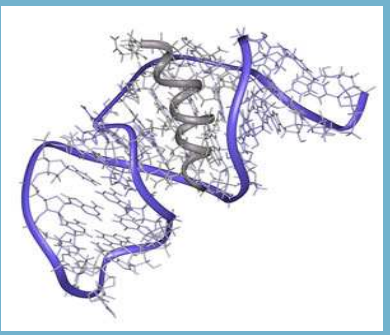
LFB
ETHICAL COMMITMENT



Aptamers: Powerful and Innovative Ligands in Affinity Chromatography

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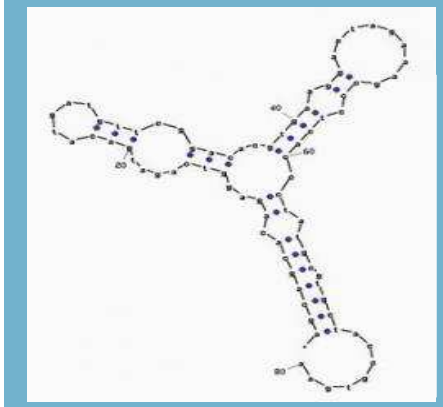
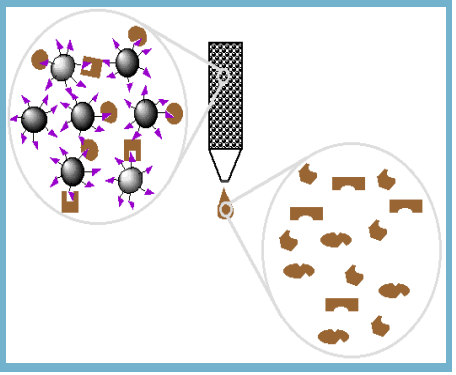
Technical Innovation Department

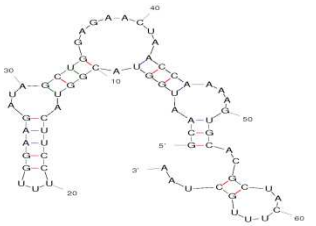




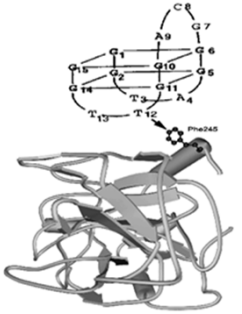
Outline

1. Aptamers as affinity ligands: definition, selection and general properties
2. Selection of an Aptamer designed for affinity chromatography application
3. Examples of aptamo-purification
4. The path to industriability
5. Conclusions

Definition and Generation



- Aptamer from « Aptus » (from the Latin ***aptus*** - fit, and Greek meros - region)
- Oligonucleotides (RNA or single-strand DNA) selected to bind a target [biological macromolecules (e.g. proteins) or little organic molecules]
- Specific 3D structures which allow aptamers to display high affinity and specificity for their targets
  - **First time reported in 1990: two publications[1,2] in Nature and Science**
  - **First selection against non nucleic acid binding protein in 1992: Thrombin[3]**

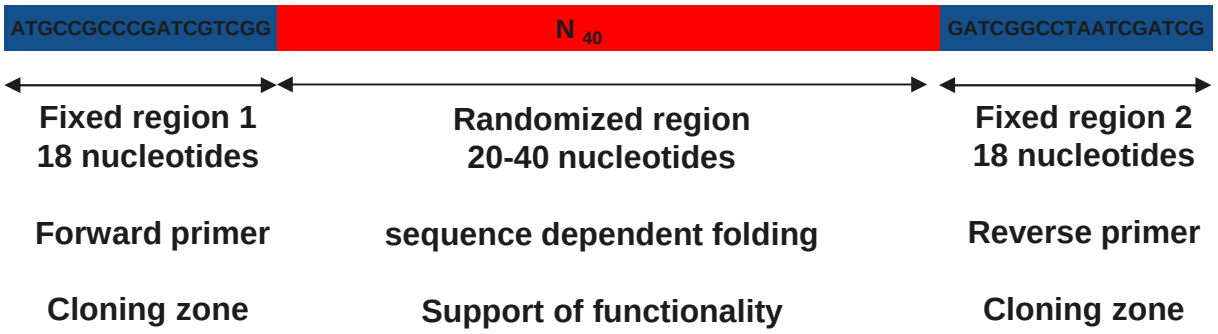


- *In vitro* Selection process inspired from natural selection : The SELEX
  - **This process enables the isolation, from a highly diverse combinatorial library of oligonucleotides, of the best binder(s) under a given set of conditions**

1: Nature. 1990 Aug 30;346(6287):818-22. In vitro selection of RNA molecules that bind specific ligands. Ellington AD, Szostak JW
2: Science: 1990 Aug 3;249(4968):505-10. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Tuerk C, Gold L.
3: Nature. 1992 Feb 6;355(6360):564-6. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ.

Definition and Generation

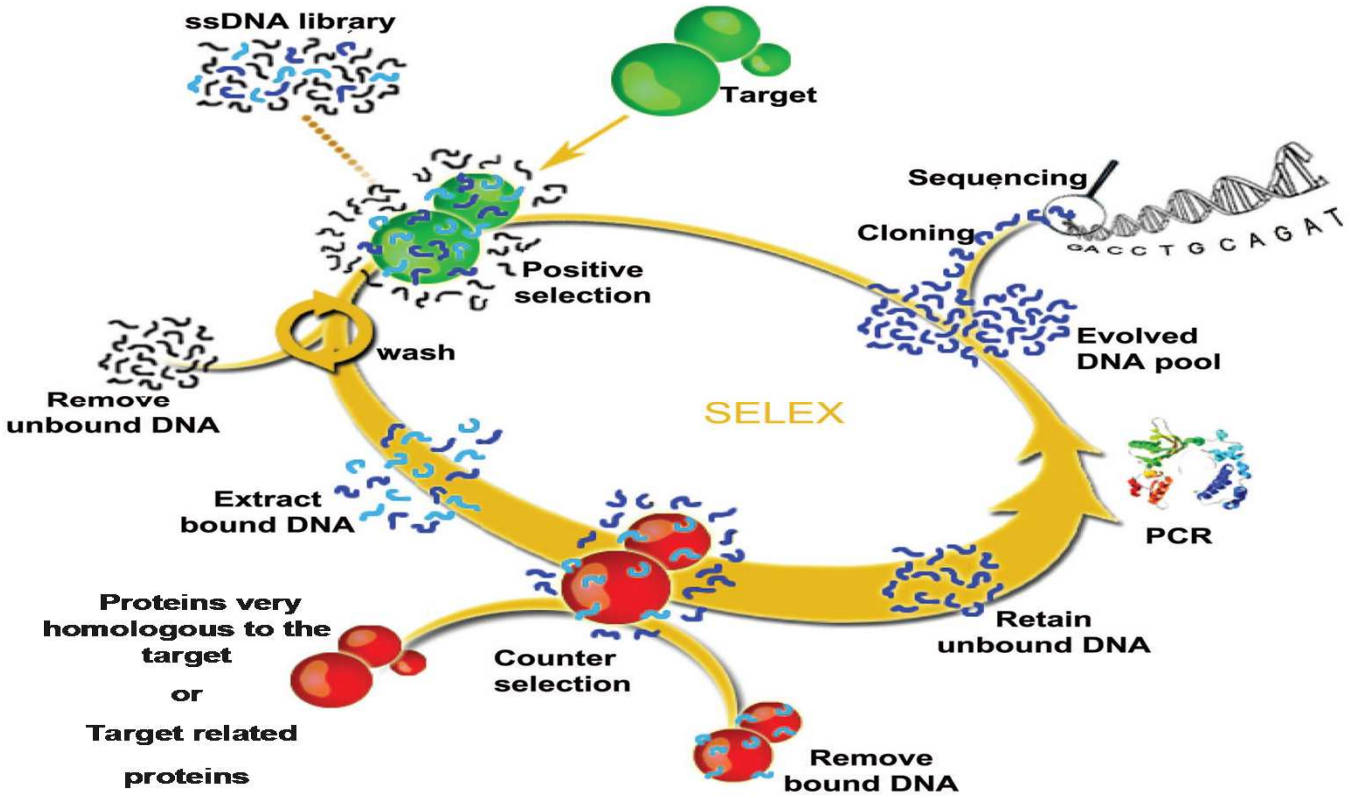
- The SELEX: Systematic Evolution of Ligand by Exponential enrichment
  - The combinatorial Library



  - Single stranded DNA chemically synthesized
  - Central randomized region of 20-40nt
    - → Huge diversity : **10^{15} to 10^{16} of different molecules**
    - → 2000 pmols of a 40nt library ≅ 47 µg ssDNA ⇒ 1.2x 10^{15} unique sequences
  - Flanked by fixed region (for PCR amplification and cloning)

Definition and Generation

- The SELEX : Systematic Evolution of Ligand by Exponential enrichment
  - Iterative process of selection/ counter-selection /amplification of the more fitting species followed by an identification step :



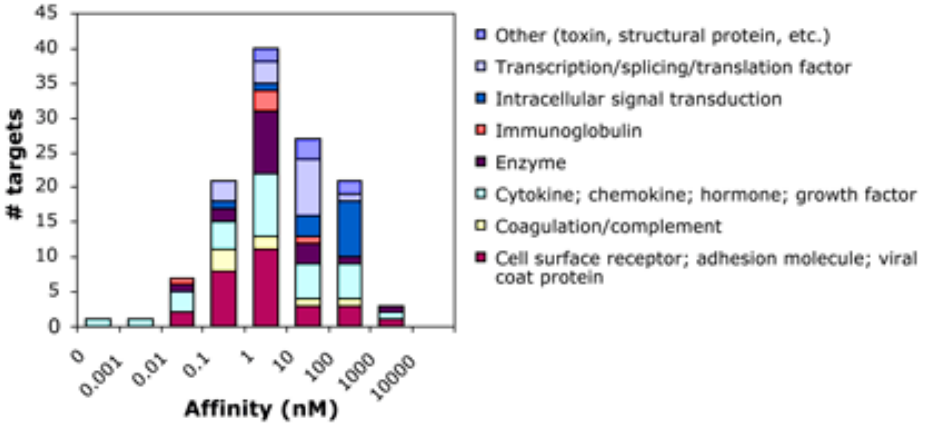
Definition and Generation

- Number of cycles needed for an efficient evolution of the library: 6 to 16
  - Stopped when the enrichment or the affinity of the pool is deemed sufficient enough
  - One SELEX leads to many specific candidates
- Sequencing performed on the evolved Aptamer pool allows
  - Identification of repeated consensus sequences
  - Characterization of the secondary structures and identification of the minimal core-sequence needed for binding
- Minimal size of the core-sequence: 10 to 60 nt
  - 15 nt anti-thrombin aptamer: GGTTGGTGTGGTTGG

  (Kd: 200nM)

Aptamers: global view

- General properties
  - Binds the target: Kd pM to µM
  - Possibly, Inhibits the biological activity of the target
- 4000+ Aptamer sequences - 370 sequences of artificial Ribozymes referenced in 500 publications and patents (DataBase : Aptamer.freebase or RiboaptDB)
- 2000+ publications on Aptamers and/or SELEX
- Some examples of target/Kd



Target	Kd
VEGF	2 nM
Adenine	8 µM
C5	2-5 nM
B12 (vitamin)	500 nM
HIV-RT	25 pM
PrPsc	40 nM
PrPc	20 nM

Aptamers : main features

- Generation of aptamers against non immunogenic, or difficult to handle targets (Prion, toxin, small molecules)
- Quick *in vitro* process: Generation in ~ 8 weeks against virtually any target
  - Less for optimized /automatized process
- Their chemical nature (5'P-3'OH) allows for precise derivatization (biotin, fluorescent reporter, spacer arm, etc.)
- Owing to their small size: anti-protein aptamers can access/contact relatively small binding pockets on their targets
  - Powerful inhibitors
- High specificity :
  - An Aptamer exhibits greater than 10,000-fold binding affinity for theophylline over caffeine
- Can be selected under a wide range of conditions
  - Select species that bind under non physiological conditions (pH, ionic strength, solvent, temperature)
  - Binding under precise conditions to meet a specific need (process, type of sample)

Affinity ligand Ideal attributes ?

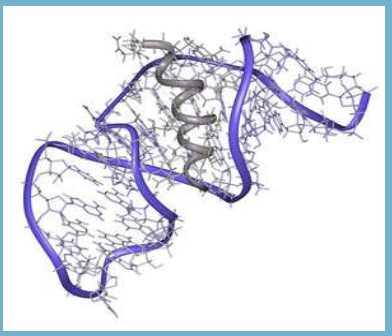
- Dedicated grafting function to allow oriented immobilization
- Capable to exclusively recognize the target protein
- Possibility to easily release the captured proteins by a chosen condition suitable for the product
- Reusable and stable under quite harsh conditions
- Neither leakage nor molecular hydrolysis
- Molecular mass as small as possible
- Non-toxic
- easy synthesis and affordable cost

Aptamer as ligand for affinity chromatography : **Ideal ligand ?**

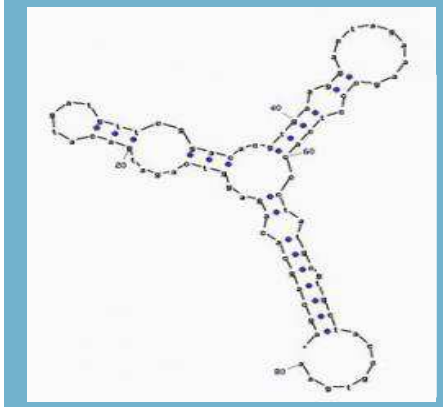
- High specificity and selectivity
  - Generally considered to be superior to antibody-derived ligands
- High physical and chemical stability: reusable and resistant to harsh sanitization
  - High physical and chemical stability of DNA chemistry
  - Modified nucleotides provide nuclease resistance
- Behavior which may perfectly fit specific process requirements
  - Behavior chosen and modulated during the SELEX process
  - Elution under optimal conditions for the product or considering the process constraints (modality of selection)
  - Improvement selectivity through counter-selection
    - → Particular interest for transgenic proteins: counter-selection against undesirable endogenous proteins

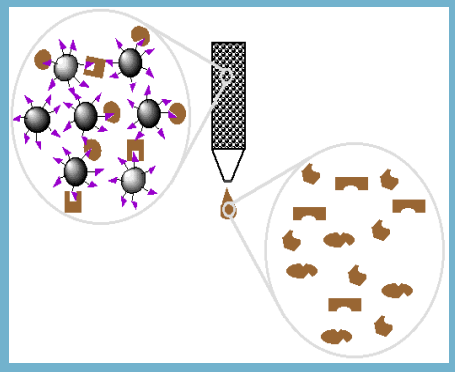
Aptamer as ligand for affinity chromatography : **Ideal ligand ?**

- Manufacturing cost: large scale production by chemical synthesis
  - High reproducibility and moderate cost: 2 to 5 K€ per gram of Aptamer with spacer and terminally-modified nucleotide (MW = 1/10 to 1/50 of Mab)
  - No potential biological contamination
- Availability of highly sensitive assays when considering aptamers as leachables: Molecular biology methods
- Lack of immunogenicity (in case of leakage)
  - Lack of immunogenicity demonstrated during pre-clinical tests for therapeutic Aptamers when 1,000-fold higher doses were administered to monkeys (by Eyetec for anti-VEGF165).



Selection of an Aptamer designed for affinity chromatography applications





Selection of an Aptamer designed for affinity chromatography applications

Case of human Recombinant FVIIa

	SELEX strategy
Selection buffer	Tris 50mM / NaCl 50mM / CaCl2 10mM / MgCl2 4mM pH = 7.5
Target	Alternatively : R FVIIa / plasmatic derived FVIIa
Counter-selections	Support : Nitrocellulose filter Recombinant rabbit FVII (85% homology)
Wash / Elution	NaCl 2M /EDTA 10mM

Significant Proportion of interesting species in round 7 confirmed in round 10

➔ Cloning and sequencing

Selection of an Aptamer designed for affinity chromatography applications

Case of human Recombinant FVIIa

Bioinformatics and SPR analysis (Biacore) for confirmation of Monoclonal Aptamer (Mapt) sequences

```
                    10         20         30         40         50
                    ....|....| ....|....| ....|....| ....|....| ....|....| ..
F10-S2T7    ------TAAG CCGCTGCGTA TTATAGCTGA ATGCCC--C- TAATGGGAAG TG
F12-S2T7    .......... .......... .......... .......... .......... ..
G11-S2T7    ......C.T. .......... .......... .........A C......... ..
E03-S2T10   ......C... .......... .......... .........A .......... ..
E09-S2T10   ......C... .......... ..-....... .........C .......... ..
A03-S2T7    ......CC.. .......... .C........ .........A .......... ..
E01-S2T7    ......C... .......... A......... .........A ......-... ..
E08-S2T10   ......C... ..T....... .......... ....T....T ......T... ..
G09-S2T10   ......C... .......... .......... ....T....T ......T... ..
H07-S2T10   ......CC.. G......... .C........ ....T....T ...C..T... ..
B08-S2T10   ......C... .........T .......... ....T....T C.....T... ..
G5-S2T7     ......C... .......... .......... ....T....A .CT...T... ..
E10-S2T10   GTGCAGCC.. ....A.TN.. AGTG...... T...AAG..A .G.G..---- --
A9-S2T10    ....ATGC.. ..-AGC..C. G.G..AG... ....AGA..A .GG.C-T... ..
B01-S2T10   ......CC.. .A.AGC..C. G.T.CAG... ....AGAT.A .GG.C-T... ..
Clustal Co          *                  ***  ***    *
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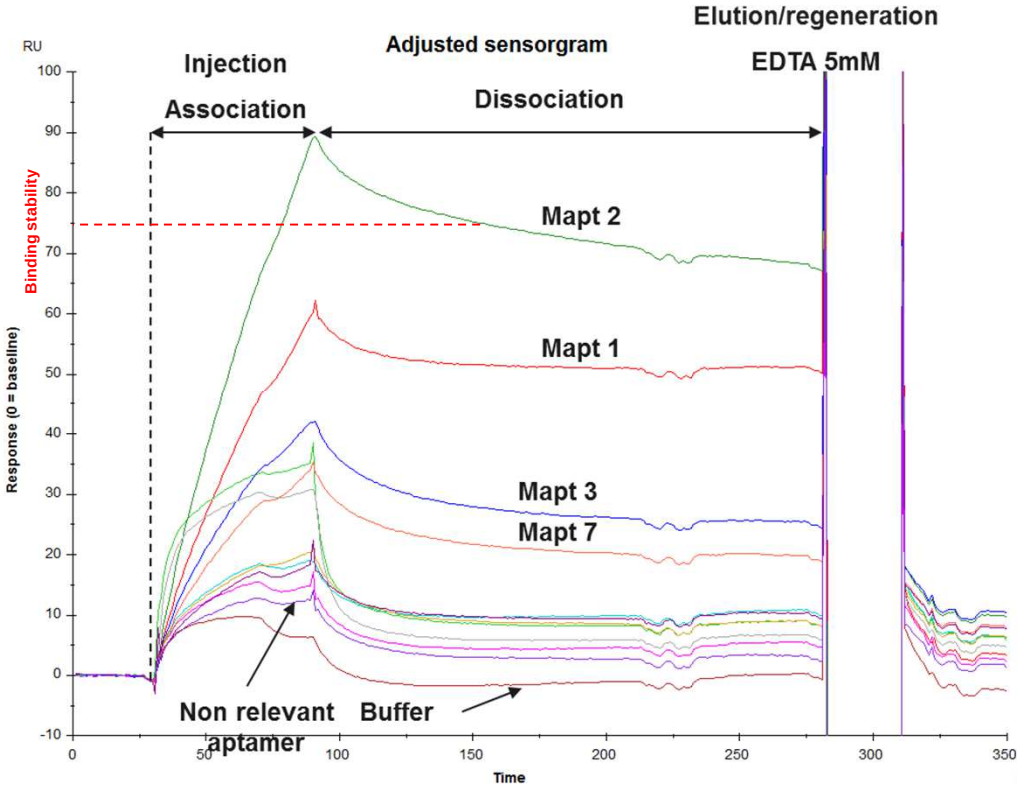
Bioinformatics alignment for family identification

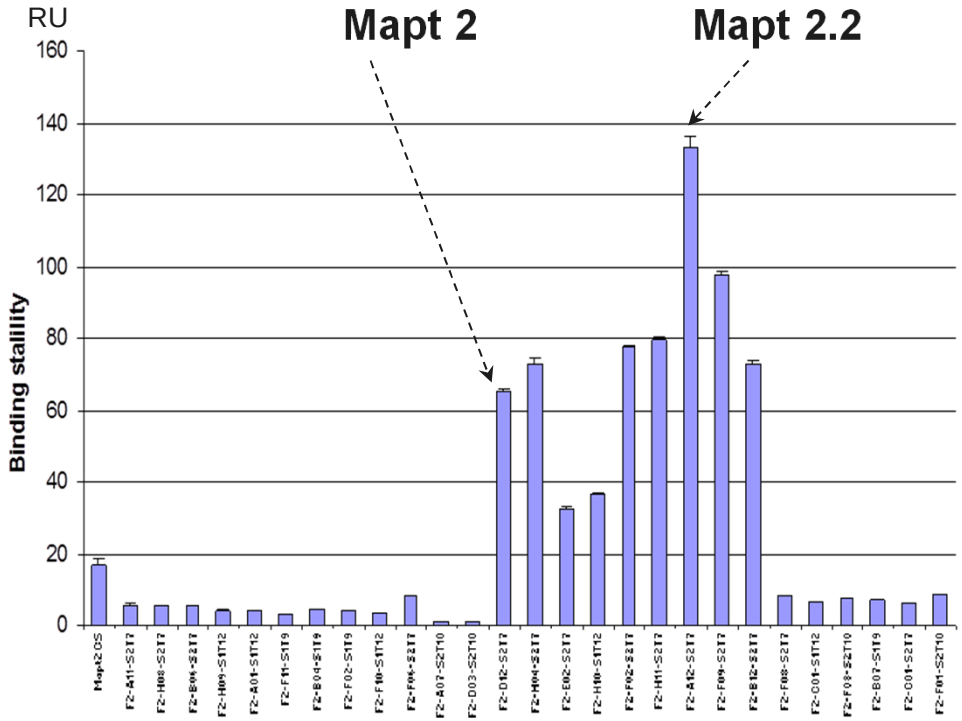


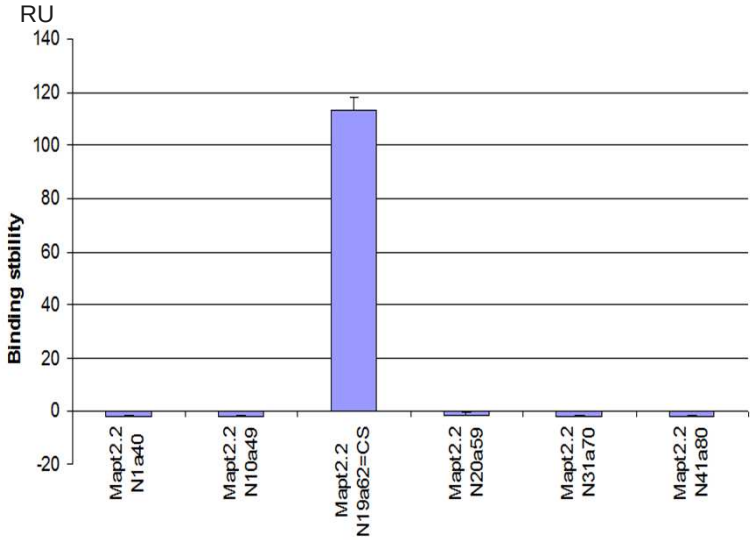
Affinity confirmation against immobilized target : R FVIIa

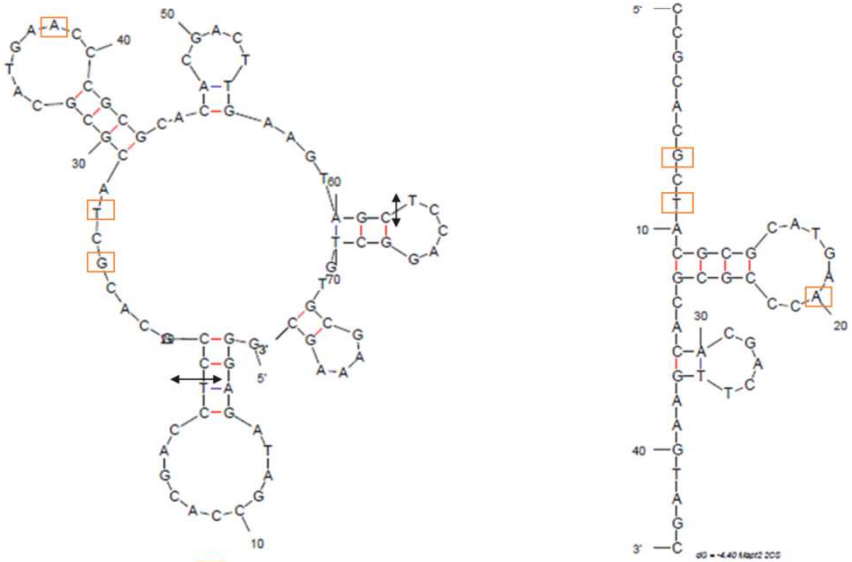
Selection of an Aptamer designed for affinity chromatography applications

Case of human Recombinant FVIIa

SPR analysis for best binder determination in a aptamer family followed by core sequence identification





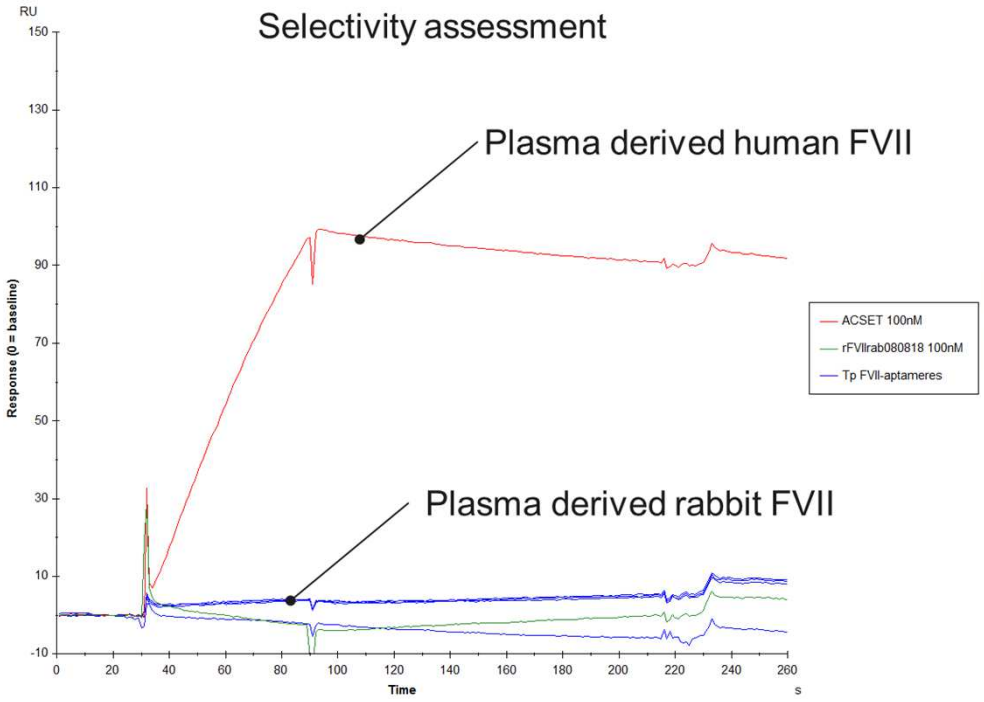


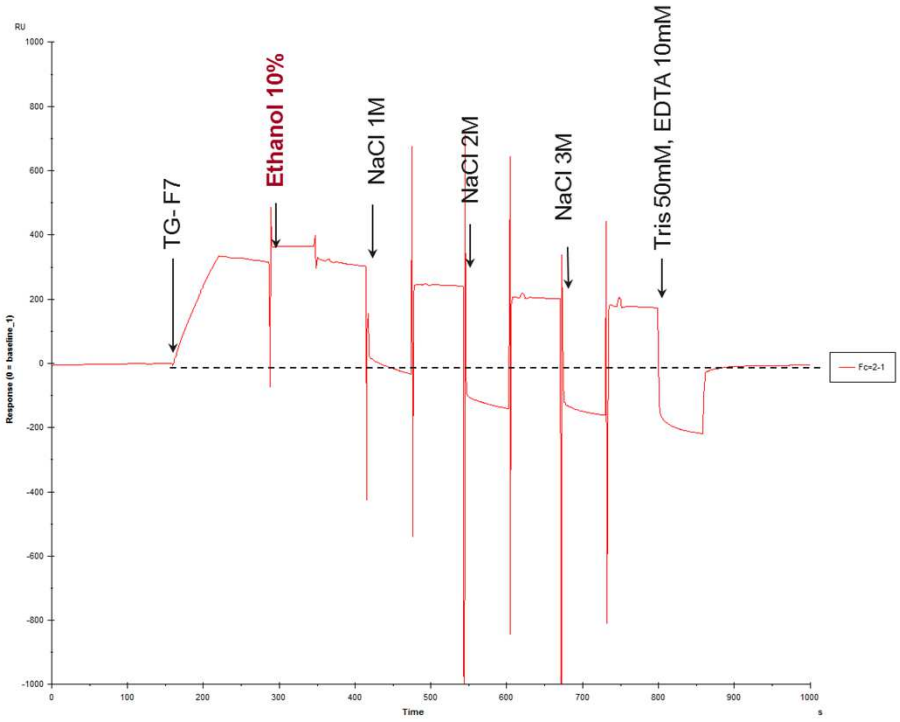
Mutation with high impact on affinity

Selection of an Aptamer designed for affinity chromatography application

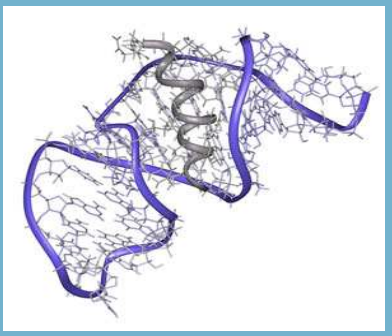
Case of human Recombinant FVIIa

Selectivity assessment and analyses of their binding properties

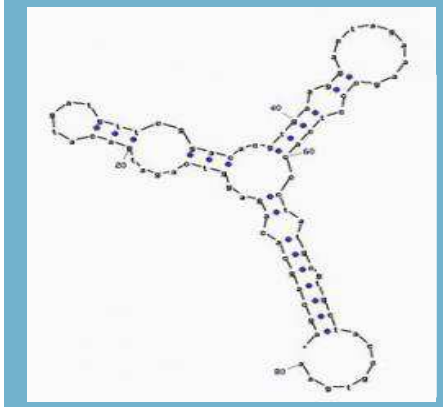


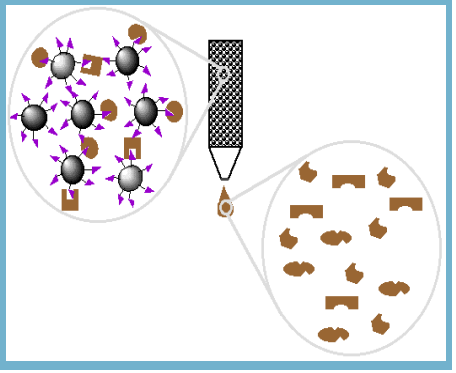


Resistance under different wash conditions evaluation with immobilized aptamer



Examples of Aptamo-purification

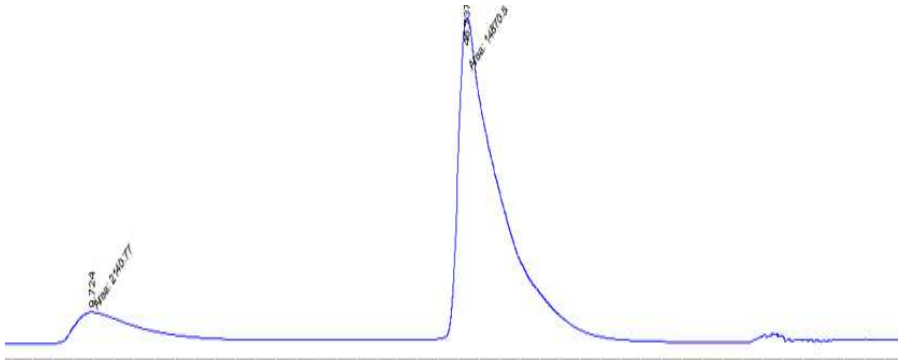




Aptamo-purification as a polishing step
Example of Recombinant FVIIa

From **highly purified** material

Purified recombinant FVIIa from slightly stressed raw materiel containing 10% of product-related impurities (other impurities less than 10ppm)



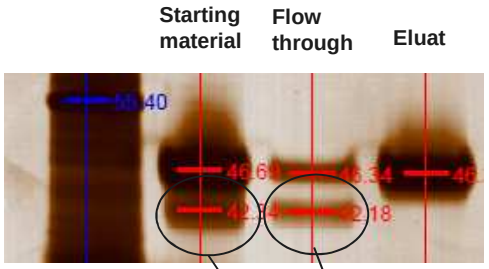
Material: Purified hRFVIIa

Equilibration buffer:

50 mM Tris-HCl (pH 7.4)
150 mM NaCl
10 mM $CaCl_2$

Washing Buffer: none

Elution: 50 mM EDTA pH 8



SDS PAGE gel - silver stainig

Apparent molecular weights indicated

⇒ Increased specific activity (ratio of chromogenic activity to antigen titer) from 0.7 to 1.0

Identified by MS as des-Gla FVII

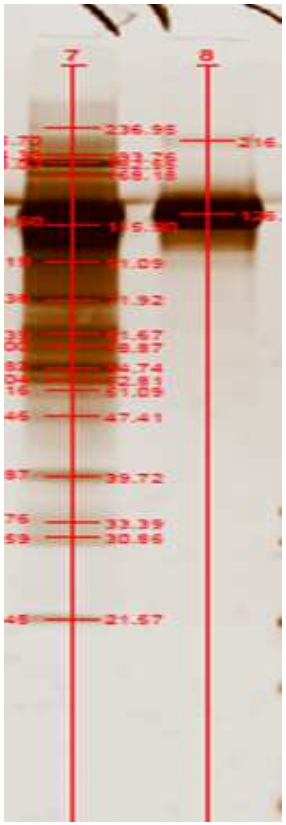
⇒ Capacity to eliminate very closely related impurities

Highly purified product by aptamo-purification
Example of Factor H protein

From **pre-purified** material
Plasmatic FH obtained after 4 chromatographic steps, including a pseudo affinity chromatography

From **crude** material
Recombinant FH in supernatant

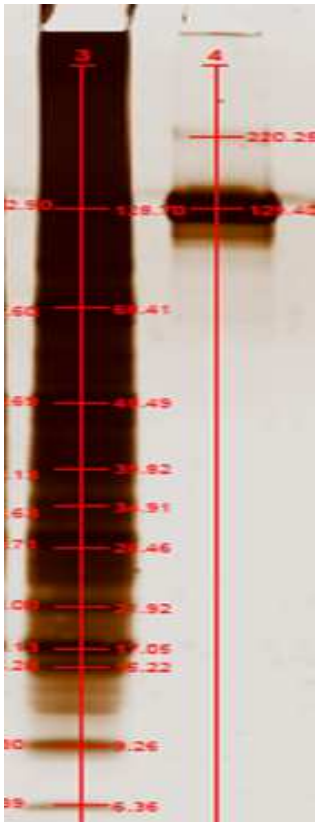
Starting material **Eluate**



SDS PAGE gel - Silver staining

Apparent molecular weights indicated

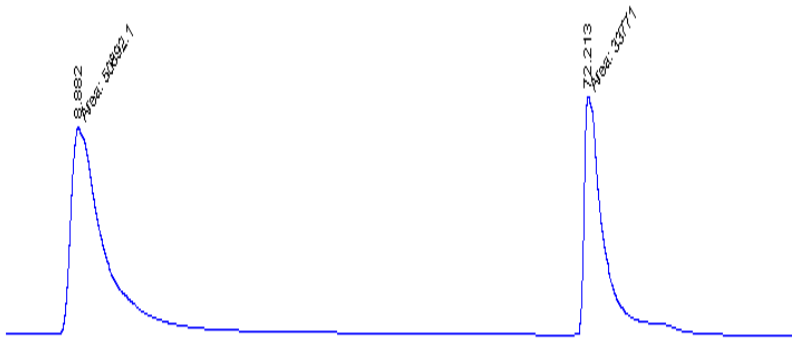
Starting material **Eluate**



➔ **Highly purified FH product obtained in on single step (with 1M NaCl wash) from pre-purified or crude material**

Highly purified product by aptamo-purification
Example of coagulation Factor IX

Anti-FIX aptamer Mapt 1 grafted – 1 mL resin

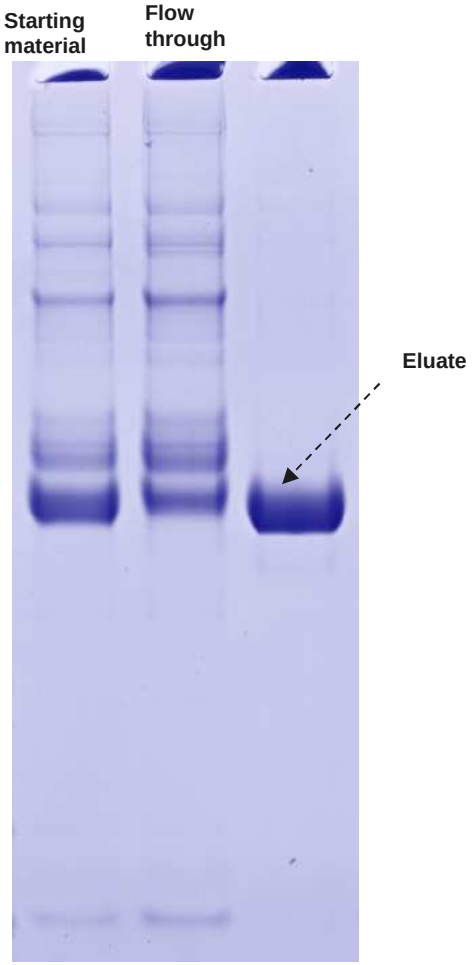


Material: Intermediate product containing 94 UI/mL of FIX

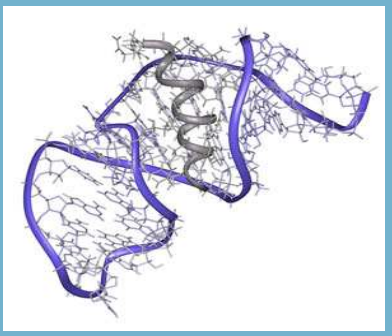
Equilibration buffer:

50 mM Tris-HCl (pH 7.4)
150 mM NaCl
2 mM $CaCl_2$
1 mM $MgCl_2$

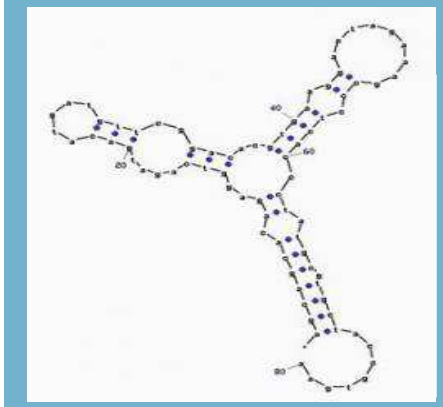
Elution: 200 mM EDTA (pH 8)

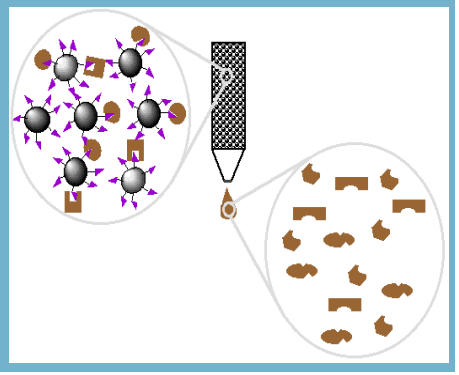


SDS PAGE gel - Blue staining



The path to Industriability: Grafting and resistance demonstration





On the path to Industriability: High grafting yield

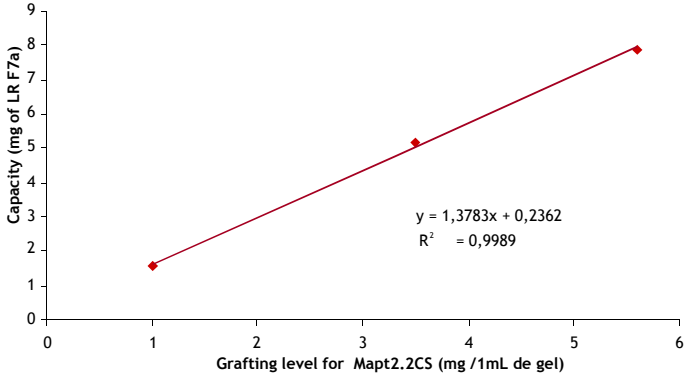
A specific concern for this technology has been addressed through the use of a proprietary immobilization approach (efficient chemical grafting of an NH2-derivatized oligonucleotide on a classical support)

Quantity targeted in µg (for 1mL gel)	100	3500	6000
Quantity grafted in µg	≈100	3498	5556
Grafting yield (%)	**100.0**	**100.0**	**92.6**

Immobilization (mg)	1.0	3.5	**5.7**
Dynamic Capacity* (mg)	1.6	5.2	**8.0**

***Capacity for a 50 KDa protein at 60 cm/H**

Immobilization level/binding capacity relationship



⇒ **New grafting approach which provides a very high yield of immobilization**

⇒ **High efficiency (functionality/grafting)**

On the path to Industriability: Specific and sensitive detection assays

- For most of affinity ligands, the development of a specific assay especially in the presence of the target remains a challenge
  - Sensitivity required less than pg/mL
  - Overcomes the probable complex formation with the target and/or the probable matrix effect by the concentrated target itself in the final product
- For aptamers different methods developed in the genomic and molecular biology fields can be adapted for this purpose
  - Highly sensitive methods available from molecular biology for DNA assay including a specific DNA extraction and/or target denaturation

On the path to Industriability : Resistance of the aptamo-affinity resin

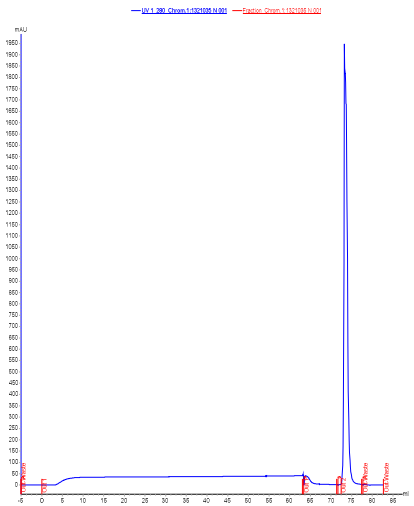
As a model : chemically immobilized anti-FVII aptamers with inverted 3'T

⇒ 2 anti-FVII aptamers with 2 different spacers (hyrophobic C6 and hydrophilic C11)

Parameters monitored:

- Capacity at 80% = 6.5-7.5 g/L (initial values)

- Specific activity of the eluted product : 0.8-1.0 (initial values)

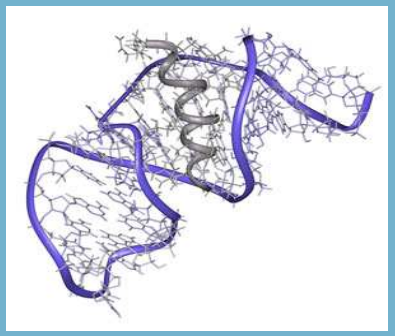


Typical chromatogram obtained on pre-purified product

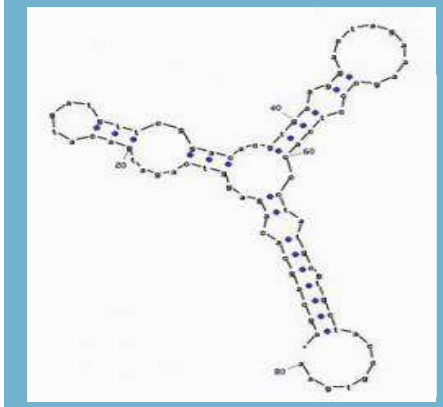
Résistance tested	Mapt 2 C11		Mapt 2,2 C6	
	Capacity 80% g/L	Specific activity (FVIIam/FVIIAg)	Capacity 80% g/L	Specific activity (FVIIam/FVIIAg)
100 Hours Sodium hydroxide 1 M	7.1	0.9	6.9	0.9
100 Hours in cryosupernatant	7.3	0.9	ND	ND
100 Hours in clarified milk	ND*	ND	7.1	0.9
+ 30 add. cycles buffer/ sodium hydroxyde 1M	ND	ND	6.9	1.0

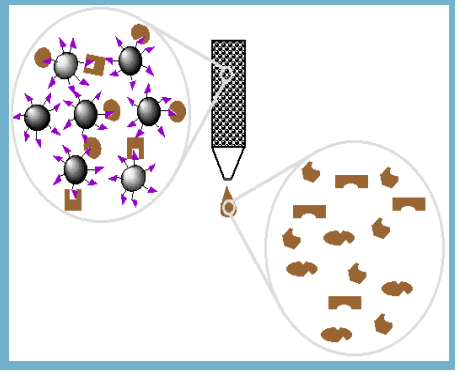
*not determined

⇒ **Very high resistance of aptamer resin**



Conclusions





Aptamer for affinity chromatography
A major interest for LFB : Aptapure®

- Most promising technology for improving yield and COGs of biotherapeutics including Plasma fractionation
  - 10 patents submitted
  - One major patent: original process for Aptamer immobilization
- The last 3 years, investment made by LFB aimed at demonstrating :
  - High selectivity (vs contaminants, related-protein contaminants, homologous proteins)
  - Capacity to discriminate proteins with correct PTMs (i.e. complete γ-carboxylation for FIX)
  - High resistance: ultimate sanitization with 1M NaOH, compatibility with biological media (serum, milk)
  - High yield of immobilization using a specific chemistry on a classical support
  - High efficiency of immobilized aptamers
  - Very sensitive assay of residual aptamers in case of leakage
- First positive feedback from EMA (Innovation Task Force)

Special Thanks

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