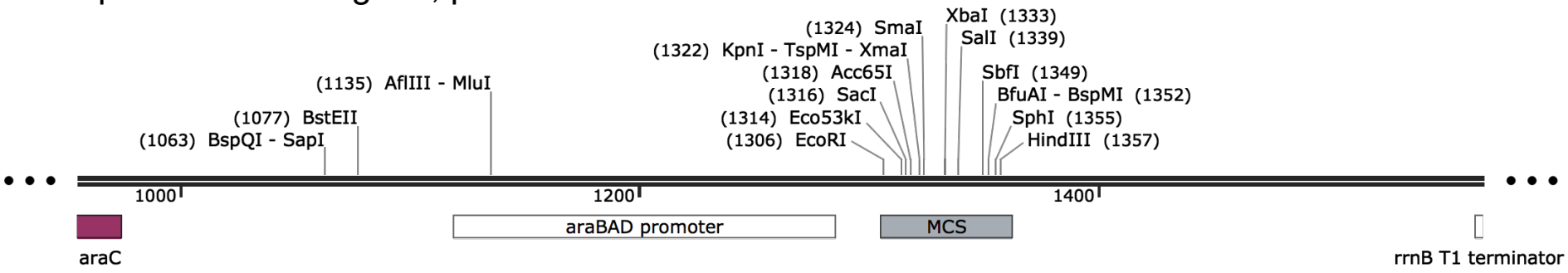
Step 3: Choosing a vector – depends on purpose, sequence type, availability….
- Your choice of vector dictates your choice of enzymes. May also influence extraction effciency/method

Hypothetical experiment - Inserting *ctxAB* in to expression vector pBAD30:

Forward Primer (EcoRI): GAGGAATTCATGGTAAAGATAATATTTGT

Reverse Primer (HindIII): TTTAAGCTTTTAATTTGCCATACTAATTG

Once primers are designed, proceed to **PCR**…

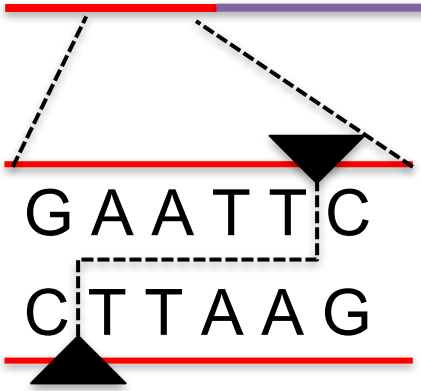


Cloning II: Digestion and Ligation Reactions

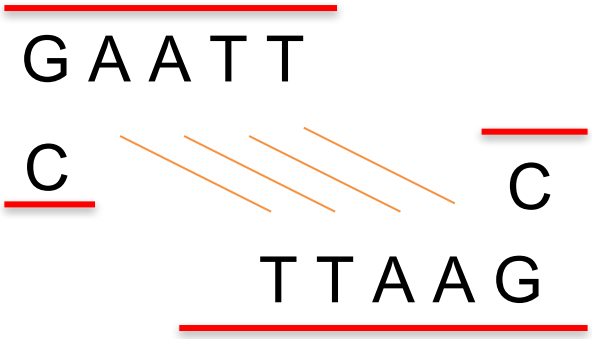
Gene is amplified by PCR

Primers with Restriction Sites/overhangs are used

Produces many copies of the gene flanked by the overhangs with restriction sites.



Restriction enzymes usually cut ('digest') asymmetrically, leaving complementary overhangs.



MCS (Left)

MCS (Right)

PCR → Digest → Ligate → Transform → Sequence

Cloning III: Common alternative approaches

A number of other methods exist that aim to remove troublesome/ tedious elements of 'classical cloning':

- **T/A Cloning:**
  - Exploits natural overhangs from certain polymerase enzymes such that restriction sites aren't needed.

- **LIC (Ligation Independent) Cloning:**
  - Uses natural ligases of host organisms to do away with the ligation step.

- **GATEWAY® Cloning:**
  - Uses viral recombinase enzymes to swap sequences between plasmids. Allows for multiple subcloning in to a variety of vectors for different applications.

- **Gibson Assembly:**
  - Single reaction that is capable of stitching multiple overlapping fragments together.

- **Recombineering**:
  - Uses direct recombination events to insert PCR products with single bp fidelity.

- **CRISPR-Cas9:**
  - Exploits a bacterial nuclease guided by an RNA to make specific base pair edits.

Cloning IV: Some considerations

Ease of cloning is HUGELY dependant on the protein/cell in question.

Some **considerations** when cloning:

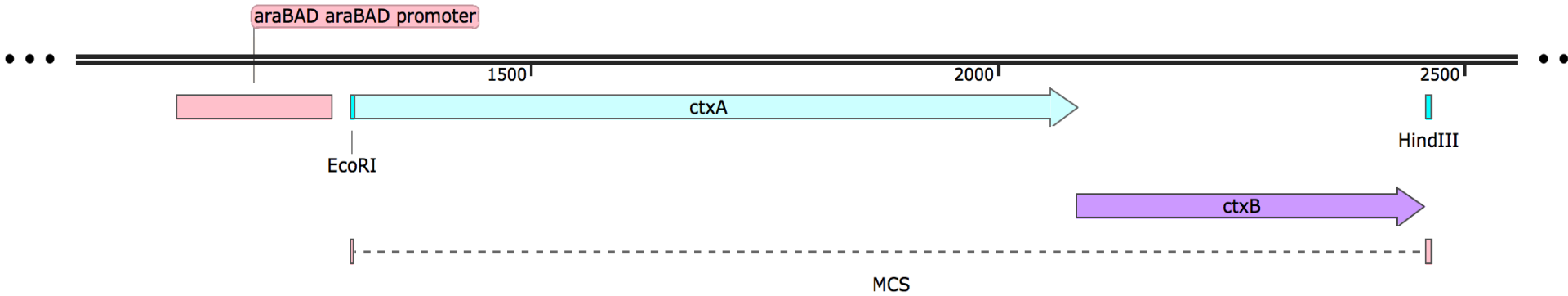
- **Mal-folded** proteins are **common**. Buffers and growth conditions often need to be **optimised**.
- **PTMs** may be *required* to get **correctly folded/active** proteins, so bacterial expression might not be an option.
- Expression (e.g. of a prok. Gene in a mammalian cell) may require **gene re-synthesis** and **codon optimisation**. Also, some bacterial strains are better than others.
- **Solubility** might be an issue – can be optimised or improved (e.g. by fusion to a more soluble partner)
- Some proteins may be **toxic** to the cell.
- Some may need **folding partners** (chaperones/foldases)

Expression I: Induction

To obtain purified protein, it must be expressed highly by the bacteria now harbouring the gene – 'switched on'.

Induction systems respond to particular compounds, AbRs, temperature etc.

In our hypothetical experiment, the **"araBAD" promotor system** can be turned on with **Arabinose** added to the culture, while glucose represses it.



Expression II: Extraction

- Depends on the specifics of the protein:
  - If secreted, **filtration of supernatants** can be sufficient
  - **Cell lysis** via sonication / homogenisation / enzymatic lysis / freeze-grinding

- Will sometimes require the **removal of DNA** if cells are lysed.

- Cultures are **centrifuged to separate proteins** from cell debris.

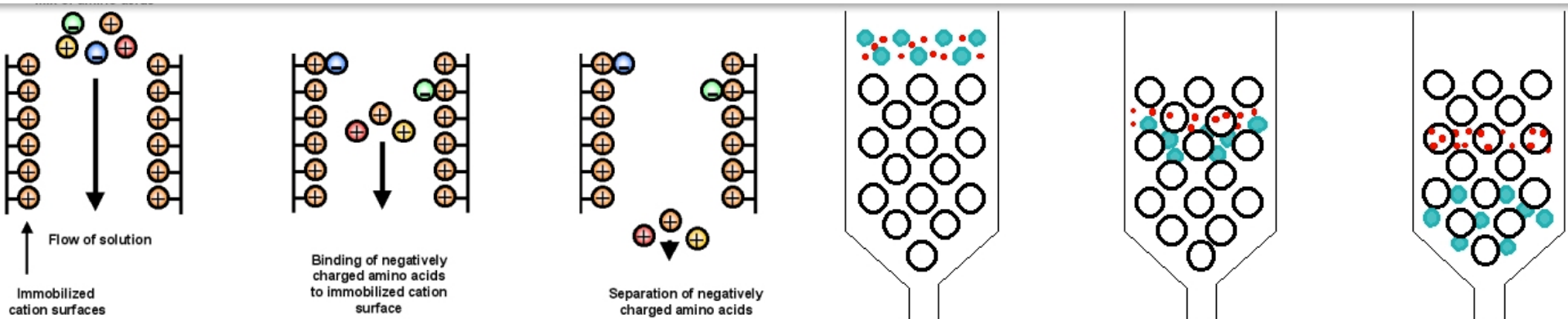
Some additional steps such as **solubility studies** and ***in vitro* refolding** might be needed for recalcitrant proteins.

Expression III: Purification

- If the protein is **tagged** (e.g. Strep or His-tagged), then **Affinity Chromatography** is the go-to method.
  - E.g. **Nickel** or **cobalt-NTA resins** bind poly-Histidine.
  - Fusions to **MBP** or **GST** etc can be used with a **binding partner**.

- **Multiple rounds** of **chromatography** with **various chemistries** can be done to achieve better quality preps.
  - **Ion Exchange** (**IEX**) can be calibrated to retain proteins with certain pI's/charges while loosely binding proteins flow through.
  - **Gel Filtration** chromatography works like electrophoresis to separate proteins by size, homogenising a prep.

IEX **Gel Filtration**



Creating Variant Proteins

For biomaterial applications and function studies, making protein variants is usually the most informative/useful:

- **Fusion proteins** & **tags** as discussed already (achieved by simple '**in-frame**' cloning.
- **Site Directed Mutagenesis (SDM)**
  - Uses a PCR and cloning strategy, whereby a **deliberate sequence change** is made in the primer sequences, which results in amino acid changes in the final protein product.

- **Non-natural amino acids**
  - Using bacterial strains with **engineered amino acid carrying tRNAs** can make use of an **expanded genetic code**.
  - Can do **site-specific chemistries** that will only react with non-naturally occuring functional groups.

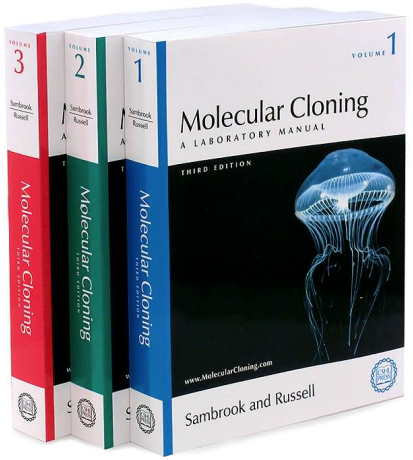
Advantages for a Chemistry Lab

Some key advantages of employing cloning/molecular bio:

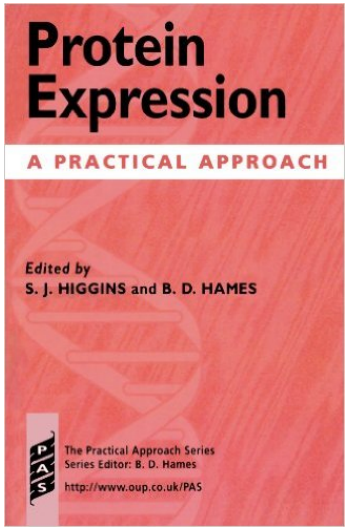
- Is *USUALLY* **easy** and **cheap**.
- Can obtain a **large amount** of **protein**.
- Protein **mutants/variants** can be **created** (that may not be commercially available).
  - **Fusions**
  - **Tags/Labels**
  - **Point mutations**
  - **Isotopic incorporation (e.g. for NMR)**
  - **Non-natural amino acids**
- **Cell-free protein synthesis** can be done (simpler, though with reduced yield).
- Creation of '**Reporter' experiments** (e.g. to test mode of action of novel compounds)

Reading Links

Some useful go-to resources/lab bibles:



- Sambrook *et al*. "**Molecular Cloning: A Laboratory Manual"**



- Higgins and Hames. "**Protein Expression: A Practical Approach"**

http://www.embl.de/pepcore/pepcore_services/index.html

http://openwetware.org/wiki/Protein_Expression_and_Purification

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