

EFFICACY AND IMMUNOGENICITY OF VACCINES MADE FROM RECOMBINANT PROTEIN TECHNOLOGIES FOR THE PREVENTION OF DISEASES CAUSED BY *STREPTOCOCCUS INIAE* IN ASIAN SEABASS (*LATES CALCARIFER*)



Quentin ANDRES, PhD Student

Center of Excellence in Aquatic Animal Health Management,
Faculty of Fisheries, Kasetsart University, October 16, 2023.

WEEK 42

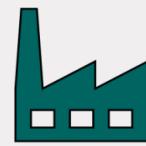


Research objectives, context and recent achievements.

Research objectives

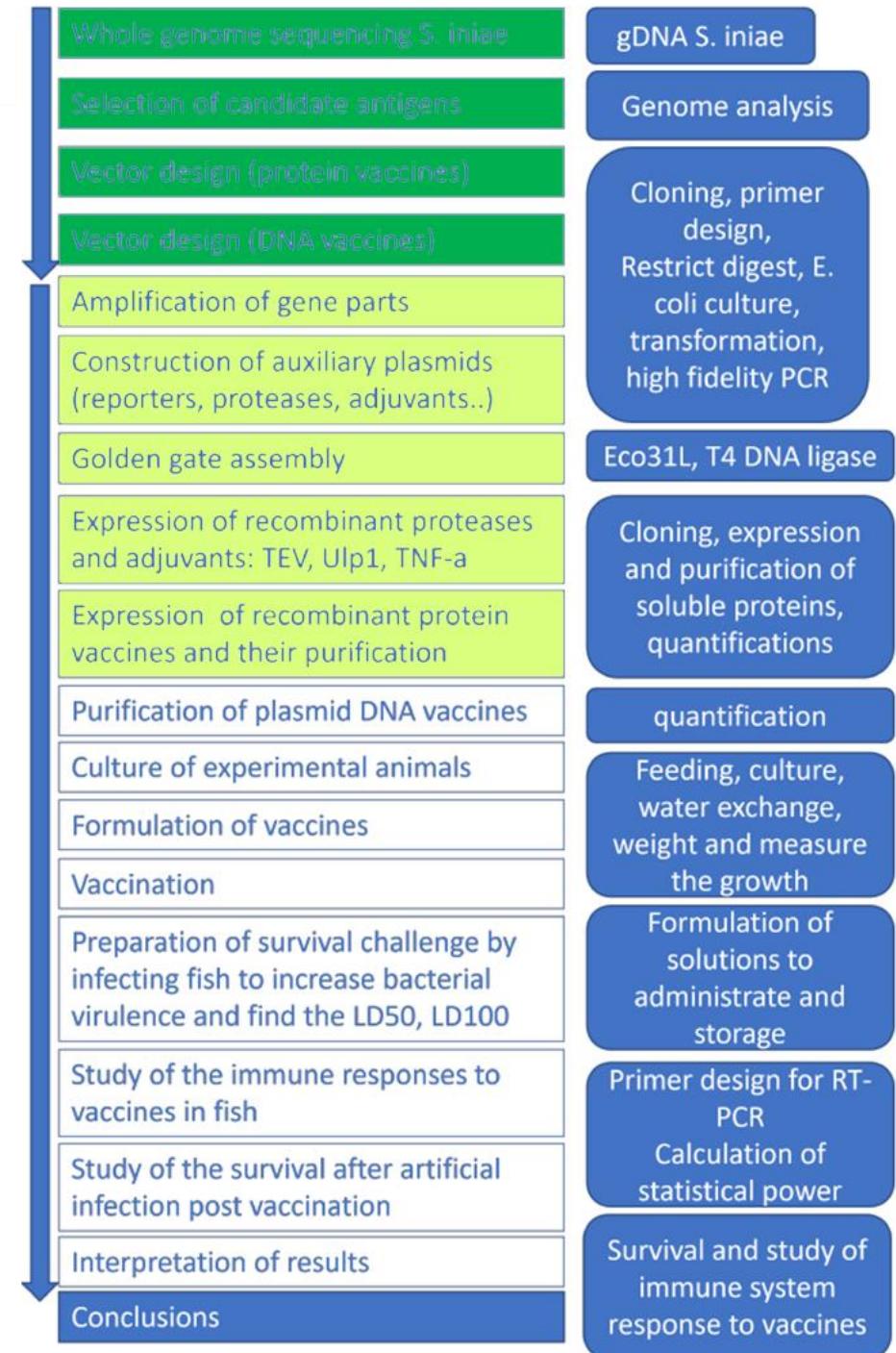
Develop cost-effective recombinant protein vaccines and plasmid DNA vaccines for the prevention of disease induced-mortality caused by *Streptococcus iniae* in Asian seabass (*Lates calcarifer*).

1. Isolation of *Streptococcus iniae* in farmed fish and DNA genome sequencing and annotation. Comparative genomic analysis and reverse vaccinology to find suitable vaccine candidates for mass vaccine production.
2. Clinical development of cost-efficient protein vaccine manufacturing strategies.
3. Clinical development of cost-efficient DNA vaccine manufacturing strategies. (bonus)
4. Evaluation of vaccines performance (in-vivo immune response + relative protective efficacy).



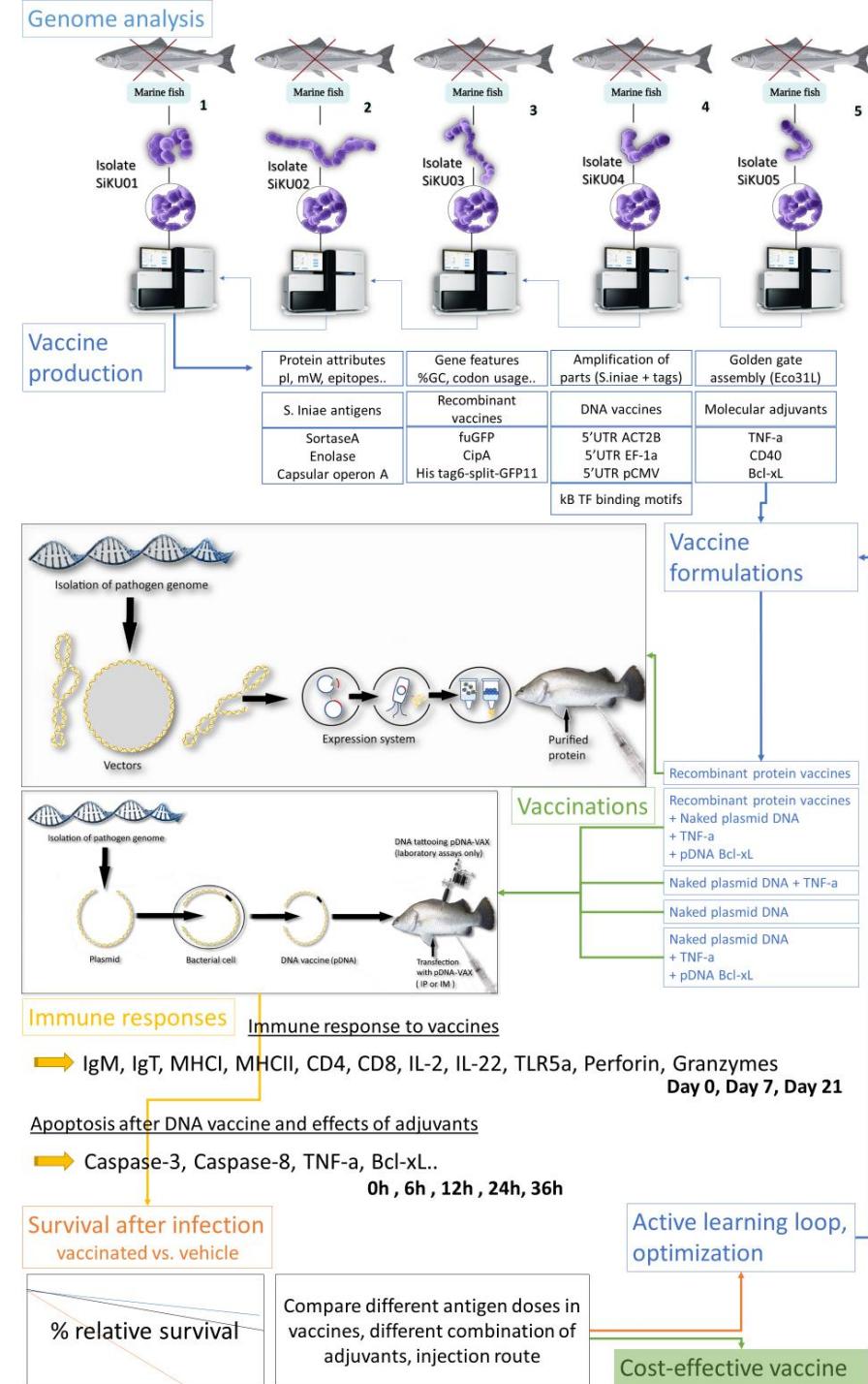
General methodology

EFFICACY AND IMMUNOGENICITY OF VACCINES
MADE FROM RECOMBINANT PROTEIN TECHNOLOGIES
FOR THE PREVENTION OF DISEASES CAUSED BY
STREPTOCOCCUS INIAE IN ASIAN SEABASS (*LATES CALCARIFER*)



Graphical summary

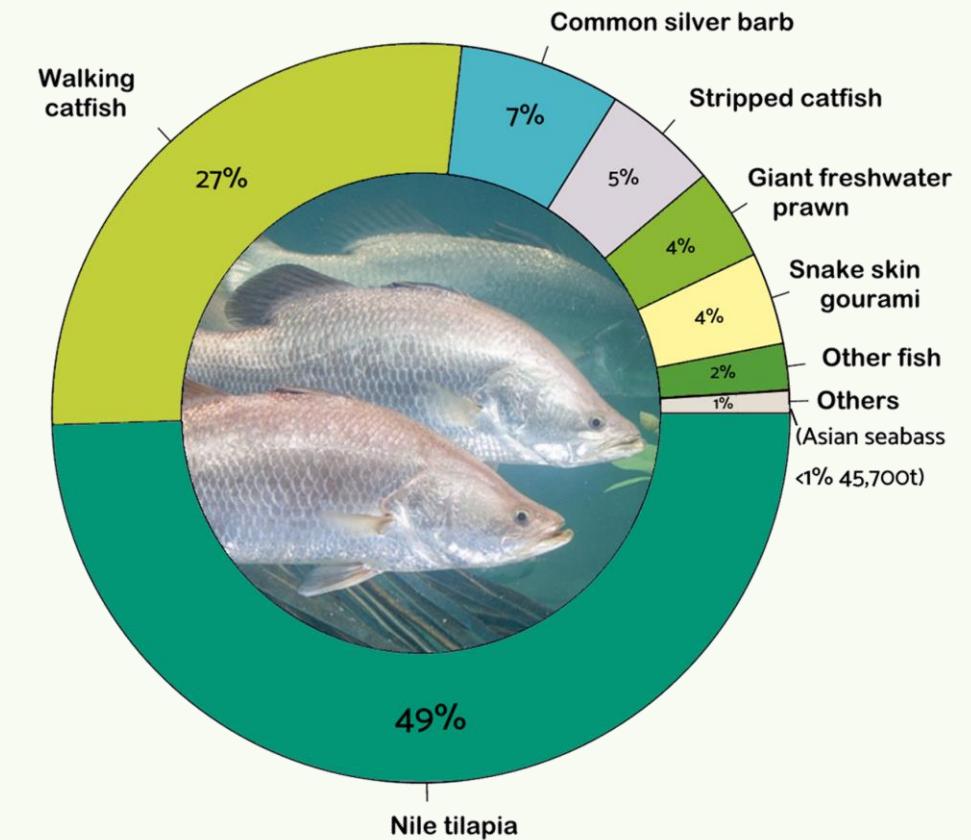
**EFFICACY AND IMMUNOGENICITY OF VACCINES
MADE FROM RECOMBINANT PROTEIN TECHNOLOGIES
FOR THE PREVENTION OF DISEASES CAUSED BY
STREPTOCOCCUS INIAE
IN ASIAN SEABASS (*LATES CALCARIFER*)**



Production of Asian seabass by Species, Types of Culture and Provinces / Relative Proportion to Freshwater Aquaculture Species in Thailand, 2020

Context of research

PROVINCE	PRODUCTION IN TONS ↓	POND (TONS)	CAGE (TONS)
ฉะเชิงเทรา Chachoengsao	14,109	14,044	65
สมุนไสห์ Samut sakhon	9,371	9,371	0
สมุทรสงคราม Samut songkhram	7,693	7,434	259
สงขลา Songkhla	3,508	247	3,261
สมุทรปราการ Samut prakan	3,016	3,016	0
สุราษฎร์ธานี Surat thani	1,723	1,560	163
ประจวบคีรีขันธ์ Prachuap khiri khan	1,547	1,547	0
ปัตตานี Pattani	1,068	125	943
นครศรีธรรมราช Nakhon si thammarat	1,040	936	104
ระยอง Rayong	429	429	0
ชุมพร Chanthaburi	321	259	62
เพชรบุรี Phetchaburi	294	294	0
สตูล Satun	220	44	176
นราธิวาส Narathiwat	180	0	180
ตราด Trat	172	141	31
กระบี่ Krabi	71	18	153



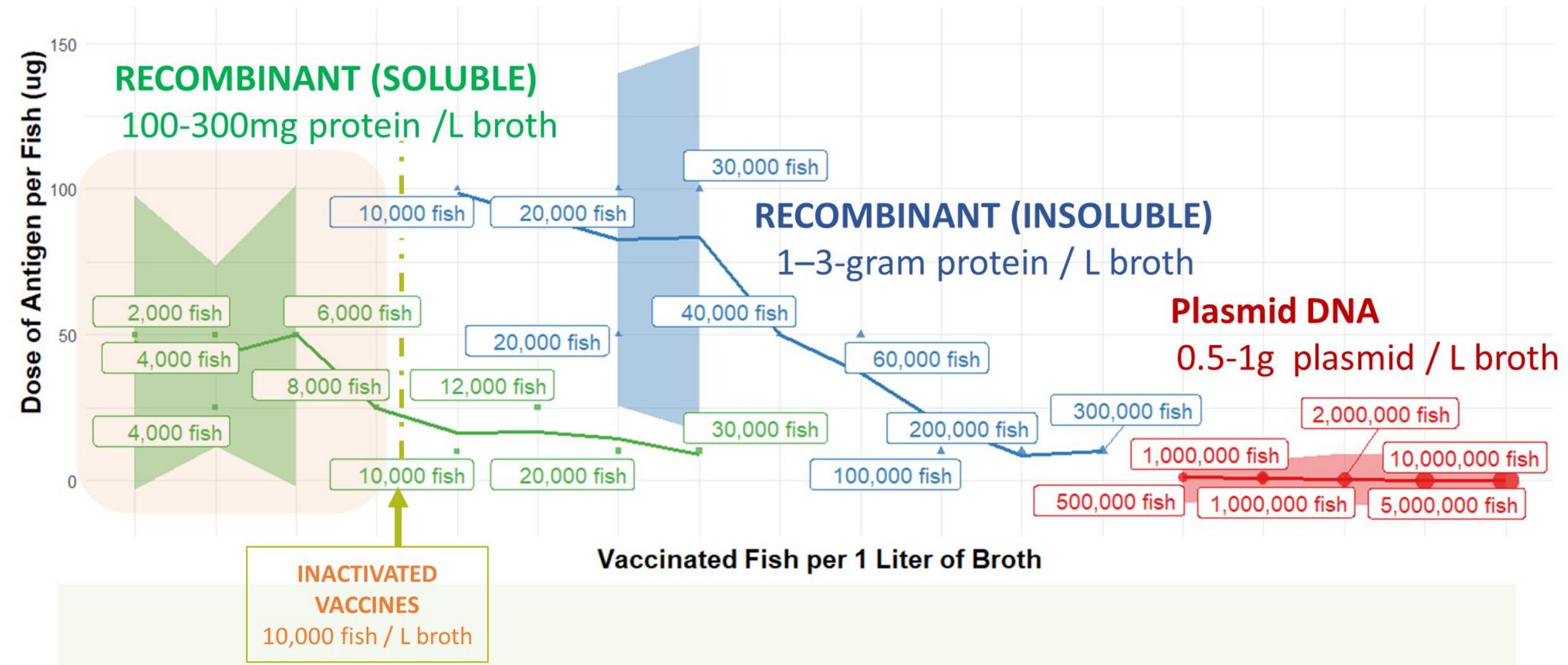
สถิติการประมงแห่งประเทศไทย พ.ศ. (Page 26) [2563 FISHERIES STATISTICS OF THAILAND 2020](#)

→ The economic value of barramundi (ปลากระพงขาว or Asian seabass) aquaculture in Thailand is 4,604.5 million THB as of 2020, doubling in the last 4 years.

Vaccinated Fish per 1 Liter of E. coli Broth and per Antigen Dose (μ g)

Context of research

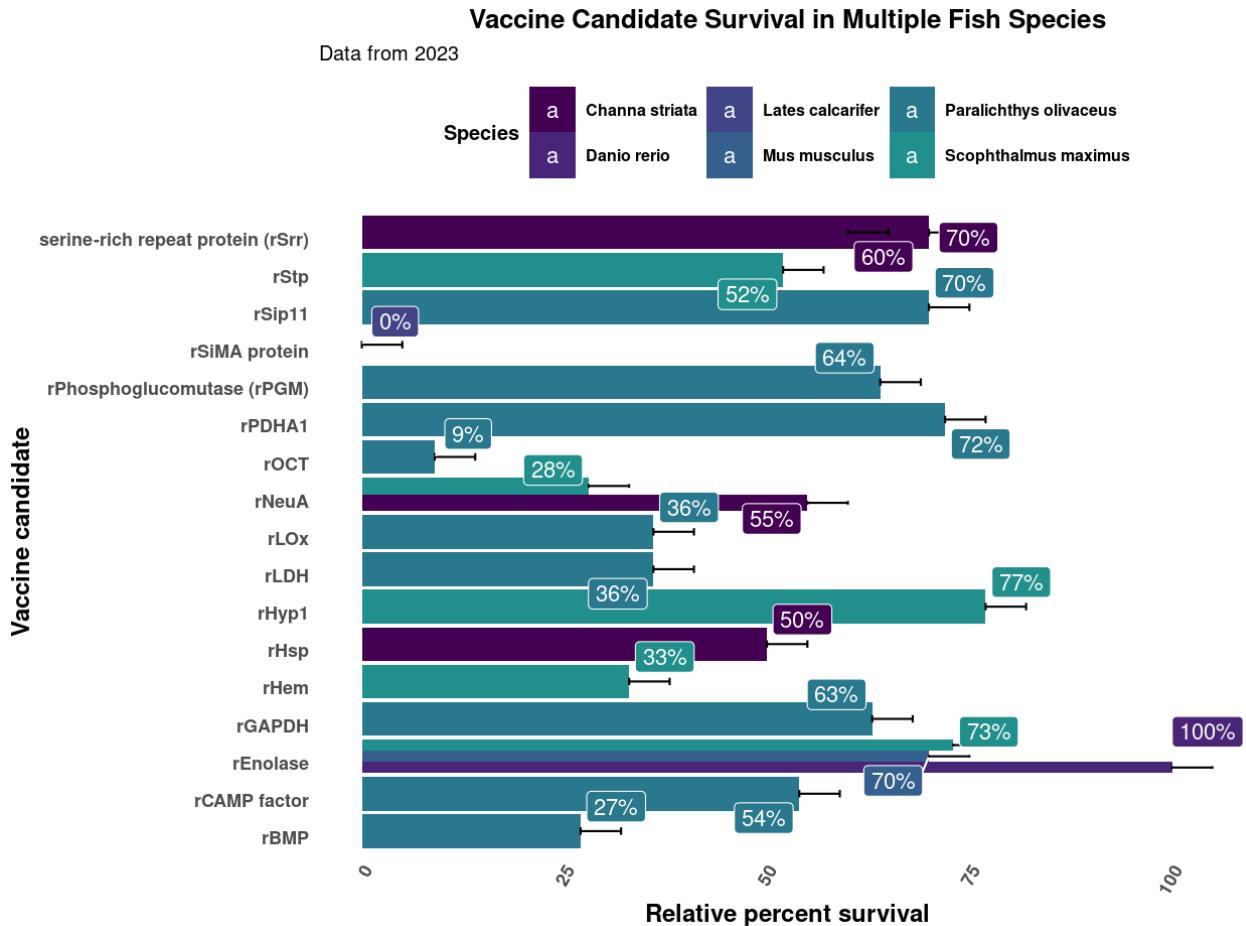
Visualization of the number of fish that can be vaccinated per liter of E. coli broth based on different antigen production methods and their respective dosages.



Context of research

Vaccine development is a work of optimization and require extensive manufacturing design

Streptococcus iniae cause diseases in seabass farms, vaccination is an alternative to antibiotics, more than 22 vaccines have already been developed over the past 20 years by academic scientists but none of the research took into consideration the manufacturing of vaccines. As a result, none of the vaccine could be used in real life situations because their design was too expensive from the start.



Recent achievements



Literature review (vaccine manufacturing, quality by design methodologies, regulatory, vaccine evaluation and analytics)



Expressed sfGFP-1-10, sonicated and prepared the stock detector solution.



Proposal writing.



Mostly focusing on genome analysis of *S. iniae* and the draft manuscript "Comparative Genomics of Streptococcus iniae: A Blueprint for Vaccine Innovations in Aquaculture"

On-going tasks



Completing the draft manuscript.



Completing the thesis proposal.

Next steps (short-term)



Finishing to set up split GFP Assay.



Build an entry pET plasmid by inserting a LacZ white blue screening cassette for negative selection on X-gal.

Next steps (mid-term)



Order primers to amplify gene components for golden gate assembly.



Set up purification systems for proteins based on silica affinity purification, cellulose affinity purification.



Crystallize vaccine protein by crosslinking in glutaraldehyde.

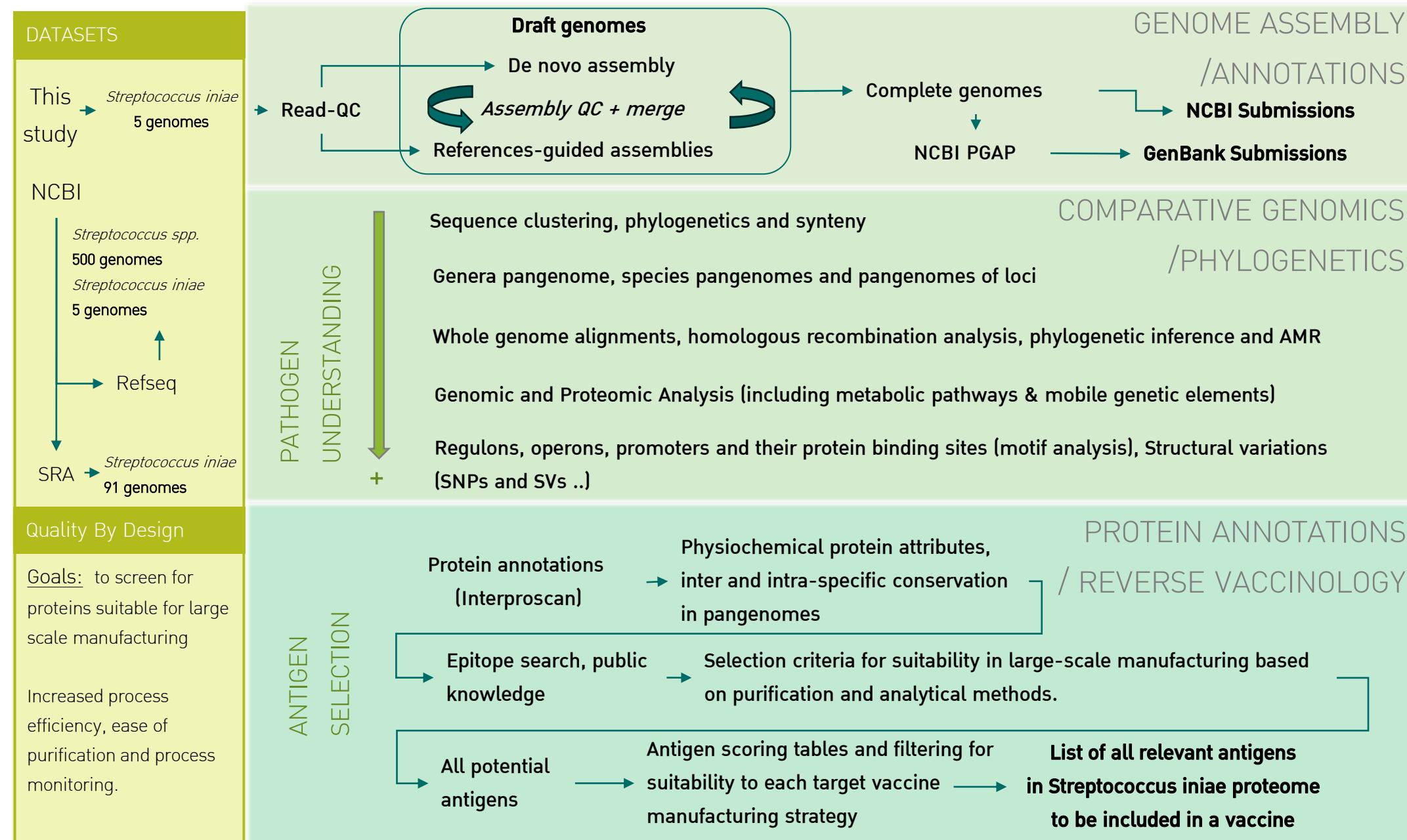


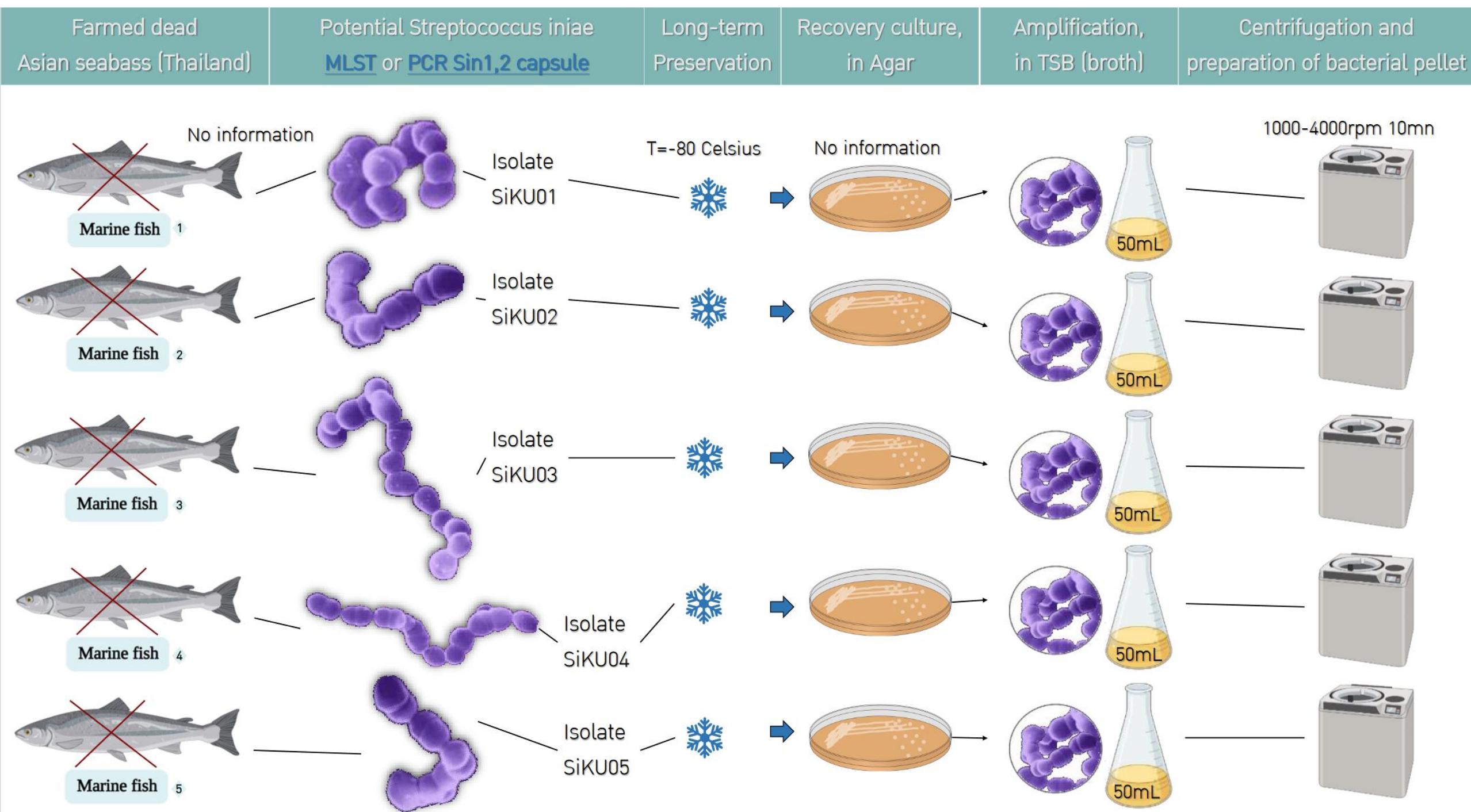
Immunization of Seabass with inactivated vaccines to produce broad serum for future analytical assays

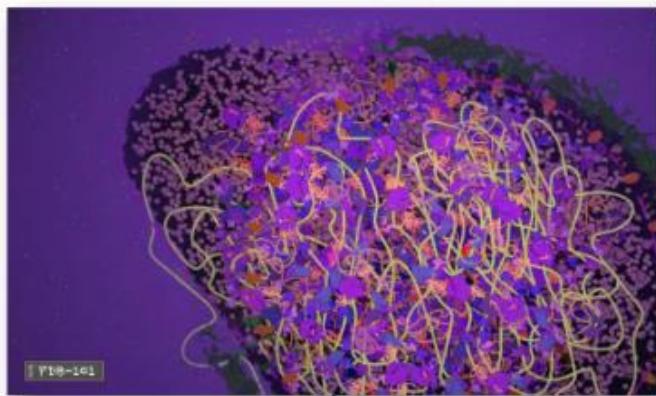


DNA sequencing of *Streptococcus iniae*, assembly, annotation
and submissions to public health databases (NCBI, EBI)

GRAPHICAL ABSTRACT: Comparative Genomics and Reverse Vaccinology of *Streptococcus iniae*: Blueprints for Affordable Aquaculture Vaccines



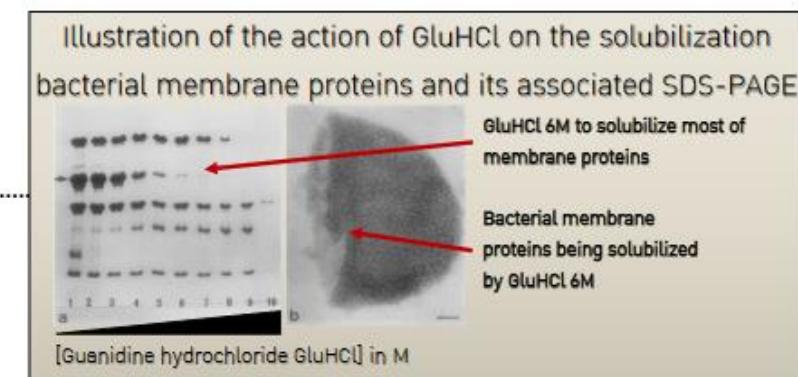
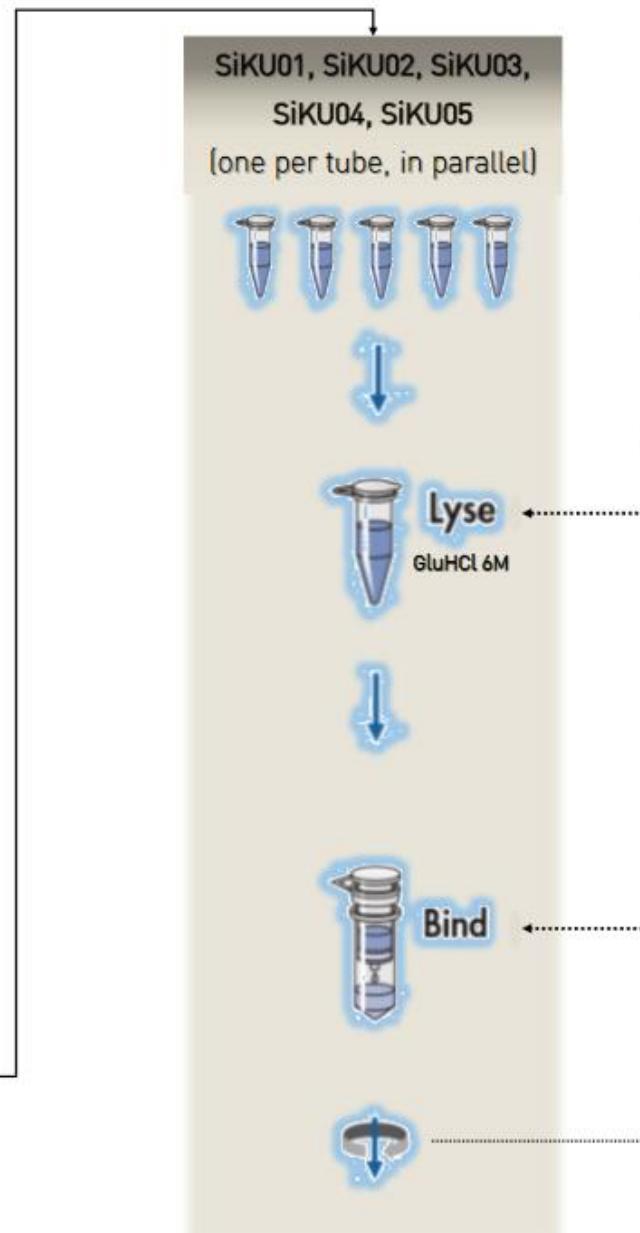




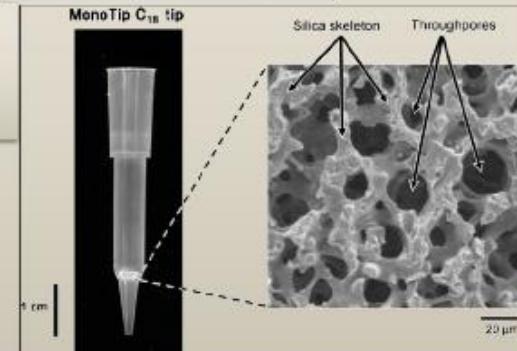
The PGN structure is destabilized, chaotropic agents can access the membrane

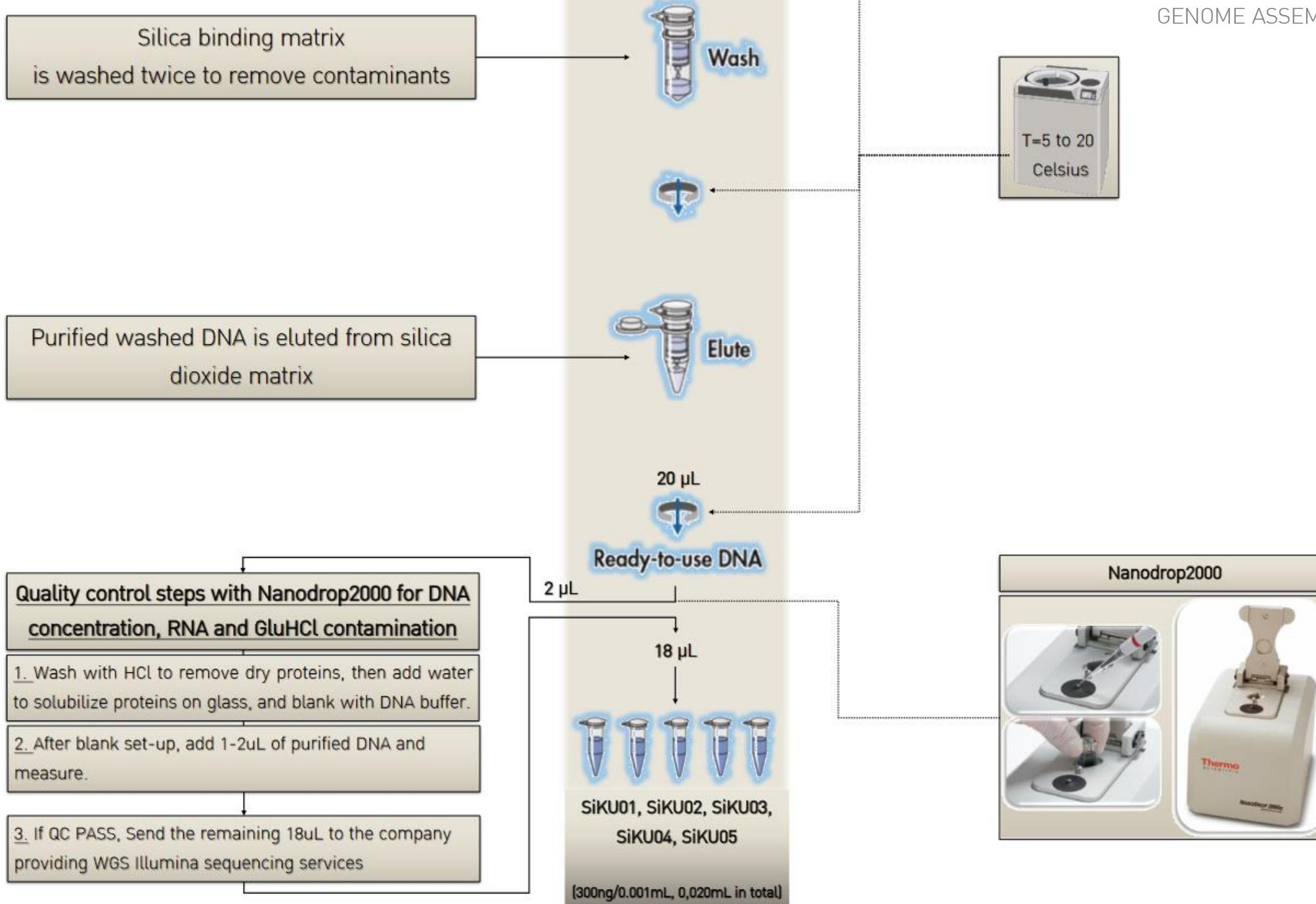
For pretreatment of Gram-positive bacteria

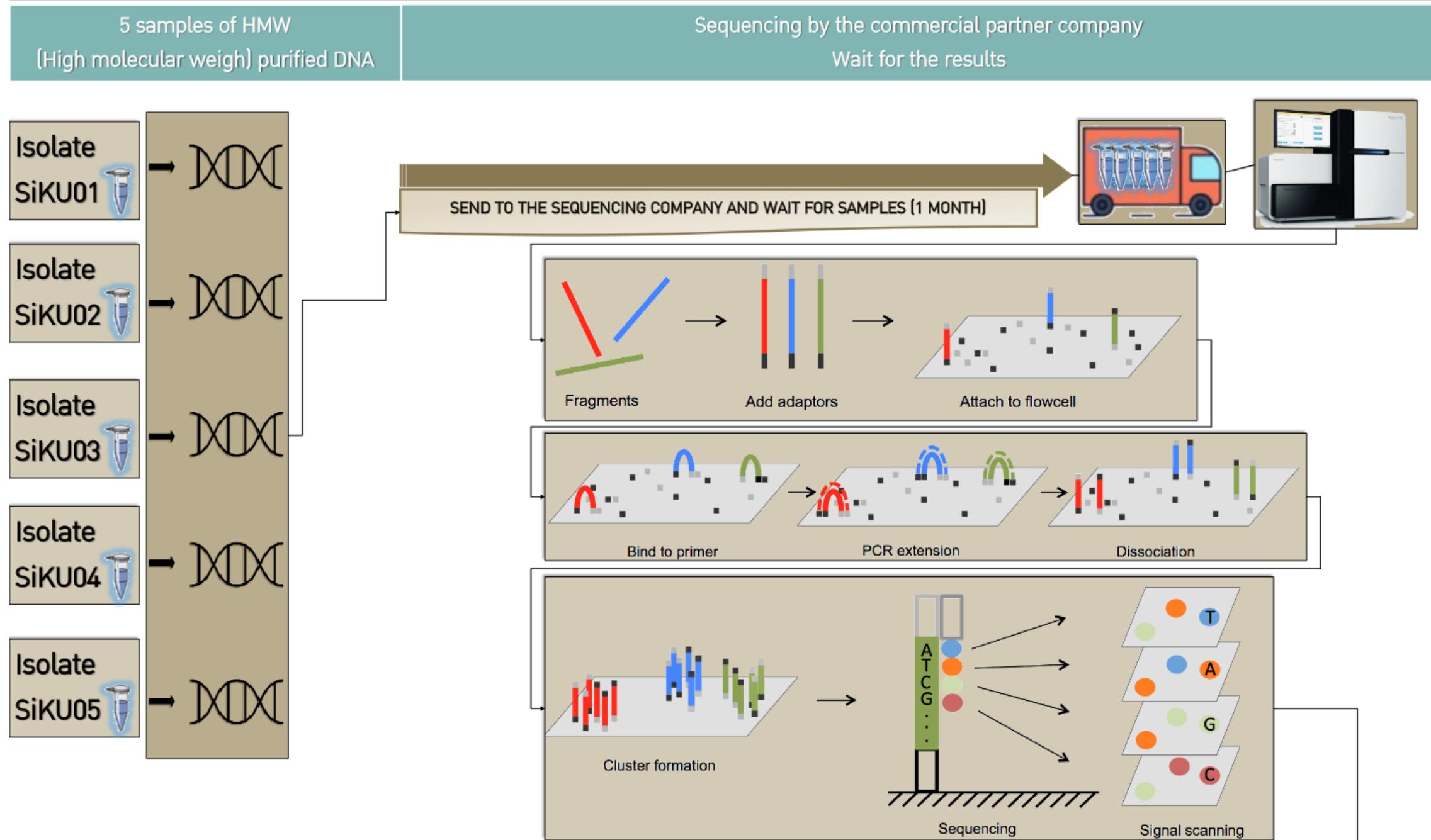
- Enzymatic lysis buffer:
 - 20 mM Tris·Cl, pH 8.0 Detergent, solubilize membranes
 - 2 mM sodium EDTA DNase inhibitor, chelate Mg²⁺
 - 1.2% Triton® X-100 Detergent, solubilize lipid membranes
 - Immediately before use, add lysozyme to 20 mg/ml

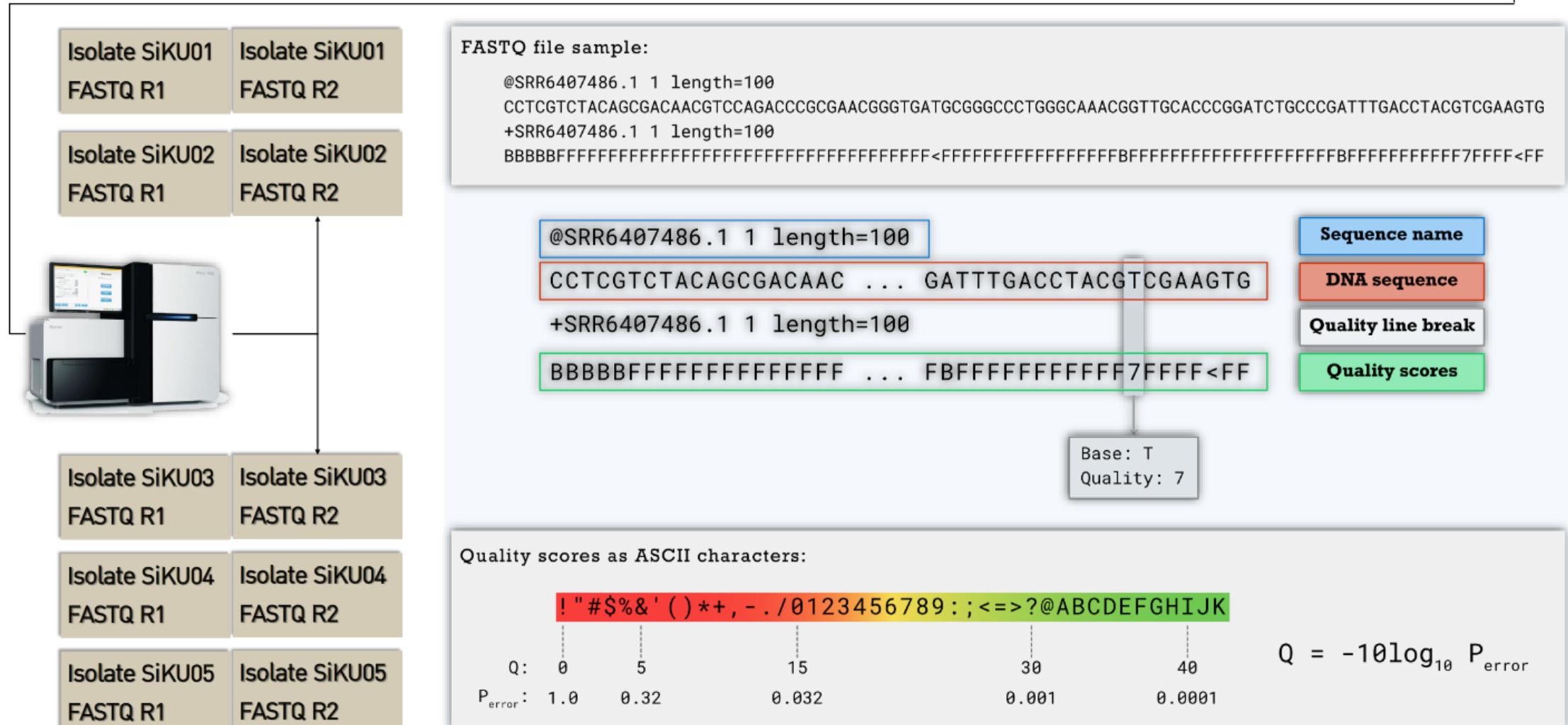


Silica binding matrix
retain genomic DNA









10 FASTQ files are obtained

2 FASTQ per Isolate, paired-reads R1 and R2 containing 3 million reads of 151bp (=2*151bp pair-end reads)

GENOME ASSEMBLY OF *Streptococcus iniae*

GENOME ASSEMBLY /ANNOTATIONS



Your submissions

GENOME ASSEMBLY /ANNOTATIONS

Start a new submission	
• GenBank	• BioProject
• Sequence Read Archive	• BioSample
• Genome	• Supplementary Files
• TSA	• API

5 submissions

Submission	Title	App	Status	Updated
SUB12934427	SIKU01	WGS	⚙️ Genomes: Processing (Details)	Apr 10
SUB12859515	Streptococcus iniae Genome sequencing and assembly	BioProject	✓ BioProject: Processed PRJNA933632 : Streptococcus iniae Genome sequencing and assembly, THAILAND (TaxID: 1346) Locus Tag Prefixes: • SIKU01 (SAMN33244442)	Mar 14
SUB12934419	New	GenBank	⌚ Unfinished at the Submission Type step	Mar 06
SUB12859633	Streptococcus iniae Genome sequencing and assembly, Feb 10 '23	Sequence Read Archive (SRA)	✓ SRA: Processed (5 objects) Download metadata file with SRA accessions View and manage my SRA submission data	Feb 10
SUB12859554	Pathogen: clinical or host-associated sample	BioSample	✓ BioSample: Processed (Details) Download attributes file with BioSample accessions	Feb 10

Submitting organization KASETSART UNIVERSITY, Faculty of fisheries
<https://fish.ku.ac.th/en/>

Project type

Sample scope Monoisolate

Target description Streptococcus iniae isolate from Kasetsart University. The Bacterium was collected from a diseased Asian seabass in a farm in the region of Samut Prakan in 2022. It is the first isolate of 5 in our collection.

BioSample None

Target

Organism name (taxid) Streptococcus iniae

Strain SIKU01

Isolate SIKU01

Label SIKU01

Locus tag prefix SIKU01

General information

Project details

Project type • genome sequencing and assembly
• raw sequence reads

Title Streptococcus iniae Genome sequencing and assembly

Description Streptococcus iniae isolate from Kasetsart University. The Bacterium was collected from a diseased Asian seabass, in a farm in the region of Samut Prakan in 2022. It is the first isolate of 5 in our collection.

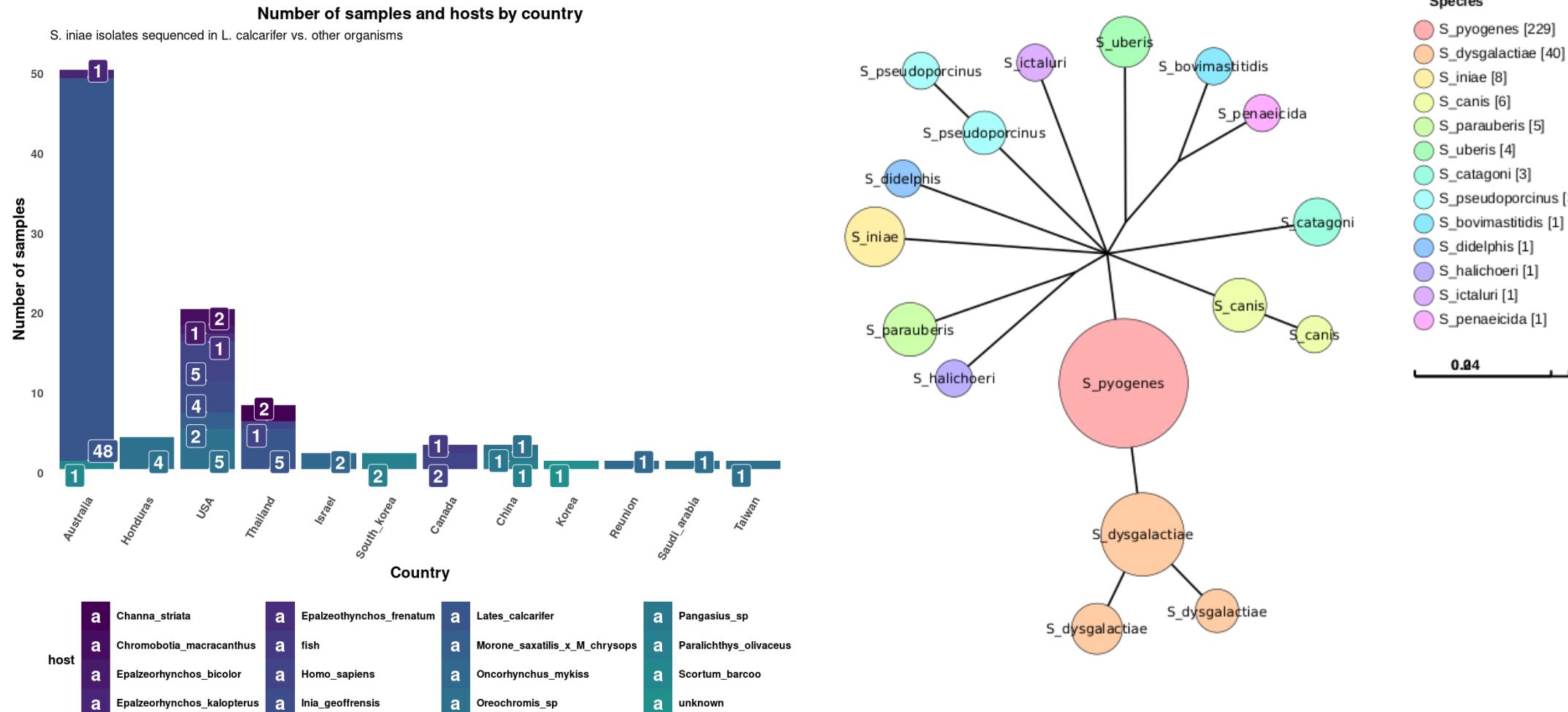
Relevance Agricultural

NCBI submissions (processed and to be released)



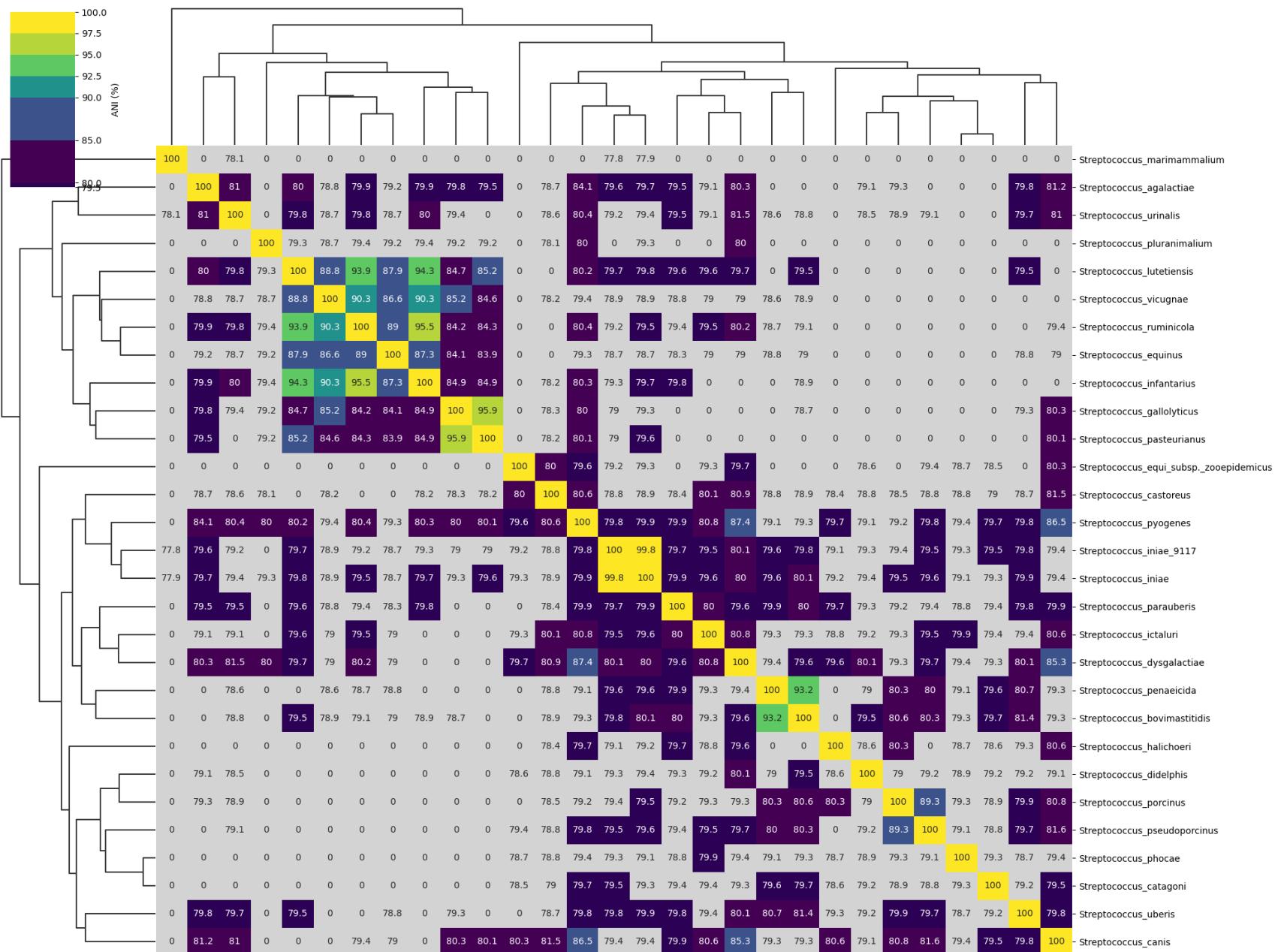
Comparative Genomics and Reverse Vaccinology of *Streptococcus iniae*

STEPTOCOCCUS GENUS, SPECIES K-MER CLUSTERING, DBSCAN, GMM-DISTANCES AND SAMPLING METADATA

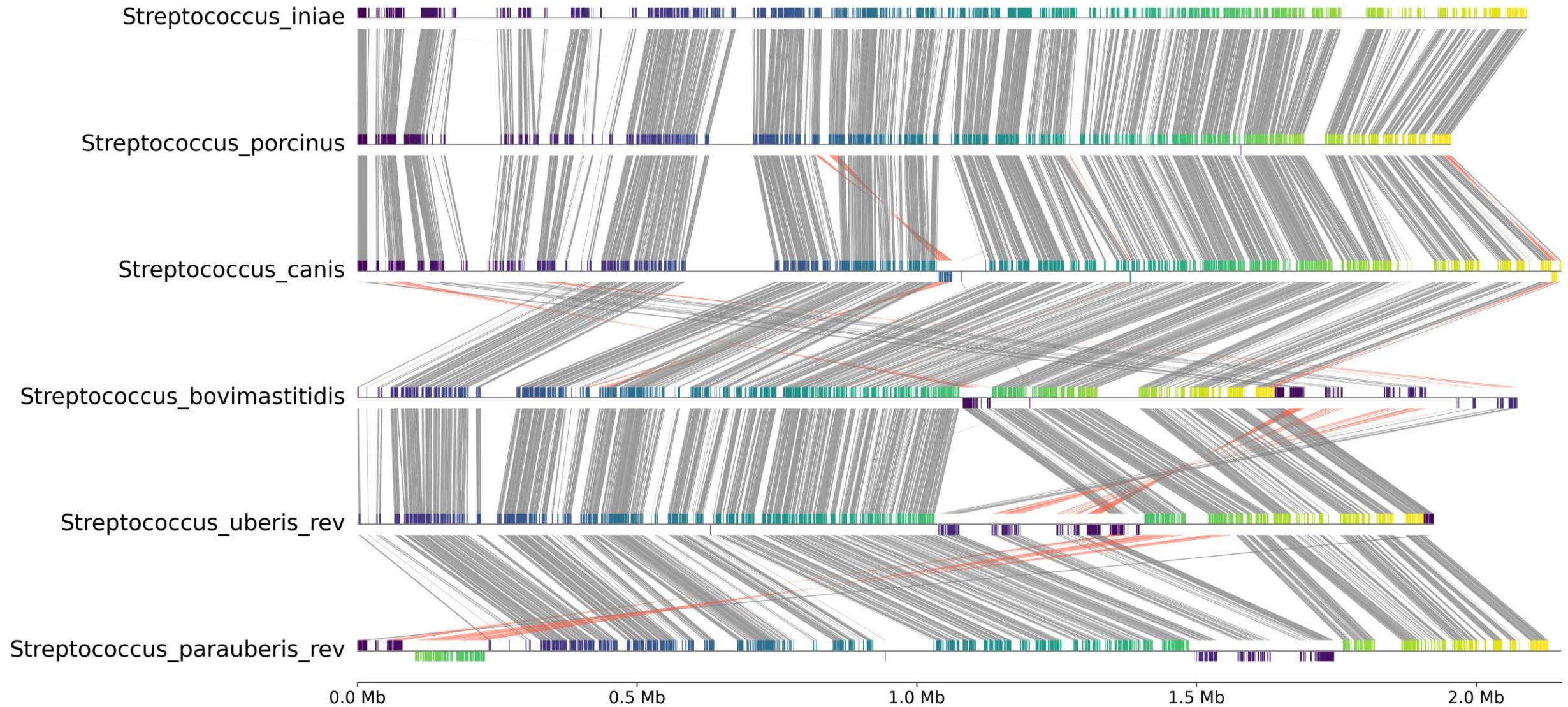


Data source: NCBI Genomes metainfo datasets

STEPTOCOCCUS FASTANI CLUSTERING AT THE GENUS LEVEL (WHOLE GENOMES)



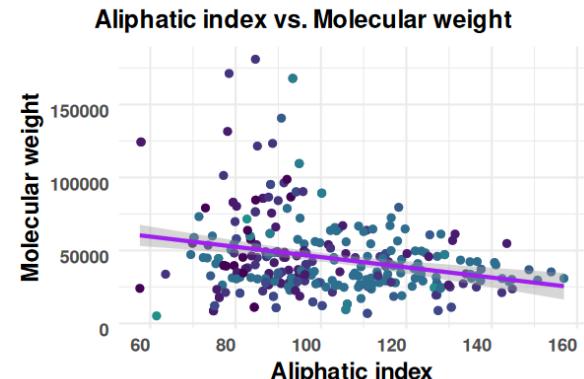
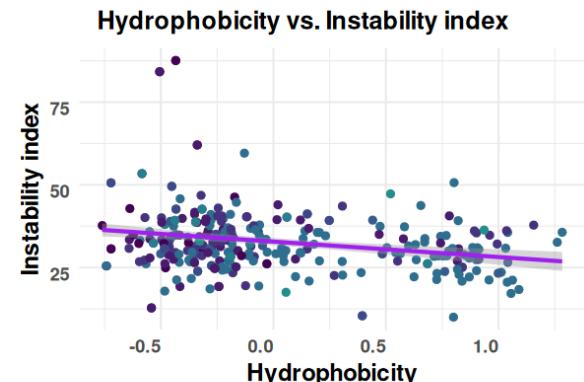
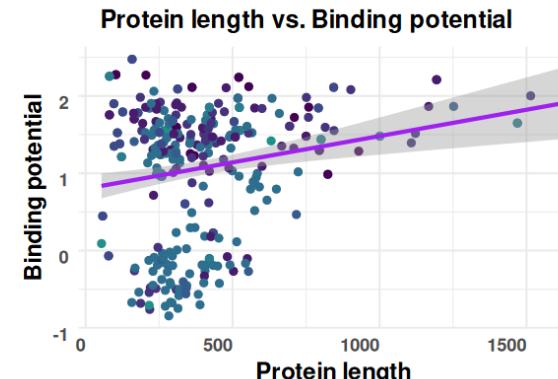
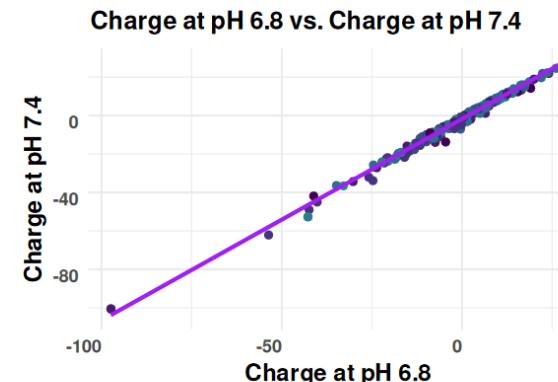
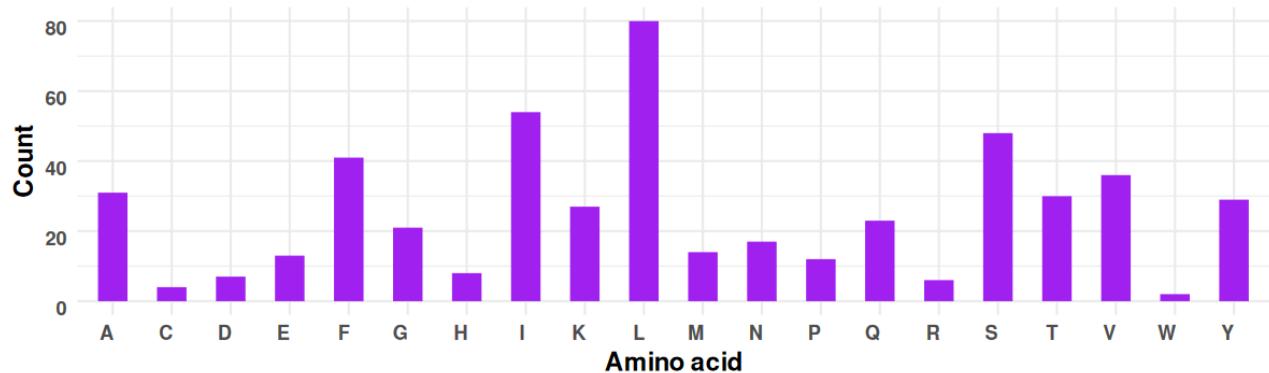
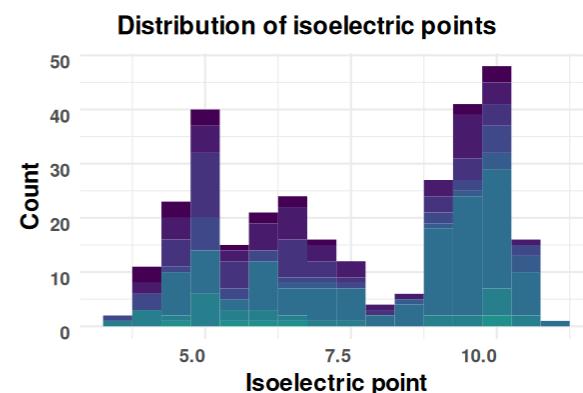
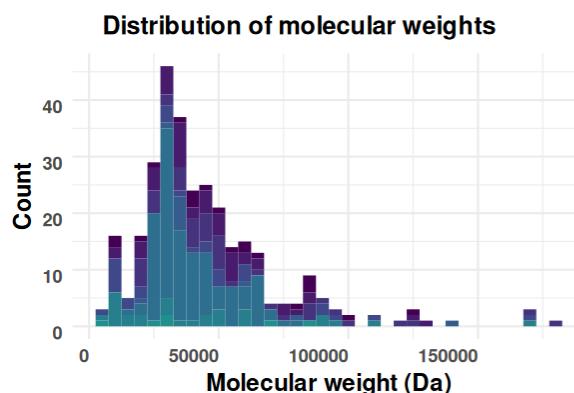
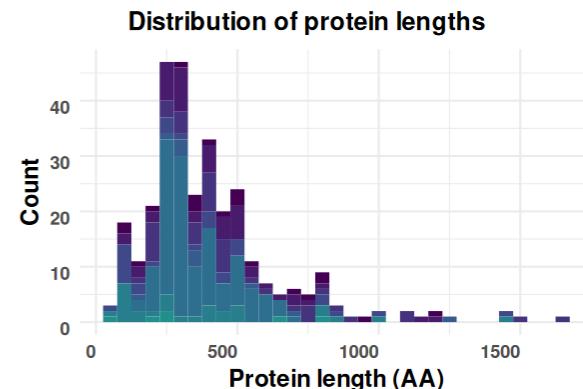
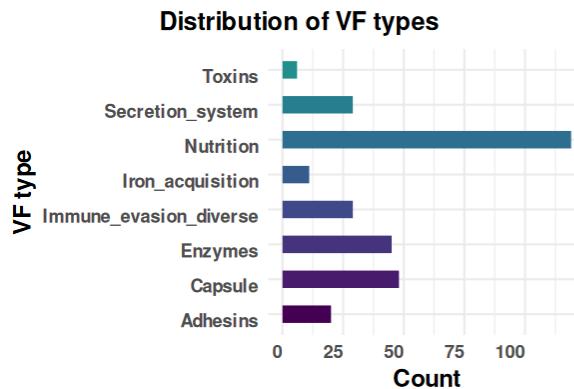
SYNTENY BETWEEN THE CLOSEST RELATIVES OF *STREPTOCOCCUS INIAE*



GENOME ANALYSIS OF STREPTOCOCCUS INIAE

PROTEIN ANNOTATIONS / REVERSE VACCINOLOGY

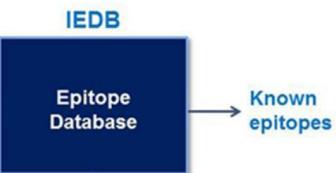
→ Goal: to find suitable antigens
to include on a vaccine:



GENOME ANALYSIS OF STREPTOCOCCUS INIAE

PROTEIN ANNOTATIONS / REVERSE VACCINOLOGY

Experimental data
Sequences
Organisms
Antigens
MHC alleles
Epitopes
Other data



bbuchfink/
diamond_docs

DIAMOND online documentation



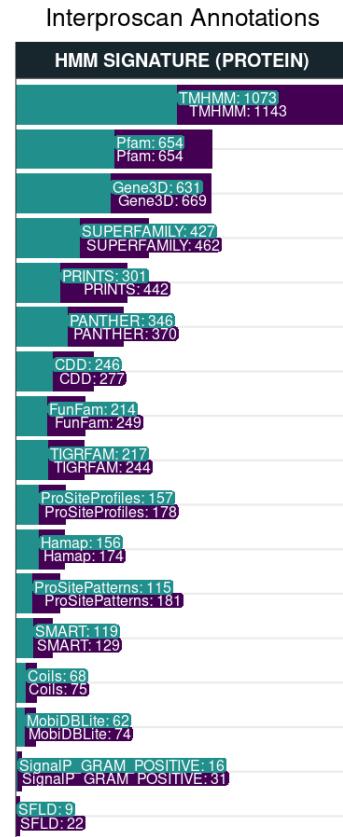
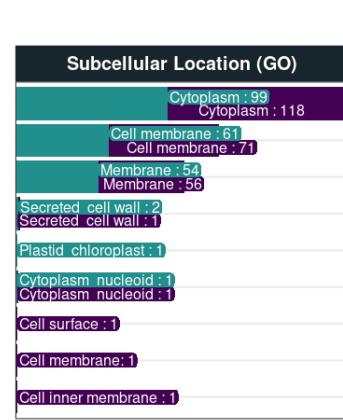
R2 - RStudio Source Editor

epitopesSIniae x

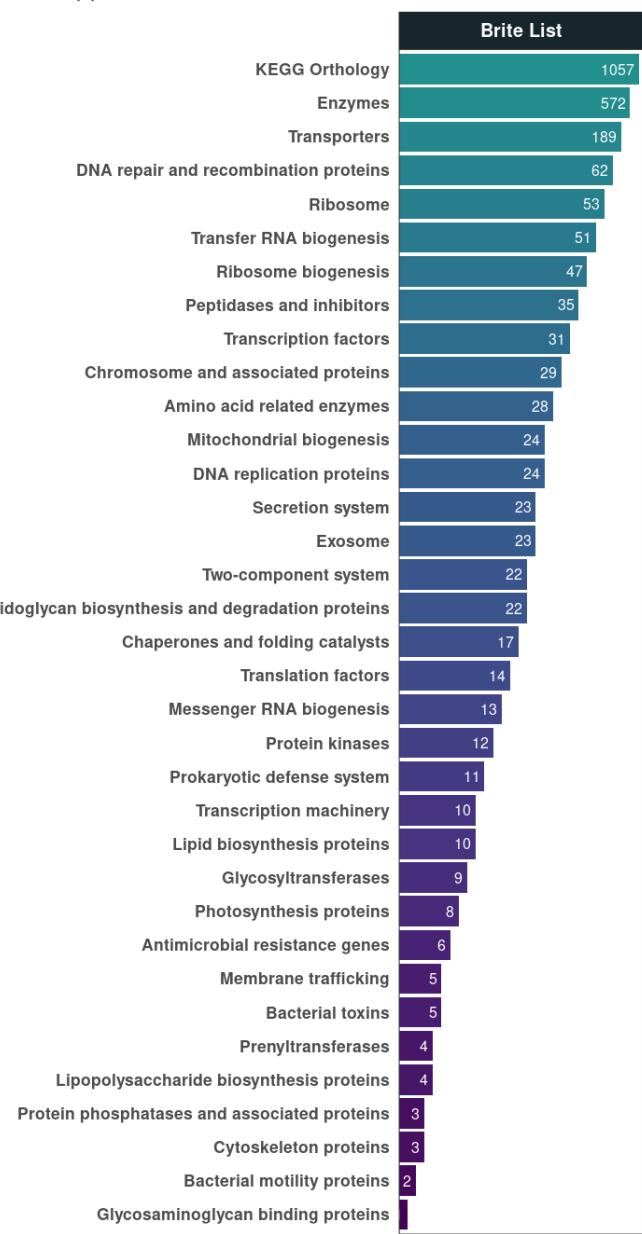
Filter

Locus_tag	Subject_id	Epitope_sequence
1	SIKU01_001181	Enolase
2	SIKU01_001378	metal_binding_protein_of_ABC_transporter_(lipoprot...)
3	SIKU01_001395	cell_envelope_proteinase_A
4	SIKU01_001681	pneumococcal_histidine_triad_protein_D
5	SIKU01_001709	immunogenic_secreted_protein
6	SIKU01_001765	Glyceraldehyde_3_phosphate_dehydrogenase
7	SIKU01_001769	surface_exclusion_protein
8	SIKU01_001890	M_protein
9	SIKU01_001902	GroEL
10	SIKU01_001991	Inosine_5'_monophosphate_dehydrogenase_[Strept...
11	SIKU01_001995	conserved_hypothetical_protein
12	SIKU01_000047	amidase
13	SIKU01_000165	DNA_directed_RNA_polymerase_subunit_beta_[Stre...
14	SIKU01_000216	pneumococcal_histidine_triad_protein_D
15	SIKU01_000259	YSIRK_signal_domain/LPXTG_anchor_domain_surfac...
16	SIKU01_000404	trigger_factor
17	SIKU01_000973	hypothetical_protein_SPy1154

Showing 1 to 17 of 17 entries, 3 total columns



KEGG Mapper



GENOME ANALYSIS OF STREPTOCOCCUS INIAE

Efficacy and Immunogenicity of Novel Multi Recombinant Protein and DNA Vaccines for the Prevention of Streptococcus iniae Diseases in Asian Seabass (*Lates calcarifer*)

The need for affordable vaccines: Profitable for the farmer : < 1-3 THB / fish – 5 cents / fish

Literature review	Fish vaccines Fish immunology	DNA vaccines	Recombinant protein vaccines	Microbial genomics
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Cost considerations: How to lower the price?

Production costs	Raw materials availability	Expression system	Ease of production	Vaccine design
Increased yields	Target antigen(s)	Delivery associated costs	Stable when stored	Tunability of the costs
Increased efficiencies	Adjuvant(s)			Ability to scale up

Candidate antigen selection: choosing the most promising antigens for the highest production

Reverse vaccinology approach	Bacterial isolation	Illumina sequencing	Genome sequences	List of genes	Pangenomes
	Operons and regulons	Protein attributes	Human epitopes	List of proteins	Orthologues

List of antigen candidates N= 308

Pangenomics

Gene absence / presence



Intergenic sequences IGR



Pangenomes *S. iniae* (species)

Pangenomes Streptococcus (genus)

Proteins important for bacterial virulence and potential for vaccine targets

Regulons	Operons for essential pathways	Gene co-regulations	Virulence factors from the literature	Toxins, secreted effectors
Redundancy	Location in the cell	Response to environment	Classification in categories (proteases..)	

Antigen candidate selection scoring system

Cross-protection / immune responses

Conserved proteins	Sequence identity	Motifs	Human T/B cells epitopes in amino acid sequences
Orthologues in other pathogenic Streptococcus species			Virulence factors from the literature

Protein features

pH	Isoelectric point	Amino acid sequence length	Hydrophobicity
Aliphatic index (stability)	Charge at pH 'X'	Molecular weight (kDa)	GRAVY
Instability index	solubility	Stability	

Gene features

Type IIS internal restriction sites (Bs1 / Eco31L)

Gene length	GC content	Codon usage in E.coli (CAI)
-------------	------------	-----------------------------

List of most promising antigens N = 30

List of most promising antigens for soluble expression N = 3

ATGCCGGGCCCCCTGGGCAAACGGTTGCACCGGG
ATCTGCCCGATTTGACCTACGTGCGAAGTG

List of most promising antigens for expression as inclusion bodies (insoluble) N = 3

ATGCCGGGCCCCCTGGGCAAACGGTTGCACCGGG
ATCTGCCCGATTTGACCTACGTGCGAAGTG

Antigen candidates DNA sequences and primer design for golden gate

Antigen structure predictions (Alphafold2 collab)

```
R - RStudio Source Editor
epitopesSiniai x Select_candidates.R x
Source on Save Run Source

10 # Define a function to score each candidate protein based on its attributes
11 score_protein <- function(protein) {
12
13   # Assign points based on certain criteria
14   points <- 0
15   if (as.numeric(protein["length_AA"]) >= 100) points <- points + 2
16   if (as.numeric(protein["mw"]) >= 20000 & as.numeric(protein["mw"]) <= 40000) points <- points + 2
17   if (as.numeric(protein["pi"]) >= 4 & as.numeric(protein["pi"]) <= 10) points <- points + 1
18   if (as.numeric(protein["hydrophobicity"]) >= -1 & as.numeric(protein["hydrophobicity"]) <= 1) points <- points + 1
19   if (as.numeric(protein["instability_index"]) <= 40) points <- points + 1
20   if (as.numeric(protein["binding_potential"]) >= 0.5) points <- points + 1
21   if (as.numeric(protein["charge_pH_7"]) >= -1 & as.numeric(protein["mw"]) <= 2) points <- points + 3
22   if (!is.na(protein["PubMed_ID"]) > 0) points <- points + 1
23   #if (as.numeric(protein["RPS"]) >= 50) points <- points + 4
24   if (as.numeric(protein["bsaI_count"]) < 1) points <- points + 4
25   if (!is.na(protein["Epitope_sequence"])) > 0) points <- points + 3
26
27   # Return the total score for the protein
28   return(points)
29 }

30
31 # Apply the scoring function to each row in the dataframe and store the results
32 scores <- apply(Virulence_factors_candidates, 1, score_protein)
33
34 # Create a new dataframe with the scores and the original data
35 results <- data.frame(Virulence_factors_candidates, score = scores)
36

43:41 (Top Level) ⇧ R Script ⇧
```

RESULTS FROM REVERSE METHODOLOGY ANTIGEN SELECTION FOR SILICA-BASED VACCINE MANUFACTURING STRATEGIES

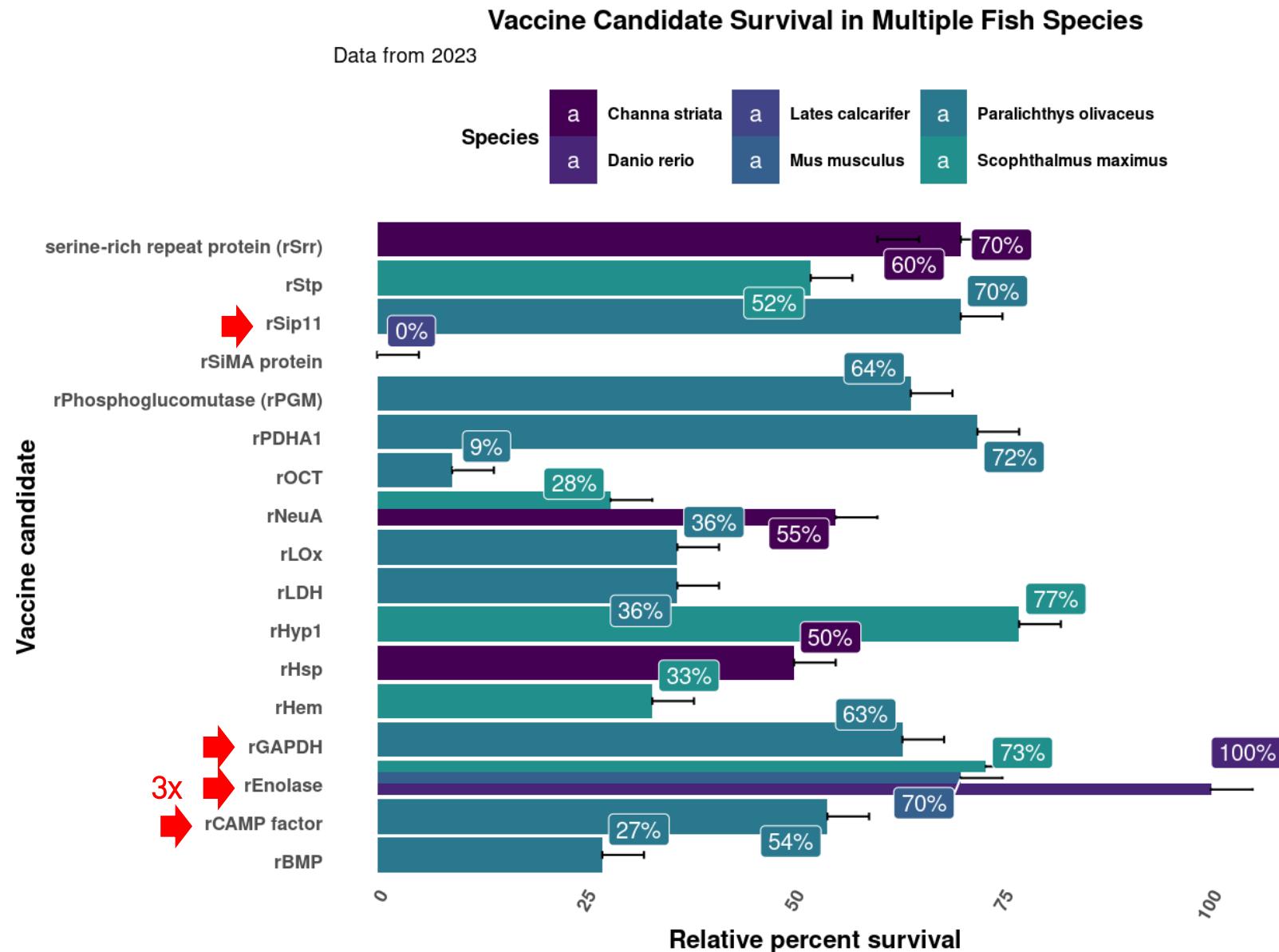
Table 3 Antigen candidates for *Streptococcus iniae* scoring more than 17 points

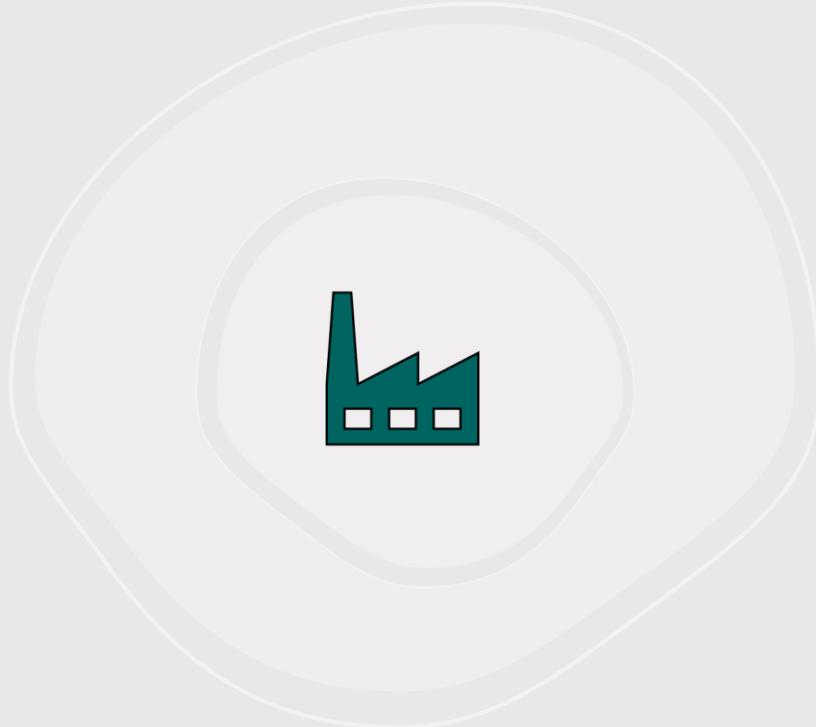
Locus (SIKU01)	Start	End	Strand	Gene name(s)	Protein name(s)	AA	mw (kDa)	pl	Score protein	PubMed ID	Epitope sequence (IEDB.org)
000047	34298	35512	+	NA	CHAP domain-containing protein	404	42.5	6.57	23	NA	EEKQAAQEAINTVA
000259	226392	227462	+	NA	Cell wall anchor protein	356	39.2	9.88	18	NA	GVNQFIPFELFGGDGMLTRL
000404	387230	388513	+	tig	Trigger factor (TF) [EC 5.2.1.8] [PPIase]	427	47.8	4.15	20	NA	ELDDELAKDIDEEV
000518	514136	515170	+	sip11	Iron ABC transporter substrate-binding protein [Sip11]	344	37.6	6.81	22	20096393	NA
000531	528303	529253	+	arcC	Carbamate kinase	316	33.5	4.46	17	32940251	NA
000801	827894	828607	+	deoD	Purine nucleoside phosphorylase DeoD-type [PNP] [EC 2.4.2.1]	237	25.7	4.793	17	17194809	NA
										17098893	
000803	829462	830382	-	cpsY	CpsY	306	35.4	6.43	17	23001668	NA
000805	832098	832829	+	cpsB	Tyrosine-protein phosphatase [EC 3.1.3.48]	243	28.2	7.12	20	17194809	NA
										17098893	
										22107596	
000862	887941	888711	+	cfi	cAMP factor	256	27.6	5.64	17	17991014	NA
000971	998521	999477	-	NA	Extracellular protein	318	34.1	5.82	17	28415641	NA
000973	1000282	1001040	-	NA	Sortae A [Sortase]	252	27.9	9.21	17	25090938	DSVLEAQMASQQLPVIGGIA

RESULTS FROM REVERSE METHODOLOGY ANTIGEN SELECTION FOR SILICA-BASED VACCINE MANUFACTURING STRATEGIES

Locus (SIKU01)	Start	End	Strand	Gene name(s)	Protein name(s)	AA	mw (kDa)	pI	Score protein	PubMed ID	Epitope sequence (IEDB.org)
001022	1054906	1055577	+	NA	Transcriptional regulatory protein	223	25.6	6.42	17	17537179	NA
001181	1219959	1221266	-	eno	Enolase (EC 4.2.1.11) [2-phosphoglycerate dehydratase]	435	47.1	4.35	20	NA	RAAADYLEVPLYNYLG
001377	1419701	1420429	-	mtsB	Metal ABC transporter ATP-binding protein (MtsB)	242	26.7	6.03	17	21122131	NA
										21111784	
001378	1420495	1421424	-	mtsA	Metal ABC transporter substrate-binding protein (MtsA)	309	34.6	5.89	23	21122131	EINTEEGTPDQISSLIEK
001681	1727136	1729517	-	NA	Pneumococcal-type histidine triad protein	793	88.1	5.70	17	NA	YVTSHGDHYHYYNGKVPYDA
001709	1759258	1760757	-	NA	CHAP domain-containing protein	499	53.3	4.80	17	NA	DASGTGKRRAEVMEKLDQWIDRHGGTP
001765	1822113	1823123	-	gapC GAPDH	Glyceraldehyde-3-phosphate dehydrogenase [EC 1.2.1.-]	336	35.7	5.06	20	NA	MVKVGINGFGRIGRLAFRRIQ
001890	1972490	1973578	-	NA	YSIRK signal domain/LPXTG anchor domain surface protein	362	39.7	4.90	22	NA	QLPSTGDSYNPFFTASAMAI
001902	1985693	1987321	-	groEL	60 kDa chaperonin	542	57.2	4.43	18	19620365	LPTLVNLKIRGTFNWWAVKAPGFGDRRKAM
001991	2077814	2079295	-	guaB	Inosine-5'-monophosphate dehydrogenase	493	52.8	5.83	17	NA	VVKVGIGPGSIC

RESULTS FROM REVERSE METHODOLOGY ANTIGEN SELECTION FOR ALL VACCINE MANUFACTURING STRATEGIES





Protein vaccine production



Protein vaccine production - manufacturing strategy 1:

Silica-based manufacturing strategies

Drug substance formulated as a complex antigens immobilized on silicon dioxide nanoparticles (adjuvant)

PROTEIN VACCINE PRODUCTION via silica-based manufacturing strategies.

Table 15 Target product profile for vaccines produced via silica-based manufacturing strategies.

Category	Description
Mechanism of Action	rSI-VAX-Silica (drug product) is a universal recombinant vaccine containing the conserved enolase/sortaseA/GAPDH antigen from <i>Streptococcus iniae</i> , in a buffer solution with silicon dioxide (SiO ₂) immune adjuvant / molecular carrier. rSI-VAX-Silica is expected to provide a cellular (Th1) and humoral (Th2), antigen-specific, protective immune response when compared with a natural <i>Streptococcus iniae</i> infection. It is possible to prepare the vaccine solution at the last moment by mixing SiO ₂ nanoparticles to the vaccine solution one day before use.
Indication	rSI-VAX-Silica is indicated for the active immunization of 2-month-old juveniles to adults freshwater and marine fish species susceptible to <i>Streptococcus iniae</i> for prevention Streptococcosis related illnesses caused by <i>Streptococcus iniae</i> .
Primary Endpoints	<ul style="list-style-type: none"> • 60% reduction of <i>S. iniae</i>-confirmed disease within one year after dosing (below 40% is a no go) in the target population • Safe and tolerable as defined by solicited symptoms, adverse events, and serious adverse events (no evidence of enhanced <i>S. iniae</i> disease), no known impacts on FCR or growth parameters.
Key Claims	<ul style="list-style-type: none"> • Has a favorable risk-benefit profile in farm • Can be dosed with other fish vaccines and probiotics • Universal recommendation for the replacement of antibiotic usage in farm • Achieves World Health Organization (WHO) stability requirements
Secondary Endpoints	<ul style="list-style-type: none"> • Analysis supportive of primary endpoint in target fish population • Reduction in <i>S. iniae</i>-specific mortality and rates of bacteria-confirmed diseases • Reduction in antibiotic use for <i>S. iniae</i>-confirmed disease • Duration of protection >6 months (with/without booster)
Formulation/Dosing	<ul style="list-style-type: none"> • Antigen in a flask + SiO₂ nanoparticles sterile powder • Antigen containers are pre-mixed with SiO₂ powder prior to injection • 1 doses administered

Proteins purified via chromatography by affinity to a support matrix made of silicon dioxide macro and nanoparticles, antigens are immobilized, and the nanoparticles are directly administrated to the fish

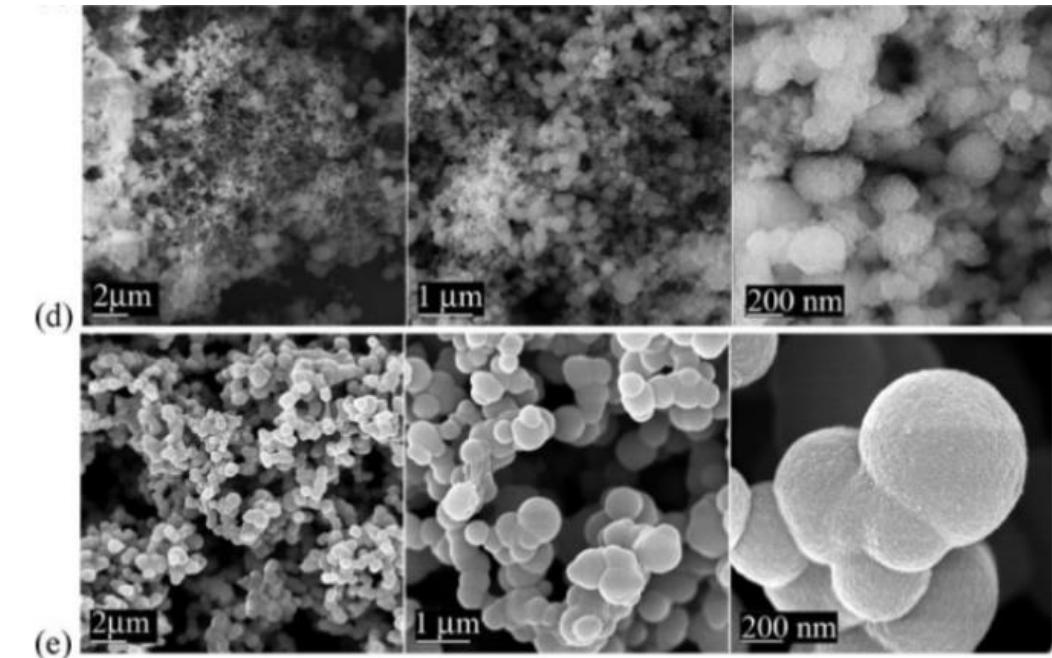


figure 1. SEM micrographs of silica matrices formed by (a) R5-Gfp, (b) R5-Pde, (c) R5(1)-Opd, and (d) R5(3)-Opd

PROTEIN VACCINE PRODUCTION risk assessment for silica-based manufacturing strategies.

Table 18 Impact score.

Impact	Score	Safety and Tolerability		
Uncertainty Score	Uncertainty			
Very High	25	Has a negative impact on FCR SGR, fish development and/or vaccine performance		
Moderate	8	Has a moderate impact on FCR, SGR, vaccine performance		
Minimal	2	No changes in FCR and SGR, vaccine performance		

Table 19 Uncertainty score.

(1)

Quality/Product Attribute	Method	I*	U*	S*
Potency				
Ability to induce antibodies (Serology)	Indirect ELISA (vaccinated fish serum as primary Ab source) (adsorbed)	25	2	50
Ability to induce antibodies (Serology)	Agglutination test (vaccinated fish serum as Ab source) (adsorbed)	8	2	16
Th1/Th2 Profile	Cytokine-panel qRT-PCR (Gene expression)	25	2	50
Animal Model (confirms correlation)	Fish Serology (adsorbed)	25	2	50
Animal Model (confirms correlation)	qRT-PCR (Gene expression IgM,IgT)	25	2	50
Animal Model (confirms correlation)	Survival infection trial	25	2	50
Purity (desorbed Ag)				
Recombinant protein level	Calculated - Split-GFP	8	3	24
Total proteins	BCA assay	25	2	50
Complexes/Aggregates	Non-reducing CGE	25	2	50
Product-derived Impurity (desorbed Ag)				
Fragments	Reducing CGE	8	3	24
Complexes/Aggregates	Non-reducing CGE	25	3	75
Process-derived Impurity				
Activation and Binding Reactants	Calculated	8	5	40
Structure/Function (Charac.)				
(SiO ₂ adsorbed unless indicated)				
Ag-SiO ₂ /free-Ag/free-SiO ₂ -Adjuvant Ratio	Calculated	8	5	40
Binding	Thermal shift assay / DSF	8	5	40
Ag Size Distribution	Microscopy	25	5	125
Size of Aggregates	Microscopy	25	5	125
Extent of Conjugation (as Ps-VLP, free Ps, and free VLP)	Reducing CGE	25	3	75

Table 20 Initial CQAs and risk assessment for reconstituted silica-based vaccines.

Other				
Quantity (as Protein Content)	Calculated	25	2	50
Quantity (as Adjuvant Content)	Calculated	25	2	50
Fill Volume in Container	Compendial	25	1	25
Endotoxin	Compendial	25	1	25
Completeness-of-Adsorption (Adsorption to SiO ₂)	Thermal shift assay / DSF	25	5	125
SiO ₂ Content	Rely on manufacturer specs, TBD.	25	1	25

* Impact = I, Uncertainty = U, and Severity = S (see Equation 1) and TABLE 19 : Uncertainty Score for rSI-VAX, TABLE 18 : Impact Score for rSI-VAX.

PROTEIN VACCINE PRODUCTION via silica-based manufacturing strategies.

11.1.5 Key Molecular Characteristics of Vaccines

Table 2-4: QTPP for rSI-VAX lists the vaccine's quality target product profile. The QTPP is a prospective summary of the desired quality characteristics of the drug product that will ideally be achieved, taking into account the safety and efficacy of rSI-VAX (ICH Q8).

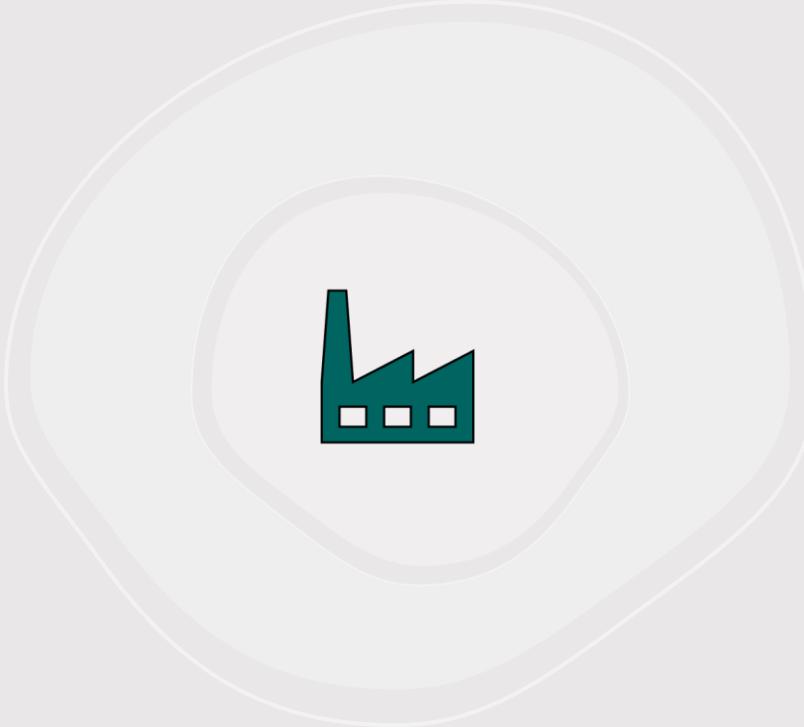
Table 17 Quality target product profile (QTPP) for silica-based vaccines.

Key Claims	Description
Easy to administer	0.1-mL intraperitoneal delivery in farm setting using a 1-mL syringe (27G × ½ inch needle)
Stability	3 months at room-temperature storage or 1 years at 2-8 °C, and 24 hours' physical and chemical stability following reconstitution at 2-8 °C or 8 hours at room temperature (achieves WHO stability requirements)
No animal-derived products	No animal- or human-derived products are used in the manufacture of rSI-VAX
Formulation/Dosing	Description
Sterile product	The drug substance (rAntigens) can be sterile filtered
1 doses	100 doses containing 40 mcg of antigen; adsorbed to 300 mcg silicon dioxide (50um diameter) adjuvant as bound antigens administered in a single vaccination)
Soluble or reconstituted	Soluble or reconstituted with standard diluents containing adjuvant: rapid reconstitution profile with viscosity of 1-3 cP
Composition	Composition includes sugar, surfactant, buffer (isotonic pH), and rAntigen
Label volume	Label volume of 10 mL filled (actual fill volume will be greater than the label volume to account for losses)
Multiple-dose container	Multiple-dose wide PET bottle with narrow neck and seal (13-mm aluminum seal with flip-off plastic button)
Secondary packaging and shipping	Allowed shipping-excursion temperature of 2-40 °C for 3 days in a carton (10 pcs./carton)

Table 21 Triage round 1 for silica-based vaccines.

Quality/Product Attribute	Method	I*	U*	S*
Potency				
Animal Model (confirms correlation)	qRT-PCR (Gene expression IgM,IgT)	25	2	50
Th1/Th2 Profile	Cytokine-panel qRT-PCR (Gene expression)	25	2	50
Animal Model (confirms correlation)	Survival infection trial	25	2	50
Purity (desorbed Ag)				
Recombinant protein level	Calculated - Split-GFP	8	3	24
Product-derived Impurity (desorbed Ag)				
Complexes/Aggregates	Non-reducing CGE	25	3	75
Process-derived Impurity				
Structure/Function (Charac.)	(SiO ₂ adsorbed unless indicated)			
Ag-SiO ₂ /free-Ag/free-SiO ₂ -Adjuvant Ratio	Calculated	8	5	40
Ag Size Distribution	Microscopy	25	5	125
Binding	Thermal Shift Assay / DSF	8	5	40
Other				
Quantity (as Protein Content)	Calculated	25	2	50
Quantity (as Adjuvant Content)	Calculated	25	2	50
Quality/Product Attribute	Method	I*	U*	S*
Completeness-of-Adsorption (Adsorption to SiO ₂)	Thermal shift assay / DSF	25	5	125

* Impact = I, Uncertainty = U, and Severity = S (see Equation (1) and TABLE 19 : Uncertainty Score for rSI-VAX, TABLE 18 : Impact Score for rSI-VAX).



Protein vaccine production - manufacturing strategy 2: Cellulose-based manufacturing strategies

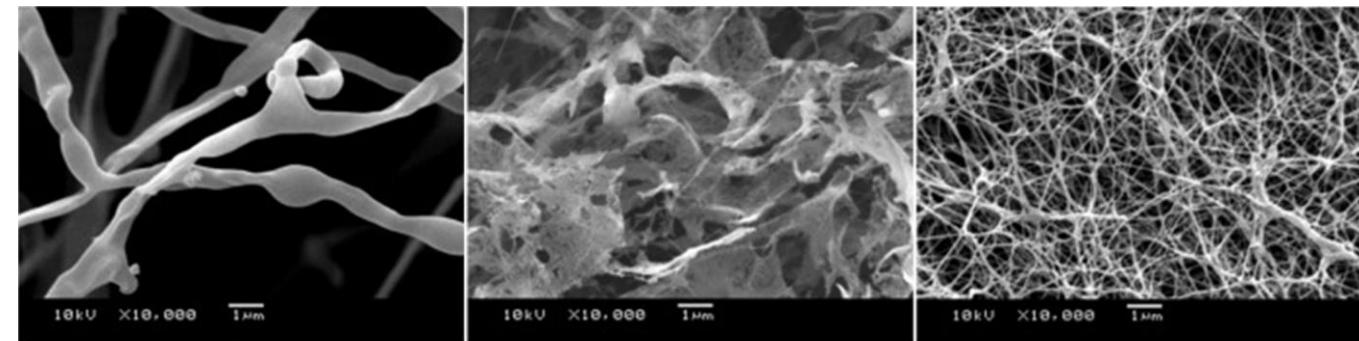
Drug substance formulated as protein crystalline inclusions.

PROTEIN VACCINE PRODUCTION via cellulose-based manufacturing strategies

Proteins purified via chromatography by affinity to a support matrix made of biodegradable cellulose polymers

Table 14 Target product profile for vaccines produced via cellulose-based manufacturing strategies.

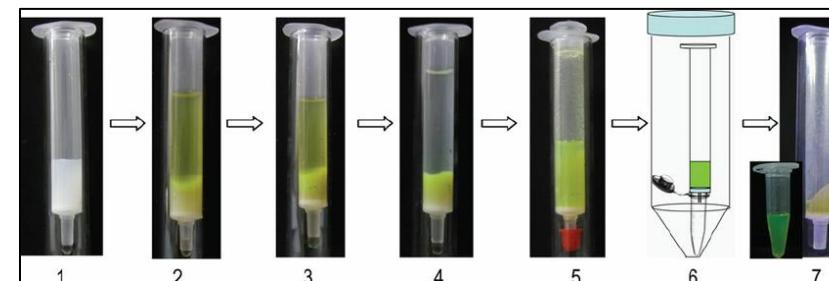
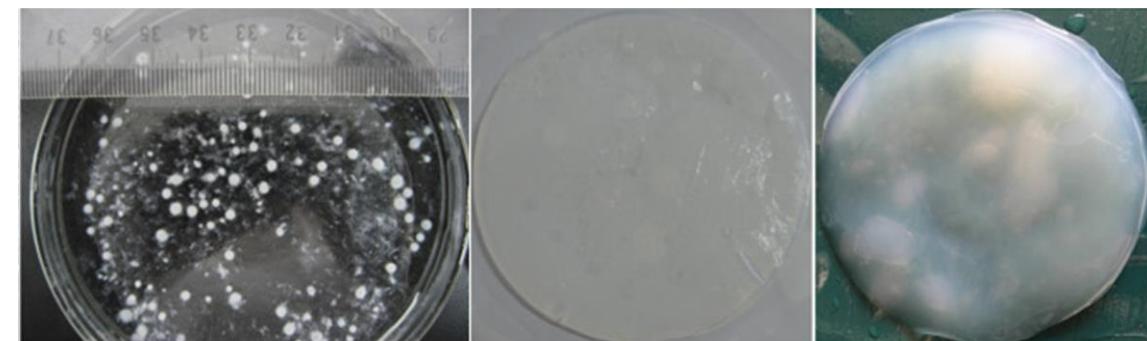
Category	Description
Mechanism of Action	rSI-VAX-Cell (drug product) is a universal recombinant vaccine containing the conserved enolase/sortaseA/GAPDH antigen from <i>Streptococcus iniae</i> , in a buffer solution with microcrystalline cellulose as an immune adjuvant. rSI-VAX-Cell is expected to provide a cellular (Th1) and humoral (Th2), antigen-specific, protective immune response when compared with a natural <i>Streptococcus iniae</i> infection.
Indication	rSI-VAX-Cell is indicated for the active immunization of 2-month-old juveniles to adults freshwater and marine fish species susceptible to <i>Streptococcus iniae</i> for prevention Streptococcosis related illnesses caused by <i>Streptococcus iniae</i> .
Primary Endpoints	<ul style="list-style-type: none">• 60% reduction of <i>S. iniae</i>-confirmed disease within one year after dosing (below 40% is a no go) in the target population• Safe and tolerable as defined by solicited symptoms, adverse events, and serious adverse events (no evidence of enhanced <i>S. iniae</i> disease), no known impacts on FCR or growth parameters.
Key Claims	<ul style="list-style-type: none">• Has a favorable risk-benefit profile in farm• Can be dosed with other fish vaccines and probiotics• Universal recommendation for the replacement of antibiotic usage in farm• Achieves World Health Organization (WHO) stability requirements
Secondary Endpoints	<ul style="list-style-type: none">• Analysis supportive of primary endpoint in target fish population• Reduction in <i>S. iniae</i>-specific mortality and rates of bacteria-confirmed diseases• Reduction in antibiotic use for <i>S. iniae</i>-confirmed disease• Duration of protection >1 year (with/without booster)
Formulation/Dosing	<ul style="list-style-type: none">• Antigen in pre-filled syringe or single-dose vial• Antigen containers are pre-mixed prior to injection• 1 doses administered



Amorphous cellulose nanostructure

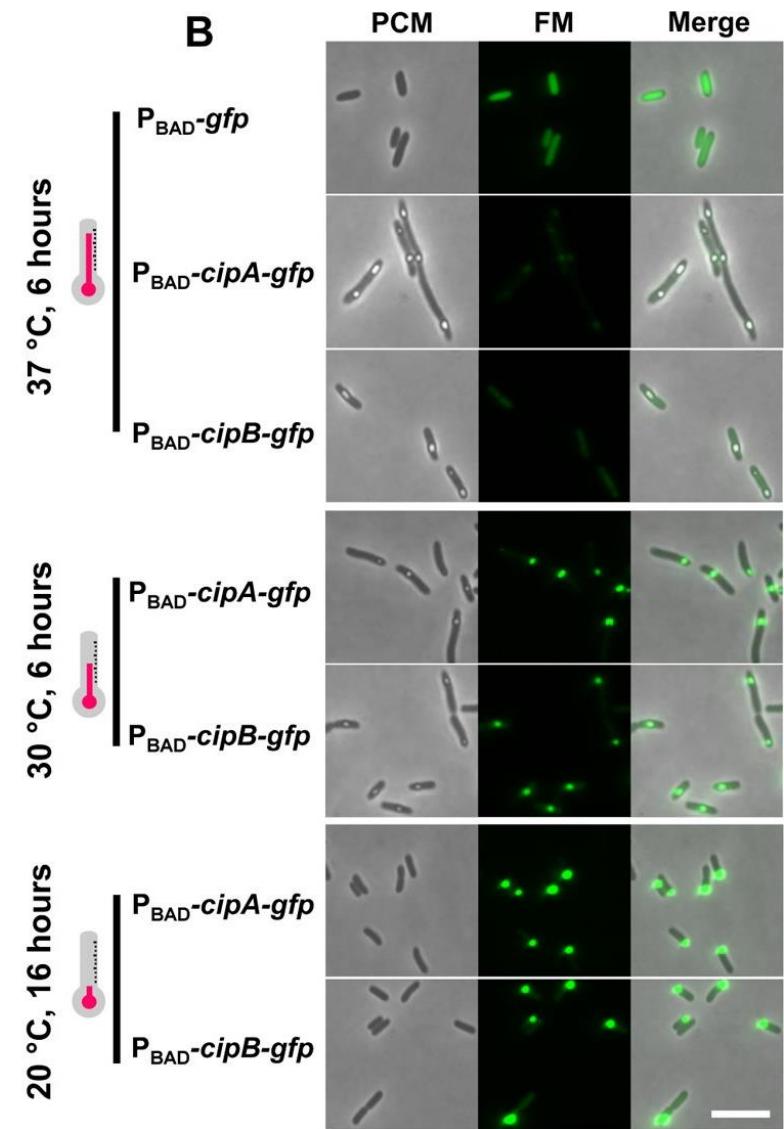
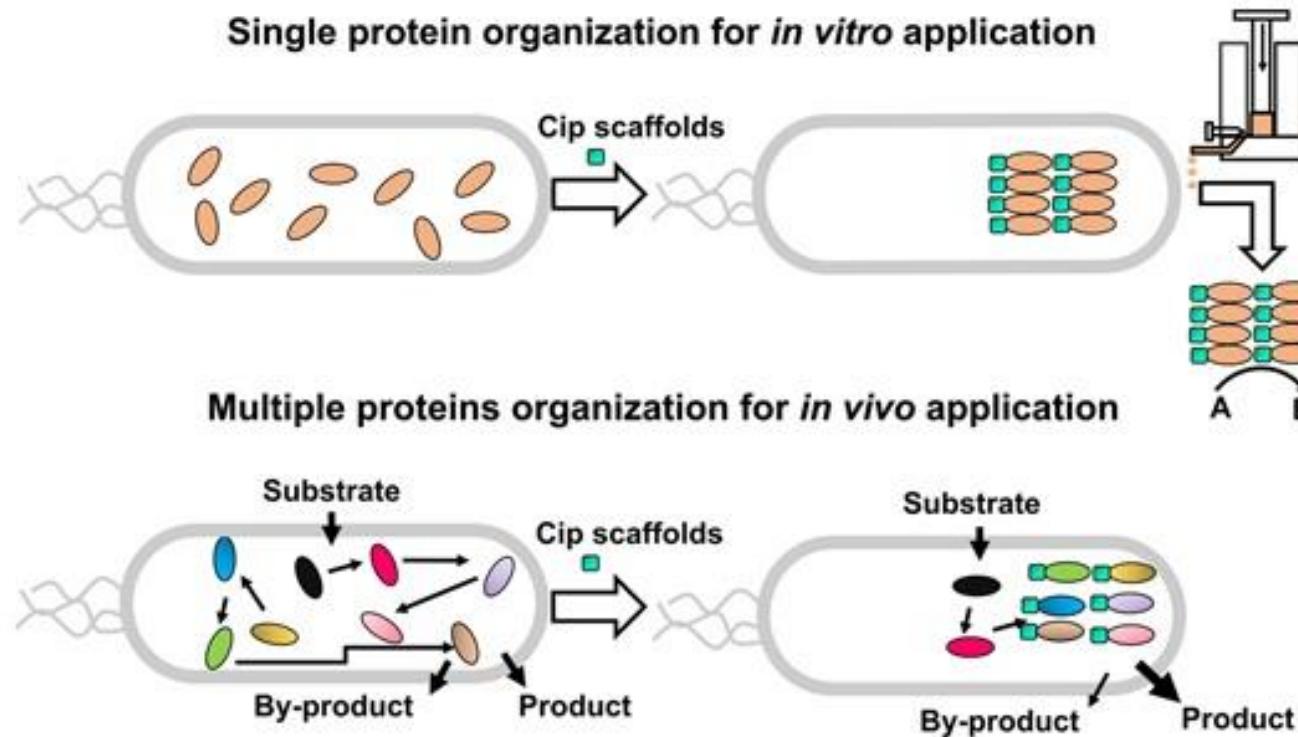
Amorphous cellulose
<https://doi.org/10.1016/j.ijbiomac.2018.06.013>

Amorphous cellulose ultrastructure



PROTEIN VACCINE PRODUCTION via cellulose-based manufacturing strategies

Proteins purified on cellulose can form protein inclusions crystals which are extremely resistant to temperature stress and highly stable, and potentially capable of inducing a sustained immune response by via extended antigen release.





Protein vaccine production - manufacturing strategy 3: Cellulose-based manufacturing strategies

Drug substance formulated as cross-linked protein crystals

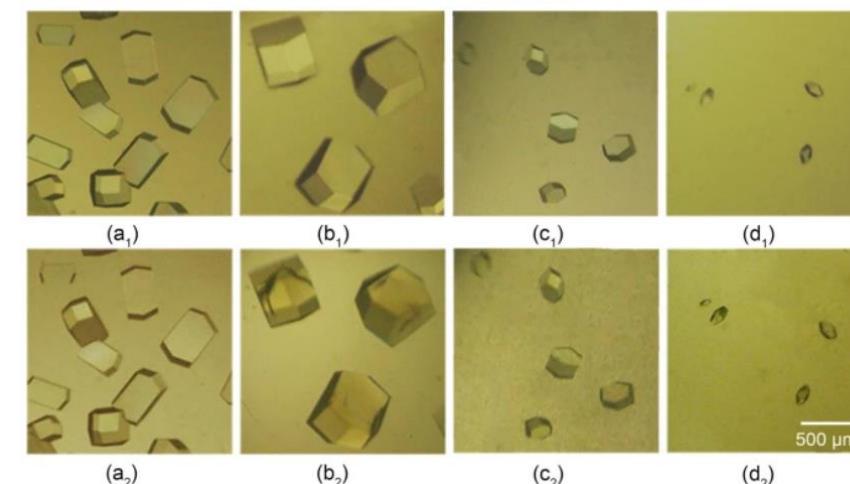
PROTEIN VACCINE PRODUCTION via cross-linking manufacturing strategy

Table 13 Target product profile (TPP) for vaccines produced via cross-linking manufacturing strategies.

Category	Description
Mechanism of Action	rSI-VAX-Sol/Crys (drug product) is a universal recombinant vaccine containing one of the conserved enolase/sortaseA/GAPDH/GroEL antigen from <i>Streptococcus iniae</i> , in a buffer solution without immune adjuvants, or available as a cross-linked protein crystal. rSI-VAX-Sol/Crys is expected to provide a cellular (Th1) and humoral (Th2), antigen-specific, protective immune response when compared with a natural <i>Streptococcus iniae</i> infection.
Indication	rSI-VAX-Sol is indicated for the active immunization of 2-month-old juveniles to adults freshwater and marine fish species susceptible to <i>Streptococcus iniae</i> for prevention Streptococcosis related illnesses caused by <i>Streptococcus iniae</i> . The crystalline version is more resistant to harsh environmental conditions but the immune response and vaccine protection takes longer to develop.
Primary Endpoints	<ul style="list-style-type: none"> • 60% reduction of <i>S. iniae</i>-confirmed disease within one year after dosing (below 40% is a no go) in the target population • Safe and tolerable as defined by solicited symptoms, adverse events, and serious adverse events (no evidence of enhanced <i>S. iniae</i> disease), no known impacts on FCR or growth parameters.
Key Claims	<ul style="list-style-type: none"> • Has a favorable risk-benefit profile in farm • Can be dosed with other fish vaccines and probiotics • Universal recommendation for the replacement of antibiotic usage in farm • Achieves World Health Organization (WHO) stability requirements
Secondary Endpoints	<ul style="list-style-type: none"> • Analysis supportive of primary endpoint in target fish population • Reduction in <i>S. iniae</i>-specific mortality • Reduction in <i>S. iniae</i>-specific rates of bacteria-confirmed diseases • Reduction in antibiotic use for <i>S. iniae</i>-confirmed disease • Duration of protection >1 year (with/without booster)
Formulation/Dosing	<ul style="list-style-type: none"> • Antigen in pre-filled syringe or single-dose vial • Antigen containers are pre-mixed prior to injection • 1 doses administered

Soluble proteins administrated in the form of crosslinked protein crystals after a short incubation in glutaraldehyde (glutaraldehyde is a cross-linking agent).

Figure 1

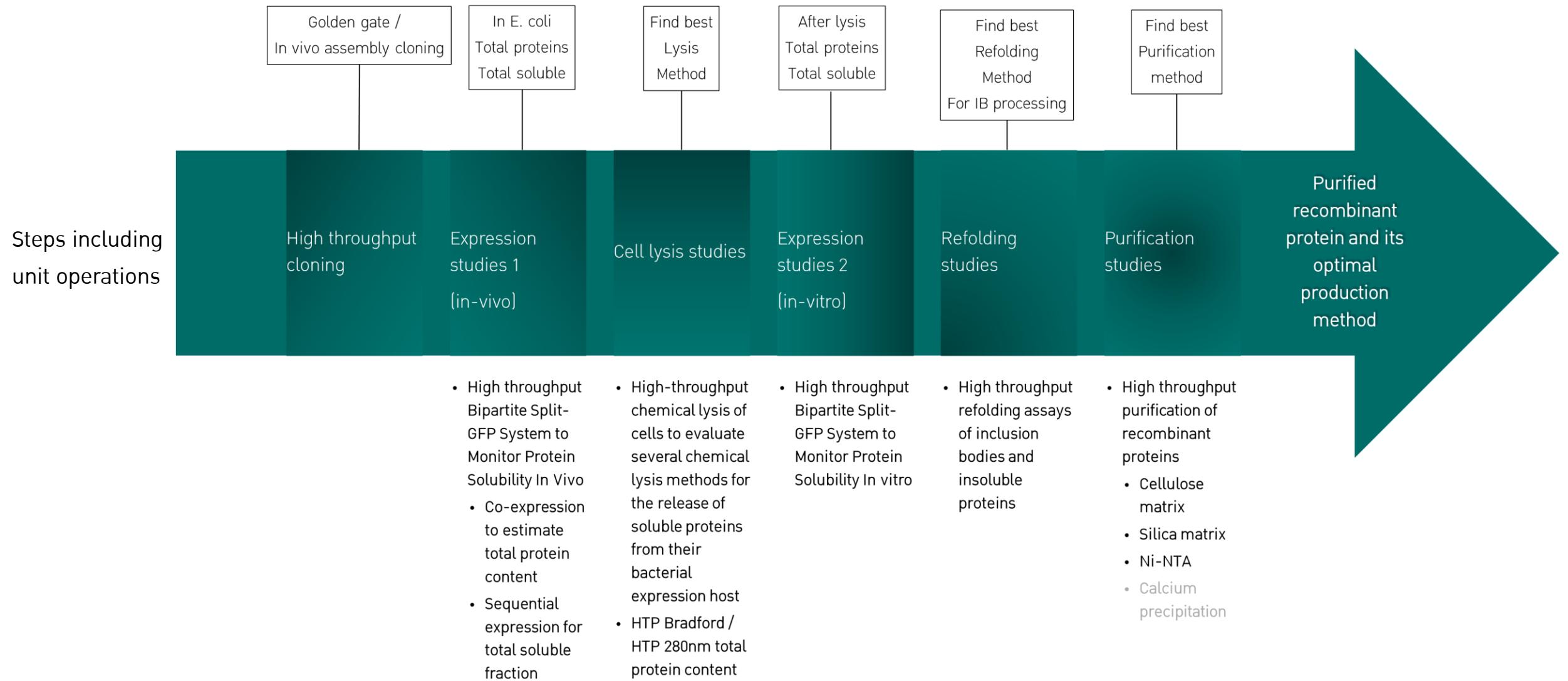


Typical examples of crystals morphology before and after chemical cross-linking at 17 °C.

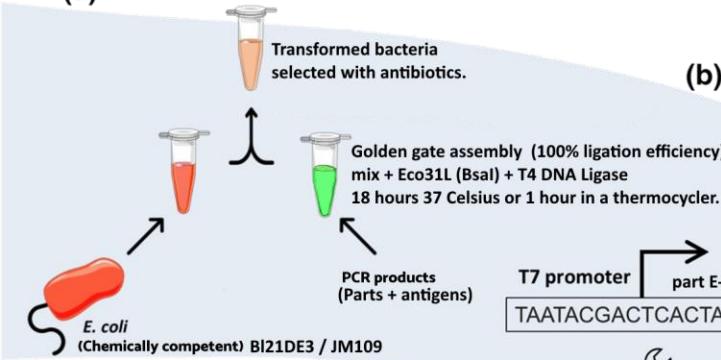
(a) 5 days, pH = 4.2; (b) 4 days, pH = 5.4; (c) 3 days, pH = 6.6; (d) 2 days, pH = 7.8. The subscripts 1 and 2 indicate the crystal images before and after cross-linking, respectively.

[Full size image >](#)

PROTEIN VACCINE PRODUCTION



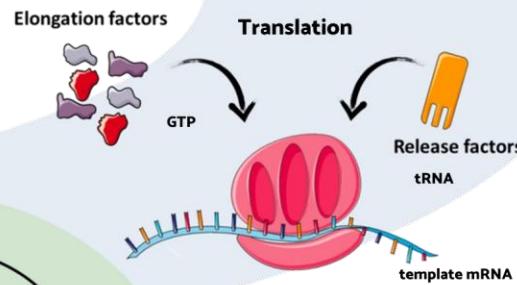
(a)



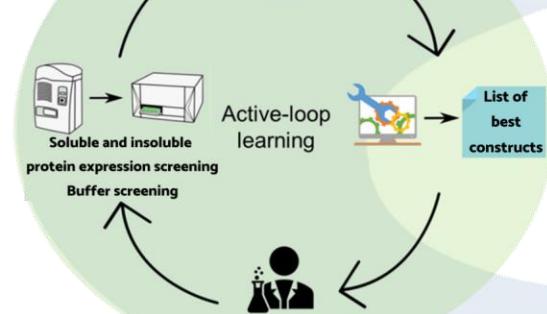
(b)

DEVELOPMENT RECOMBINANT PROTEIN VACCINES

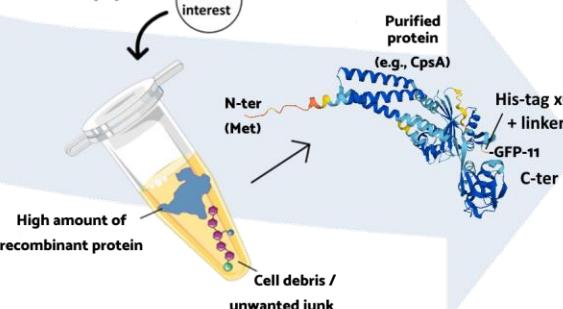
(c)



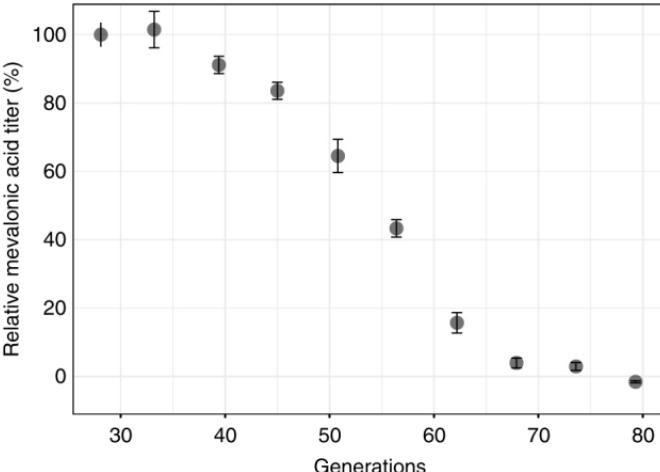
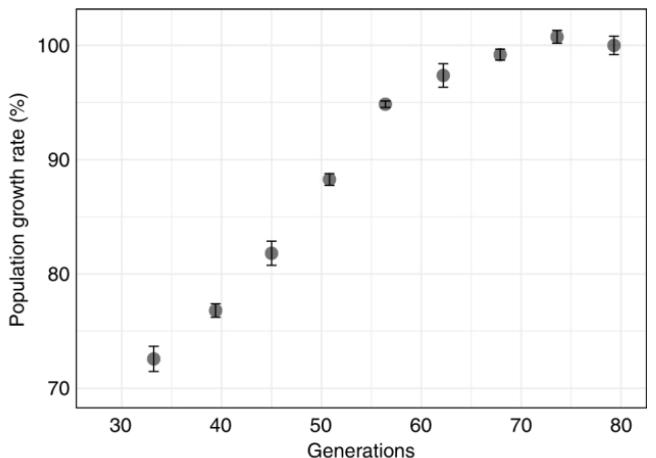
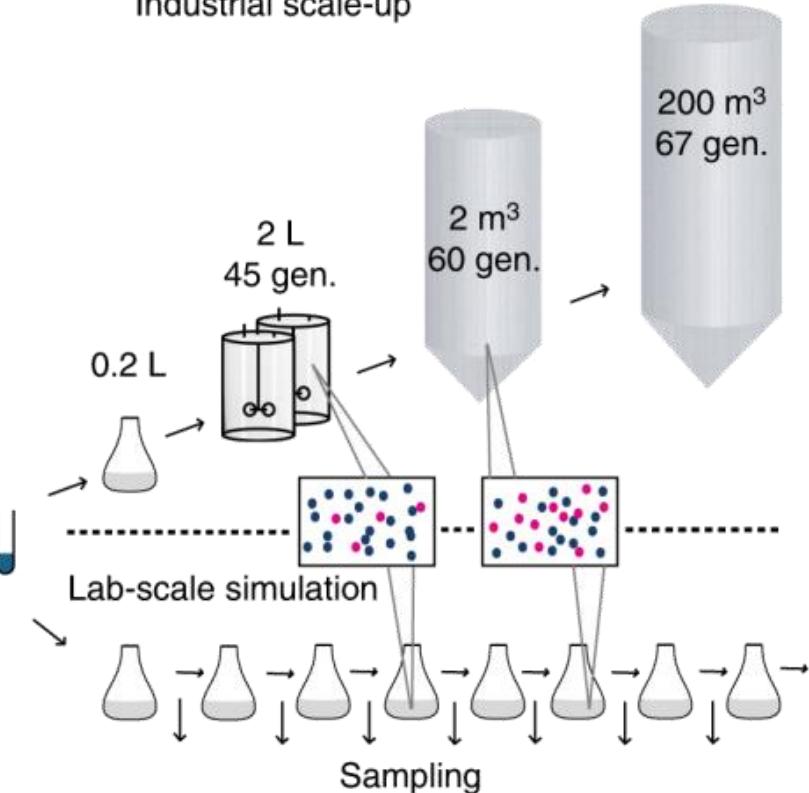
(d)



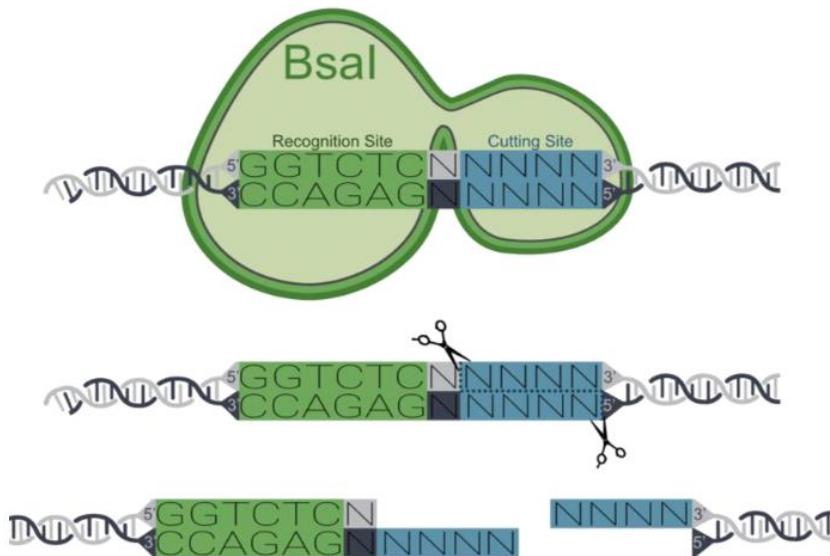
(e)



Industrial scale-up



Cloning / golden gate assembly



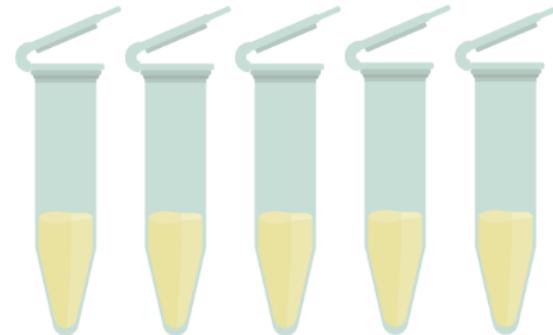
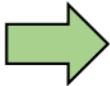
Type IIs restriction enzyme:
Golden Gate cloning/assembly

During the assembly reaction, the DNA containing the recognition site is removed, creating a product that lacks a cut site.

Protocol.



PCR amplification of parts



Assembly reaction

Pipette all your parts together

Golden Gate assembly allows for cloning in a one pot reaction.
Parts, restriction enzyme, and ligase are pipetted together

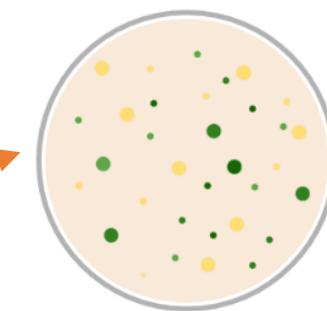
Bsal Cut-Ligation Protocol	
Backbone (100ng/ μ l)	1.0 μ l
Insert(s) (100 ng/ μ l)	1.0 μ l
10x CutSmart Buffer	1.5 μ l
10 mM ATP	1.5 μ l
Bsal	0.75 μ l
T4 Ligase	0.75 μ l
H ₂ O	
Total	15.0 μ l

Incubate 18h at room temperature (e.g., 37C).



Transformation

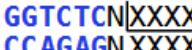
1. Transform **5uL** into **50uL** E.coli (competent)
2. incubate 10mn on Ice, heat-shock 42C 30 seconds,
3. Incubate on ice 5mn.
4. Add 950uL media, shake, incubate
5. 37C RT for one hour, then plate on agar.

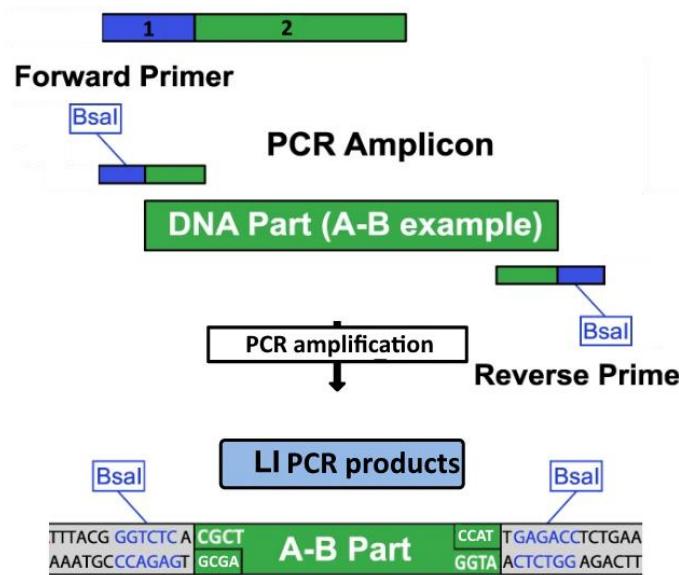


LI Amplicon

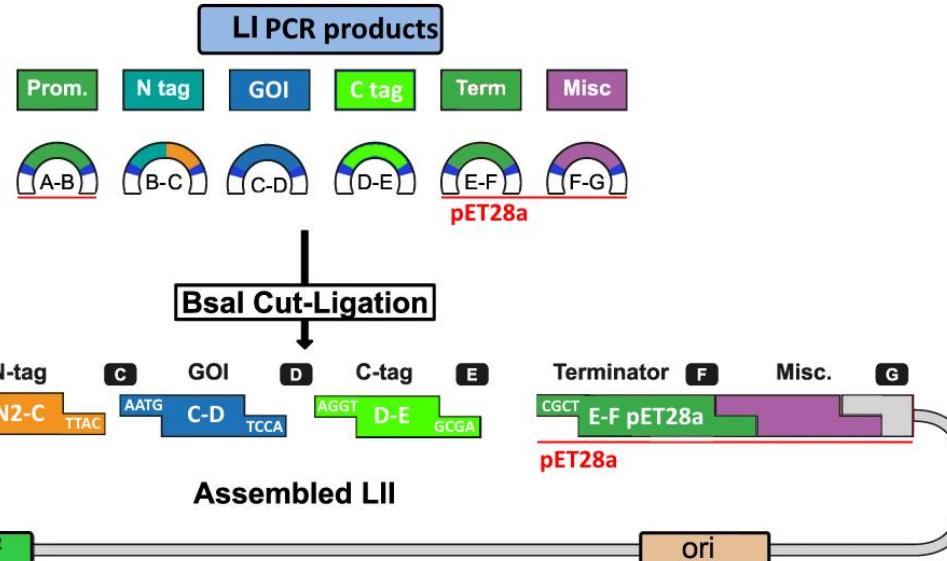
Primer Components:

1. Bsal Site: Directional cloning
2. Part homology (A-B Example)

Bsal 



LII Assembly



Cloning / golden gate assembly

Supporting Table S8. Predicted high fidelity four-base overhang sets for use with Golden Gate assembly methods (based on 18 h at 37°C).

Set	Number of overhangs	Estimated fidelity	Overhang sequences
1	10	100%	CTTA, CTCC, ACTA, GGTA, TCCA, CGAA, AATG, AGCG, ATGG, AGAT
2	15	99.8%	AGAG, ACAT, GACA, AGCA, ATTC, GGTA, CAAA, CCAA, AACG, CTGA, CCTC, ACGG, TCCA, CAGC, ACTA
3	20	99.3%	GACA, ACTA, CGGA, ATTA, AGAG, AACG, CCAA, GGTA, CTGA, AGGA, CAGC, ACGG, CAAA, GAAC, AGAT, CCTC, CTAC, AGCA, AATC, ATGA
4	25	98.5%	AGCA, GACA, GTAA, CAGC, AATC, ATAG, GAAC, ATGA, AACT, CAAA, CTTC, CGTA, ATTA, CTGA, TCCA, ACTC, AATG, GCGA, ACAAA, AGGG, CTCA, ACCG, CCAA, GGTA, AGAT

Overhangs:

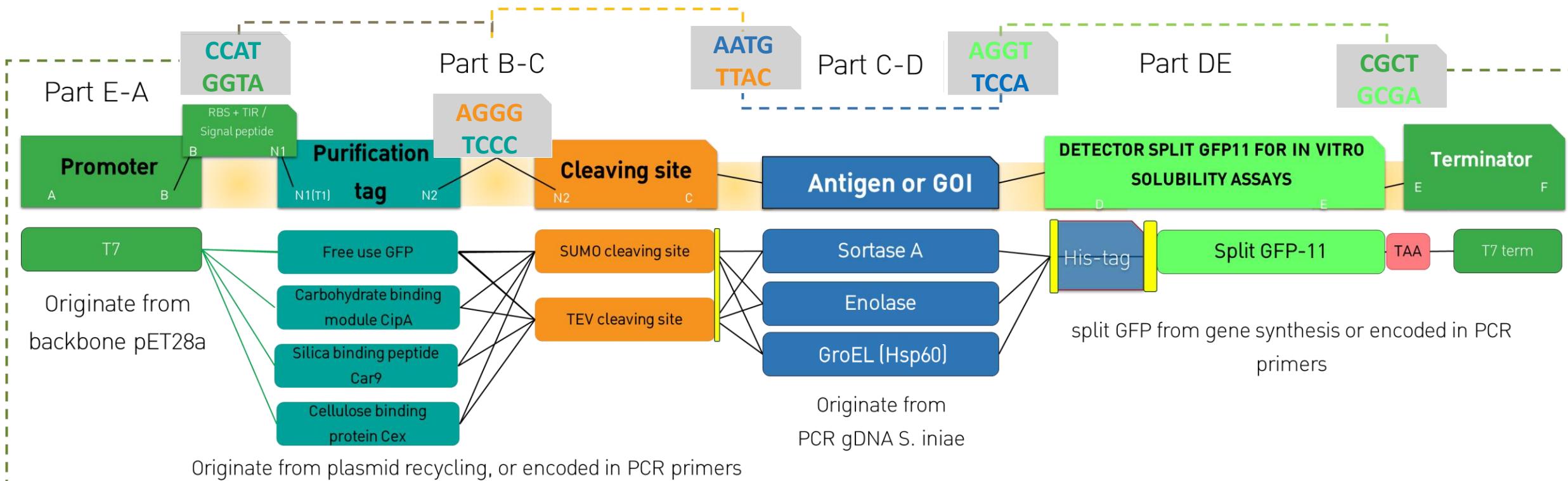


Component Fragments

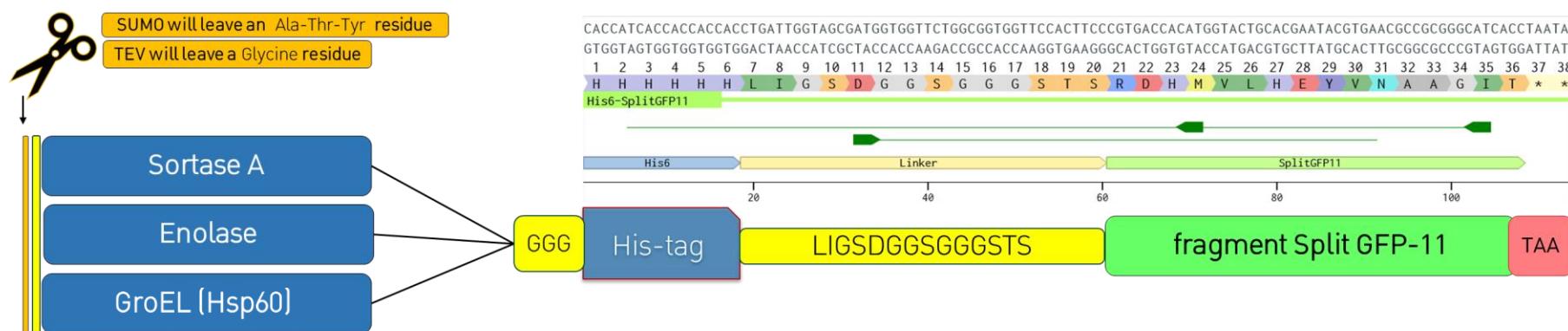
pET28a_linear_2 N1N2_Npur_Cex N2C_SUMO Part_CD_SortaseA His6_SplitGFP11

Name	Length	Produced by	5' End Overhang	3' End Overhang
pET28a_linear_2	5231 bp	Restriction Digest	CGCT	ATGG
N1N2_Npur_Cex	322 bp	Restriction Digest	CCAT	CCCT
N2C_SUMO	309 bp	Restriction Digest	AGGG	CATT
Part_CD_SortaseA	759 bp	Restriction Digest	AATG	ACCT
His6_SplitGFP11	127 bp	Restriction Digest	AGGT	AGCG

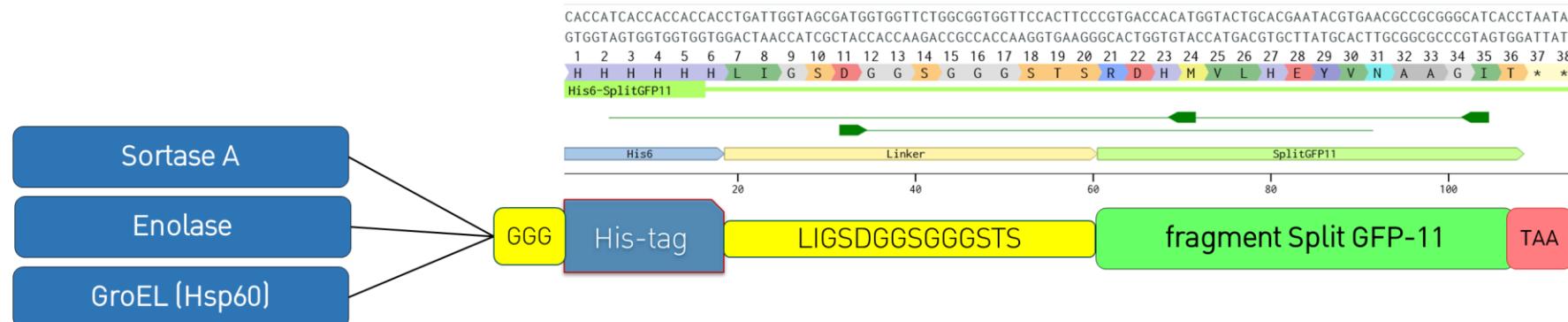
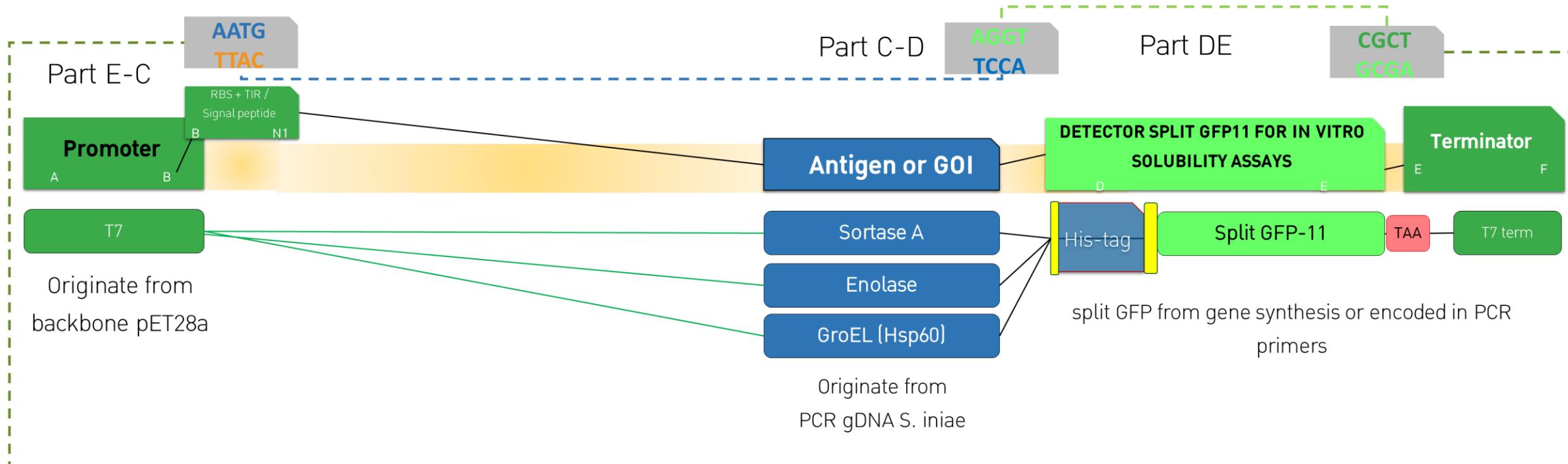
DEV. PROTEIN VACCINES: Plasmid design for high expression.



The starting construct will always result in a similar protein vaccine after the digestion with TEV or Ulp1 (SUMO protease).

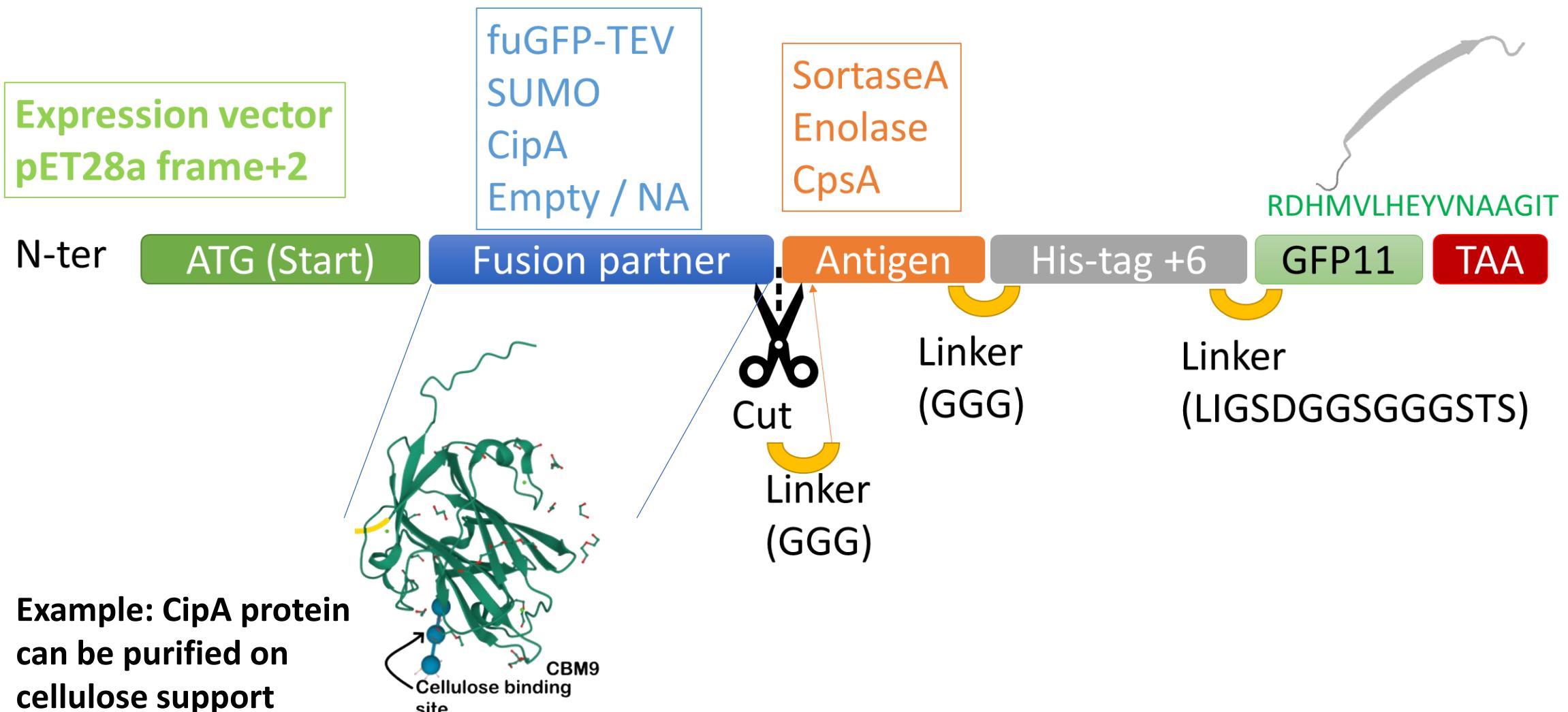


DEV. PROTEIN VACCINES: One single primer pair, to remove/add parts.

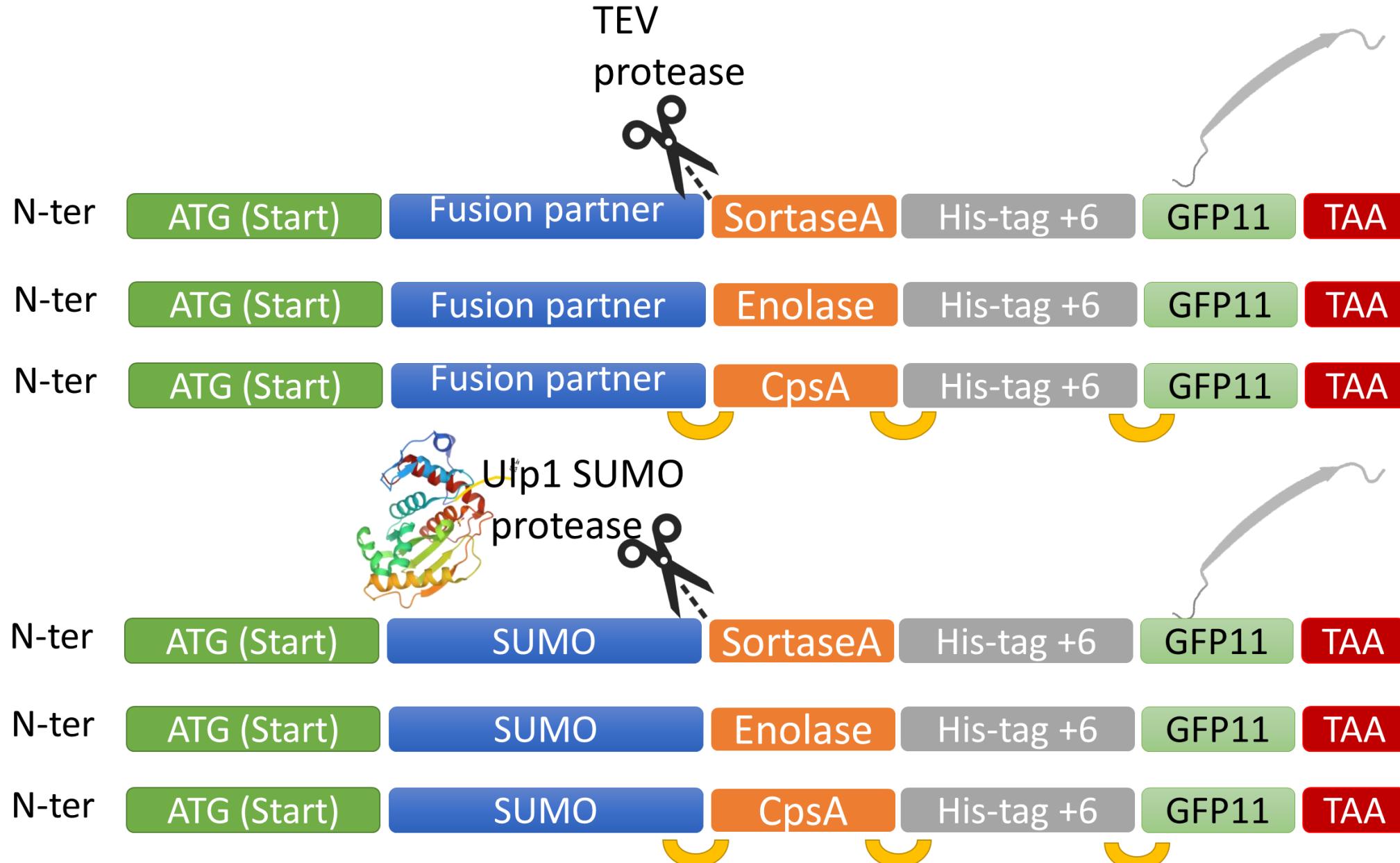


DEV. PROTEIN VACCINES: Plasmid design for high expression.

Soluble antigen production with a solubility enhancer as fusion partner



DEV. PROTEIN VACCINES: Plasmid design for high soluble expression.

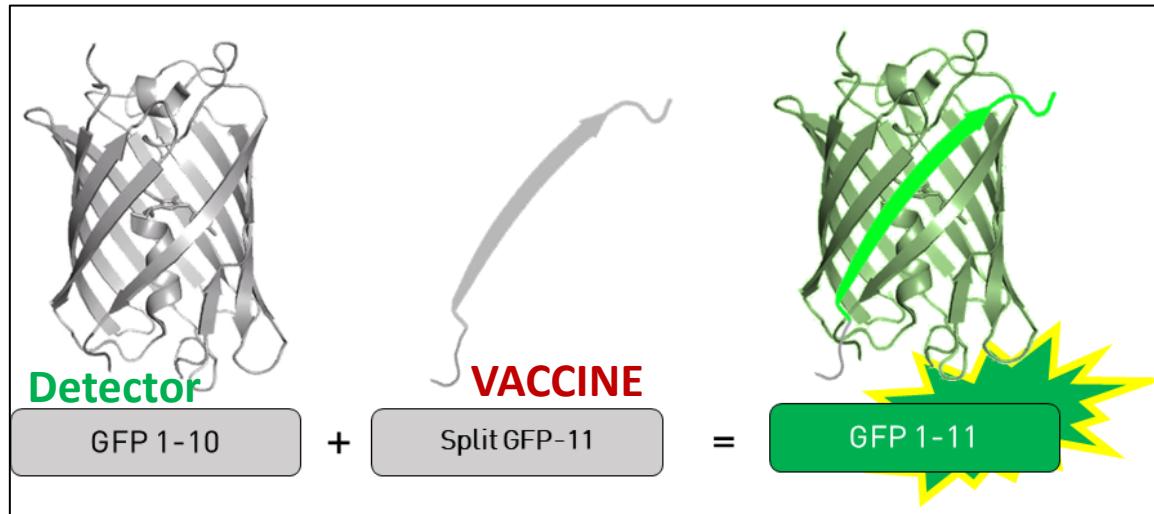


DEV. PROTEIN VACCINES: Plasmid design for (insoluble IB) expression.

Antigen production without a solubility enhancer as fusion partner

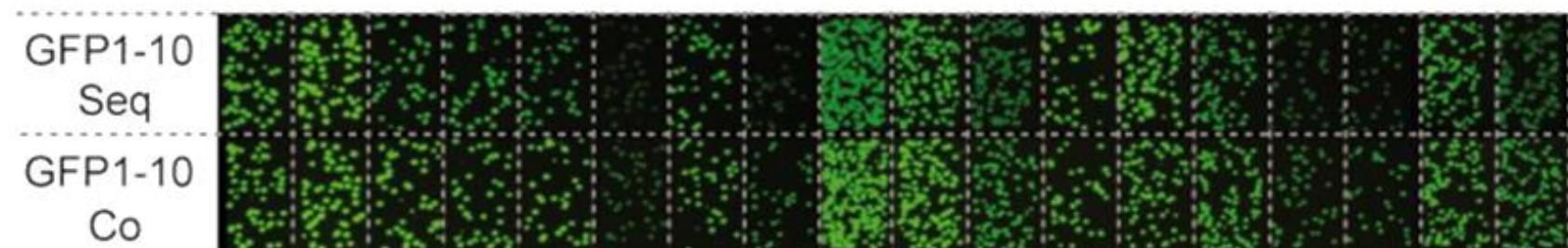
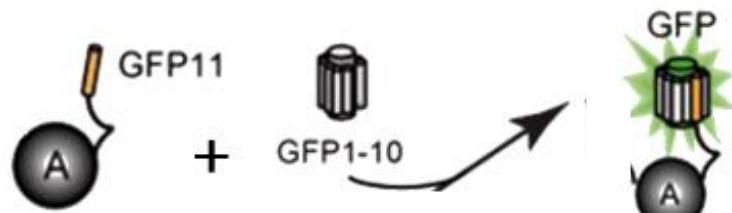


DEV. PROTEIN VACCINES: Split-FP complementation to replace SDS-PAGE



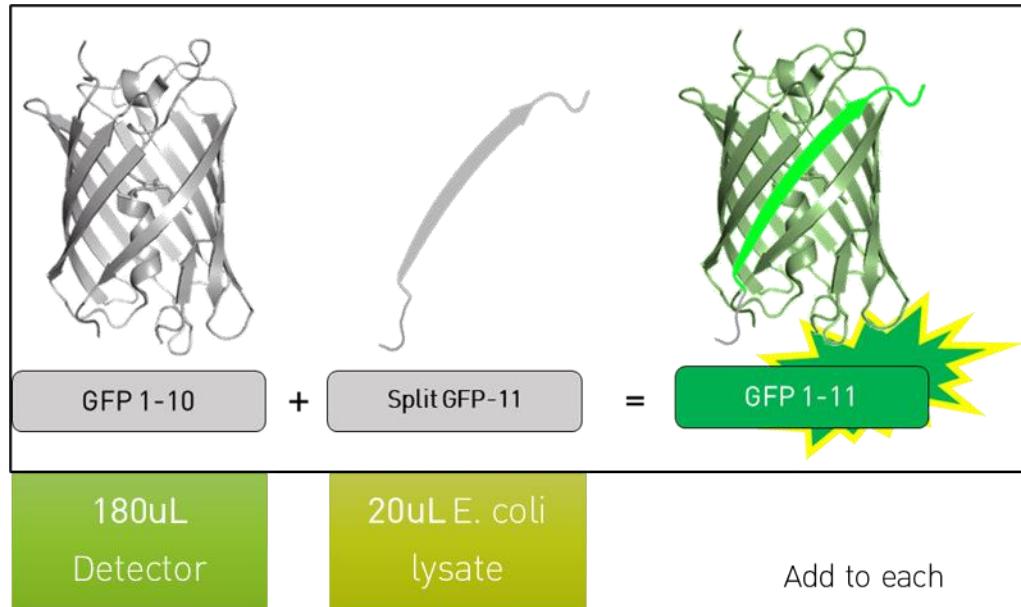
The nascent polypeptide CpsA-GFP11 can directly bind in the cytoplasm to GFP1-10 constitutively expressed. Because the binding is quick, and irreversible, it measures the total of recombinant proteins produced, even if they shortly become insoluble and dark.

Faster and more sensitive than BCA assay, alternative to SDS-PAGE.

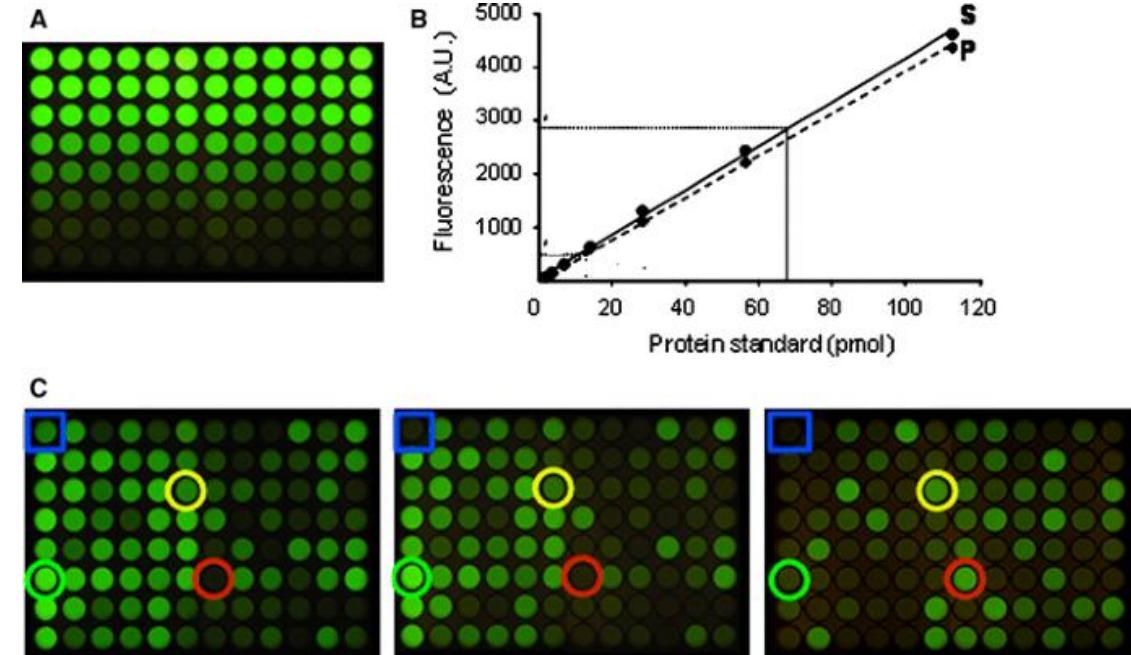


DEV. PROTEIN VACCINES: Analytical assay to quantify and optimize yields

SPLIT-FLUORESCENT PROTEIN COMPLEMENTATION

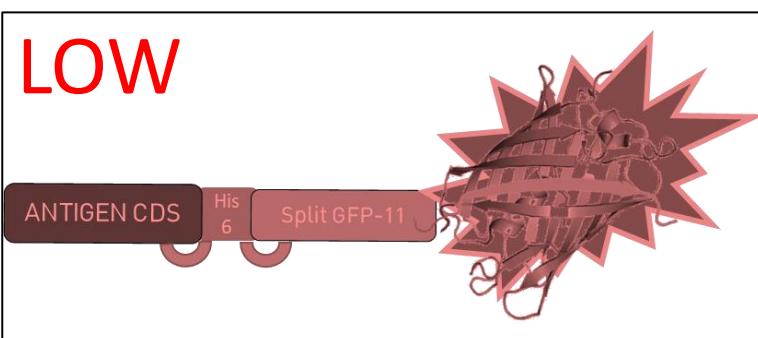


Add to each
well and wait 2
hours at 20-25C

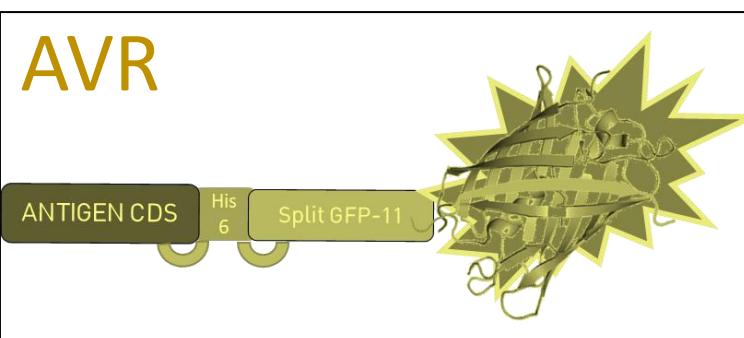


RESULTS

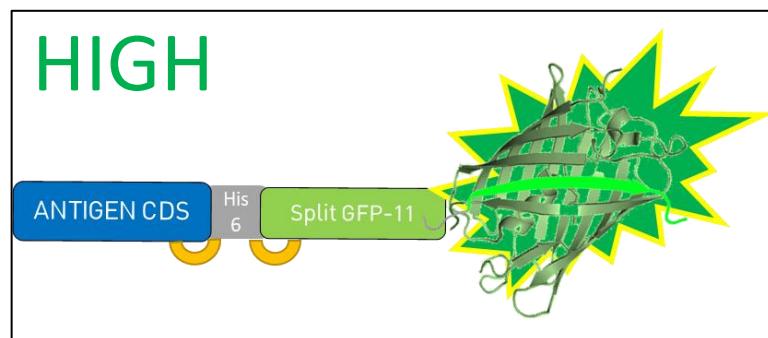
LOW



AVR



HIGH



DEV. PROTEIN VACCINES (Cytoplasmic E. coli proteins = SOLUBLE frac.)

Unit operation 1: Fermentation and production in E. coli:

LOW PRODUCTION



- ✓ DOUBLE SELECTION OF COLONIES (plate on dish then select best CFU * twice)

PROTEIN SEQUENCE – HYDROPHOBICITY



- ✓ SELECT SOLUBLE ANTIGENS
- ✓ ADD SOLUBILITY PARTNERS (SUMO, GFP..)

OPTIMIZE FERMENTATION MEDIA



- ✓ RICH MEDIA, 1% GLUCOSE
- ✓ STABILIZERS DURING GROWTH
- ✓ AND INDUCTION (NaCl, Sorbitol, Glycyl betaine)

OPTIMIZE FERMENTATION TEMPERATURE, DURATION

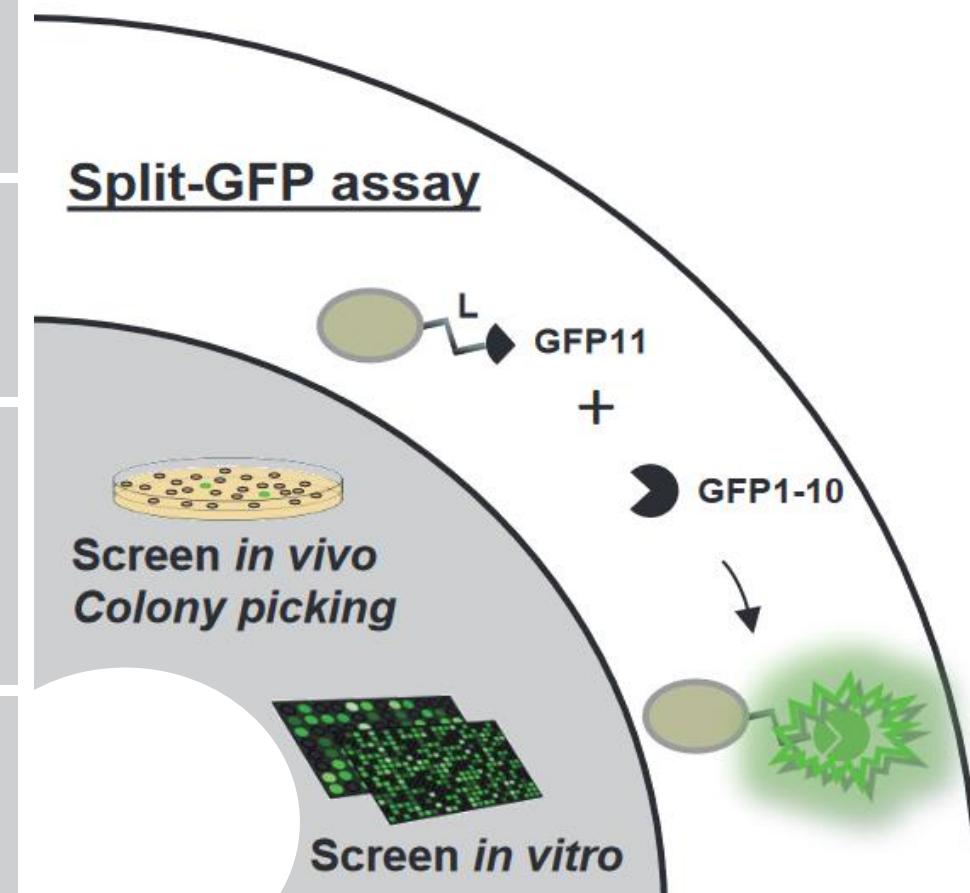


- ✓ LOW TEMPERATURE 18 - 23C,
- ✓ LONG EXPRESSION 24-36H+
- ✓ 18C, 20C, 23C, 27C, 30C, 32C
- ✓ 8h, 12h, 18h, 24h, 30h, 36h, 48h

SHAKING, AERATION, OD AT INDUCTION



- ✓ 5*50mL IN 250mL FLASKS



GOAL = INCREASE E. COLI CYTOPLASMIC SOLUBLE PROTEIN CONTENT

DEV. PROTEIN VACCINES (Cytoplasmic E. coli proteins = SOLUBLE frac.)

Unit operation 1: Fermentation and production in E. coli:

SELECT A HIGH PRODUCER CFU



PROPER STARTER CULTURE

Grow a starter inoculum in rich media + glucose



Inoculate fresh LB or minimal media + NaCl, start induction



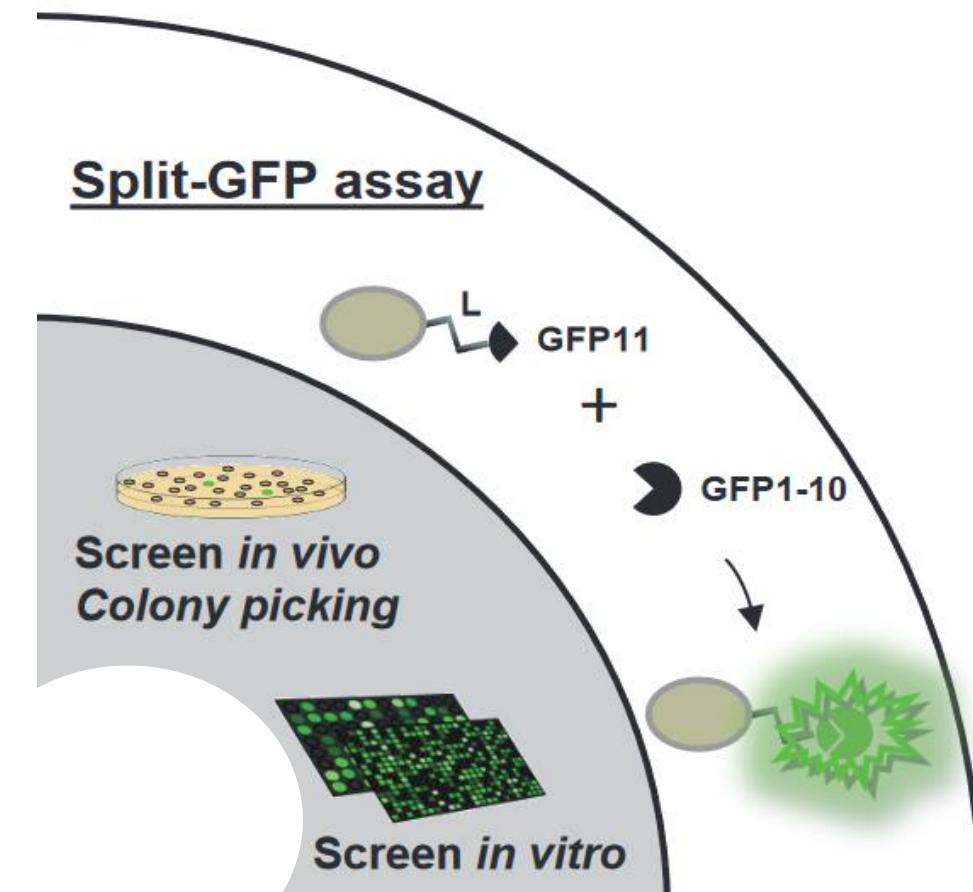
Check target protein yield regularly = Screen in vitro



ADD ADDITIVES OR ANTIBIOTICS if necessary

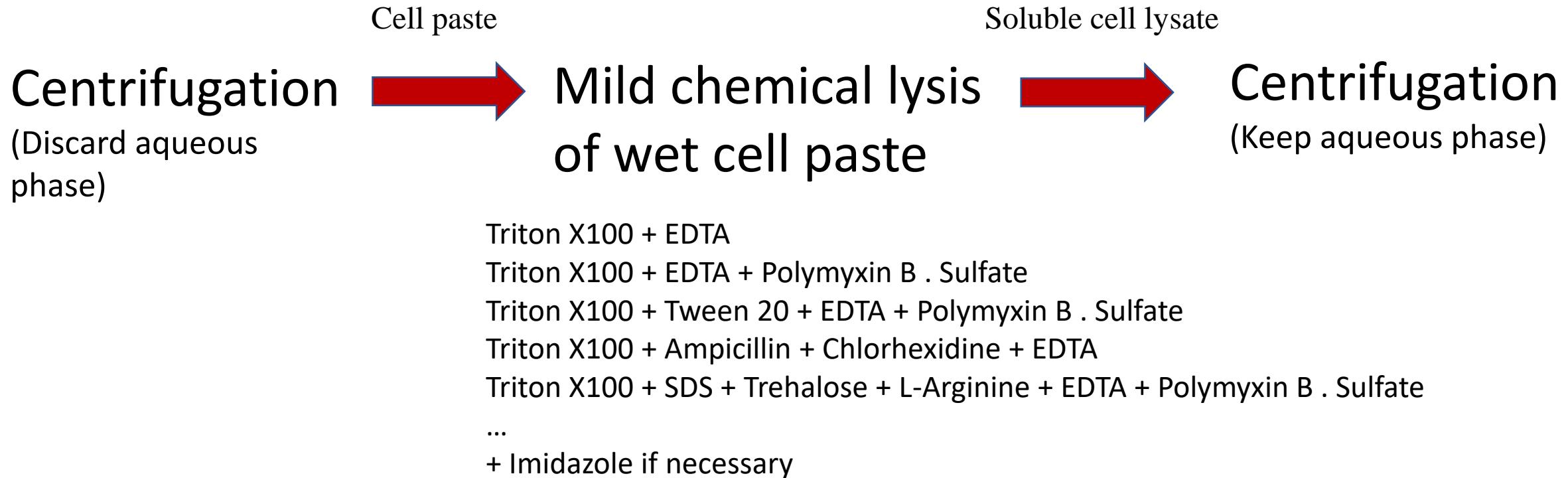
Take 200–500 μ L of cell suspension after expression, spin down,
UREA 9M, take 20 μ L to add to 180 μ L GFP detector

Time	24 h	28 h	32 h	36 h	40 h	44 h	54 h
OD ₆₀₀	2.5	3.9	7.2	9.1	8.4	8.0	8.1
pH	6.6	6.5	6.3	6.0	6.0	6.0	6.1
Protein yield	-	+	++	+++	++	++	-

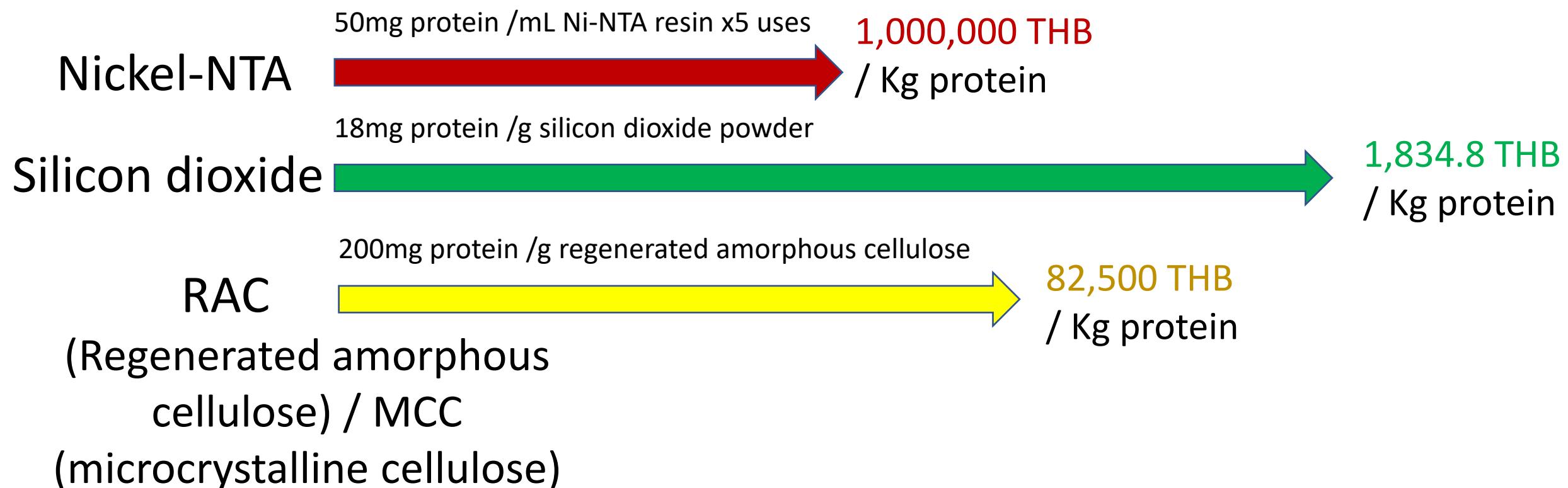


GOAL = INCREASE E. COLI CYTOPLASMIC SOLUBLE PROTEIN CONTENT

Unit operation 2: E. coli cell paste harvest and lysis: methods:

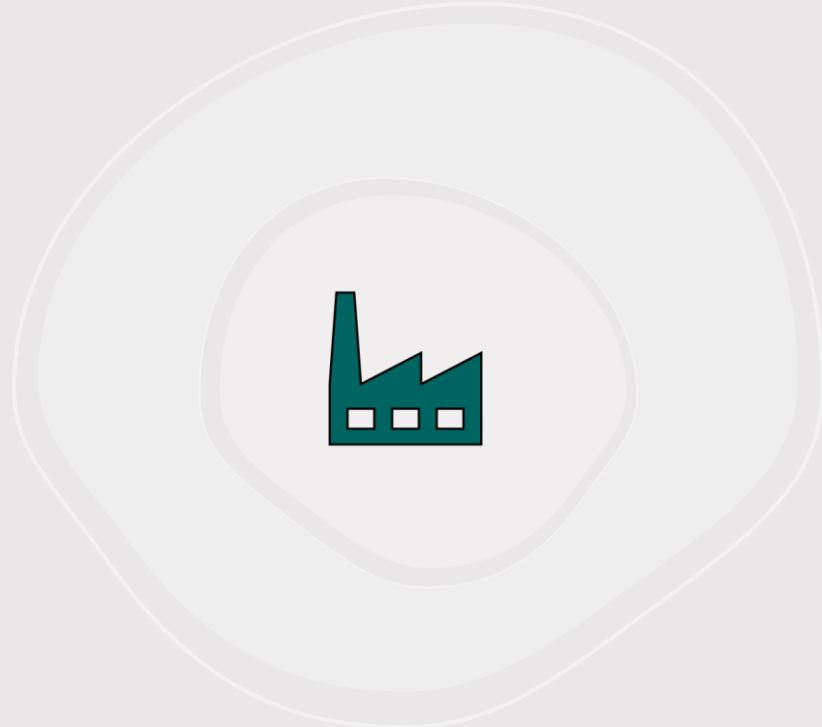


Unit operation 3: Separation / purification of proteins by affinity purification:
3 methods:



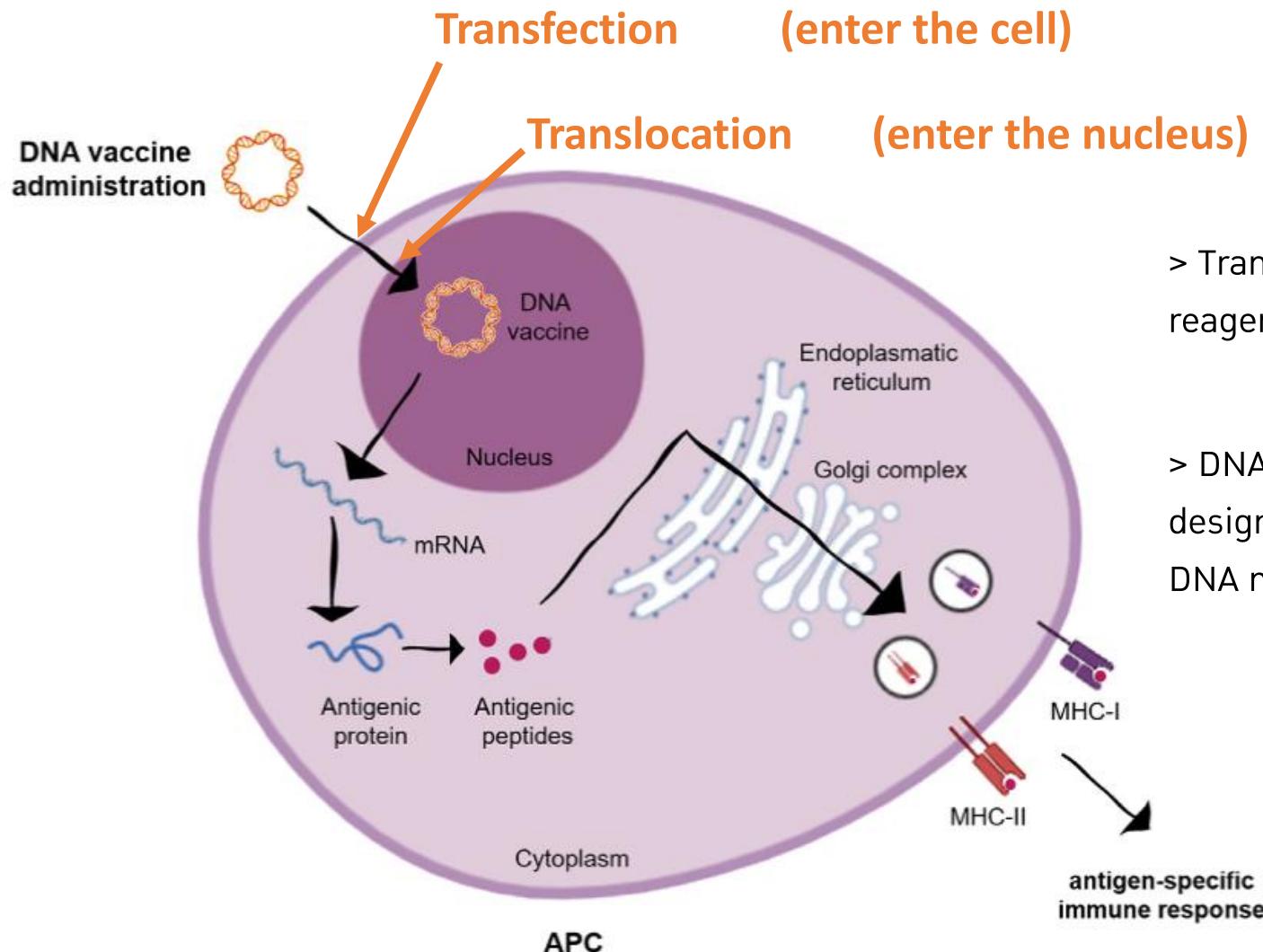
Unit operation 4: Formulation, QC and
conservation :

Sterile filtration through a membrane?
Lyophilization?
Stabilizers (sucrose, trehalose)?
Additives? Glycerol, ethylene glycol, EDTA..



pDNA vaccine production

pDNA VACCINES: Transfection and Translocation



- > Transfection conditions, transfection reagents, buffers, DNA condensation
- > DNA translocation sequences (DTS), vector design, pCMV, transcription factor binding DNA motifs (kB motifs)

Optimization of fish cell transfection

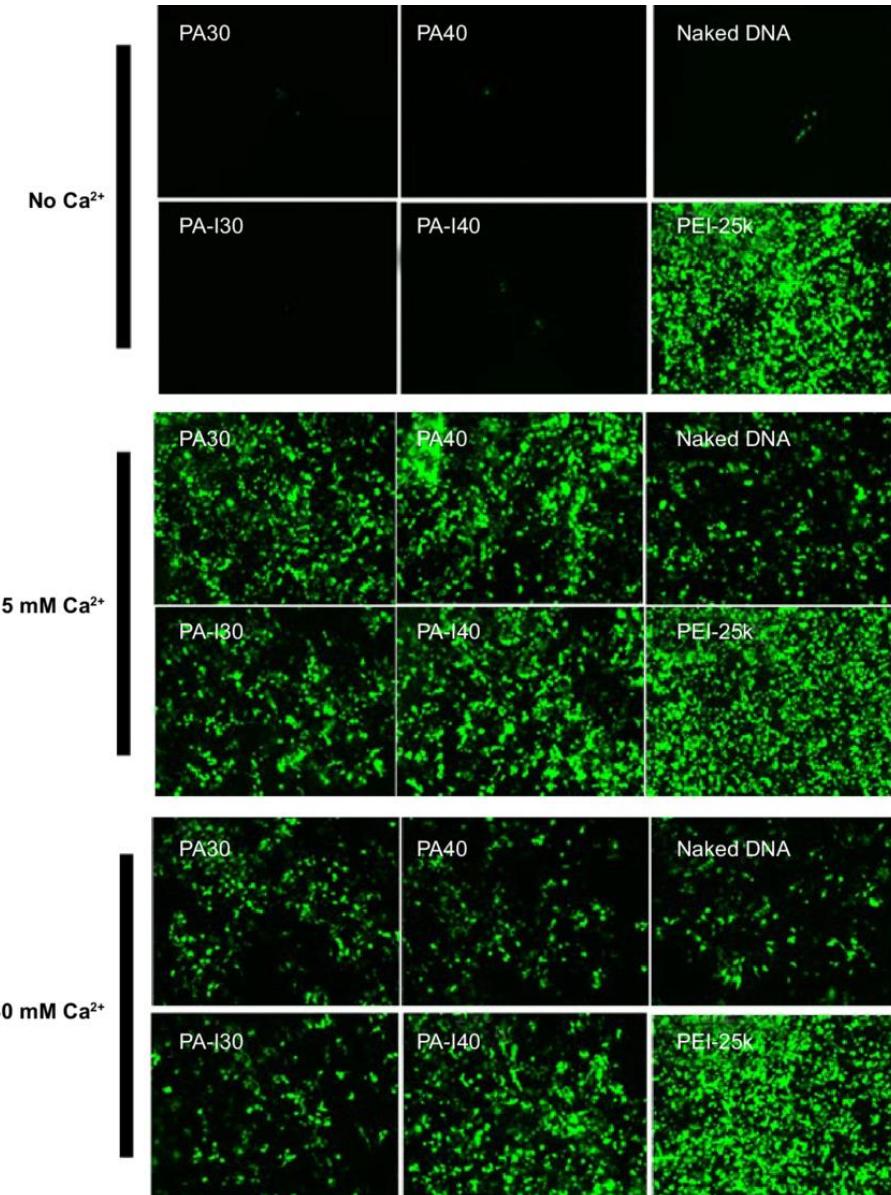
- Goal: increase pDNA vaccine efficacy

Problem: fish cells are poorly characterized,

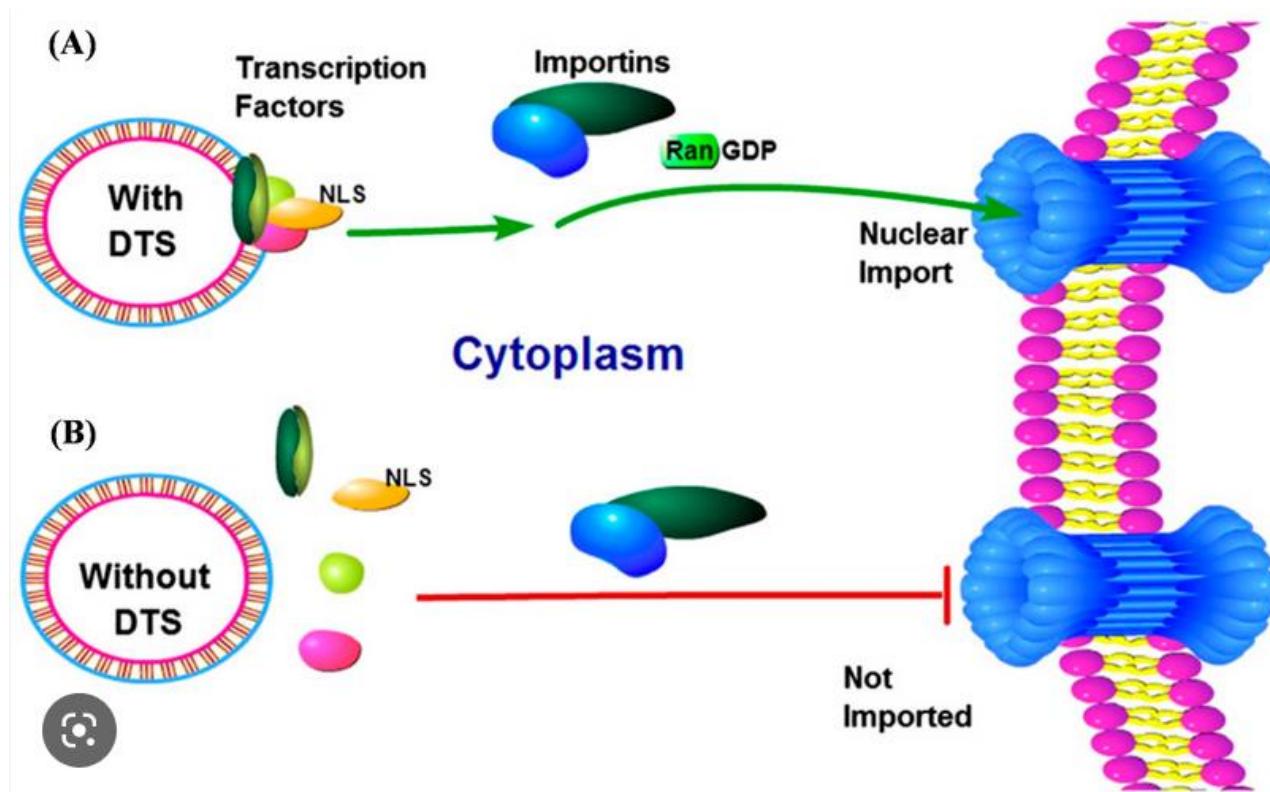
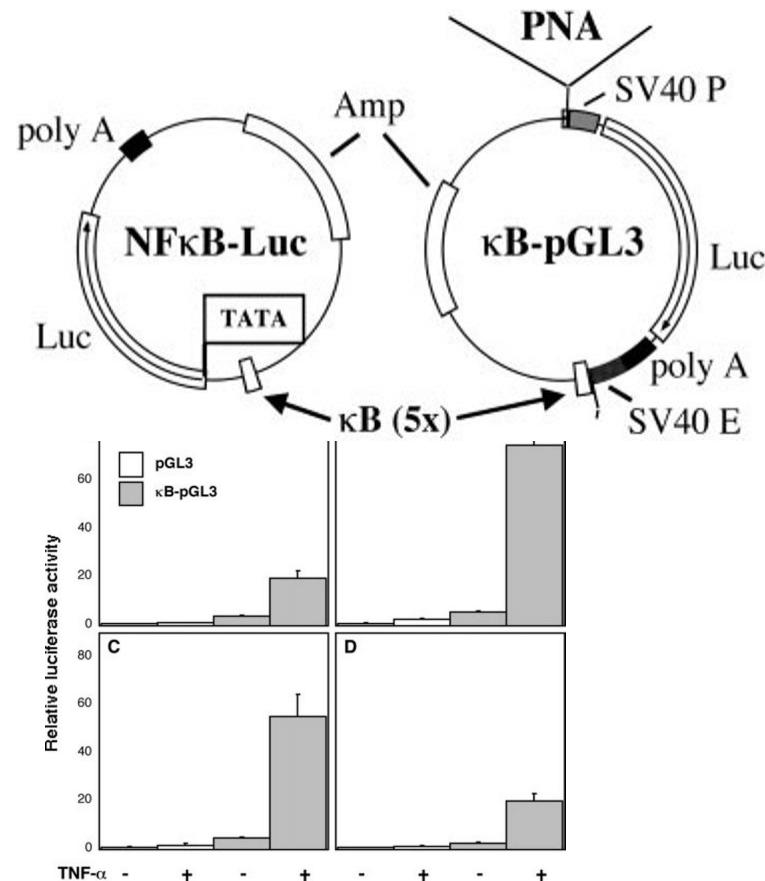
Transfection efficacy is cell-specific / species specific.

Some studies have shown that the optimization of
transfection buffers and reagents increased the total
Transfection efficacy from 10 % to 90 %.

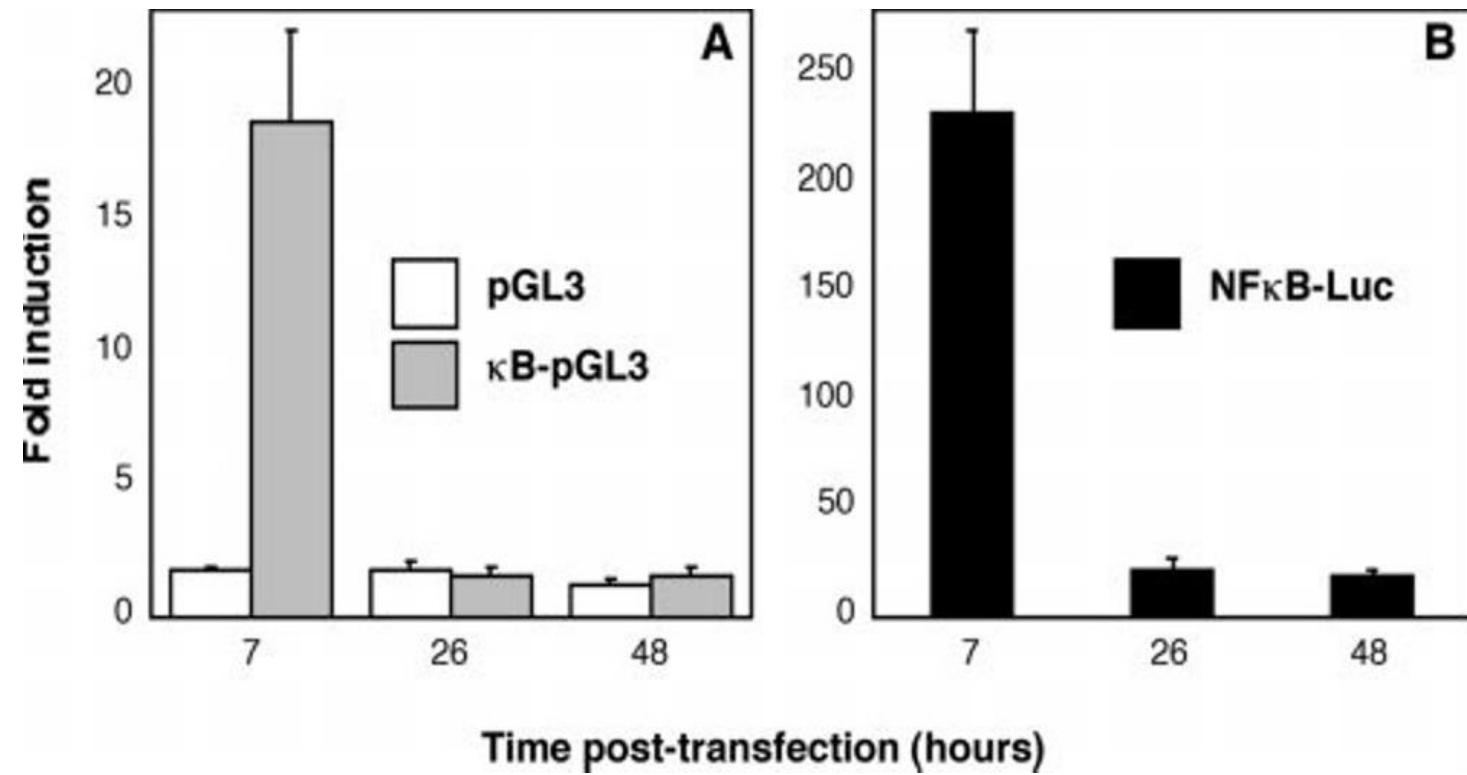
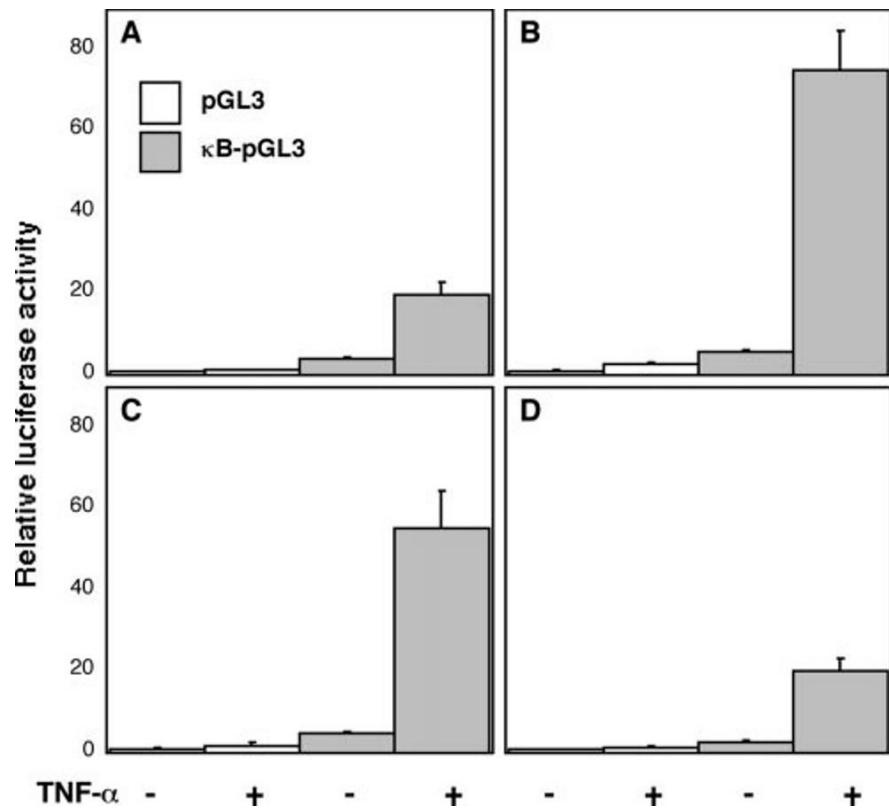
Cell must be isolated and cultured, and viable.



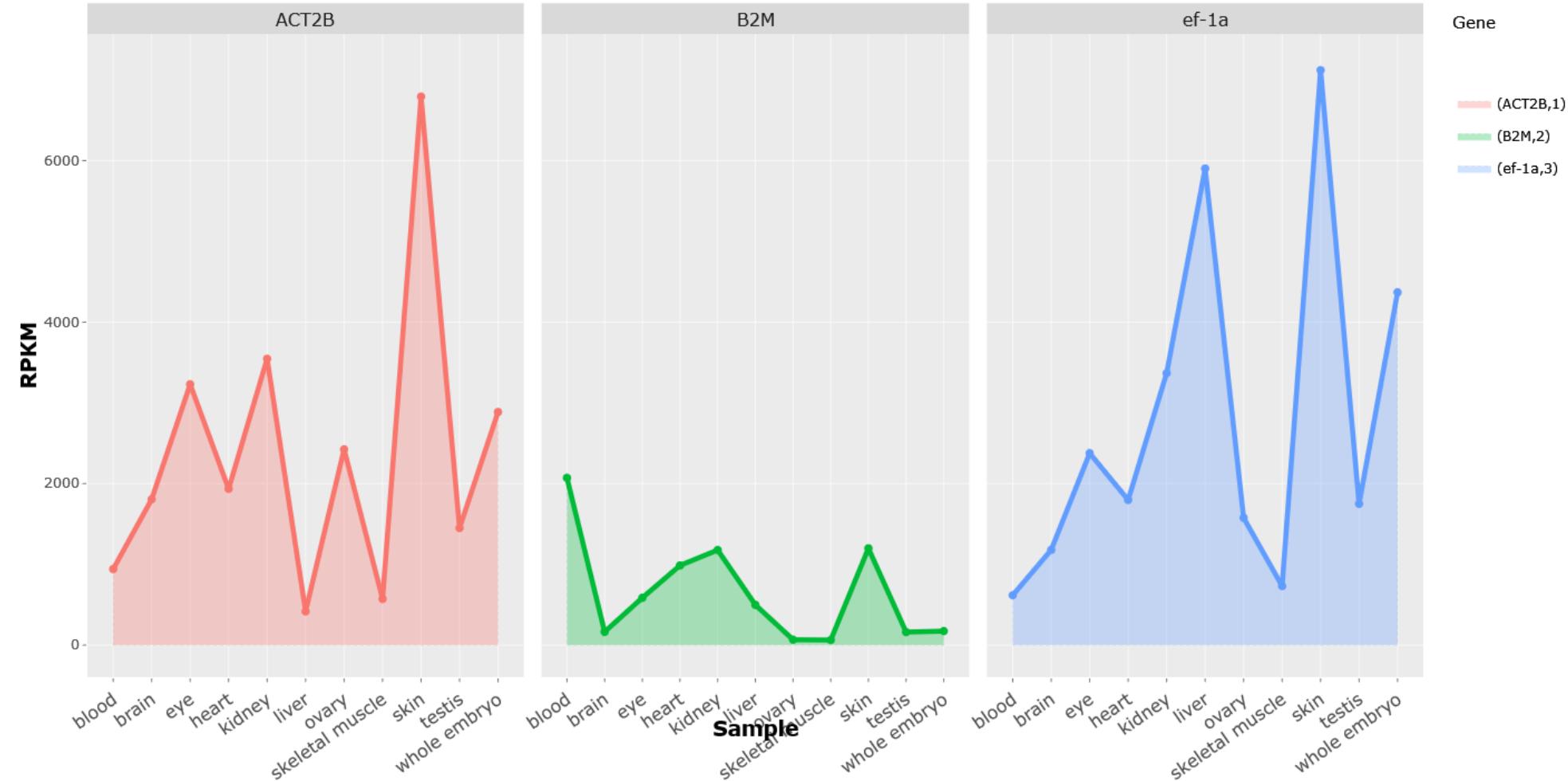
pDNA VACCINES: Translocation / DTS / NLS sequences



pDNA VACCINES: Translocation / DTS / NLS sequences



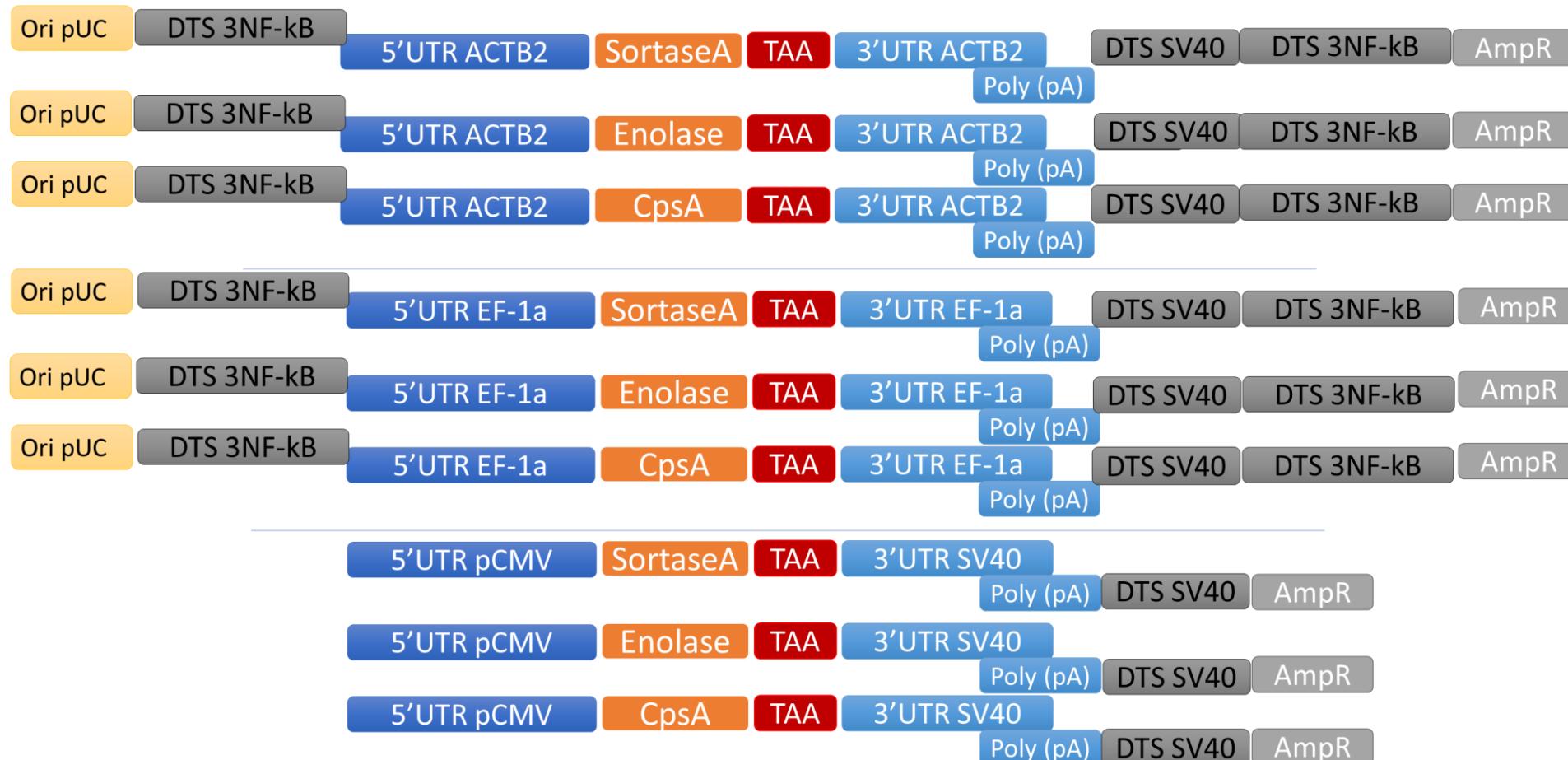
pDNA VACCINES: Untranslated regulatory DNA sequences



DEVELOPMENT OF PLASMID DNA VACCINES



Different regulatory sequences are evaluated in order to reach maximum transgene expression in fish cells.



pDNA VACCINE MANUFACTURING DEVELOPMENT

Unit operations

Establishment of Master Cell Bank (MCB) and Whole Cell Bank (WCB)

Fermentation and Recovery

Alkaline Lysis, sodium acetate, NaOH, Tween 20

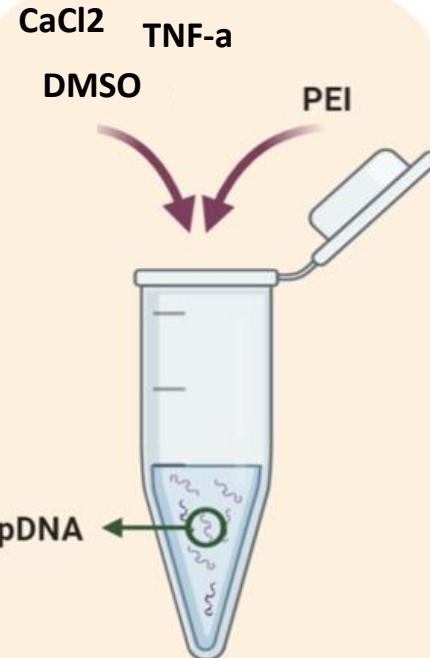
Filtration on synthetic woven fabric

Pre-clarification and Endotoxin Removal

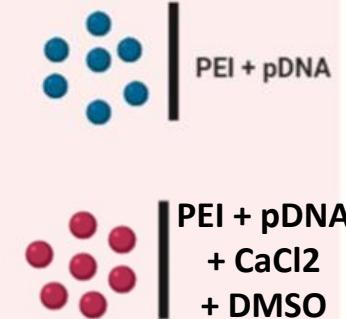
Isolation of the plasmid DNA and purification by PEG-8000 precipitation

Quality control + Stabilization

I. Production

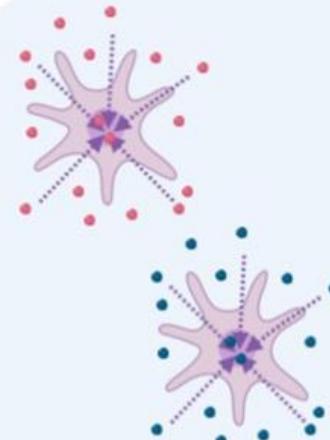


II. Optimization



Parameters evaluated:
Encapsulation efficiency

III. Biological assessment



Parameters evaluated:
Cytotoxicity
Transfection efficiency
Protein expression

IV. Main conclusions



PEI ratio 10
↑32% of protein expression



DMSO 25%
↑54% of protein expression

Formulations and quality control:

methods:

Formulation
with adjuvants
and stabilizers
(sodium azide..)



Quality control



Examination of the nucleic acid for Requirement/Limit
Endotoxins <300 I.U./mg of DNA
E. coli genomic DNA <50 μ g/mg of DNA
Protein <100 μ g/mg of DNA
Supercoiled DNA >90%
A260/280 1.75-1.85
Residual salt scan from A220 to A320
RNA <1%
Sterility no colonies after 14 days
of tryptose culture

Storage of S.C
pDNA in
plastic
containers in
freezer

III. BIOLOGICAL ASSESSMENT OF TNF-a / Bcl-xL / transfection reagents toxicity in fish cells (in-vitro)

Cell death analysis: Fish cells were transfected and after 24 hours / 36 hours / 48 hours, cells were detached by pipetting, incubated with 1 µg/ml PI which specifically stains dead cells and analysed on an automatic cell counter in red fluorescence or counted with a microscope fluorescence.

13.3.3.2 Measurements of Apoptosis Related Genes in Fish Cell Extracts Following Transfection

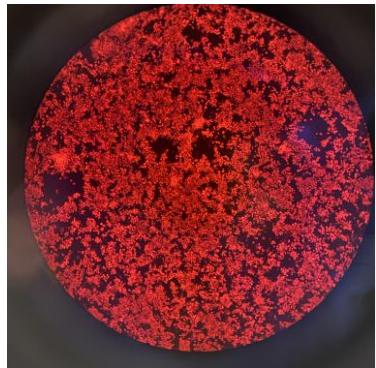
Gene	Hour 0	Hour +6	Hour +12	Hour +36
Caspase-3				
Bax				
Bcl-2				
Bcl-xL				
Fas				
FasL				
Caspase-8				

Table 24 In-vitro measurements of apoptosis-related gene transcription

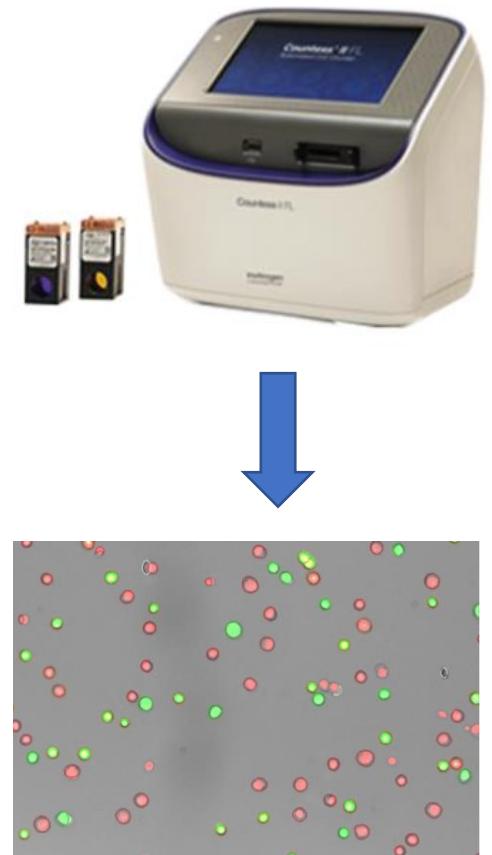
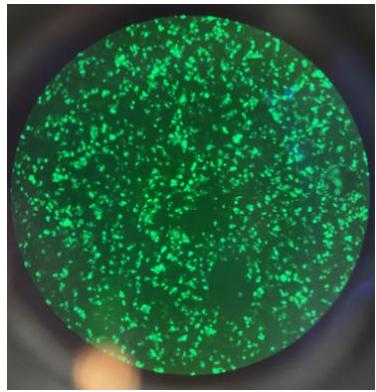
Biomarker	Description and Importance in Assessing Vaccine Efficacy
Caspase-3	An executioner caspase and a central player in the execution phase of apoptosis. Monitoring its expression and activation will provide insights into the progression of apoptosis.
Bax	A pro-apoptotic member of the Bcl-2 family and plays a significant role in the intrinsic mitochondrial pathway of apoptosis. It can induce mitochondrial outer membrane permeabilization, leading to the release of cytochrome c and activation of caspases.
Bcl-2	An anti-apoptotic protein that can inhibit Bax and other pro-apoptotic proteins. Monitoring its expression can help assess the balance between pro-apoptotic and anti-apoptotic signals in the cells.
Bcl-xL	Used in this research as anti-apoptotic protein adjuvant, it is important to monitor its expression to evaluate its effects on apoptosis.
Fas and FasL	Involved in the extrinsic death receptor pathway of apoptosis. Monitoring their expression can provide insights into this specific apoptotic pathway's activation in response to TNF-alpha.
Caspase-8	An initiator caspase involved in the extrinsic death receptor pathway. Monitoring its expression and activation can help evaluate the involvement of this pathway in apoptosis.

III. BIOLOGICAL ASSESSMENT OF TNF-a / Bcl-xL / transfection reagents toxicity in fish cells (in-vitro)

PI +



GFP +



TRANSFECTION
EFFICIENCY

TOTAL APOPTOSIS
LEVEL

VIABLE CELL COUNT

pDNA VACCINE KINETICS
OF EXPRESSION

$$\% \text{ Transfected cells} = \frac{\text{Number of green cells} = \text{number of transfected cells}}{\text{Number of red nucleus} = \text{Number of cells}} * 100$$

13.3.1.1 Transitory Transfection of Fish Cells

Transfection of fish cells with a vector allowing cytoplasmic eGFP expression under the control of an actin promoter or any promoter from the above designs, the technique principle is as follows:

Transfection protocol (part I)

- **Day 1:** 4 wells labteks are available. 5.10e5 cells have been seeded in each well. First carefully remove the complete media and replace it with 600 µl fresh minimal media (media without antibiotics and without serum).
- Then prepare as many microtubes as needed (2 or 3 experiments + 1 control*). For each experiment use one microtube in which:

3 µl plasmid (200ng/µl) + 100 µl PEI buffer

Mix by tapping with fingers

Incubate at Room Temperature (RT) 2 min

Add 10 µl PEI, pipet up and down 5 times

Incubate at RT 5 min

Add 600 µl minimal media then add everything to the cells

*empty plasmid (8)

Transfection protocol (part II)

- **Day 2:** Remove the transfection media and replace it with fresh media containing antibiotics and serum.

-
- **Day 3:** Carefully remove the media and fix the cells 10 min with PBS1X containing 4% of Formaldehyde. (Careful dangerous) (5)

- Recover the formaldehyde solution and trash in a special chemical trash

- Carefully wash the cells three times with PBS 1X ; 0,1% Tween-20 (alternatively 0.1% Triton X-100 can be used, or 15 seconds in 0.005% saponin..) (5),(9)

- Incubate the cells for 5 minutes with PBS 1X ; 0,1% Tween-20 ; Propidium Iodide (PI) to color cell nucleus in red (Careful dangerous) (10)

- Carefully wash the cells three times with PBS 1X ; 0,1% Tween-20

- Mount the cells, seal the coverslip wait before analysis.



Immunological responses and protective efficacy to
vaccines in Asian seabass

IMMUNOLOGICAL ASSAYS AND COMPARATIVE PERFORMANCE OF DNA VACCINES AND RECOMBINANT PROTEIN VACCINES IN ASIAN SEABASS

6.3 Fish Immune Responses to Vaccines

In Asian seabass, primary immunoglobulins to monitor are IgM and IgT. The immune gene expression levels of several other most important genes involved in the fish immune system are being measured to determine the efficacy of the vaccines. These genes include:

Marker	Importance in Assessing Vaccine Efficacy
TCR (T-cell receptor)	Crucial for T cell activation and immune response as they recognize and bind to antigens. Measuring TCR expression helps determine if the vaccine effectively stimulates T cell responses.
MHC I and MHC II	Molecules that present antigens to T cells, essential for initiating adaptive immune responses. Monitoring their expression helps determine if the vaccine effectively stimulates antigen presentation.
IgM and IgT	Immunoglobulins produced by B cells in response to infection, playing significant roles in humoral immunity. Measuring their expression helps assess the vaccine's ability to stimulate B cell responses and antibody production.
TLR5a (Toll-like receptor 5a)	Part of the innate immune system, recognizing specific pathogen-associated molecular patterns (PAMPs) such as those found in Streptococcal flagellin. Monitoring TLR5a expression helps determine if the vaccine effectively activates the innate immune response.
IL-22 and IL-2b	Cytokines involved in immune response regulation, including T cell activation and differentiation. Measuring their expression helps assess the vaccine's ability to stimulate specific immune cell responses.
TNF-α (Tumor Necrosis Factor-alpha)	A cytokine with a vital role in inflammation and immune system regulation. Monitoring TNF-α expression provides insights into the vaccine's ability to trigger appropriate inflammatory responses.
Granzymes	Enzymes inducing apoptosis in infected cells, a crucial aspect of cell-mediated immunity. Measuring granzyme expression helps assess the vaccine's ability to stimulate cytotoxic immune responses.

- qRT-PCR (preferred) or ELISA
- Agglutination tests
- Artificial infection challenges

DNA VACCINE TOXICITY? H&E staining and microscopic examination

System	Organs	Changes/Pathology	Naïve	Control 1	Vaccine
Circulatory	Heart	Lymphoid foci	0/5	0/5	1/20
	Kidney	Renal thrombosis	0/5	0/5	2/20
	Liver	Hyperemia	0/5	1/5	2/20
	Spleen	Hyperemia	0/5	0/5	0/20
Reproductive	Ovary	Atresic follicles	0/5	1/5	6/20
Nervous	Brain	Lymphoid foci	0/5	0/5	3/20
Digestive	Intestine	-	0/5	0/5	1/20
Urinary	Kidney	Presence of pigments, tubular	0/5	0/5	2/20
		and Bowman capsule structural integrity loss			
Fotoreceptor	Eye	-	0/5	0/5	0/20
Endocrine	Langehans islands	-	0/5	0/5	0/20
Tegumentar	-	-	0/5	0/5	0/20
Respiratory	Gills	-	0/5	0/5	0/20

Table 30 Summary of histopathological findings in different organs of Asian seabass and zebrafish injected with DNA vaccines and protein vaccines against *Streptococcus iniae* (vaccine enolase, sortaseA, CpsA). Number of fish with histopathological alterations out of total fish injected. Fish were injected either with Naïve control ($n = 5$), Control 1 (protein buffer) ($n = 5$), or *Streptococcus iniae* vaccines ($n = 20$).

ANIMAL IMMUNIZATIONS

Experiment / Group	Vaccine type	Dose (volume injected / total protein)	Route of administration	Injection site(s)	Adjuvant(s)
Vaccination 1: Comparison of protein antigens and determination of optimal dose					
1 / 1Eno10	Protein	100 μ l Enolase 10 μ g	IP.	Belly	None
1 / 1Eno20	Protein	100 μ l Enolase 20 μ g	IP.	Belly	None
1 / 1Eno40	Protein	100 μ l Enolase 40 μ g	IP.	Belly	None
1 / 1SrtA10	Protein	100 μ l SortaseA 10 μ g	IP.	Belly	None
1 / 1SrtAB20	Protein	100 μ l SortaseA 20 μ g	IP.	Belly	None
1 / 1SrtAB40	Protein	100 μ l SortaseA 40 μ g	IP.	Belly	None
1 / 1CpsA10	Protein	100 μ l CpsA 10 μ g	IP.	Belly	None
1 / 1CpsA20	Protein	100 μ l CpsA 20 μ g	IP.	Belly	None
1 / 1CpsA40	Protein	100 μ l CpsA 40 μ g	IP.	Belly	None
Vaccination 2: Effect of fusion partners on vaccine performance					
2 / 2fuGFP-Eno20	Protein	100 μ l fuGFP-Enolase 20 μ g	IP.	Belly	None
2 / 2CipA-Eno20	Protein	100 μ l CipA-Enolase 20 μ g	IP.	Belly	None
2 / 2Eno20	Protein	100 μ l Enolase 20 μ g	IP.	Belly	None
2 / 2fuGFP-SrtA20	Protein	100 μ l fuGFP-SortaseA 20 μ g	IP.	Belly	None
2 / 2CipA-SrtA20	Protein	100 μ l CipA-SortaseA 20 μ g	IP.	Belly	None
2 / 2SrtA20	Protein	100 μ l SortaseA 20 μ g	IP.	Belly	None
Vaccination 3: Combinaison of antigens in a single vaccine					
3	Protein	100 μ l Enolase 10 μ g	IP.	Belly	None
3	Protein	100 μ l Enolase 20 μ g	IP.	Belly	None
3	Protein	100 μ l SortaseA 10 μ g	IP.	Belly	None
3	Protein	100 μ l SortaseA 20 μ g	IP.	Belly	None
3	Protein	100 μ l Enolase 5 μ g SortaseA 5 μ g	IP.	Belly	None
3	Protein	100 μ l Enolase 10 μ g SortaseA 10 μ g	IP.	Belly	None

ANIMAL IMMUNIZATIONS

Experiment / Group	Vaccine type	Dose (volume injected / total protein)	Route of administration	Injection site(s)	Adjuvant(s)
Vaccination 4: Effect of antigen-bound silicon dioxide as a vaccine adjuvant					
4	Protein	100 μ l Car9-Enolase 10 μ g	IP.	Belly	None
4	Protein	100 μ l Car9-Enolase 20 μ g	IP.	Belly	None
4	Protein	100 μ l Silicon-dioxide-bound Car9-Enolase 10 μ g	IP.	Belly	SiO2
4	Protein	100 μ l Silicon-dioxide-bound Car9-Enolase 20 μ g	IP.	Belly	SiO2
Vaccination 5: Comparison of plasmid DNA vaccines and determination of optimal dose and injection route					
5	Plasmid DNA	100 μ l 50 ng Enolase	IM.	Back	None
5	Plasmid DNA	100 μ l 75 ng Enolase	IM.	Back	None
5	Plasmid DNA	100 μ l 100 ng Enolase	IM.	Back	None
5	Plasmid DNA	100 μ l 200 ng Enolase	IM.	Back	None
5	Plasmid DNA	100 μ l 50 ng Enolase	IP. (skin)	Belly	None
5	Plasmid DNA	100 μ l 75 ng Enolase	IP. (skin)	Belly	None
5	Plasmid DNA	100 μ l 100 ng Enolase	IP. (skin)	Belly	None
5	Plasmid DNA	100 μ l 200 ng Enolase	IP. (skin)	Belly	None
5	Plasmid DNA	100 μ l 50 ng Enolase	IP. + IM.	combined	None
5	Plasmid DNA	100 μ l 75 ng Enolase	IP. + IM.	combined	None
5	Plasmid DNA	100 μ l 100 ng Enolase	IP. + IM.	combined	None

Note: In each vaccination group, all fish ($N=40$) were from the same hatchery and randomly assigned to experimental groups, each group was performed in duplicate (R1, R2). **Abbreviations:** IP. = intraperitoneal injection (injection in the belly), IM. = intramuscular injection performed in the upper-back muscles, Skin tattooing = refer to the procedure of injecting a liquid through a multiple needle, see link, TNF- α = recombinant tumor necrosis factor alpha from Asian seabass, Bcl-xL = plasmid DNA expressing Bcl-xL from Asian seabass and co-administrated with plasmid DNA vaccines to counteract the side effects from TNF- α , CD40 = recombinant cluster of differentiation 40 from Asian seabass, pDNA-Flagellin = plasmid DNA expressing Streptococcal flagellin from *Streptococcus iniae* and co-administrated with plasmid DNA vaccines.

ANIMAL IMMUNIZATIONS

Vaccination 6: Comparison of plasmid DNA vaccines adjuvanted with TNF- α and determination of optimal dose

6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	None
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	5ng TNF α
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	15ng TNF α

Vaccination 7: Evaluation of DNA tattooing for plasmid DNA delivery in fish epithelium

7	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α
7	pDNA Tattoo	100 μ l 100 ng Enolase 1 sec. 1cm	Skin	Belly	10ng TNF α
7	pDNA Tattoo	100 μ l 100 ng Enolase 2 sec. 2 cm	Skin	Belly	10ng TNF α
7	pDNA Tattoo	100 μ l 100 ng Enolase 4 sec. 4 cm	Skin	Belly	10ng TNF α

Vaccination 8: Comparison of plasmid DNA vaccines co-adjuvanted with rTNF- α + pDNA Bcl-xL

6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α

					10ng Bcl-xL
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α 50ng Bcl-xL
6	Plasmid DNA	100 μ l 100 ng Enolase	Skin	Belly	10ng TNF α 100ng Bcl-xL

Vaccination 9: Combination of recombinant protein vaccines and pDNA vaccines
co-adjuvanted with rTNF- α + pDNA Bcl-xL in various tissues

Vaccination 10: Replicate the results in adult fish (for the best vaccine)

Note: In each vaccination group, all fish ($N=40$) were from the same hatchery and randomly assigned to experimental groups, each group was performed in duplicate ($R1, R2$). **Abbreviations:** IP. = intraperitoneal injection (injection in the belly), IM. = intramuscular injection performed in the upper-back muscles, Skin tattooing = refer to the procedure of injecting a liquid through a multiple needle, see link, TNF- α = recombinant tumor necrosis factor alpha from Asian seabass, Bcl-xL = plasmid DNA expressing Bcl-xL from Asian seabass and co-administrated with plasmid DNA vaccines to counteract the side effects from TNF- α , CD40 = recombinant cluster of differentiation 40 from Asian seabass, pDNA-Flagellin = plasmid DNA expressing Streptococcal flagellin from Streptococcus iniae and co-administrated with plasmid DNA vaccines.



THANK YOU FOR YOUR ATTENTION