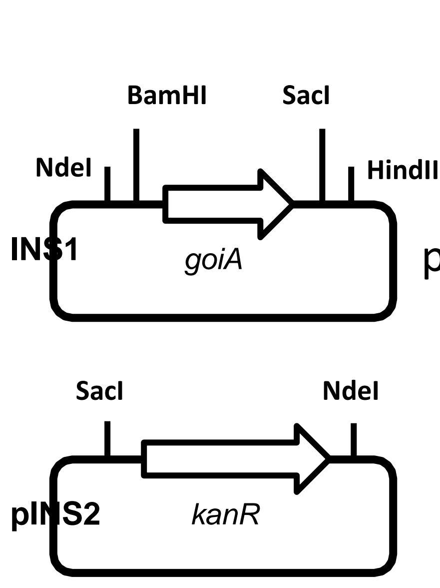
# Pharmaceutical biotechnology (B-KULK09J3A)

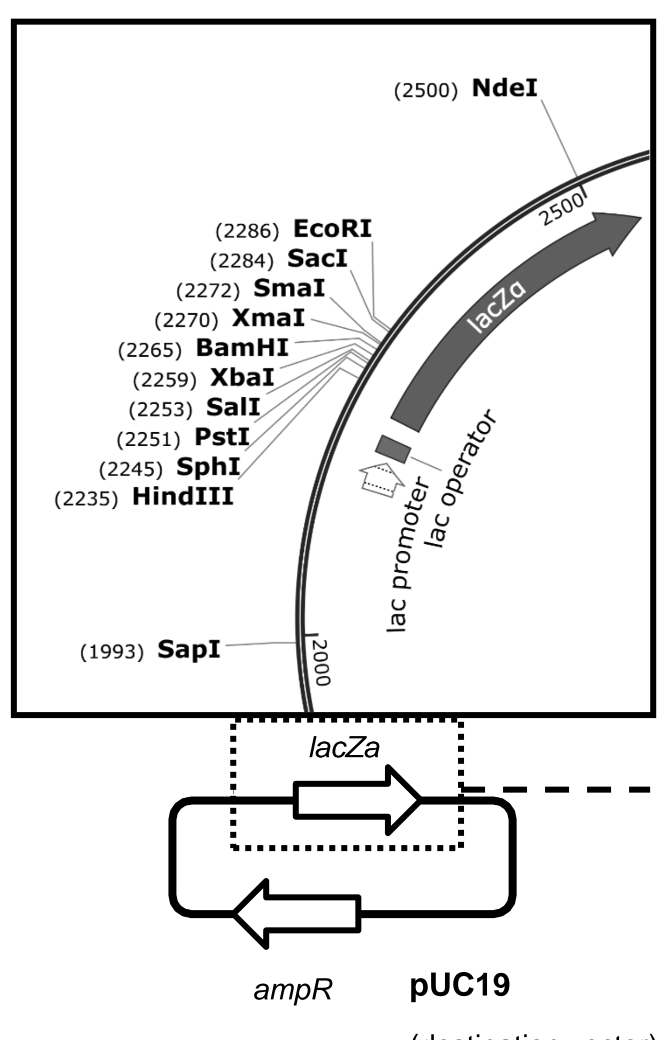
**Examenvragen** (prof. Pinheiro)

*Noot: deze examenvragen zijn doorgestuurd door de prof zelf.*

## Part I – Short answer questions

Q.1 – You are tasked with subcloning two genes (from plasmids pINS1 and pINS2) into pUC19 for IPTG-induced expression. Using the plasmid maps given, describe a viable cloning strategy.




*poiA* – gene of interest A

*kanR* – resistance to kanamycin

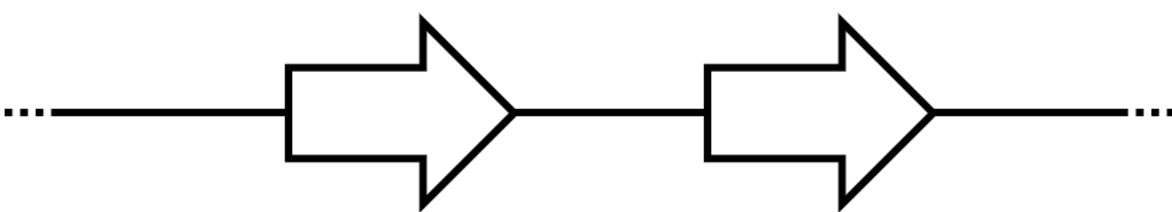
*ampR* – resistance to ampicillin

*lacZa* – alpha-fragment of LacZ

(destination vector)

Q.2 – Considering the construct you generated in Q.1, draw the necessary DNA elements required for the high-level expression of both genes (*goiA* and *kanR*) in *E. coli*. Use any relevant SBOL symbols shown below.

| | | | | | | |
|---|---|---|---|---|---|---|
| **A.** | Origin of replication | | **E.** | Terminator |
| **B.** | Recombination site | | **F.** | Operator |
| **C.** | Promoter | | **G.** | Translation stop site |
| **D.** | Ribosomal binding site | | **H.** | Primer binding site |



Q.3 – Explain how LacZα can be used as a reporter gene in cloning. Considering your proposed cloning strategy in Q.1, would LacZa be an effective cloning reporter? If yes, highlight which cloning step the *lacZa* gene would report on. If not, suggest an alternative reporter system that could be used.

Q.4 – Match the terms on the left-hand side column to the right-hand-side definitions for a biotherapeutic being manufactured in *P. pastoris* cells in liquid culture:

a. Harvesting
b. Clarification
c. Capturing
d. Polishing

**1.** Early stage of the protein production process where small cultures (up to 1 L) are used to inoculate larger volumes for large-scale production.

**2.** Using diafiltration or porous membranes, change the buffer in which proteins were purified to introduce buffers and additives suitable for long-term storage and therapeutic use.

**3.** Lysed fungal cells are treated with nucleases, protease inhibitors and crude separation is carried out by tangential filtration or centrifugation.

**4.** Based on biophysical properties of the protein of interest, large-scale purification approaches (normally affinity-based) are used to remove as many of the other proteins in the mixture with the goal of obtaining a low complexity mixture highly enriched for the protein of interest.

**5.** Isolating the cells from the culture by centrifugation or tangential filtration after the induction of protein expression.

**6.** Lysed fungal cells are treated with proteases and heated to near boiling temperatures (95°C) for extended period of time (1 h) before crude separation by tangential filtration or centrifugation.

**7.** Having allowed *P. pastoris* cells to reach the desired cell concentration, methanol is titrated into the culture to limit the amplification of biomass and to induce the expression of the protein of interest.

**8.** Low volume (up to 1 L) protein purification stage where the protein of interest is already the majority of the sample. The goal is to separate the protein of interest from as many close variants as possible to increase the homogeneity of the sample.

| a. Harvesting | b. Clarification | c. Capturing | d. Polishing |
|---|---|---|---|
| | | | |

Q.5 Explain Biobrick cloning and give one potential advantage of the method over classic molecular biology cloning strategies (using endonucleases).

Q.6 – List and briefly justify 3 important considerations in primer and probe design fora qPCR assay.

Q.7 – Given the choice of the following available reagents, draw a viable immunoassay topology for the detection of a yeast cytoplasmic protein, called EX1.

**1.** Protein A-coated immunoassay plate

**2.** Biotinylated EX1

**3.** rabbit Fab anti-EX1

**4.** luciferase-Protein A fusion

**5.** Streptavidin-coated immunoassay plate

**6.** horseradish peroxidase

**7.** EX1

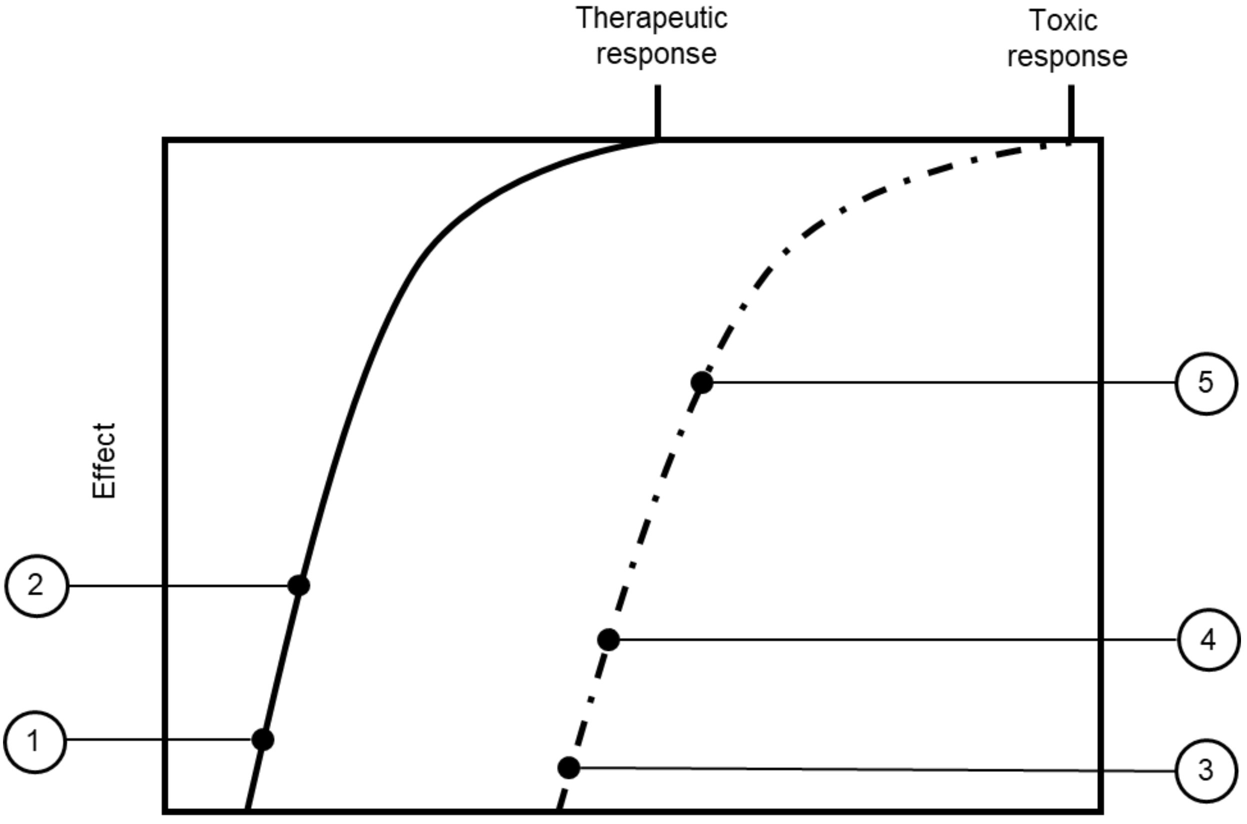
**8.** mouse anti-EX1 IgG

Q.8 – Having set up a viable assay in Q.7 for the detection of EX1, you notice that even in the absence of EX1 your assay output is high (i.e. high background). Using a mass action model, identify and justify 1 experimental parameter that can be changed to reduce assay background.

Q.9 – List and briefly explain two differences between bacterial and mammalian hostsfor the large-scale production of a therapeutic IgG.

Q.10 – List two Critical Quality Attributes for a protein-based biotherapeutic and briefly explain how they can affect its therapeutic function.

Q.11 – Draw in the graph below the therapeutic concentration range of the biotherapeutic EX2. Briefly explain how therapeutic drug monitoring can be used to optimise personal dosing.




Biotherapeutic concentration

**1.** Limit of detection for biological activity

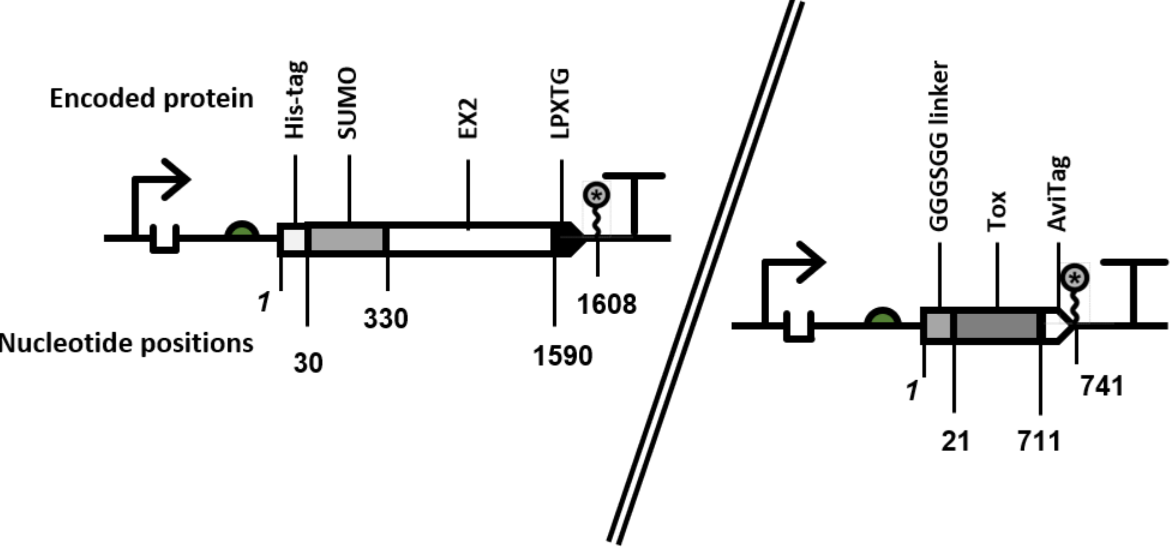
**2.** Minimally-anticipated biological effect level

**3.** Limit of detection for toxicity

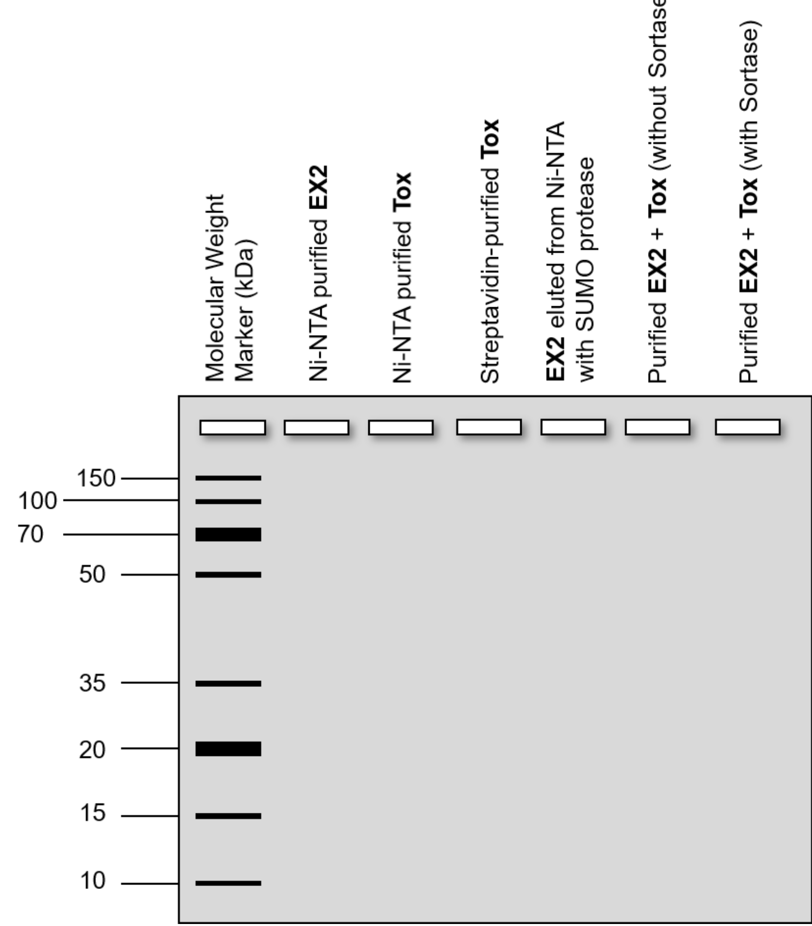
**4.** Toxicity-related side effects reported

**5.** Clinical complications from side effects

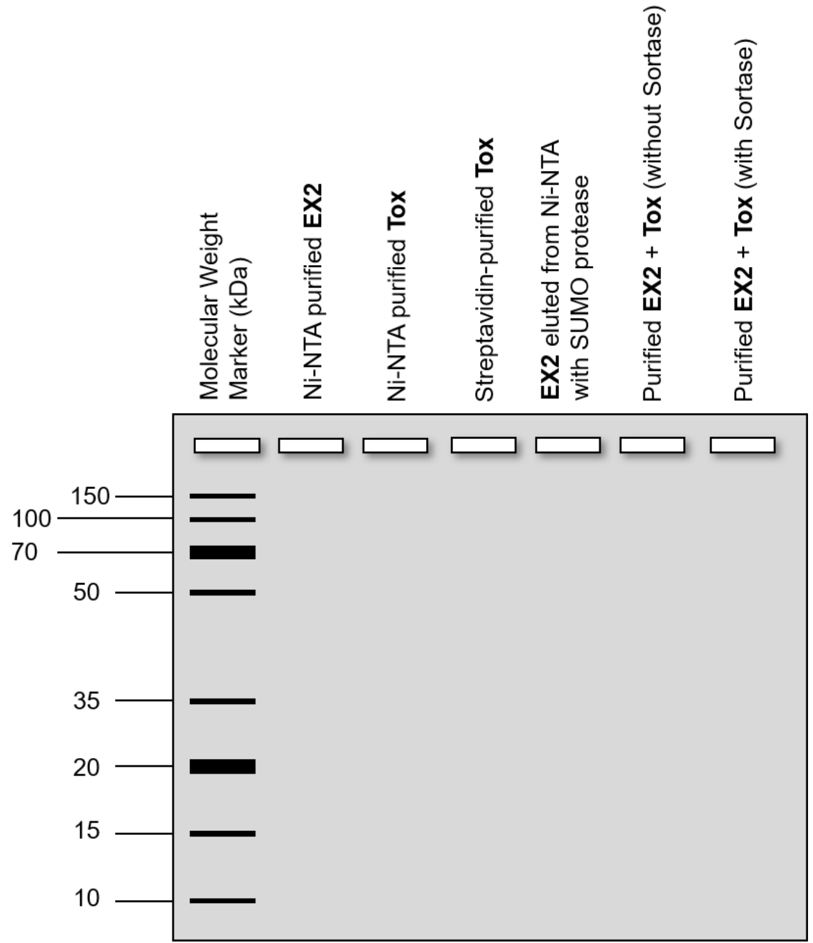
Q.12 – Preparation of a novel biotherapeutic, called EX2-Tox, requires sortase-mediated ligation of EX2 and Tox. The components are expressed (in separate cells) from the annotated genetic circuits depicted below. Using the knowledge that an average amino acid is 110 Da, complete the SDS-PAGE and Western blot.




SDS-PAGE




**Western Blot:** Developed with mouse anti-His-tag IgG




Q13. – In relation to the Schmit et al. paper, explain how the authors used the following constructs depicted in Figure 1. (What was the purpose of the experiment? What was the key finding of those experiments?)

| Monomer Size (kDa) | Pentamer Size (kDa) | Nomenclature used in this paper | |
|---|---|---|---|
| 50 | 250 | Toxin-emGFP | Stx2B – ETA-II – emGFP –6xHis–KDEL |
| 53 | 265 | Toxin-SNAP-monobody | Stx2B – ETA-II – SNAP – Monobody –6xHis–KDEL |

Furin cleavage site (between Stx2B and ETA-II)

Q.14 – In relation to the Schmit et al. paper, explain the experimental rationale behind Figure 5a. (What was the experiment? What are the controls? What is its main conclusion?)

Q.15 – Subsequent to Schmit et al. publication, *in vivo* testing of the Toxin-VHL-monobody construct (subcutaneous delivery) showed poor tissue penetration and high immunogenicity. Give a possible reason for that observation and propose a modification to the Toxin-VHL-monobody construct that could improve its tissue penetration.

Q.16 – Schmit et al. used an *E. coli* host for recombinant Toxin-VHL-monobody expression and, subsequent to publication, the same host was considered for scaling up manufacturing of the toxin. State 2 possible contaminants in the production of that biotherapeutic and describe (for each) a viable strategy for their detection.

Q.17

**Part II – Essay questions**

Q.1. – Define a biosimilar and compare its development and approval pathway to a generic drug.

Q.2 – Define phenotype-genotype linkage and explain its role in the directed evolutionof novel high-affinity biotherapeutics by phage display.

**Examen januari 2022**

**Part I – Short answer questions**

**Q.1 –** DNA polymerases in the absence of nucleotide triphosphates can work as exonucleases, digesting DNA from its 3'-end. In the presence of a single nucleotide triphosphate, for instance dCTP, the exonuclease function proceeds until the first C is removed, upon which it is readily re-incorporated.

a. Given the DNA sequence below, write down the opposite strand and cross out the bases that would be removed from the DNA.

```
5'- GAA GTA TCG … TGT ACG CTA – 3'

3'-                 …                  – 5'
```

b. This method can be used for DNA assembly. Explain how it can be used for cloning and compare it to Type IIS or Golden Gate cloning.

**Q.2 –** Using the SBOL symbols below draw a plasmid for the expression of a single-chain Fragment variable protein labelled at its C-terminus with a Strep-tag. Highlight in your construct the boundaries between tags, linkers and immunoglobulin domains.

| | | | | | | |
|---|---|---|---|---|---|---|
| **A.** | ◯ | Origin of replication | | **E.** | T | Terminator |
| **B.** | ▷ | Recombination site | | **F.** | ⊔ | Operator |
| **C.** | ↱ | Promoter | | **G.** | | Translation stop site |
| **D.** | ◠ | Ribosomal binding site | | **H.** | ⬠ | Open Reading Frame |

**Q.3** – List two Critical Quality Attributes for a protein-based biotherapeutic and briefly explain how they can affect its therapeutic function.

**Q.4** – Given the choice of the following available reagents, write down a viable immunoassay topology for the detection of a yeast cytoplasmic protein, called EX1

**1.** EX1-containing sample

**2.** mouse anti-EX1 IgG

**3.** rabbit Fab anti-EX1

**4.** Streptavidin-coated immunoassay plate

**5.** horseradish peroxidase-Protein A fusion

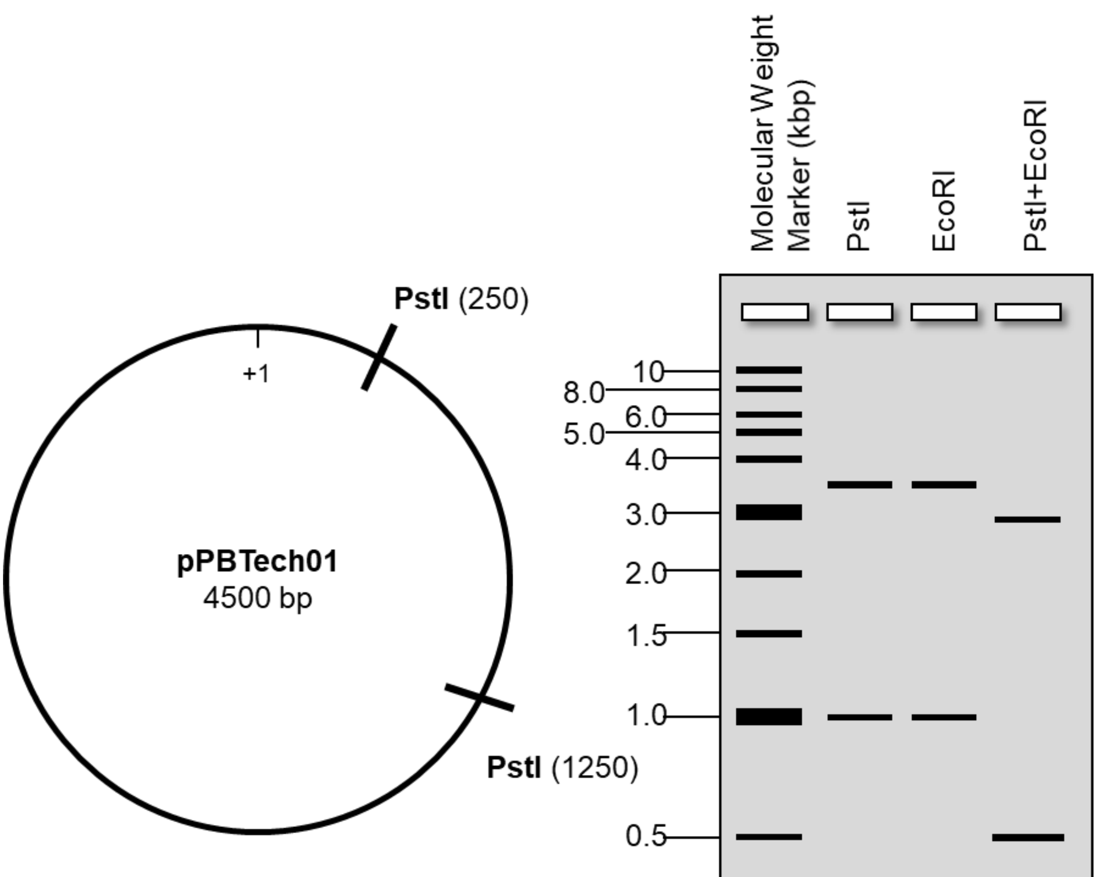
**6.** Bovine milk casein (typically used as a blocking agent)

**7.** Uncoated immunoassay plate (i.e. will bind any protein)

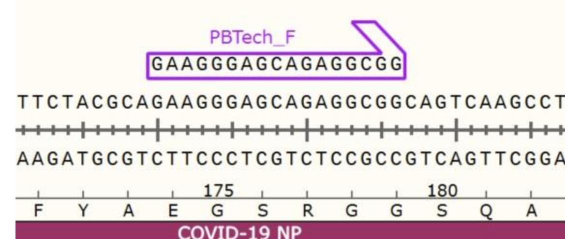
**8.** luciferase

**Q.5** – Define a reporter gene. Explain, giving an example, how reporter genes can be used to monitor cloning. Propose a possible reporter gene for monitoring cloning in eukaryotic hosts.

**Q.6** – Given the restriction patterns observed below in an agarose gel, map the positions of the cut sites to the plasmid backbone. A PstI restriction site (and its position) is already given on the plasmid map. Is the information given sufficient for unambiguous assignment? Justify your answer.




**Q.7** – The following primers (PBTech_F and PBTech_R) were designed for the detection of COVID from patient samples.

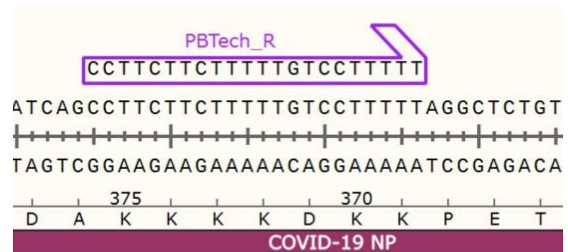



**PBTech_F (17-mer):**

Tm: 58.5°C

Most stable self-dimer: ΔG = - 3.61 kcal/mol

GC content: 70%



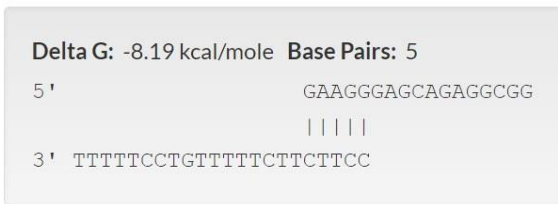

**PBTech_R (22-mer):**

Tm: 49.5°C

No significant stable self-dimers

GC content: 32%

**Heterodimer predictions:**


**Delta G:** -8.19 kcal/mole **Base Pairs:** 5

```
5'                      GAAGGGAGCAGAGGCGG
                        |||||
3' TTTTTCCTGTTTTTCTTCTTCC
```

Unfortunately, initial experiments using the following conditions yielded only non-specific amplifications

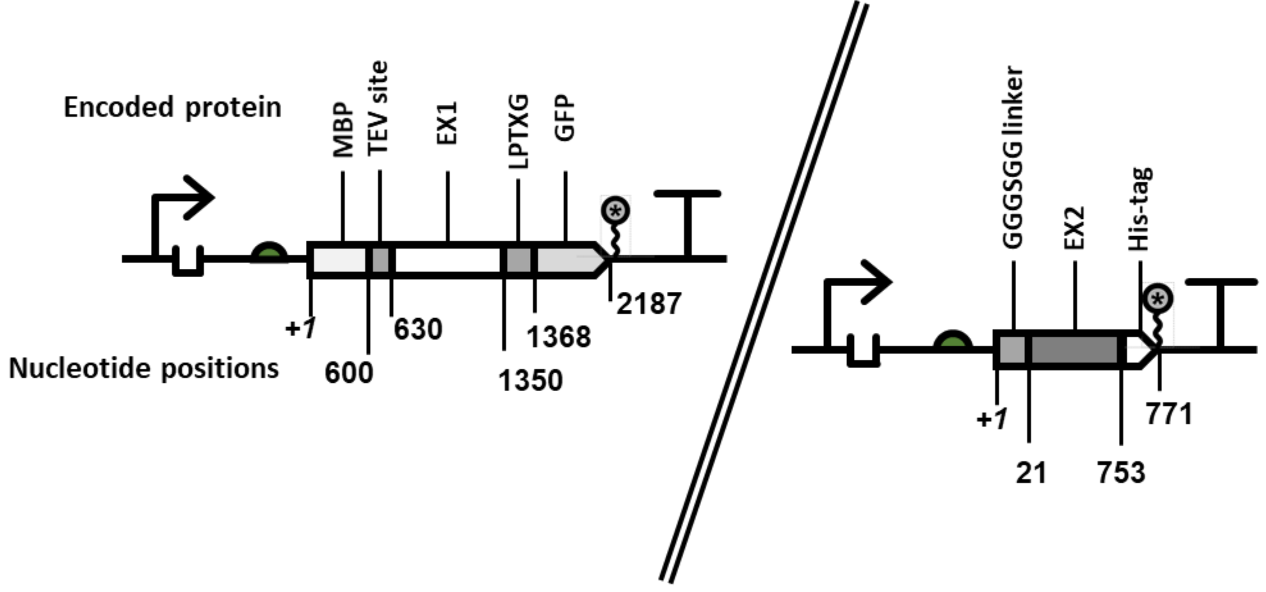
| Denaturation | 5 min @ 95°C | |
|---|---|---|
| Denaturation | 1 min @ 95°C | X10 |
| Annealing | 10 s @ 57°C | X10 |
| Extension | 10 s @ 68°C | X10 |

Propose, and briefly justify, two possible changes to the experiment to improve its success as a diagnostic platform.

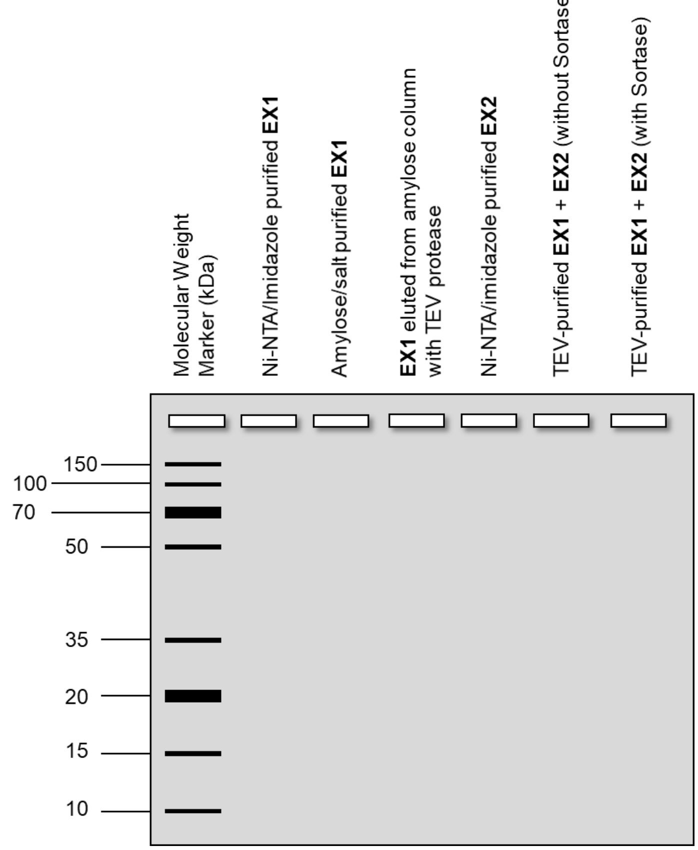
**Q.8** – You have been tasked with expressing and purifying mouse IgGs from human hybridoma cells in culture for a clinical trial. With respect to other possible hosts (e.g. bacterial cells or mouse hybridoma cells in culture), is this a good choice of host for IgG expression? Briefly justify your answer.

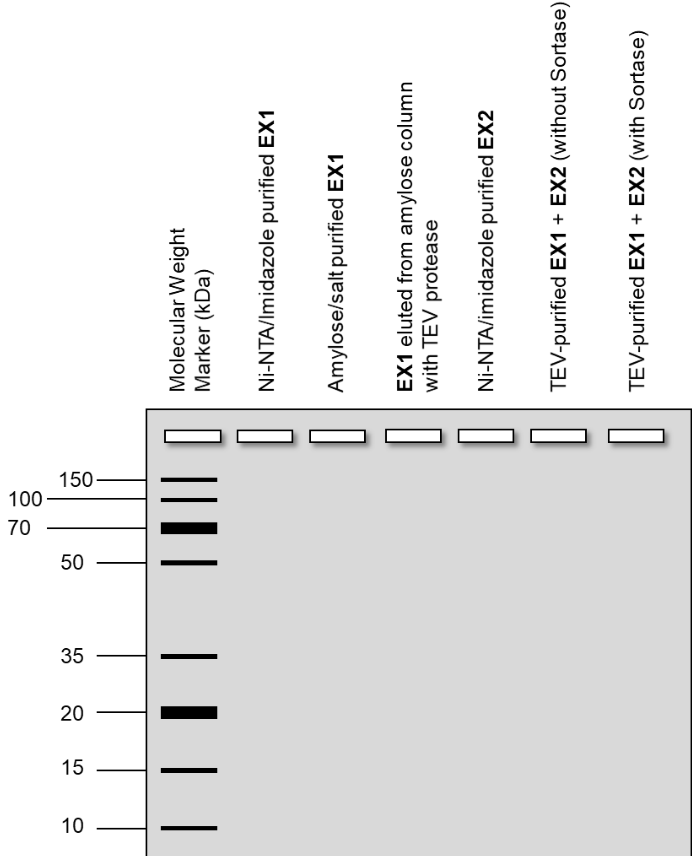
**Q.8** – Preparation of a novel bivalent scFv, called EX1-EX2, requires sortase-mediated ligation of EX1 and EX2. The components are expressed (in separate cells) from the annotated genetic circuits depicted below. Using the knowledge that an average amino acid is 110 Da, complete the SDS-PAGE and Western blot. (Show your calculations)

Construct:




**SDS-PAGE:**






**Western Blot:** Developed using a rabbit anti-GFP IgG and a goat anti-rabbit IgG conjugated to HRP.

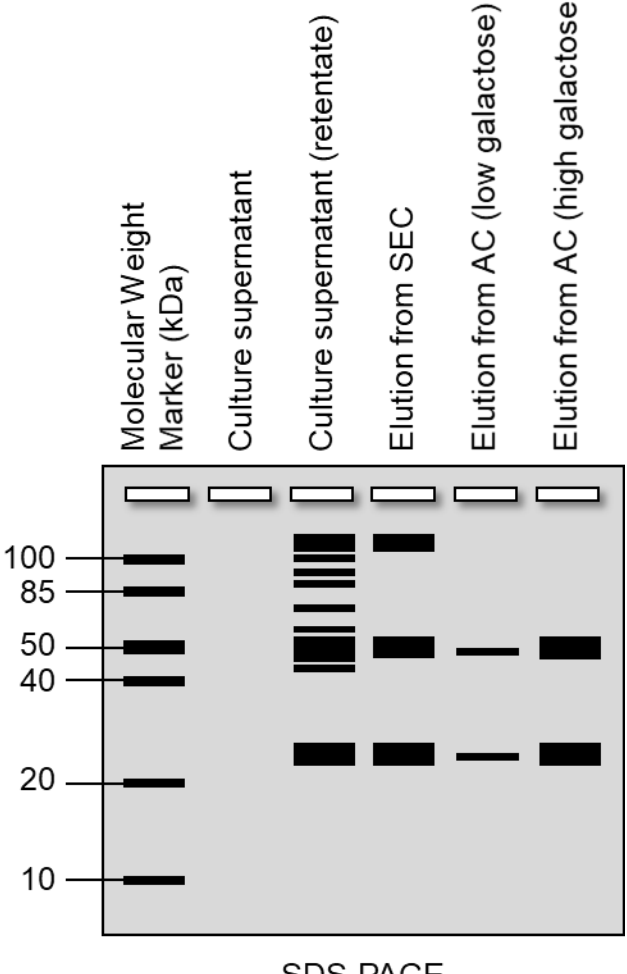
**Q.10** – Continuing your task from Q.8, you express the IgGs, recovering the culture supernatant and proceeding with the following purification procedure:

1. Concentration through a 100 kDa filter

2. Size exclusion chromatography (SEC)

3. Affinity chromatography using a column containing lectins with affinity for galactose

The results are shown in the following reducing SDS-PAGE:




a. Based on the SDS-PAGE, was purification successful? Briefly justify your answer.
b. Although you filtered the sample using a 100 kDa filter, why are there proteins lower than that cut-off in the sample?
c. Propose an alternative purification approach for the IgGs.

**Q.11** – Still with regard to the IgG purification above, list two possible contaminants and briefly describe how they can be removed.

**Q.12** – Explain what genotype-phenotype linkage is in directed evolution. Is it important? Briefly justify.

**Q.13** – Regarding Park et al., explain the experimental rationale behind Figure 7. (What is the experiment? What is its main conclusion? What are the experimental controls?)

**Q.14** – Regarding Park et al., the authors state that only 11.8% of the MCF cells were HER2 positive. How did the authors came to that conclusion? Does that agree with the findings shown in Figure 7? Justify your answer

**Q.15** – Regarding Park et al., what (molar) concentration of HER2(scFv)-GFP was used for the flow cytometry experiments? The scFv used is known to have an affinity for HER2 of approximately 20 pM. Given the definition fractional binders [$\theta = [A]/(K_d + [A])$], what fraction of the scFv is expected to be bound?

* as shown in Figure 6, the molecular weight mass of HER2(scFv)-GFP is approximately 50 kDa.

**Q.16** – Regarding Part et al., what is the *E. coli* shuffle strain and why was expression in it attempted?

**Part II – Essay questions**

**E.1** – Explain the impact of variability of personal pharmacokinetics and pharmacodynamics on biotherapeutic dosing and monitoring.

**E.2** – Describe available strategies to engineer a nanobody (mass 12.5 kDa) against a cancer cell-surface marker ($k_{on} = 10^8$ $M^{-1}$ $s^{-1}$; $k_{off} = 10^{-1}$ $s^{-1}$) to increase its therapeutic potential (i.e. better production yield, higher affinity, greater potency and longer half-life if relevant).