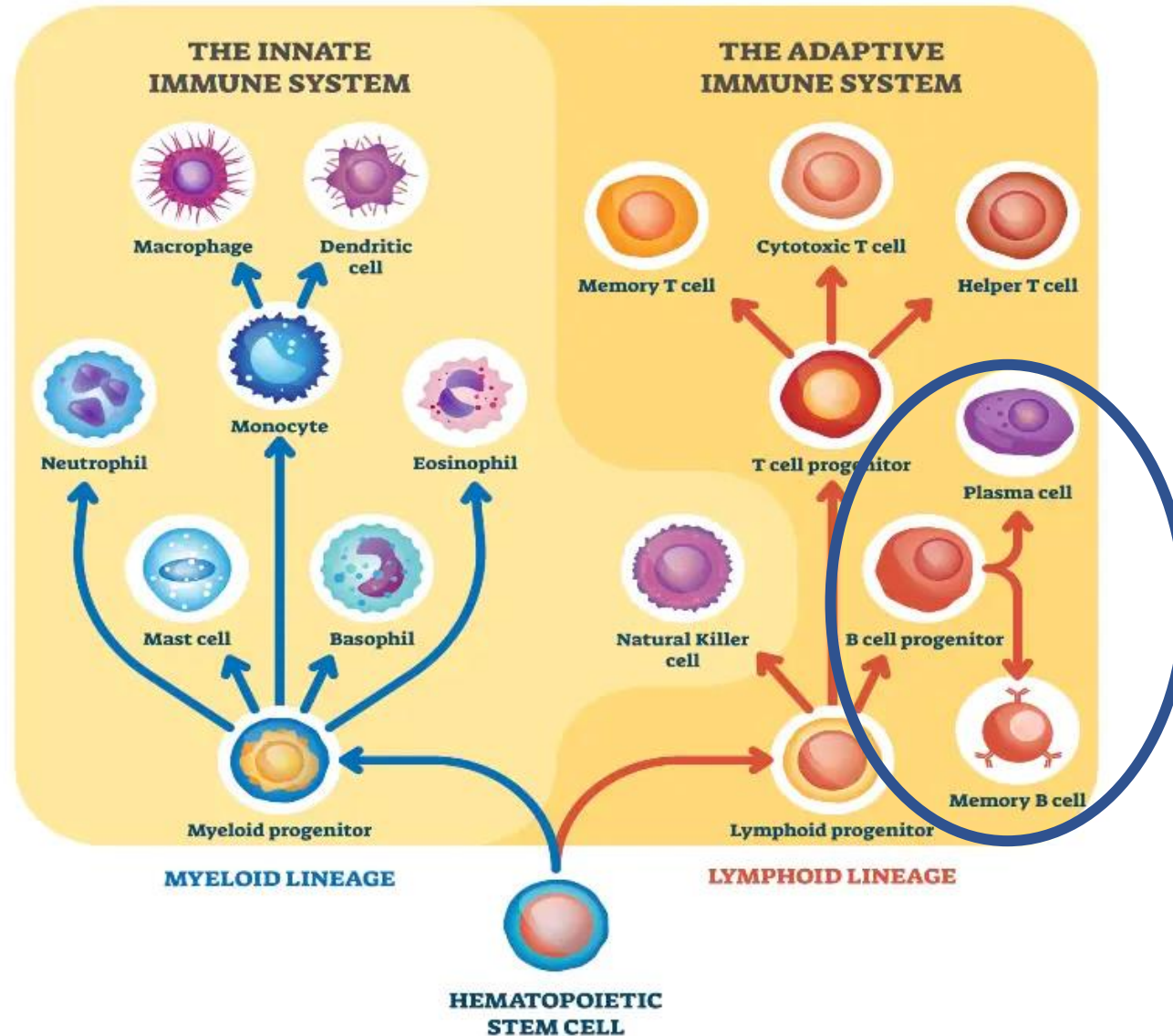


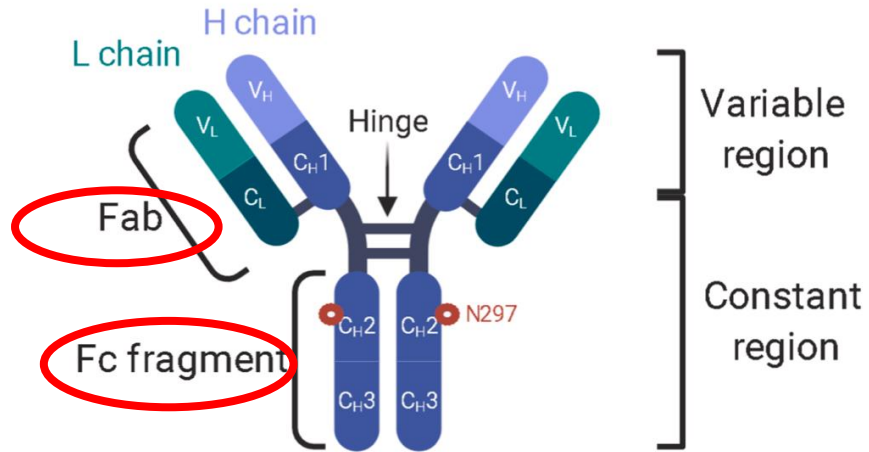
Produzione di proteine terapeutiche

Gli anticorpi monoclonali (mAb)

CELLS OF THE IMMUNE SYSTEM



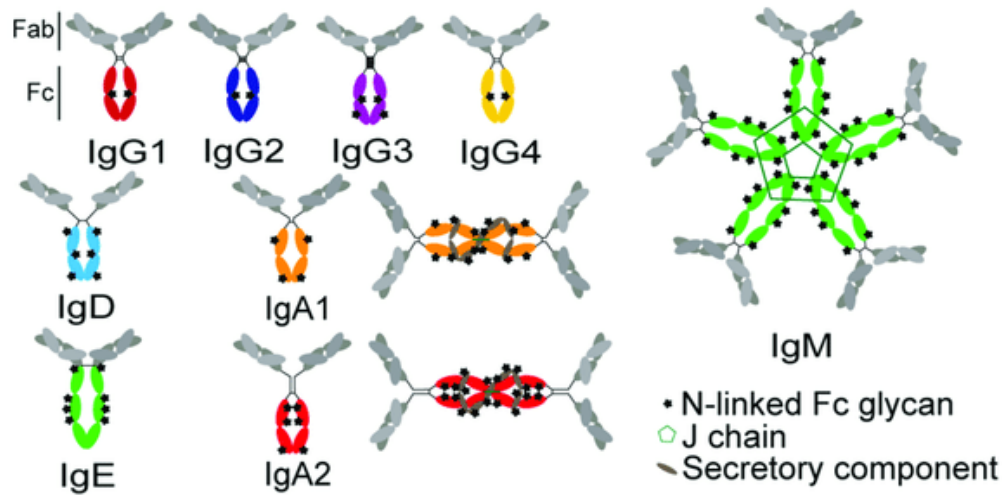
Struttura degli anticorpi



Le immunoglobuline (Ig) sono formate da 4 catene polipeptidiche:

- 2 catene pesanti (Heavy)
- 2 catene leggere (Light)

Ogni catena contiene un dominio variabile V nel quale si trova il sito di riconoscimento per l'antigene (**CDR: complementarity-determining region**) e domini costanti C necessari per le funzioni effettrici.



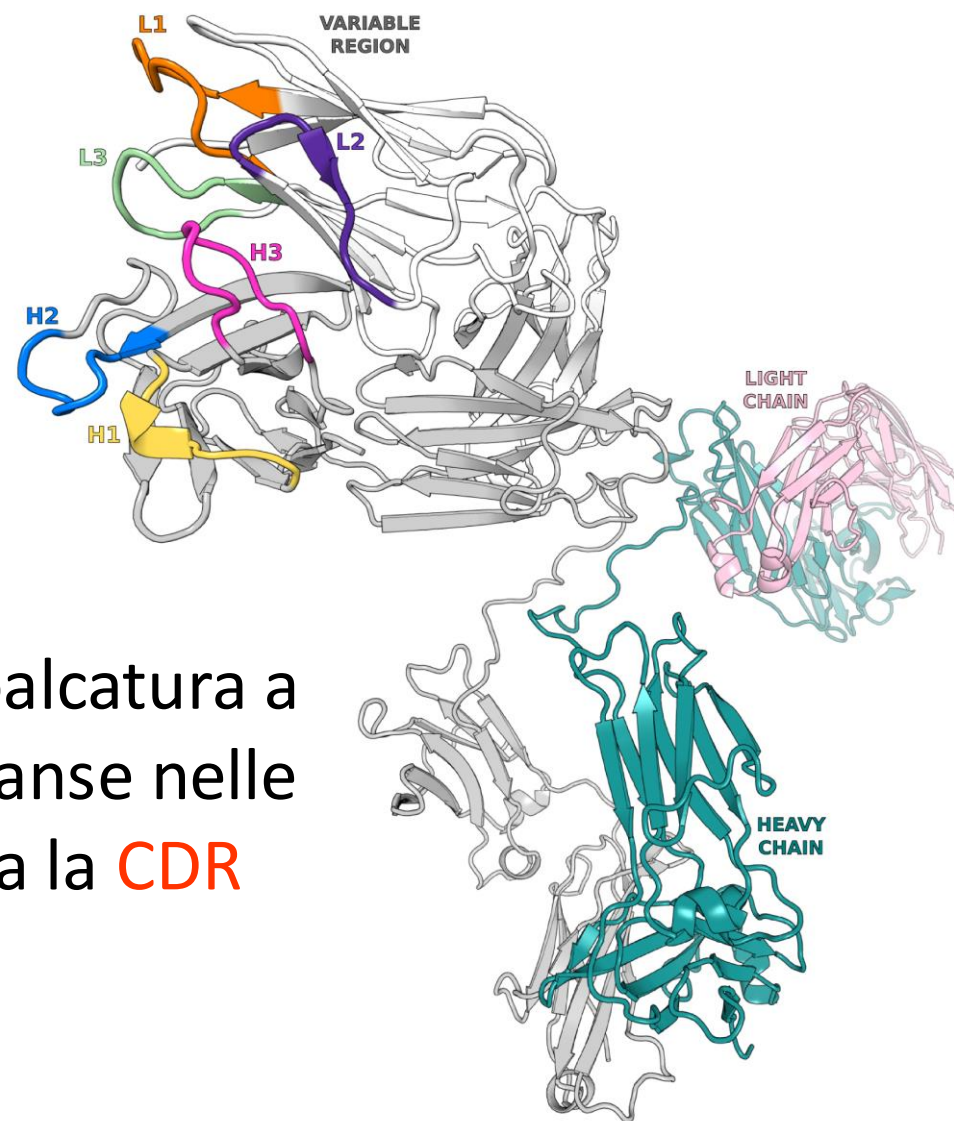
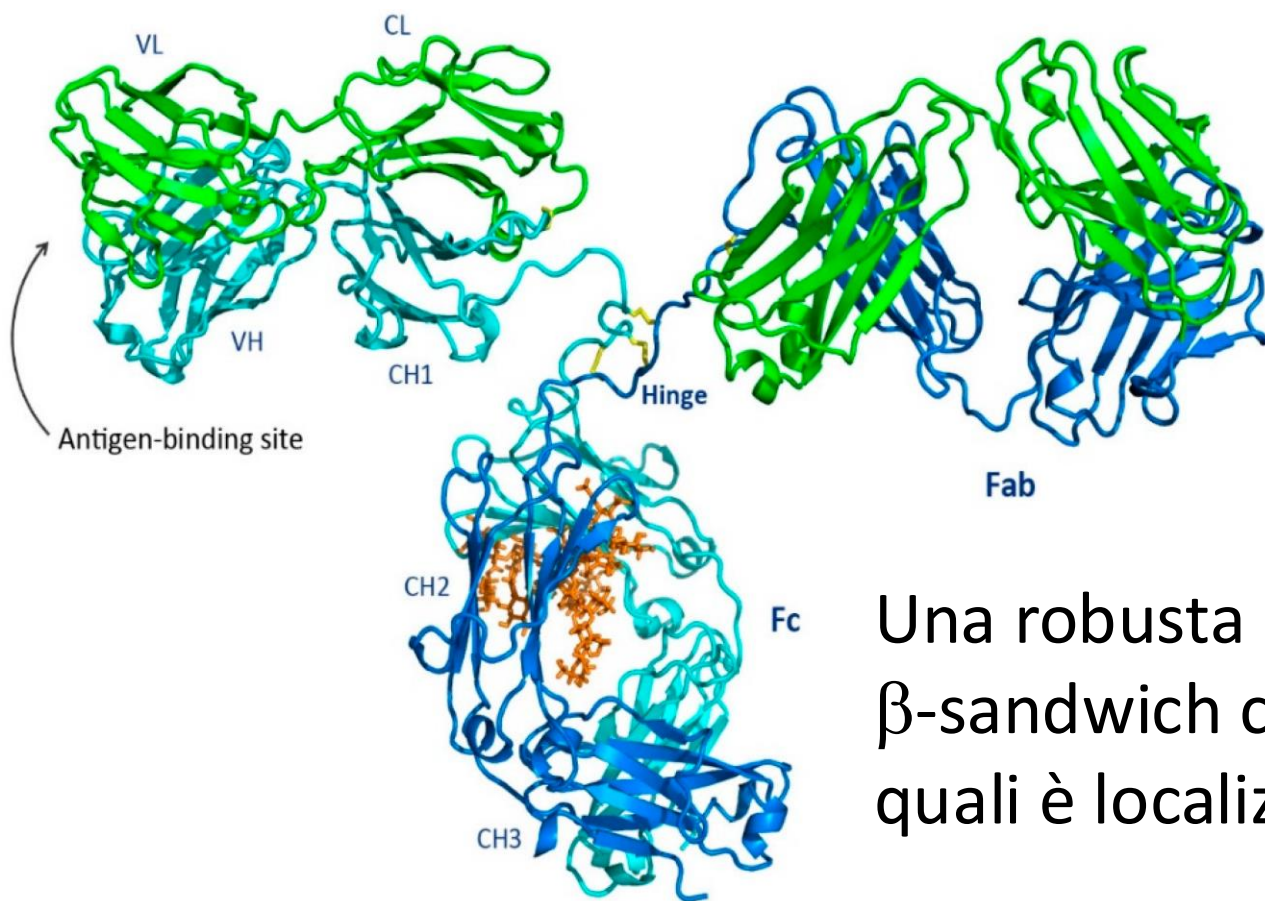
In base al tipo di catena pesante:

- **IgG**, IgE, IgD monomeriche
- IgA dimeriche
- IgM pentameriche

Modifiche post-traduzionali:

- ponti disolfuro
- glicosilazione

Struttura degli anticorpi



Una robusta impalcatura a β -sandwich con anse nelle quali è localizzata la **CDR**

Funzioni effettrici degli anticorpi

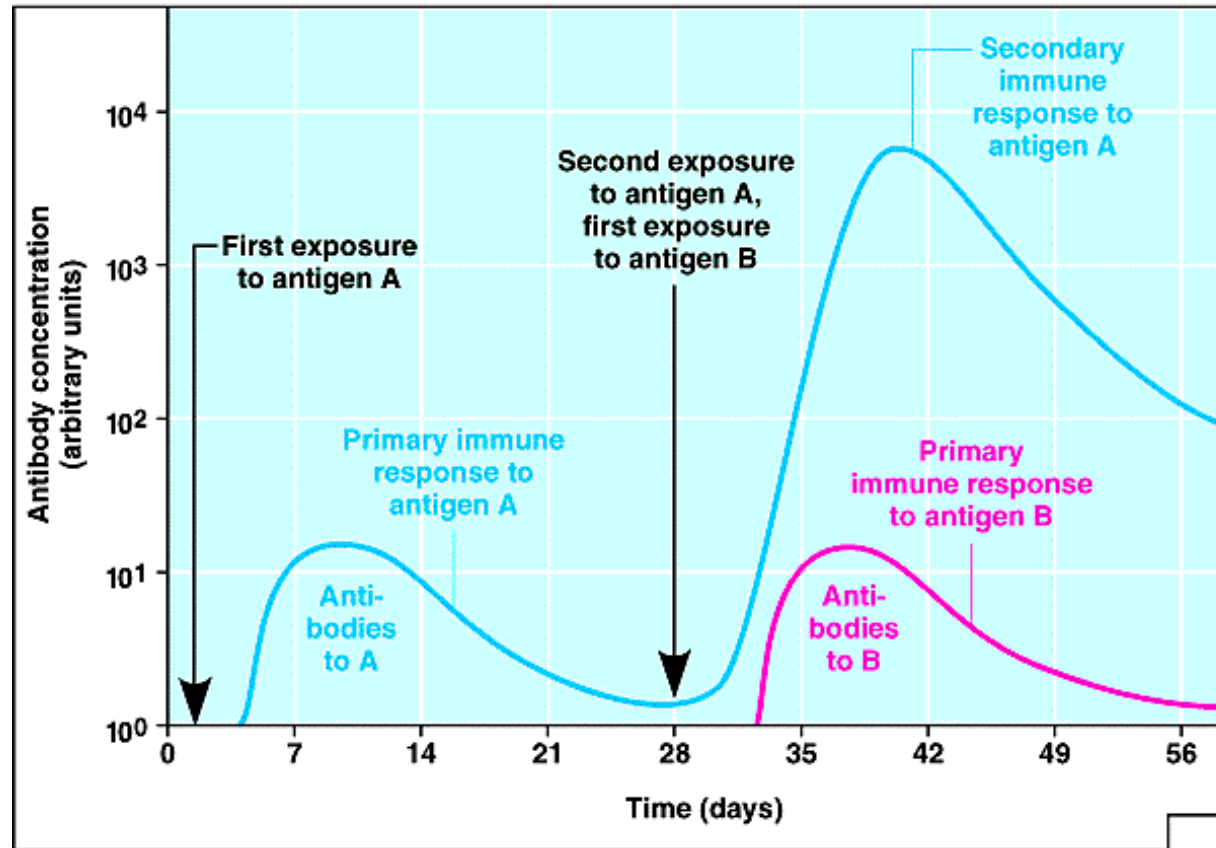
- **Citotossicità complemento-mediata**

proteine del complemento legano la **Fc** dell'anticorpo che si è legato alla cellula bersaglio e la cellula va incontro a lisi

- **Citotossicità cellulare anticorpo-dipendente (ADCC)**

la **Fc** dell'anticorpo legato alla cellula bersaglio è riconosciuta da recettori su cellule effettrici ed avviene lisi cellulo-mediata

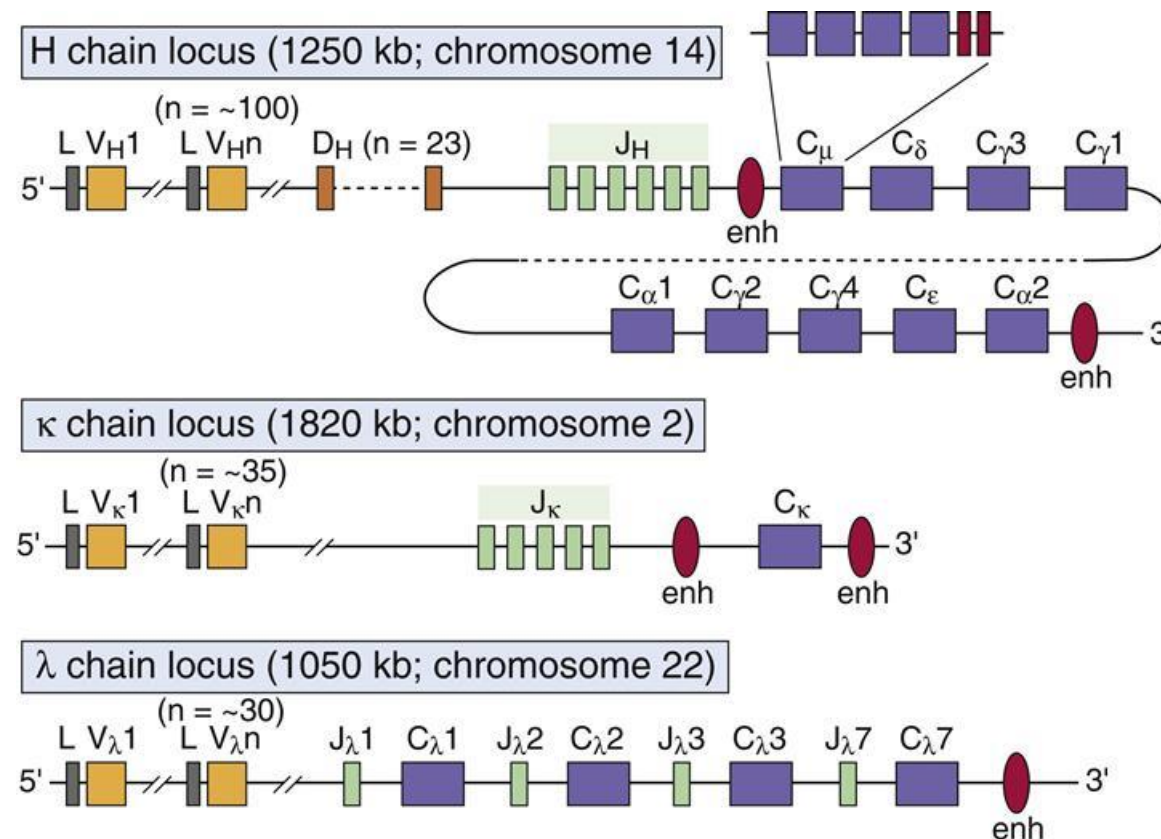
La risposta immunitaria è **policlonale**!



Come viene generata la diversità anticorpale?

Organizzazione genica delle Ig

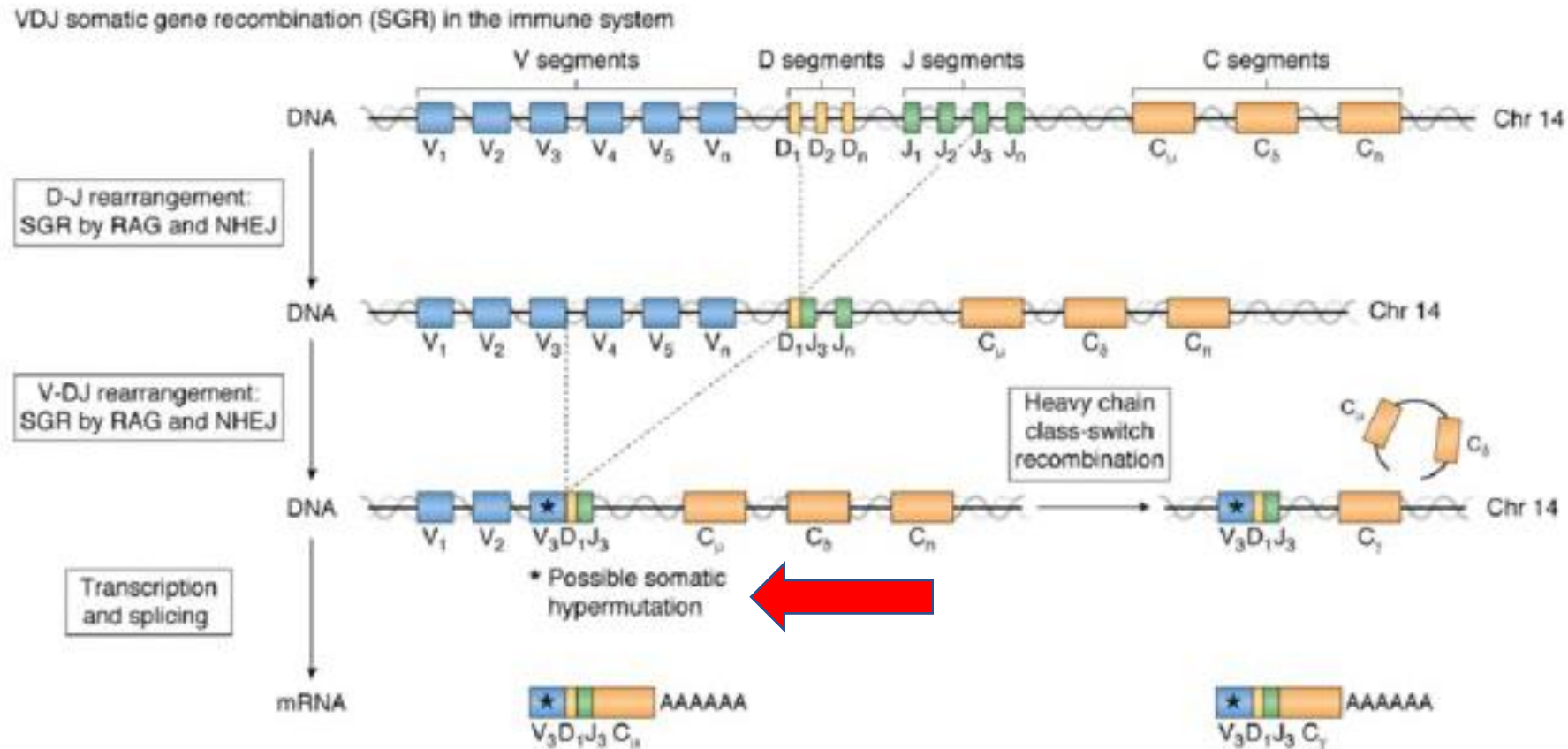
I geni per la catena pesante sono localizzati sul cromosoma 14 (14q32.33), i geni per la catena leggera si trovano in due loci distinti, kappa e lambda, sul cromosoma 2 (2p11.2) e sul cromosoma 22 (22q11.2)



Number of functional gene segments in human immunoglobulin loci			
Segment	Light chains		Heavy chain
	κ	λ	H
Variable (V)	40	30	40
Diversity (D)	0	0	25
Joining (J)	5	4	6

Organizzazione genica delle Ig

La **ricombinazione somatica** delle regioni V-D-J genera la diversità anticorpale che porta alla produzione di anticorpi specifici per l'antigene



1. Come selezionare anticorpi **monoclonali** specifici?

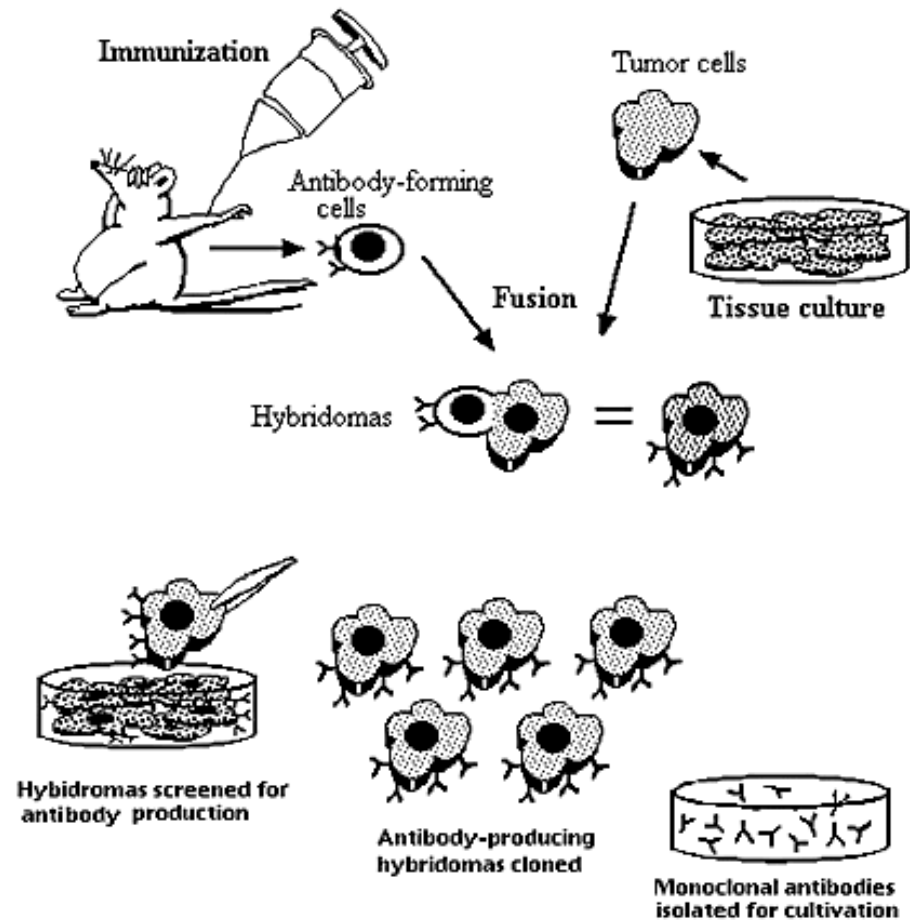
- Tecnologia degli **ibridomi** (1975)
 - G. Kohler e C. Milstein
- Tecnologia **phage display** (1990)
 - G. Smith e G. Winter



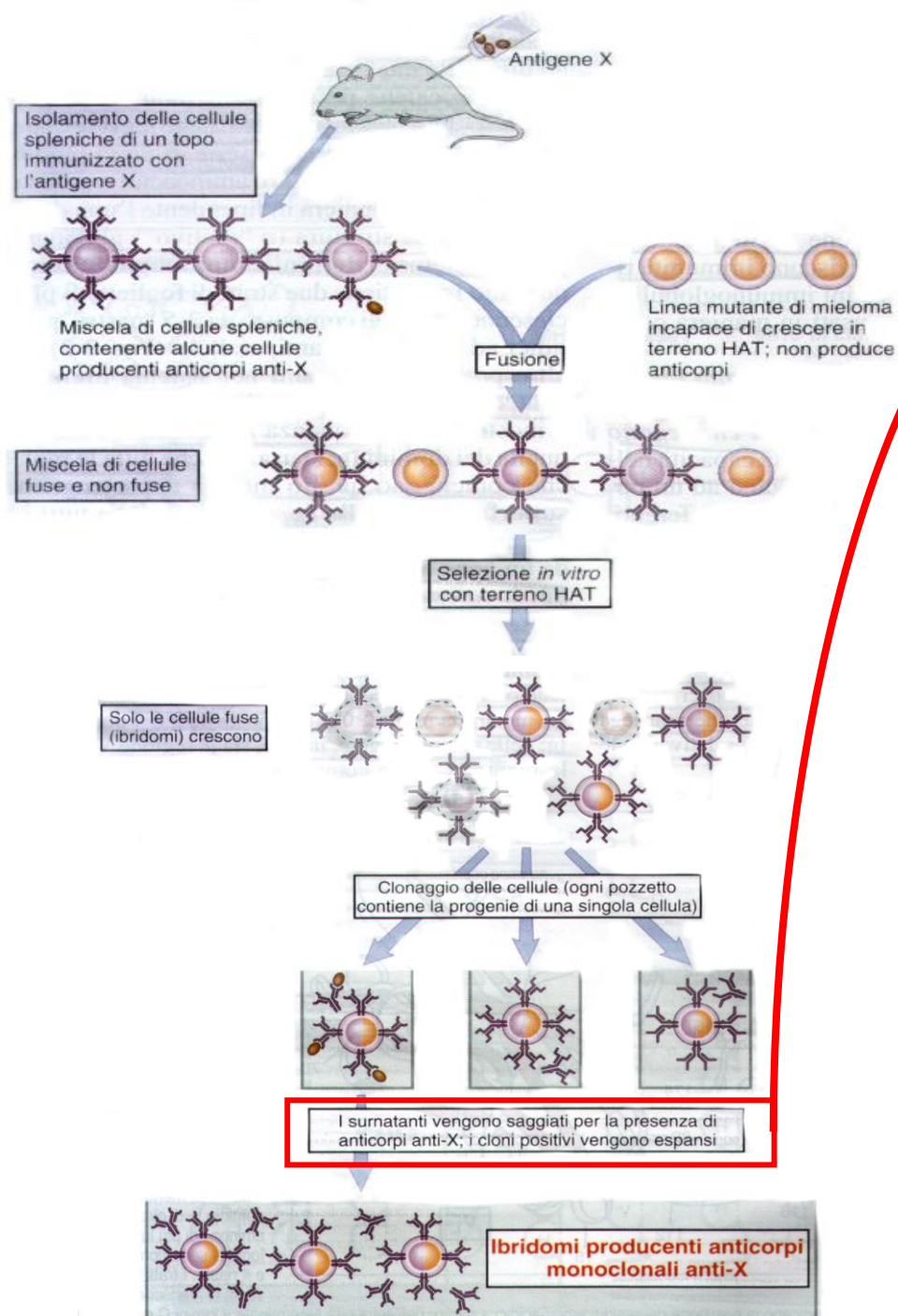
Anticorpi monoclonali prodotti in linee cellulari stabilizzate (**ibridomi**)

- immunizzazione di un topo con l'antigene
- linfociti B che producono anticorpi + cellule di mieloma murino HGPRT⁻ in presenza di un agente di fusione
- crescita su terreno selettivo HAT (hypoxantina, aminopterina, timina)
- l'aminopterina è un antibiotico che inibisce la sintesi *de novo* degli acidi nucleici. Ipoxantina e timina permettono di sopravvivere a cellule HGPRT⁺, che possono usare le vie di recupero

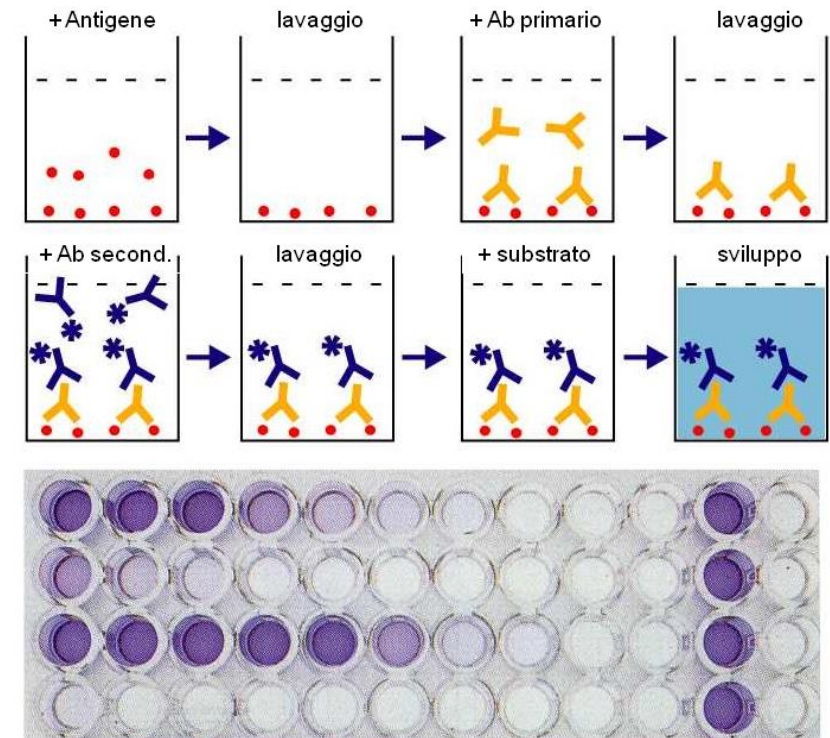
HGPRT è un enzima della via di recupero delle purine.



Monoclonal Antibody Production



Saggio ELISA per identificare mAb con elevata affinità di legame per l'antigene



Alcuni differenti 'formati' di anticorpi

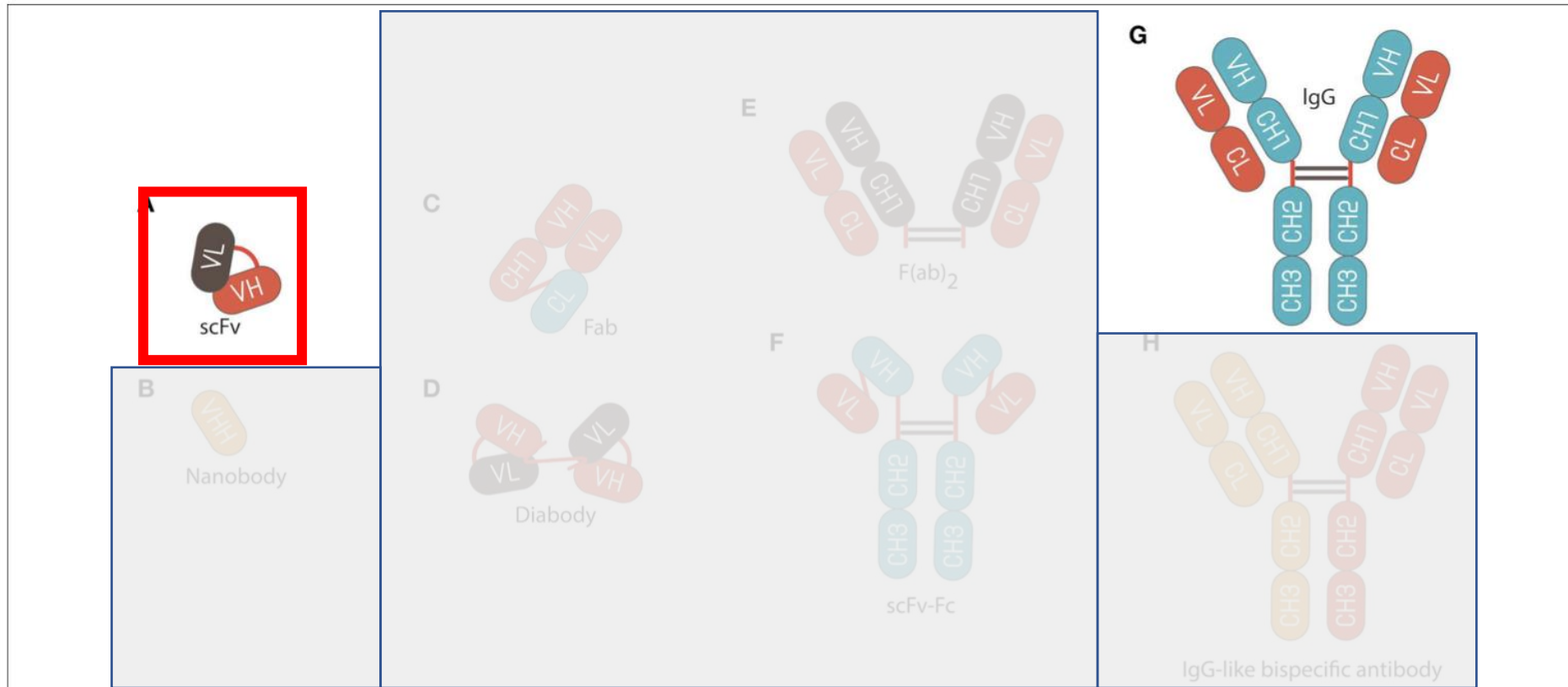


FIGURE 1 | Schematic representation of different antibody formats. **(A)** Single chain fragment variable (scFv) composed of variable regions of the light chain (V_L) linked to variable regions of the heavy chain (V_H) by a flexible glycine-serine linker $(Gly_4Ser)_3$. **(B)** Nanobody fragments. **(C)** Fragment of antigen binding (Fab) composed of V_L and a constant domain of the light chain (CL) linked to V_H and constant domain 1 of the heavy chain (CH1) by a disulphide bond between the CL and CH1 domains. **(D)** Diabody composed of V_L linked to variable heavy V_H by a pentameric (Gly_4Ser) . **(E)** $F(ab)_2$ fragment composed of $2 \times$ Fab fragments joined by an Immunoglobulin G (IgG) hinge region. **(F)** scFv fusion with an Fc IgG. **(G)** IgG composed of constant fragment (Fc), which is able to bind and stimulate immune effector cells, and Fab, which comprises the variable domains that contain the antigen binding regions. **(H)** Bispecific IgG antibody.

Phage display per ottenere anticorpi scFv specifici

- Display di peptidi o proteine sulla superficie di un fago
- Le sequenze codificanti **VH** e **VL** sono fuse alla sequenza di una proteina di superficie del fago
- Il fago filamentoso M13 e la proteina pIII sono i più utilizzati
- Le sequenze **VH** e **VL** degli anticorpi sono facilmente amplificate per RT-PCR con primer fiancheggianti e clonate in vettori per produrre le proteine di fusione con pIII

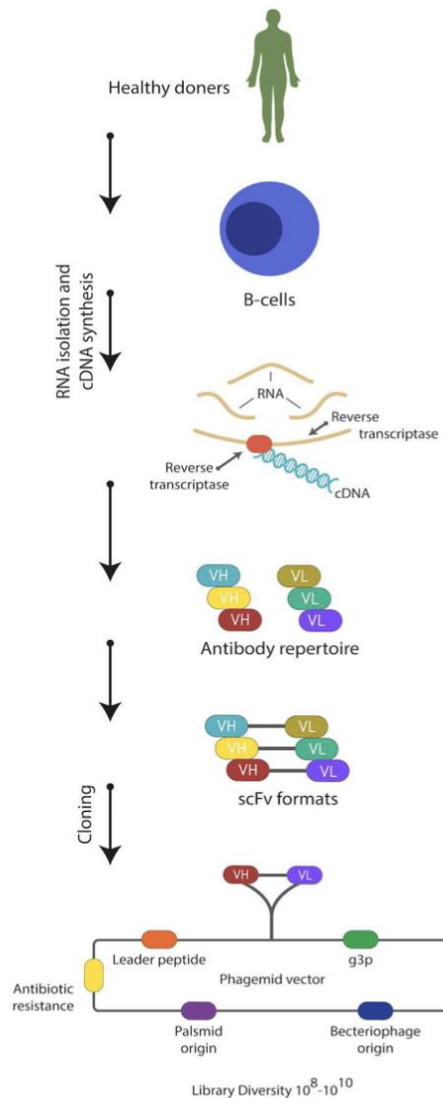
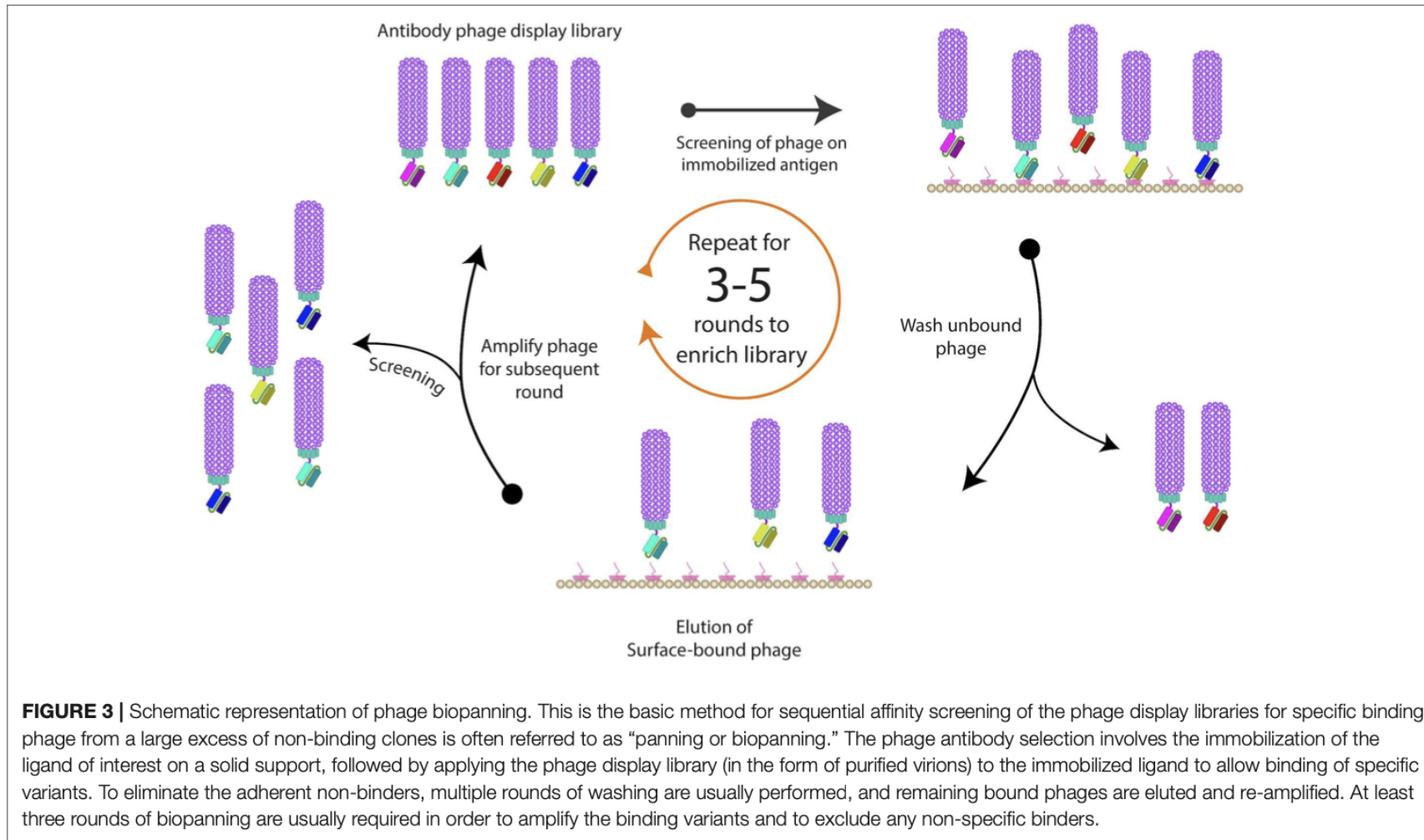


FIGURE 2 | Strategy used for construction of naïve scFv-phage display libraries. Total RNA is isolated from B-lymphocytes from non-immunized healthy donors. Then cDNA is synthesized from the isolated RNA using reverse transcriptase enzyme. Then the repertoire of the V_H and V_L genes is amplified from the cDNA using forward and reverse primers hybridizing to the variable domains. scFvs are constructed and cloned into phagemid vector and a naïve phage library of 10^8 - 10^{10} is usually generated.

scFv: single-chain variable domains

Phage display per ottenere anticorpi scFv specifici: procedura di screening della libreria fagica

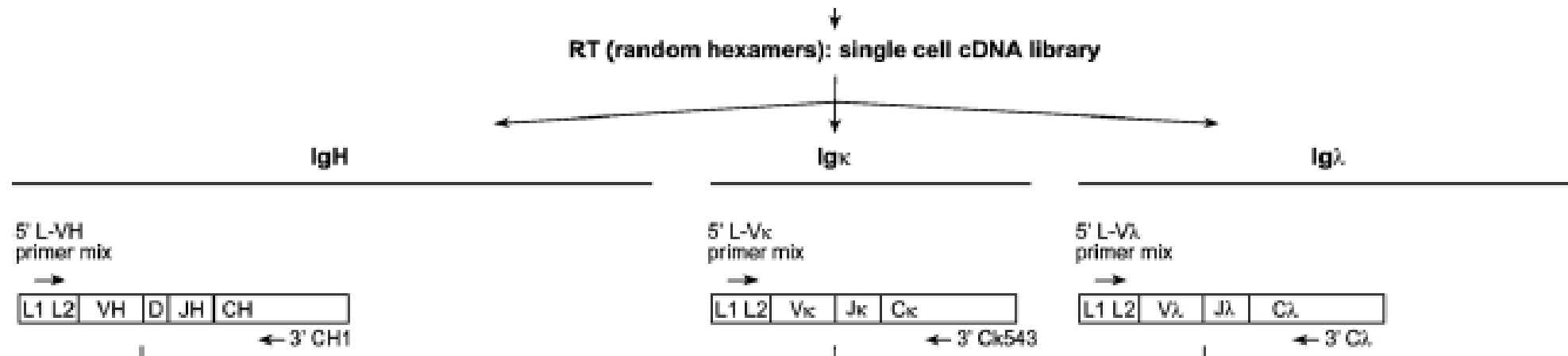


2. Come produrre anticorpi monoclonali ricombinanti?

- Clonaggio della sequenza codificante nel vettore di espressione
- Scelta del 'formato' dell'anticorpo
- Scelta del sistema di espressione
- Purificazione

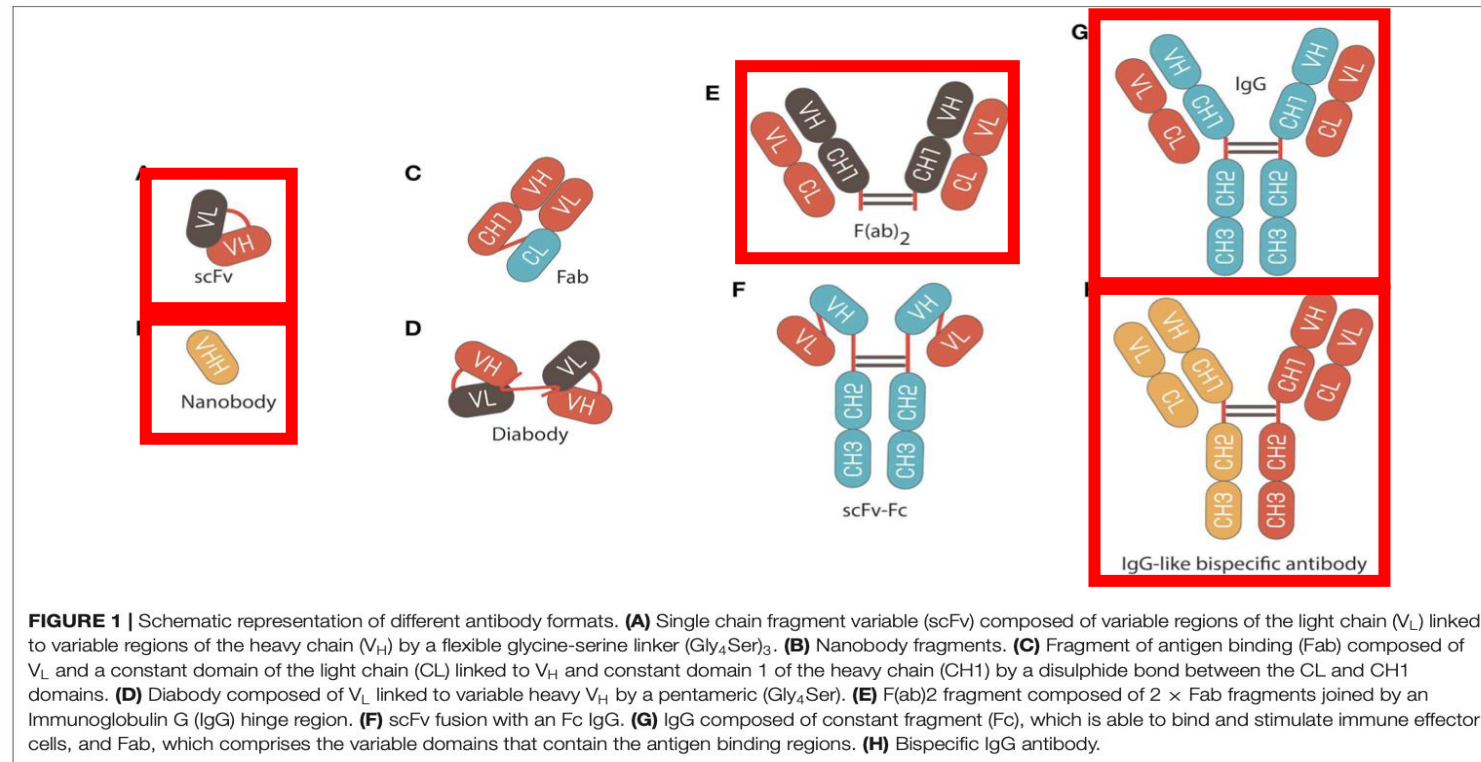
2. Come produrre anticorpi monoclonali ricombinanti?

- Clonaggio della sequenza codificante
 - RT-PCR della sequenza codificante delle catene H e L o di VH e VL, da cloni identificati nello screening di ibridomi o librerie fagiche
 - Potrebbe essere necessario utilizzare miscele di primer



2. Come produrre anticorpi monoclonali ricombinanti?

- Scelta del 'formato' dell'anticorpo - 1
 - intero, scFv, **nanobody**, anticorpi bispecifici



2. Come produrre anticorpi monoclonali ricombinanti?

- Scelta del 'formato' dell'anticorpo - 2
 - mAb murini, chimerici, umanizzati e umani

La scelta del 'formato' dell'anticorpo dipende dall'applicazione

- Applicazioni terapeutiche
- Applicazioni in diagnostica e ricerca

Gli anticorpi monoclonali sono tipicamente murini!

- **Anticorpi chimerici** (80% umani): i domini costanti della molecola murina sono sostituiti con le loro rispettive controparti umane utilizzando le sequenze geniche della catena leggera κ e della catena pesante delle IgG1, le Ig più efficienti ad attivare il complemento e la citotossicità delle cellule effettrici
- **Anticorpi umanizzati** (95% umani): le CDR murine sono inserite in anticorpi umani
- **Anticorpi umani**: topi transgenici in cui i loci genici delle Ig sono sostituiti con quelli umani (HuMabMouse e XenoMouse)
- La nomenclatura degli anticorpi monoclonali indica se sono murini (-omab), chimerici (-ximab), umanizzati (-zumab) o umani (-umab).



2. Come produrre anticorpi monoclonali ricombinanti?

- Sistemi di espressione

- Ibridomi

- Linee cellulari di mammifero: CHO (criceto) e NS0 (topo)

- Lievito: *Pichia pastoris*

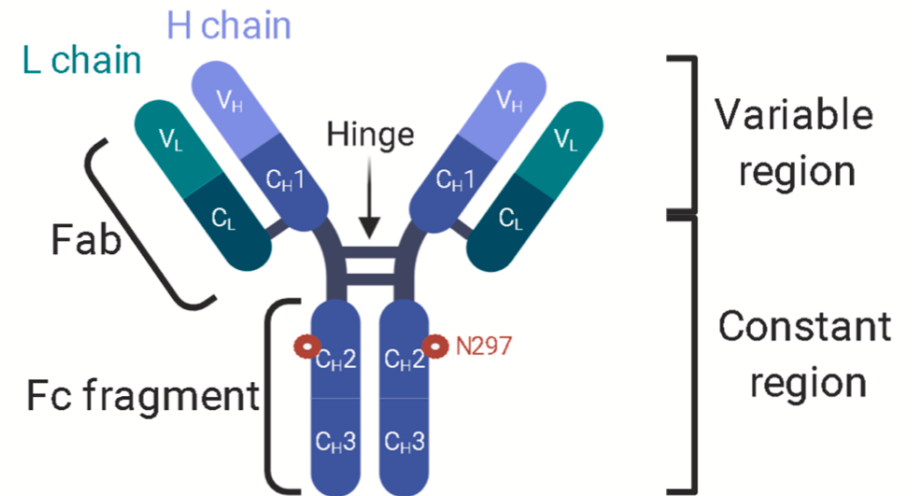
- Batteri: *E. coli*

Gli anticorpi sono glicoproteine

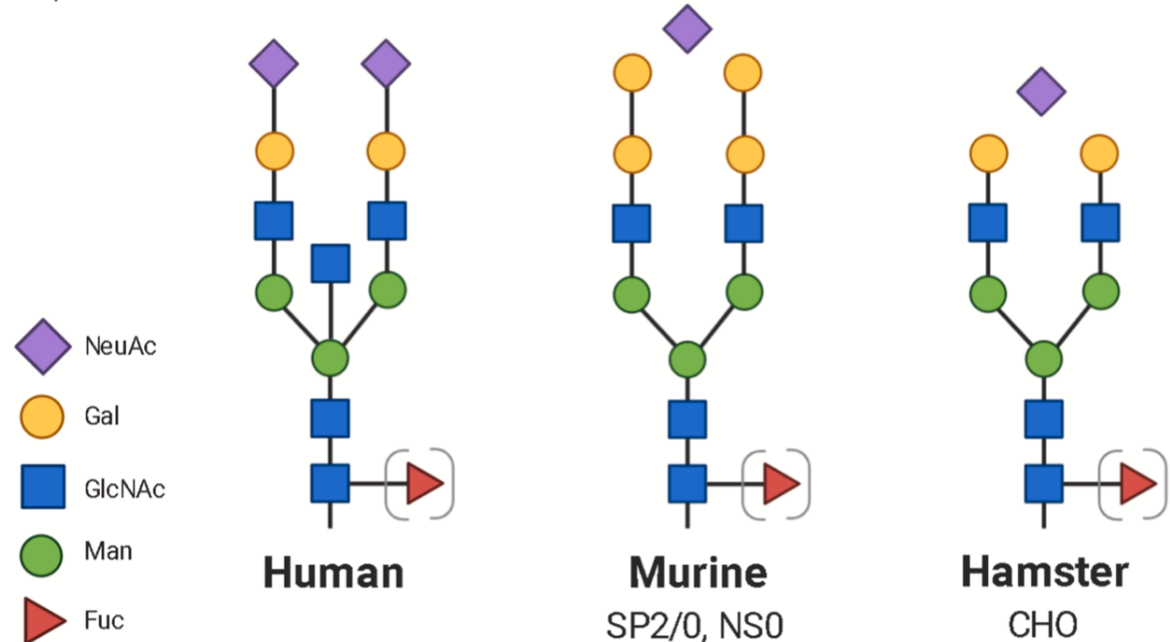
La glicosilazione avviene su Asn297 del dominio C_H2 ed è necessaria per la funzionalità dell'anticorpo.

Le cellule murine e di criceto sintetizzano oligosaccaridi con strutture leggermente differenti da quelle umane.

A)



B)



Sviluppo di linee cellulari: le cellule CHO e NS0

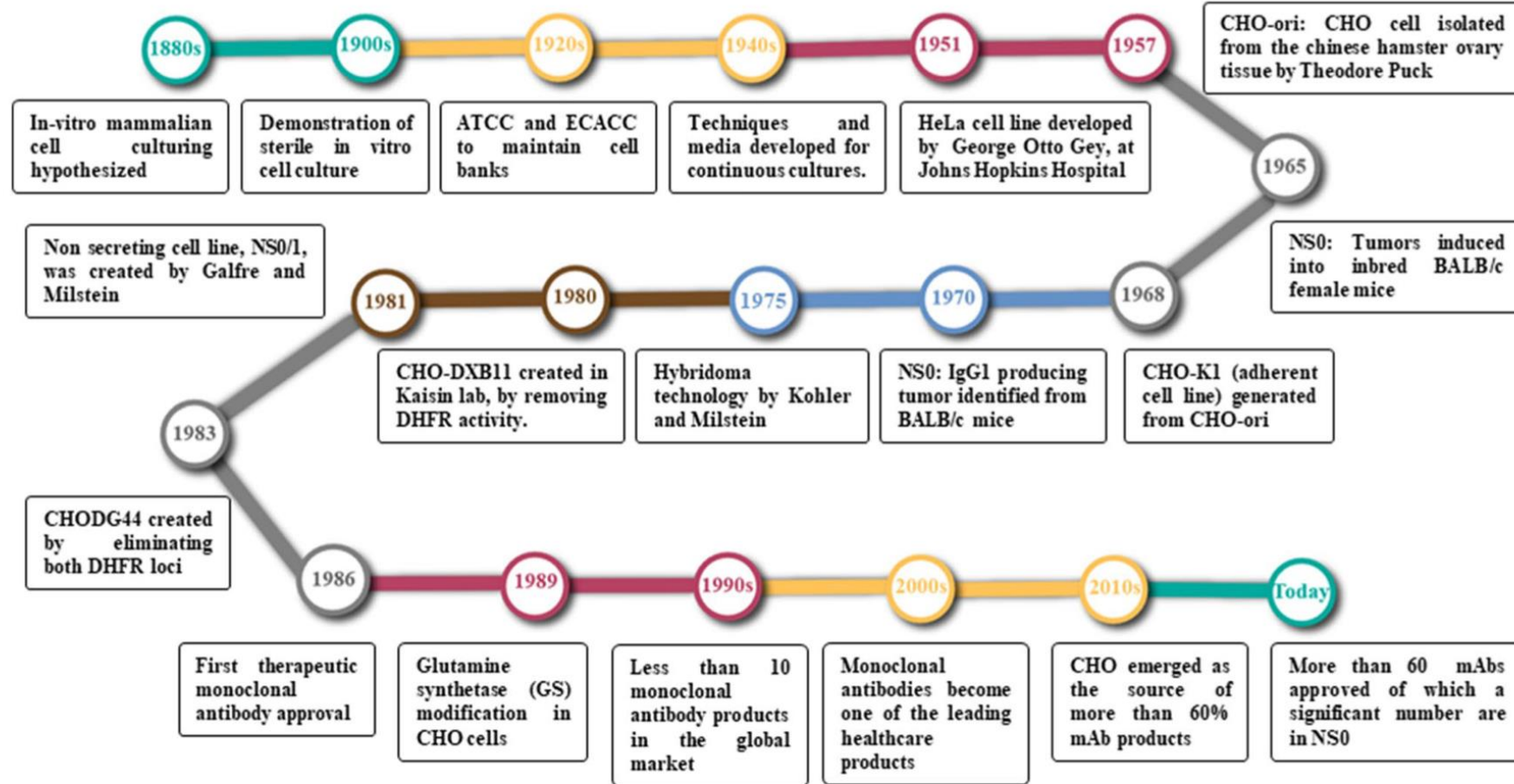


Fig. 2 Timeline for the history of mammalian cell culture for the production of therapeutic monoclonal antibody production [16, 17]

Marcatori di selezione: DHFR e GS

Table 2 Comparison of the dihydrofolate reductase and glutamine synthetase expression platforms [26, 37–40]

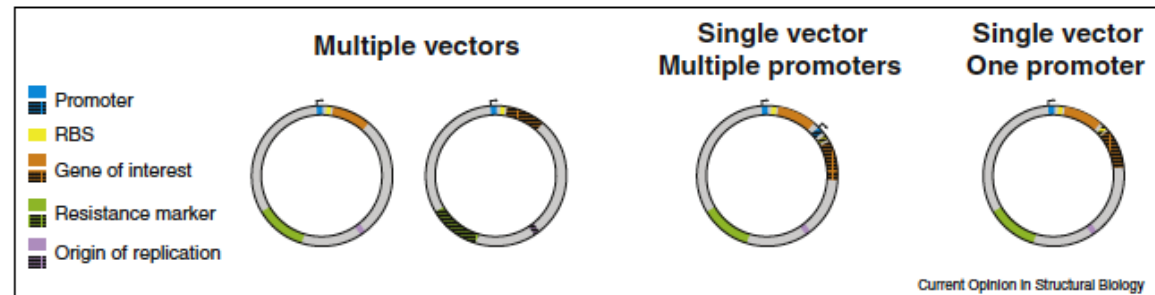
Properties	DHFR system	GS system
Enzyme function	The DHFR enzyme catalyzes the conversion of folate to tetrahydrofolate, which is necessary for de novo synthesis of purines and pyrimidines, as well as glycine	The GS enzyme catalyzes the production of glutamine from glutamate and ammonia
Drug resistance	MTX inhibits DHFR activity, thereby pushing the cells to amplify the <i>DHFR</i> gene, leading to co-amplification of the recombinant protein gene	MSX binds to GS and inhibits the production of glutamine. Cells are then cultured in increasing levels of MSX, resulting in amplification of the gene for the recombinant protein and as well as the <i>GS</i> gene
Host cells commonly used	CHO-DG44 host cells are used for DHFR selection of recombinant cell lines because in DG44 cells, both copies of the <i>DHFR</i> gene are non-functional as compared with DuxB11 cells that have one functional and one non-functional copy of the <i>DHFR</i> gene	CHO-K1 host cells containing the active endogenous <i>GS</i> gene and NS0 cells containing extremely low levels of endogenous GS activity
Amplification	Multiple rounds	Single round
Dominancy	DHFR acts as a non-dominant selection marker	GS acts as a dominant selection marker
Media requirements	Cells use glutamine supplemented in the media	Cells are grown in glutamine-free media since they can produce their own glutamine. This causes lower accumulation of ammonia, which is toxic for cells

CHO Chinese hamster ovary, *DHFR* dihydrofolate reductase, *GS* glutamine synthetase, *MSX* methionine sulfoxamine, *MTX* methotrexate

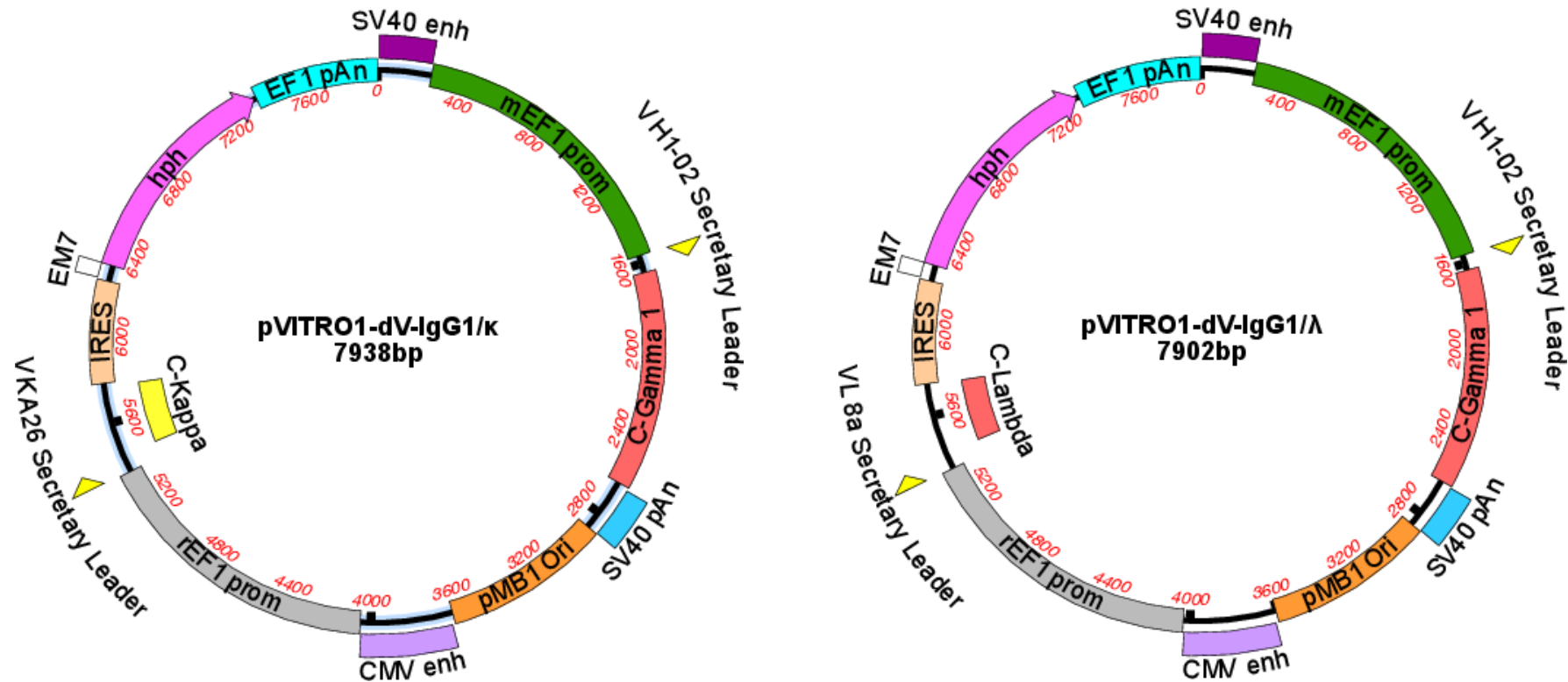
Vettori di espressione

- Plasmidi
 - promotori forti costitutivi (CMV, EF1a)
 - marcatori di selezione: DHFR, GS, resistenza ad antibiotici
- Vettori basati su virus
 - lentivirus

Espressione di catena leggera e catena pesante utilizzando due vettori oppure un solo vettore con due promotori o un promotore solo e una IRES (internal ribosome entry site)



Un esempio di vettori per la co-espressione della catena pesante e leggera delle IgG



I vettori sono privi delle sequenze dei domini variabili **VH** e **VL** che vengono aggiunte con la specificità di riconoscimento per l'antigene scelto

Purificazione mAb:

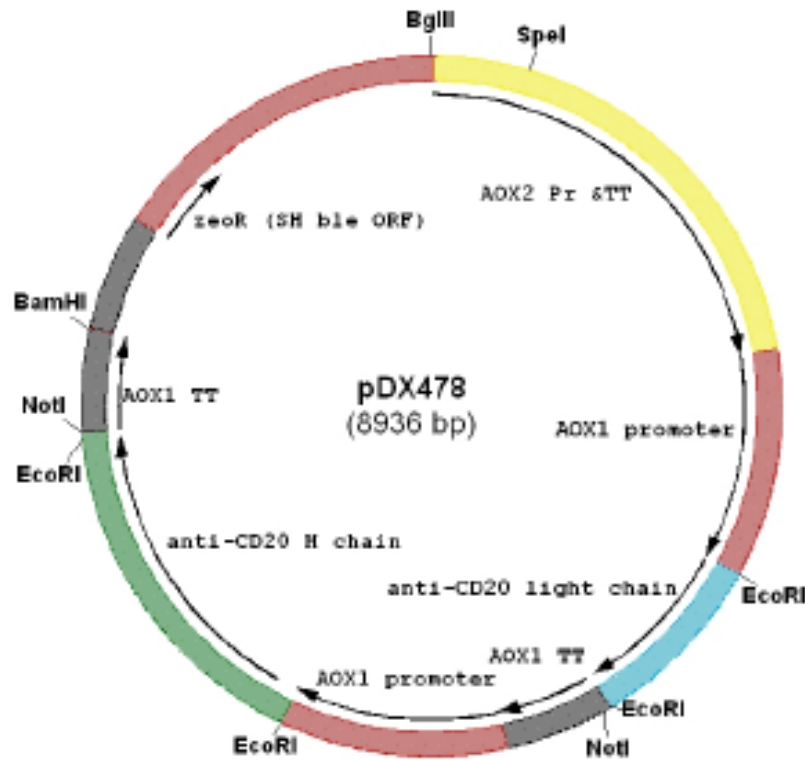
cromatografia di affinità su protein A, protein G, protein L
(proteine batteriche che legano la regione **Fc** degli anticorpi)

Table 2

Binding characteristics of different immunoglobulin-binding proteins.

Species	Antibody Class	Protein A	Protein G	Protein A/G	Protein L [§]
Human	Total IgG	+++	+++	+++	+++
	IgG ₁	+++	+++	+++	+++
	IgG ₂	+++	+++	+++	+++
	IgG ₃	+	+++	+++	+++
	IgG ₄	+++	+++	+++	+++
	IgM	+	—	+	+++
	IgD	—	—	—	+++
	IgE	++	—	++	+++
	IgA	+	—	+	+++
	IgA ₁	+	—	+	+++
	IgA ₂	+	—	+	+++
	Fab	+	+	+	+++
	scFv	+	—	+	+++
Mouse	Total IgG	+++	+++	+++	+++
	IgG ₁	+	++	++	+++
	IgG _{2a}	+++	+++	+++	+++
	IgG _{2b}	+++	+++	+++	+++
	IgG ₃	+++	+++	+++	+++
	IgM	—	—	—	+++

Produzione di anticorpi (IgG) nel lievito *Pichia pastoris*



- Ceppo glicoingegnerizzato per produrre oligosaccaridi del tipo $\text{Man}_5\text{GlcNAc}_2$
- Espressione sotto il controllo del promotore AOX1
- Resa **1.26 g/l** dopo 146 ore di coltura con biomassa circa 400 g/l peso fresco di cellule (8.6 mg/l/ora)

Produzione di anticorpi (IgG) nel lievito *Pichia pastoris*

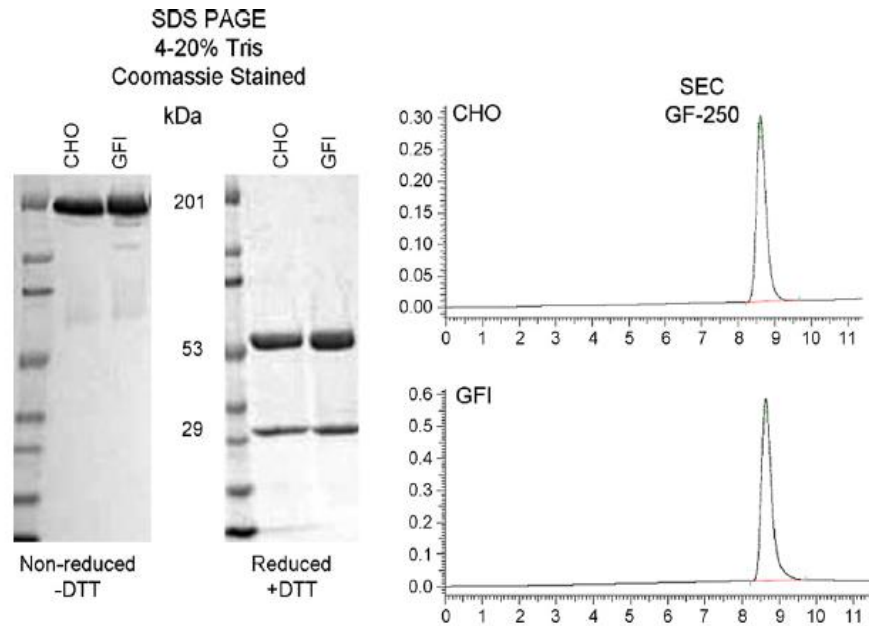


Fig. 6. Coomassie blue stained SDS-PAGE under reduced and non-reduced conditions of IgG1 produced by YGLY4140 (GFI) compared to marketed therapeutic IgG1 produced by CHO cells (CHO). The left lane of each gel is a molecular weight standard from Bio-Rad (Catalog #161-0374, Hercules, CA). The non-reduced condition shows intact antibody while the reduced condition shows the heavy and the light chains. Antibody assembly is compared by the size exclusion chromatograms (SEC) from a GF-250 column.

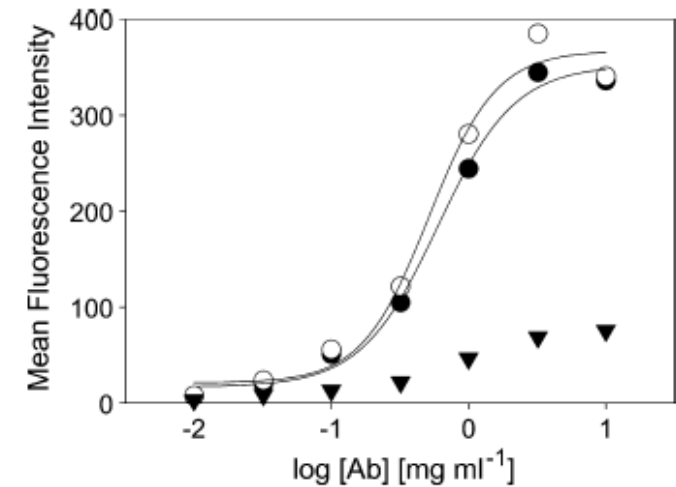
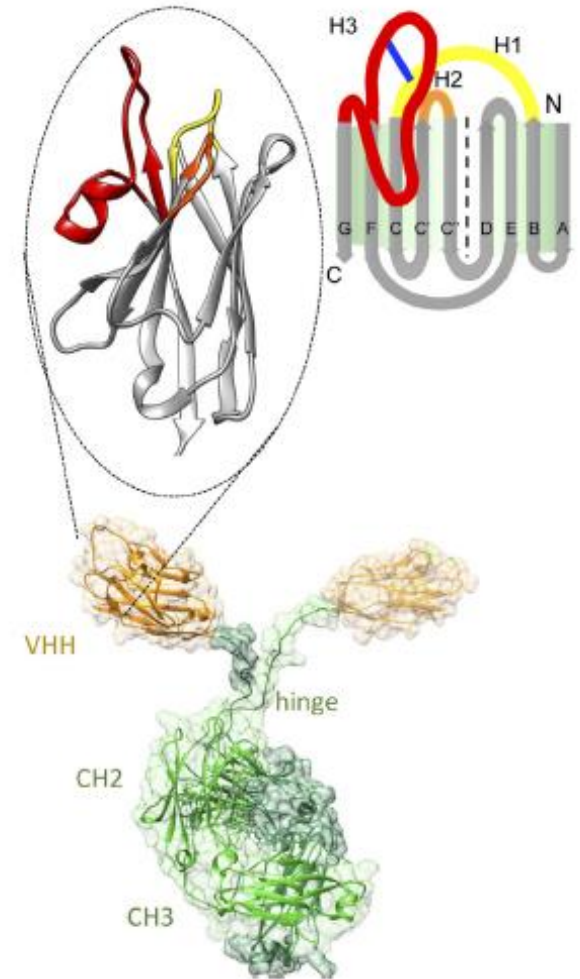


Fig. 7. Comparison of antigen binding affinity of glycoengineered yeast and commercially available CHO cell produced IgG1 by mean fluorescence intensity of antibody binding to target antigen expressed on mammalian cell surface. The filled circles (●) refers to the IgG1 produced by CHO cells, the open circles (○) refers to the glycoengineered yeast strain produced IgG1 while the filled triangles (▲) refers to the IgG1 isotype negative control.

Produzione di nanobodies (VHH) in *E. coli*

- Single-domain (120-140 aa) con un ponte disolfuro, sono derivati da Variable Heavy chain Homodimer IgG di camelidi
- Espressione citoplasmatica
 - Ceppo ottimizzato per la formazione di ponti disolfuro nel citoplasma
- Espressione periplasmatica
 - Sequenza segnale



RESEARCH

Open Access

High-yield production of functional soluble single-domain antibodies in the cytoplasm of *Escherichia coli*

Kristof Zarschler^{1*}, Stefanie Witecy¹, Franz Kapplusch¹, Christian Foerster^{1,2} and Holger Stephan¹

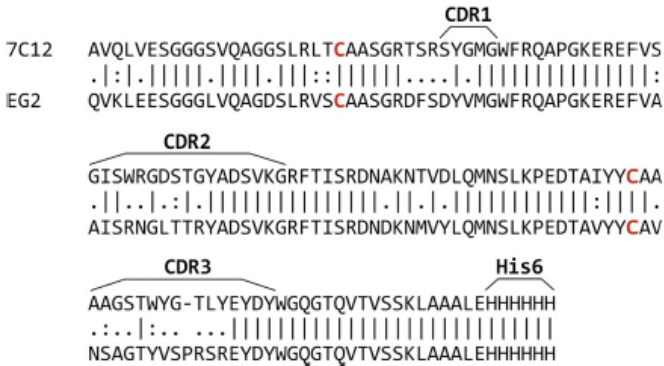


Figure 1 Protein sequence alignment of investigated single-domain antibodies. The amino acid sequences of sdAbs 7C12 and EG2 were aligned using the EMBOSS Pairwise Alignment Algorithms. The two cysteine residues forming an intradomain disulfide bridge are highlighted in red.

Table 1 Growth and pH characteristics of different *E. coli* cultures

Strain	Medium	OD ₆₀₀ at induction	pH at induction	OD ₆₀₀ at harvest	pH at harvest	CDW (g/L) at harvest
SHuffle® T7 Express [pET-28b:7C12]	LB	1.0	7.3	1.4	8.8	0.38
	TB	1.1	7.2	8.3	8.4	2.24
	EnPresso	10.6	6.5	13.1	6.9	3.54
SHuffle® T7 Express [pET-28b:EG2]	LB	1.0	7.3	1.8	8.7	0.49
	TB	1.0	7.1	10.7	8.1	2.89
	EnPresso	10.2	6.6	19.8	7.0	5.35

Samples from each cultivation were taken at the time point of induction and harvest and subsequently, OD₆₀₀ and pH were determined. Cell dry weight (CDW) was calculated at the end of cultivation, whereas one unit of OD₆₀₀ corresponds to a dry cell weight of 0.27 g/L [52].

Nanobody	Medium	Yield (mg/L)
7C12	LB	13
	TB	120
	EnPresso	130
EG2	LB	37
	TB	175
	EnPresso	200

E. coli SHuffle T7 Express
Δgor Δtrx cDsbC
Derivato da BL21(DE3)



Improving the yield of recalcitrant Nanobodies® by simple modifications to the standard protocol

Christopher K. Kariuki ^{a,b,*}, Stefan Magez ^{a,c,d}

lacUV5 promoter: IPTG induction
 pelB leader for expression in the periplasm
 His-tag for purification

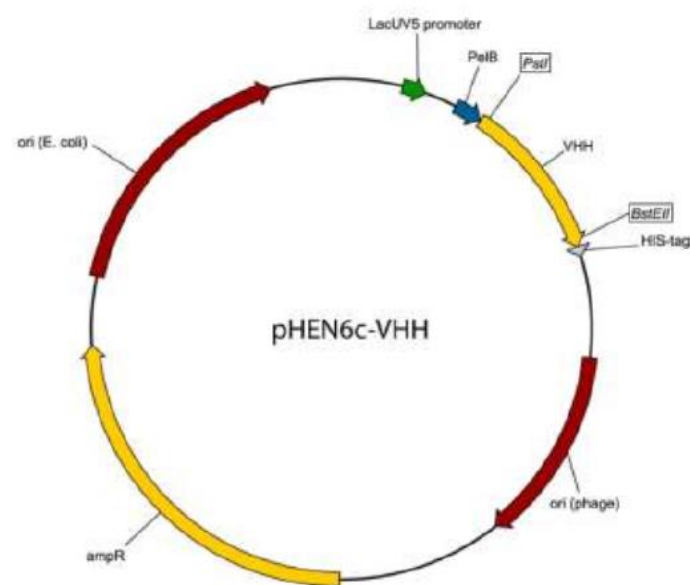


Table 1

Comparison between the standard and modified expression protocols for Nanobody® production.

	Standard Protocol	Modified Protocol
Media used	Terrific Broth	2xTY
Pre-culture temperature	37 °C	30 °C
Pre-culture/Culture ratio	1:300	1:30
IPTG concentration used	1 mM	0.25 mM
Culture temperature after induction	28 °C	37 °C
Culture time after induction	>14 h	Max 4 h
Osmotic shock after TES	TES/4 (4-fold diluted TES)	5 mM MgSO ₄
IMAC column volume (per liter of culture)	1 mL	0.5 mL

Protein	Vector	Yield (mg/liter of culture) after Standard SOP	Yield (mg/liter of culture) after modified SOP	Fold increase
Nb_ 1174	pHEN6c	0.7	4.3	6
Nb_ 1175	pHEN6c	5	5.6	1*
Nb_ 1176	pHEN6c	0.5	1.4	3
*Nb_ 60	pHEN6c	0.25	2.21	9

Utilizzo di anticorpi in terapia

- Campi di applicazione clinica
 - Malattie cardiovascolari
 - Malattie infiammatorie e autoimmuni
 - Immunosoppressione
 - Neoplasie ematologiche ed epiteliali
- Uso in terapia anticancro: proprietà (ideali) dell'antigene bersaglio
 - Espressione stabile e omogenea nelle cellule tumorali, trascurabile in cellule sane
 - Elevata densità di espressione
 - Mancanza di forme circolanti
 - Capacità di attivare i processi di morte cellulare

Table 5

Characteristics (route of administration, mode of delivery, and dose strength) of several therapeutic mAbs approved by the FDA. **Abbreviations:** RoA: route of administration, IV: intravenous, SC: subcutaneous, IVP: intravenous pyelogram, PFS: pre-filled syringe, AI: autoinjector. Information gathered from [MedScape \(2020\)](#) and [Drugs@FDA \(2020\)](#).

INN (Trade Name)	Target	Indication	RoA	Dose strength	Mode of delivery
Erenumab (Alimovig®)	CGRP receptor	Migraines	SC	70 or 140 mg/ml	PFS, SureClick AI
Sarilumab (Kevzara®)	IL-6 receptor	Rheumatoid arthritis	SC	150 mg/1.14 ml, 200 mg/1.14 ml	PFS, pre-filled pen
Emicizumab (Hemlibra®)	Factor IXa / X	Haemophilia A	SC	30 mg/ml, 150 mg/ml	Injection, IV solution vial
Denosumab (Prolia®)	RANK-L	Postmenopausal osteoporosis	SC	Prolia: 60 mg/ml Xgeva: 70 mg/ml	PFS or vial
Adalimumab (Humira®)	TNF- α	Rheumatoid arthritis	SC	40 mg/0.8 ml	PFS, pre-filled pen or vial
Trastuzumab (Herceptin®)	HER2	Breast cancer	IV/SC	IV: 21 mg/ml SC: (120 mg/2000units)/ml	IV: lyophilized powder, vial SC: single-dose vial
Rituximab (Mabthera)	CD20	Non-Hodgkin's lymphoma	IV/SC	IV:10 mg/ml (10 and 50 ml vials) SC: (120 mg/2000units)/ml	IV: prepare and dilute in IV bag SC: Single-dose vial
Teprotumumab (Tepezza®)	IGF-R1	Thyroid eye disease	IV	500 mg/single-dose vial	Lyophilized powder
Belimumab (Benlysta®)	BLyS	Systemic lupus erythematosus	IV/SC	IV: 120 or 400 mg/vial SC: 200 mg/ml	IV: lyophilized powder SC: PFS or AI
Palivizumab (Synagis®)	RSV	Respiratory syncytial virus	IM	100 mg/ml	Powder for reconstitution
Muromonab (OKT3) (<i>discontinued</i>)	CD3	Transplantation rejection	IVP	1 mg/ml	Injectable solution
Gemtuzumab (Mylotarg®) (<i>discontinued</i>)	CD33	Acute myeloid leukaemia (AML)	IV	4.5 mg/ml	Single-dose vial

Anticorpi: come uccidono le cellule (tumorali)

Azione diretta

- **Citotossicità complemento-mediata**
proteine del complemento legano la **Fc** dell'anticorpo che si è legato alla cellula bersaglio e la cellula va incontro a lisi
- **Citotossicità cellulare anticorpo-dipendente**
la **Fc** dell'anticorpo legato alla cellula bersaglio è riconosciuta da recettori su cellule effettrici ed avviene lisi cellulo-mediata o fagocitosi della cellula tumorale
- **Apoptosi**

Agisce in questo modo **Rituximab**

Azione indiretta

- **Inibizione della crescita**
- **Alterazioni del ciclo cellulare**

L'anticorpo si lega al recettore per un fattore di crescita sulla cellula tumorale in modo da bloccare il legame del ligando endogeno. La trasduzione del segnale è inibita.

Sfrutta questo meccanismo **Trastuzumab**

Rituximab

Anticorpo IgG1 *chimerico* diretto contro **CD20**. E' stato il primo anticorpo monoclonale ad avere successo nella terapia per il linfoma non-Hodgkin e ad ottenere la licenza dalla FDA nel 1997.

CD20 è una proteina transmembrana con le seguenti proprietà:

- è espressa a livelli elevati sul 95% delle cellule B affette da linfoma, ma non su altre cellule del corpo
- è espressa sui linfociti B sani ma non sui loro precursori immaturi (permettendo la ricostituzione di un compartimento di cellule B dopo la terapia) e sulle plasmacellule (permettendo una produzione continua di immunoglobuline)
- dopo il legame con l'anticorpo non va in circolo nel plasma e non viene internalizzata
- è essenziale per il differenziamento e la proliferazione dei linfociti B

L'attivazione del complemento è fondamentale nell'efficacia dell'anticorpo ma è molto importante anche l'induzione dell'apoptosi mediante influsso di calcio.

STRUCTURAL BIOLOGY

Structure of CD20 in complex with the therapeutic monoclonal antibody rituximab

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cryoEM: CD20 + RTX Fab

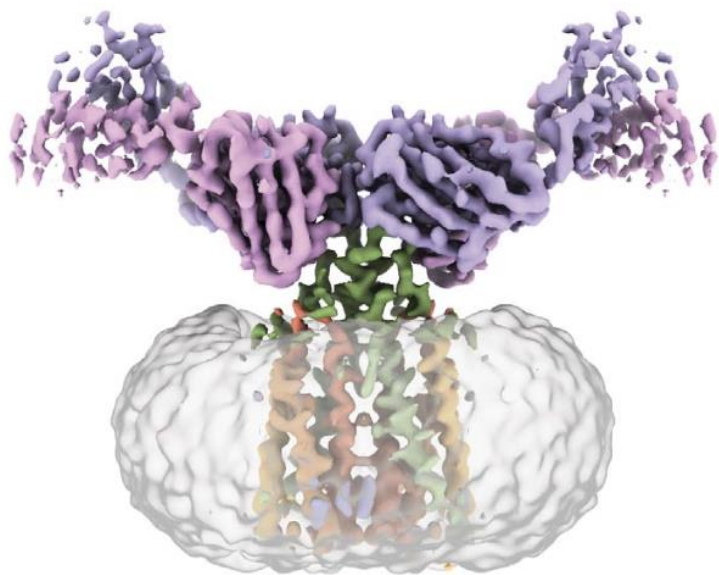


Fig. 3. Key molecular interactions between CD20 and RTX. Ribbon diagrams of the CD20:RTX Fab structure, with key amino acid side chains involved in CD20:RTX or RTX:RTX shown in stick representation. In the center diagram, the gray bars indicate the boundaries of the membrane region. (A) Top view of the center of the complex, where HC.Tyr⁹⁷ mediates Fab:Fab and Fab:CD20' contacts. (B) The canonical RTX epitope ¹⁷⁰ANPSE¹⁷⁴, in addition to being recognized by RTX's heavy chain (left, purple), is also involved in a hydrogen-bond network with Tyr⁹⁷ from the distal RTX Fab (right, gray). (C) Additional Fab:Fab contacts between heavy-chain loops H3 and H1 and light-chain loop L2. (D) A secondary epitope consisting of ECL1 and ECL2 is contacted by RTX's LC loop 1.

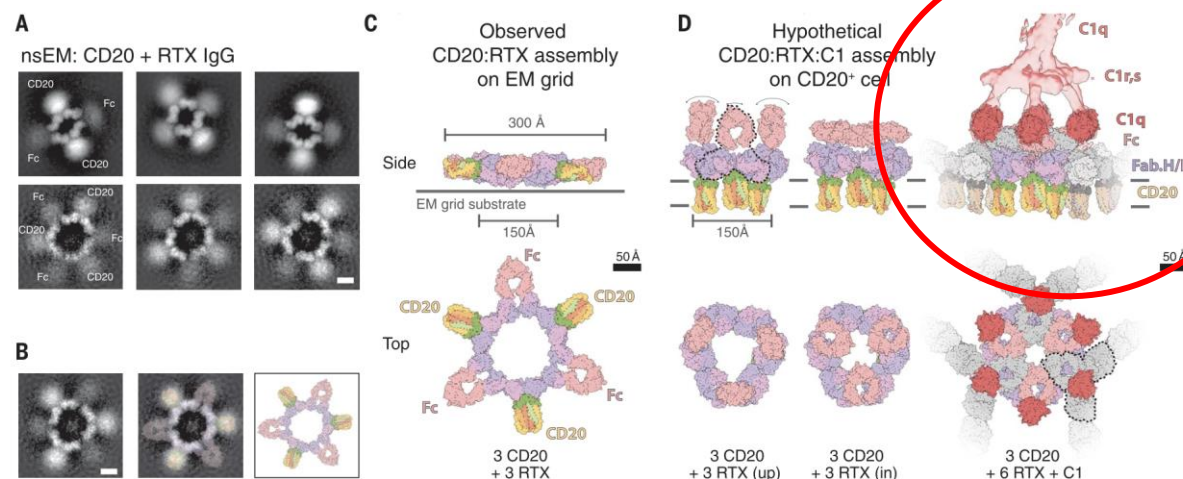
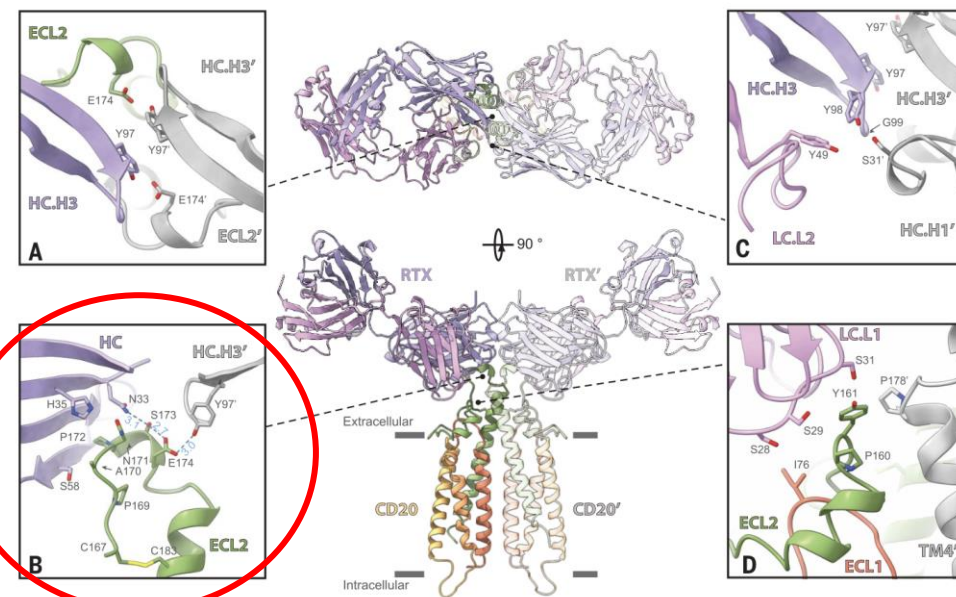


Fig. 5. RTX cross-links CD20 into circular superassemblies. (A) Average nsEM images of CD20 incubated with full-length RTX show cyclical higher-order structures of involving 2-to-2 (top row; diameter of 250 Å) or 3-to-3 (bottom row; diameter of 300 Å) CD20-to-RTX complexes. The RTX Fc domains appear disordered, presumably because of IgG hinge flexibility. Scale bar, 50 Å. (B and C) Interpretation of an nsEM class average of a 3-to-3 assembly. Scale bar in (B), 50 Å. (D) Proposed model for CD20:RTX supercomplex formation and complement recruitment. During nsEM experiments, the IgGs and solubilized CD20s are coplanar (C). Modeling these higher-order assemblies as they might

occur at the surface of CD20⁺ cells requires rotating the CD20:Fab complexes 90° [(D), left]. Given the flexibility provided by the IgG hinges, it is then possible to position Fc domains (pink) in a common plane [(D), middle]. The addition of three further Fc domains possibly contributed by neighboring CD20:IgG assemblies (gray) would complete the Fc hexamer formation and enable recruitment of C1q [(D), right]. Dashed lines outline IgG molecules. The following models were used: structure from present work (RTX Fab:CD20 complex), EMDB-4232 (EM map of C1q:Fc complex), and Protein Data Bank (PDB) 6FCZ (Fc domains and C1q head domains) (25).

Trastuzumab (Herceptin)

Anticorpo IgG1 *umanizzato* diretto contro **HER2**

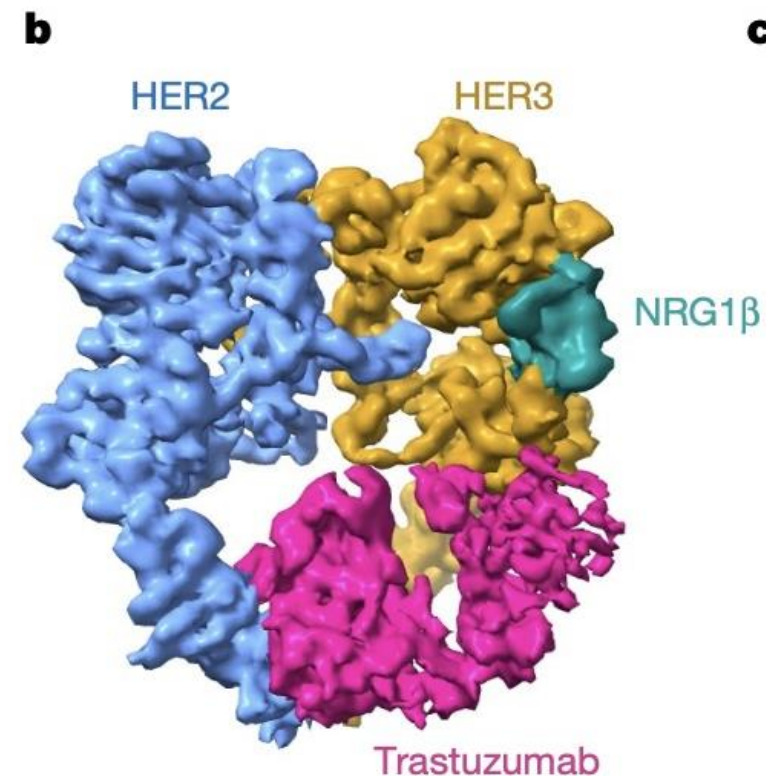
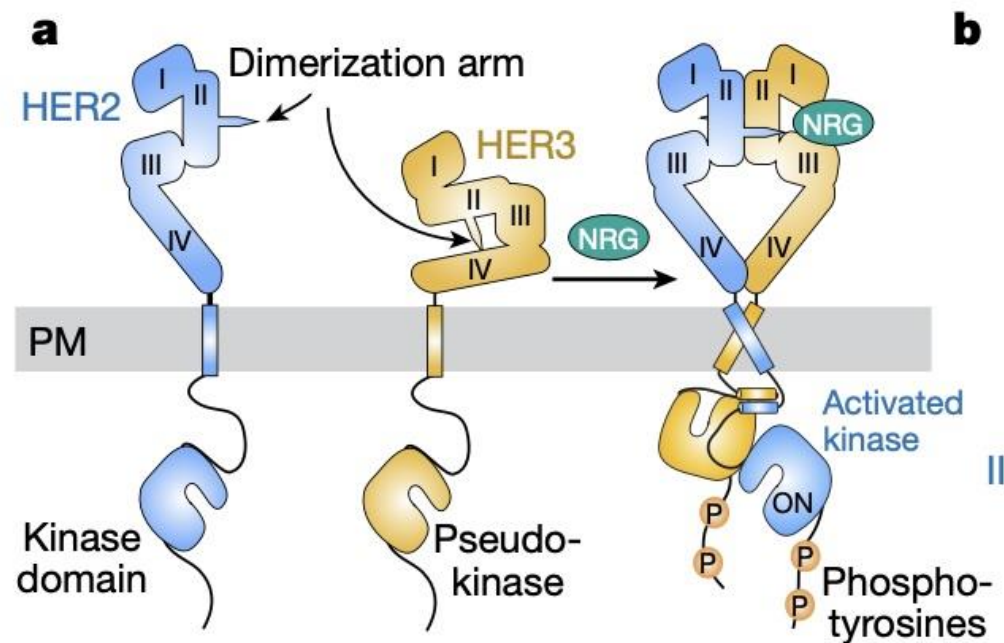
- HER2 è un proto-oncogene che codifica un recettore tirosin-chinasico della famiglia EGFR, il ligando fisiologico di HER2 non è noto
- Il recettore HER2 è una proteina transmembrana che si trova su numerosi tessuti epiteliali e che viene iper-espressa nel **20-30%** dei casi di tumore al seno (>2 milioni di copie contro 20000-50000 nelle cellule epiteliali normali). L'iper-espressione di solito è dovuta ad una amplificazione del gene
- Trastuzumab lega con alta affinità HER2 ed induce l'internalizzazione del recettore e quindi il blocco della trasduzione del segnale
- I tumori con iper-espressione di HER2 vengono identificati o mediante analisi immunoistochimica (positività a HER2-3) o con la FISH (evidenzia la presenza di amplificazione genica)
- È stato il primo anticorpo monoclonale contro tumori solidi ad essere approvato dalla FDA. E' riconosciuto come agente singolo nella terapia di seconda linea nei tumori al seno HER2⁺ metastatici

Structures of the HER2–HER3–NRG1 β complex reveal a dynamic dimer interface

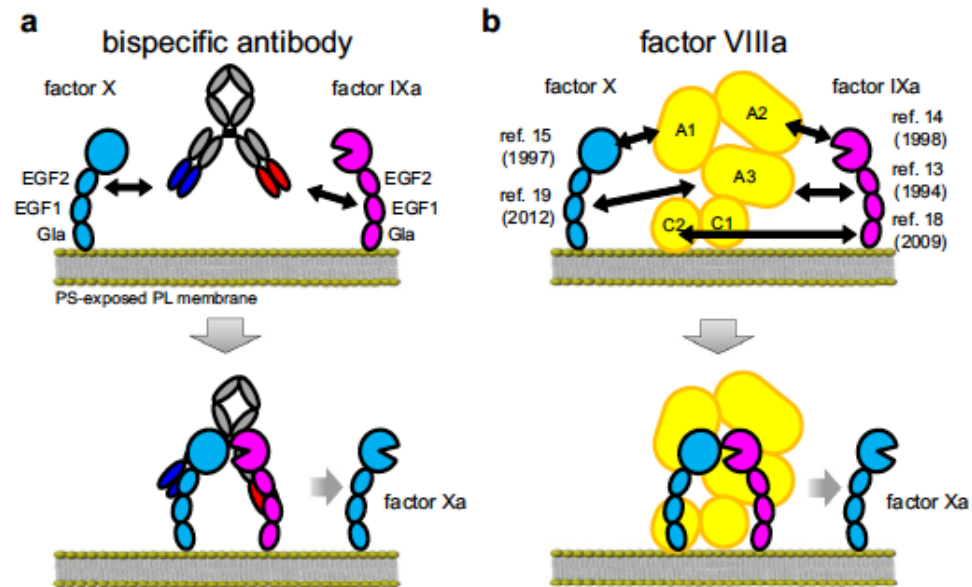
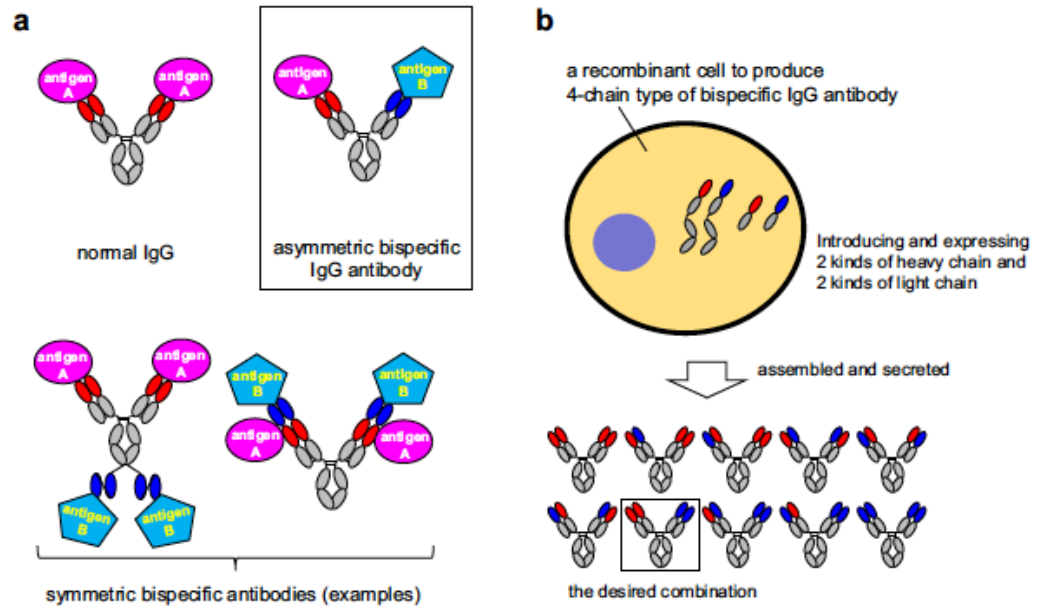
<https://doi.org/10.1038/s41586-021-04084-z>

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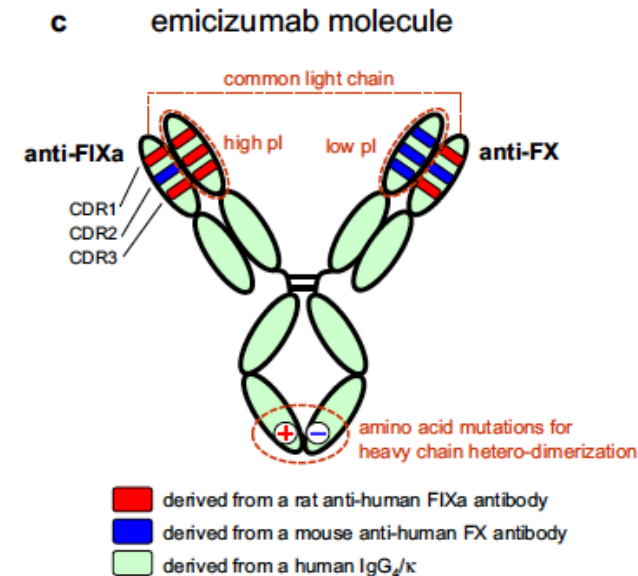
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Emicizumab: un anticorpo **bispecifico** per il trattamento dell'emofilia



Anticorpo bispecifico per FIXa e FX
mima il ruolo di FVIIIa



Rapid Generation of Stable Cell Lines Expressing High Levels of Erythropoietin, Factor VIII, and an Antihuman CD20 Antibody Using Lentiviral Vectors

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H. Trent Spencer,² Andre Roy,¹ Hatem Zayed,¹ and Boro Dropulic¹

Abstract

Lentiviral vectors (LVs) are widely recognized as the most efficient method for the stable delivery of nucleic acid sequences into mammalian cells. Using erythropoietin (EPO), recombinant factor VIII (fVIII), and an anti-CD20 antibody as model proteins, we demonstrate advantages of LV-based gene delivery to achieve high production levels by transduced cells. Highly productive cell clones were able to incorporate up to 100 vector copies per cellular genome, without selection or gene amplification, and were isolated without extensive screening of a large number of clones. The LV transgenes were shown to be distributed throughout the genome, as visualized by fluorescent *in situ* hybridization. High-expressing clones producing 100–200 pg/cell/day of EPO were isolated and characterized. EPO production was demonstrated for at least 5½ months of continuous culture without selection, during which all the clones displayed high levels of glycosylation despite production levels at 10–20 g/liter. To demonstrate the utility of LV technology for multiple classes of proteins, cell lines producing fVIII and an anti-CD20 antibody were also developed. Cell clones demonstrating high levels of fVIII (100 clot units/ml and anti-CD20 antibody as high as 40–100 pg/cell/day) were isolated and characterized. LV-transduced cells and plasmid-transfected cells were compared for protein production per transgene copy. LV-transduced cells produced significantly higher levels of protein per copy of transgene than plasmid-transfected cells did. This study demonstrates the utility of LV technology for rapid generation of highly productive and stable cell lines over conventional plasmid transfection methods, significantly decreasing the time, cost, and risk of the manufacture of proteins and other complex biological molecules.

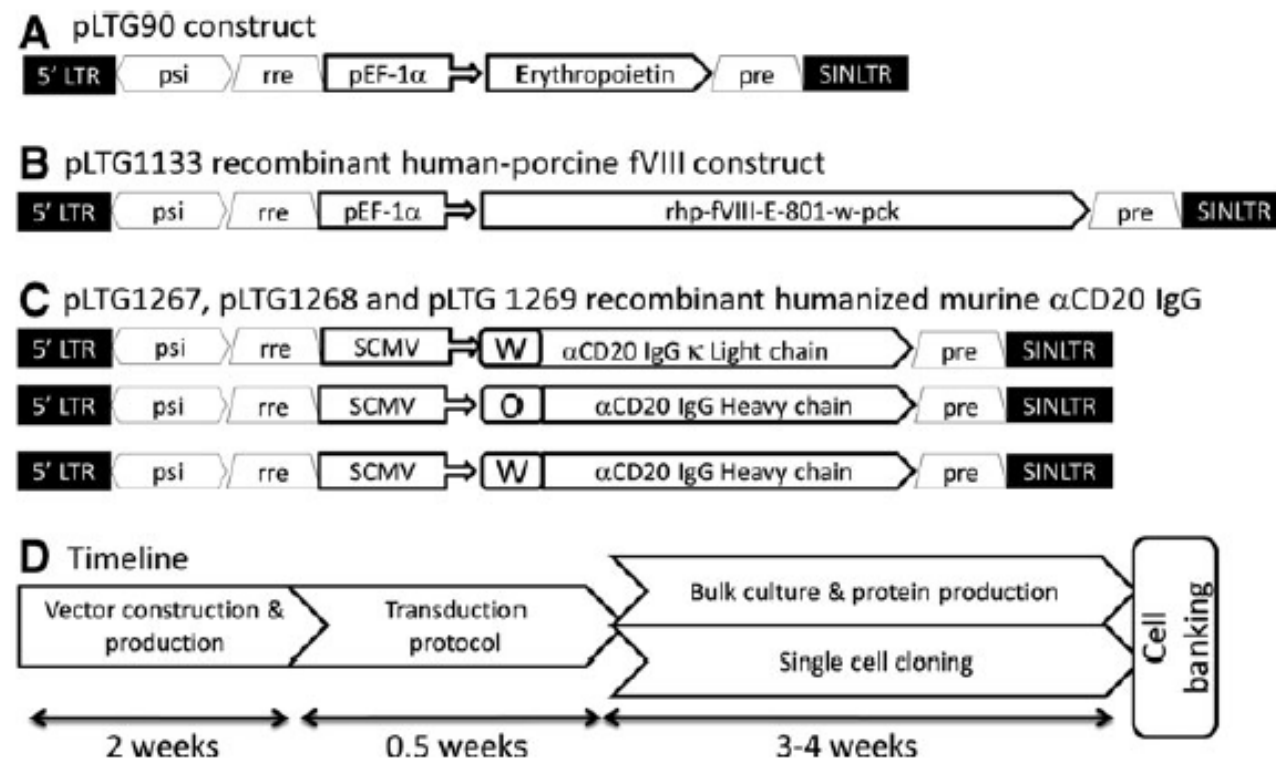


FIG. 1. Construction of lentiviral vectors and cell line development. **(A)** The erythropoietin (UniProtKB/Swiss-Prot entry P01588) encoding gene was amplified using the TC12534 as a template vector (Origene): forward primer, GATCATGCGGC GCGCCACCATGGGGGTGCACGAATGTCCT; reverse primer, CAGCTATGACCG CGGCCGCAACTAGAGTCGAGCCT. Polymerase chain reaction products were cut with *AscI*/*NotI* and gel-purified. The purified product was directionally cloned into a Self-Inactivating lentiviral expression vector (Lentigen) in frame with elongation factor-1 alpha promoter. **(B)** The recombinant human-porcine FVIII (*rhpFVIII*) gene was cloned into lentiviral backbone along with human elongation factor 1 promoter, without additional antibiotic selection marker to use for cell line development. **(C)** Three vectors were developed for the recombinant anti CD20 humanized murine immunoglobulin production: one vector encoding the light chain and the Simian Cito-megalo Virus promoter; and two constructs for the gamma heavy chain containing either a wild-type (W) murine leader peptide (MRAPA-QIFGFLLLFPGTRCDI) or an (O) encoded an optimized leader peptide (MRAPAQIFGFLLLFPGTCFA). **(D)** Timeline for vector construction, production, cell transduction, and cloning. Vector cloning and production is performed over a 2-week period. The cells are then transduced multiple times for 3–4 days, after which the cells are either grown in bulk or undergone limiting dilution and single-cell cloning. Single-cell clones were isolated and banked using standard procedures.