

单框架全合成纳米抗体文库的构建与鉴定

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罗颖, 李燕萍, 何庆华, 涂追. 单框架全合成纳米抗体文库的构建与鉴定[J]. 生物工程学报, 2025, 41(4): 1500-1514.

LUO Ying, LI Yanping, HE Qinghua, TU Zhui. Construction and characterization of single-framework fully synthetic nanobody libraries[J]. Chinese Journal of Biotechnology, 2025, 41(4): 1500-1514.

摘 要: 本研究旨在提供一种非动物源纳米抗体文库设计、合成及筛选方案, 探索框架区的选择及互补决定区(complementarity determining region, CDR)的设计对于合成纳米抗体文库的影响, 为增加合成纳米抗体文库的有效性、多样性及实用性奠定理论和技术基础。本研究基于天然纳米抗体的高通量测序结果, 确定了 1 个新的框架(IGHV3S65*01-IGHJ4*01), 依据 CDR 中每个位置氨基酸的出现频率, 分别设计简并引物, 通过重叠延伸 PCR (overlap PCR)合成纳米抗体编码片段。经过 40 次电转化, 获得了包含 6×10^9 个克隆的全合成纳米抗体文库(single-frame synthesized nanobody library, SS-Library), 经辅助噬菌体 M13K07 救援后, 展示文库滴度为 10^{13} PFU/mL。随机挑取 48 个单菌落进行菌落 PCR 验证, 插入率达 95.8%, Sanger 测序结果显示 38 个克隆序列完整, 均未见半胱氨酸以及终止密码子, 且未出现完全相同的序列, 提示文库多样性良好。以牛血清白蛋白(bovine serum albumin, BSA)、乙酰胆碱酯酶(acetylcholinesterase, AchE)、小鼠 IgG (immunoglobulin G, IgG)为靶标, 对文库进行筛选验证, 分别获得了 2 种针对 BSA、10 种针对 AchE 以及 15 种针对 IgG 的纳米抗体。每种抗原分别挑取了 1 个阳性克隆进行重组表达, 结果表明 3 种纳米抗体均为可溶性表达。采用间接酶联免疫吸附实验(enzyme-linked immunosorbent assay, ELISA)和生物膜干涉技术(bio-layer interferometry, BLI)对重组表达纳米抗体结合活性进行了测定, 结果表明, 抗 AchE 和 IgG 的纳米抗体可以特异性结合相应抗原, 亲和力常数分别为 294 nmol/L 和 250 nmol/L。本研究提出的纳米抗体合成文库制备方法简单易行、偏好性低, 有望成为一种通用的纳米抗体发现平台, 用于先导特异性纳米抗体的制备与开发。

资助项目: 国家自然科学基金(32260248, 31860260, 62362050); 江西省双千计划(jxsq2023201029); 江西省自然科学基金(20212BAB205035, 2023ACB205027)

This work was supported by the National Natural Science Foundation of China (32260248, 31860260, 62362050), the Jiangxi Double Thousand Plan (jxsq2023201029), and the Natural Science Foundation of Jiangxi Province (20212BAB205035, 2023ACB205027).

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Received: 2024-11-30; Accepted: 2025-01-24; Published online: 2025-01-26

关键词: 纳米抗体; 全合成文库; 噬菌体展示; 抗体设计; 设计与构建

Construction and characterization of single-framework fully synthetic nanobody libraries

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Abstract: This study is designed to address the development, synthesis, and screening of non-animal-derived nanoantibody libraries. Furthermore, it seeks to elucidate the impact of framework region selection and complementarity-determining region (CDR) design on the characteristics of synthesized nanoantibody libraries. These investigations aim to establish a robust theoretical and technical foundation for enhancing the efficacy, diversity, and practical applicability of synthetic nanoantibody libraries. In this study, a new framework (IGHV3S65*01-IGHJ4*01) was identified based on the high-throughput sequencing results of natural nanobodies, and degenerate primers were designed based on the frequency of amino acids at each position in the complementarity-determining region (CDR) region to synthesize the coding fragments of nanobodies by overlap PCR. After 40 times of electro-transformation, a single-frame synthesized nanobody library (SS-Library) containing 6×10^9 clones was obtained, and the titer of the library was demonstrated to be 10^{13} PFU/mL after rescue by the helper phage M13K07. Random 48 single colonies were picked for PCR, which revealed an insertion rate of 95.8%. Sanger sequencing results showed that 38 clones had complete sequences, none of which showed cysteines or stop codons, and no identical sequences appeared, suggesting that the library had higher diversity. The library was screened and validated with three antigens, including bovine serum albumin (BSA), acetylcholinesterase (AChE), and immunoglobulin G (IgG). Finally, 2 nanobodies against BSA, 10 against AChE, and 15 against IgG were obtained. One positive clone of each antigen was singled out for recombinant expression, and the results showed that all the three nanobodies were expressed in a soluble form. The binding activity of recombinantly expressed nanobodies was evaluated using indirect enzyme-linked immunosorbent assay (ELISA) and bio-layer interferometry (BLI). The results demonstrated that the anti-AChE and anti-IgG nanobodies exhibited specific binding to their respective antigens, with affinity constants (KD) of 294 nmol/L and 250 nmol/L, respectively. The nanobody synthetic library preparation method proposed in this study is simple and easy to use with low preference, and it is expected to be a universal nanobody discovery platform for the preparation and development of lead specific nanobodies.

Keywords: nanobodies; fully synthesized libraries; phage display; antibody design; design and construction

纳米抗体(nanobody, Nb)是1993年Hamers-Casterman等^[1]首次在骆驼科动物的血清中发现的一种全新的抗体,是迄今为止所报道的具有完整功能且稳定的可识别靶标抗原的最小抗体分子^[2-4]。与传统抗体相比,其具有低免疫原性^[5]、高稳定性^[6-7]、较强的组织穿透性^[8]、高亲和力^[9]、易于表达^[10-11]、人源化简单^[12]、易于改造^[13-14]等优势。基于上述特性,纳米抗体在生物成像^[15-16]、诊断学^[17]、免疫分析^[18-19]以及生物制药^[20-22]等方面得到了广泛应用。在过去的几十年内,通常是通过各种抗体展示库来发现纳米抗体^[23]。虽然近些年单细胞分选^[24]以及高通量测序^[25]等新技术被用于抗体发现,但目前噬菌体展示文库技术仍然是广泛使用的方法。

依据抗体基因的来源,抗体文库可分为免疫文库、天然文库以及合成文库^[26]。其中,免疫文库及天然文库的构建需开展动物实验,故通常不适用于毒性抗原、自身抗原以及某些不具有免疫原性的抗原^[27]。合成文库的制备不涉及动物,能够克服动物源性抗体文库的一些弊端,成为抗体发现方案的一种重要补充^[28]。合成文库具有组分明确、可操作性强等优点,文库的多样性和可控性得到了增强^[29],实验室仅需具备基本分子克隆技术,便可进行文库构建与筛选。合成库中的序列未经动物免疫系统的筛选,因此可能从其中筛选得到一些针对某些特定抗原(如自身抗原)的高亲和力抗体^[27,30],近年来,大量的文献报道证明了合成文库这一策略可用于纳米抗体的筛选与生产^[21,30-39]。确定合适的框架序列是构建合成纳米抗体文库的首要步骤,与动物源性抗体文库不同,合成文库内的所有纳米抗体共享相同的框架序列。Saerens等^[40]提出的首个经典的人源化框架cAbBCII10,通常被认为是合成文库框架的良好选择。2016年,Moutel等^[31]提出了另一个框架

Hs2dAb,并引入了4种不同长度的互补决定区3(complementarity determining region three, CDR3),设计构建了NaLi-H1文库。2018年,Zimmermann等^[32]基于3种已报道的纳米抗体序列设计了3种不同的框架,构建了3个不同的文库。

基于天然纳米抗体文库的高通量测序结果^[41],课题组前期从88个V胚系基因与7个J胚系基因的组合中确定了1个最常用的组合方式(IGHV3S65*01-IGHJ4*01)作为新框架,以此框架为基础,根据CDR3多样性设计合成了文库S-Library^[30],并使用5种抗原对文库进行了筛选验证,结果表明在天然纳米抗体文库中发现的特征对文库设计具有很好的指导意义。在此基础上,本研究基于天然纳米抗体CDR1、CDR2和CDR3区域的氨基酸序列偏好性,构建了基于单框架的全合成纳米抗体文库(single-frame synthesized nanobody library, SS-Library),并使用3种不同的抗原对文库进行筛选验证。

1 材料与方法

1.1 材料与试剂

大肠杆菌(*Escherichia coli*) TG1、BL21(DE3)和噬菌粒 pComb3XSS 为本实验室保存^[30];感受态细胞为实验室自制^[30];载体 pUC57-IGHV3S65*01 由生工生物工程(上海)股份有限公司合成;高保真 PCR 酶 PrimeSTAR[®] HS DNA Polymerase、限制性内切酶(*Sfi* I、*Not* I、*Nco* I)、连接酶 T4 DNA Ligase 均购自宝生物工程(大连)有限公司;异丙基- β -D-硫代半乳糖苷(isopropyl- β -D-thiogalactopyranoside, IPTG)、3,3',5,5'-四甲基联苯胺(tetramethylbenzidine, TMB)显色液、牛血清白蛋白(bovine serum albumin, BSA)、乙酰胆碱酯酶(acetylcholinesterase, AchE)、小鼠 IgG 干粉(immunoglobulin G, IgG)、聚乙二醇 PEG 8000、

吐温-20 均购自北京索莱宝科技有限公司; High Affinity Ni-NTA Resin 购自金斯瑞生物科技股份有限公司、Anti-M13 Bacteriophage, AlpHcAbs[®] Rabbit antibody (HRP)、Anti-His tag, AlpHcAbs[®] Rabbit antibody (HRP)均购自成都阿帕克生物科技有限公司; 96 孔酶标板购自 Corning 公司; 2×Rapid Taq Master Mix 购自南京诺唯赞生物科技股份有限公司; 脱脂乳购自生工生物工程(上海)股份有限公司; 文库构建过程所用引物由金斯瑞生物科技股份有限公司合成(表 1)。

1.2 仪器与设备

NanoDrop 1000 微量分光光度计、多功能读数仪、低温高速离心机(赛默飞世尔科技公司); 电泳仪(北京六一仪器厂); 超声细胞破碎仪(宁波新艺超声设备有限公司); PCR 仪、凝胶成像仪、电转仪[伯乐生命医学产品(上海)有限公司]。

1.3 方法

1.3.1 纳米抗体合成文库的构建及鉴定

通过 3 轮 PCR 获得完整的重链抗体可变区(variable domain of heavy-chain antibodies, VHH)全长片段, 具体实验原理如图 1 所示。第

1 轮 PCR (PCR1)和第 2 轮 PCR (PCR2)以合成的基因 *pUC57-IGHV3S65*01* 为模板, 使用引物 SyF1 和 SyR1 对 CDR1 进行多样化, 引物 SyF2 和 SyR2 对 CDR2 进行多样化。第 3 轮 PCR (PCR3)以纯化后的 PCR1 和 PCR2 产物作为模板, 使用引物 SyF1-Sfi I 和 SyR3-Sfi I 进行 overlap PCR, 获得两端带有 Sfi I 酶切位点的 VHH 片段。

使用限制性内切酶 Sfi I 过夜酶切 VHH 片段以及噬菌粒 pComb3XSS, 胶回收试剂盒回收目的条带, T4 DNA 连接酶 16 °C 连接 16 h, 62 μg 连接产物分 40 次电转化入大肠杆菌 TG1 感受态细胞, 37 °C 复苏 1 h, 留 10 μL 菌液用于文库库容测定, 其余分别涂布于 30 块直径 150 mm 的 2×YT 固体平板[含 100 μg/mL 氨苄青霉素(ampicillin, Amp)], 30 °C 过夜倒置培养。使用 2×YT 培养基刮洗收集平板上的菌苔, 混匀后加入终浓度为 25% 的甘油, 分装保存于 -80 °C。随机挑取 48 个单菌落进行菌落 PCR 验证, 阳性克隆送至生工生物工程(上海)股份有限公司测序。利用 MEGA 11 软件^[42]对测序结果进行多序列比对分析以验证其多样性。用于文库构建及验证的 PCR 条件见表 2。

表 1 本研究所用引物

Table 1 Primers used in this study

Primer name	Sequence (5'→3')
SyF1	CAGCTGCAGCTGGTTGAA
SyR1	CTGACGGAACCAGCCGATNGYGTARKWATCCAGNGWGAAGCCGCTCGCCGCGCAGCTCAG
SyF2	ATCGGCTGGTTCCGTCAGGCGCCGGGCAAAGAACGTGAAGGCGTTAGCTGCATCAGCRSNAGCGR YGGTASCACCAACTACGCGGATAGCGTT
SyR2	TGTGCCCTGTCCCCAATAATCATAWTCAYYASBAMBAMBRDNNNBNNBAMBACBACBNNBAMBAS BASBABSACCCGCCGCGCAGTAGTAAAC
SyF1-Sfi I	CATGCCATGACTGTGGCCAGGCGGCCAGSTGCAGCTGGTTGAA
SyR3-Sfi I	CATGCCATGACTCGCGGCCGCTGGCCACTTGAGACAGTGACTTGTGTGCCCTGTCCCCAATA
Omp	AAGACAGCTATCGCGATTGCAG
gIII ^F	GCCCCCTTATTAGCGTTTGCCATC

下划线碱基表示限制性内切酶识别位点。使用 IUPAC 简并符号代表某个位置出现的核苷酸集合, 其中 R=A/G; Y=C/T; M=A/C; K=G/T; S=G/C; W=A/T; H=A/T/C; B=G/T/C; V=G/A/C; D=G/A/T; N=A/G/C/T。

Underlined bases indicate restriction endonuclease recognition sites. The set of nucleotides occurring at a given position is represented using the IUPAC concatenation notation, where R=A/G; Y=C/T; M=A/C; K=G/T; S=G/C; W=A/T; H=A/T/C; B=G/T/C; V=G/A/C; D=G/A/T; N=A/G/C/T.

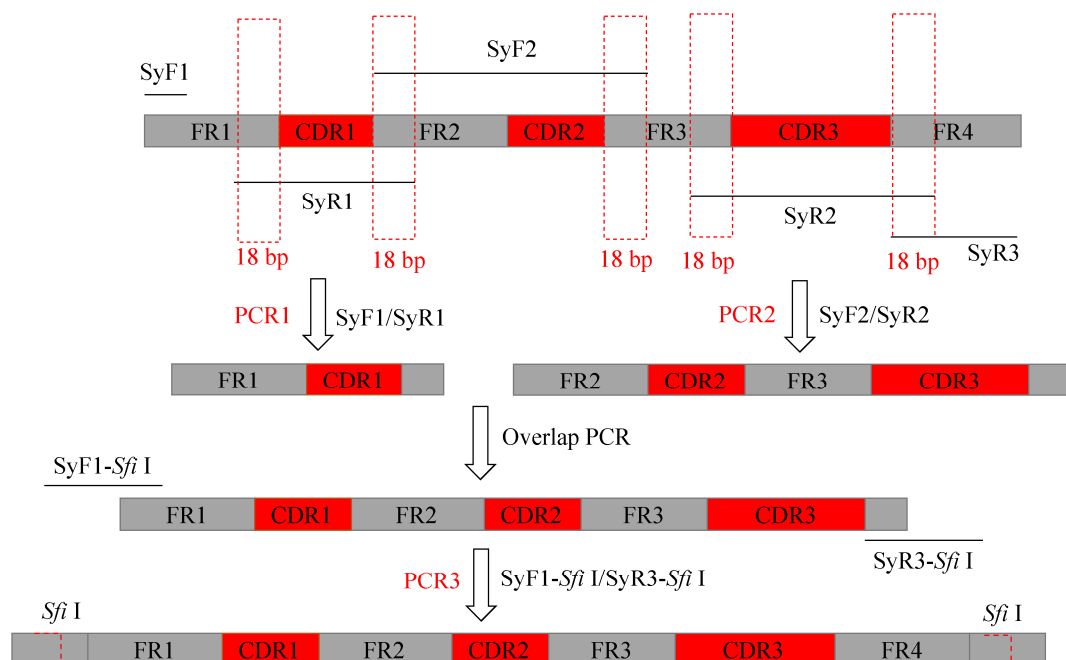


图1 VHH 全长片段构建示意图

Figure 1 Schematic diagram of VHH full-length fragment acquisition.

表2 文库构建的 PCR 条件

Table 2 PCR conditions for library construction

PCR	Template	Oligos	Enzyme concentration	Denaturation	Annealing	Extension	Cycles	Re-extension
PCR1	<i>pUC57-IGHV3</i>	SyF1 (10 pmol/L)+ S65*01 SyR1 (10 pmol/L)	1.25 U	98 °C, 10 s	65 °C, 15 s	72 °C, 15 s	30	72 °C, 5 min
PCR2	<i>pUC57-IGHV3</i>	SyF2 (10 pmol/L)+ S65*01 SyR2 (10 pmol/L)	1.25 U	98 °C, 10 s	54 °C, 15 s	72 °C, 20 s	30	72 °C, 5 min
PCR3	PCR1 product+	SyF1-Sfi I (10 pmol/L)+	1.25 U	98 °C, 10 s	54 °C, 5 s	72 °C, 30 s	5	72 °C, 5 min
	PCR2 product	SyR3-Sfi I (10 pmol/L)	1.25 U	98 °C, 10 s	50.5 °C, 5 s	72 °C, 30 s	20	72 °C, 5 min

1.3.2 噬菌体展示文库的救援与筛选

噬菌体展示文库的救援、滴度测定和固相筛选参考 Liu 等^[30]的方法进行。采用 3 种抗原 (BSA、AchE、IgG) 对文库进行了 4 轮固相筛选, 抗原包被浓度依次为 100、50、25、12.5 $\mu\text{g/mL}$, 使用 5% 脱脂乳或 4% 卵清白蛋白 (ovalbumin, OVA) 交替封闭, 4 轮筛选所用吐温-20 浓度依次为 0.05%、0.1%、0.15%、0.2%。取 10 μL 测定 Tris-HCl (pH 8.0) 中和后的洗脱液滴度, 其余洗脱液进行扩增后将其用于下一轮筛选。

1.3.3 间接噬菌体酶联免疫吸附实验鉴定阳性克隆

通过噬菌体酶联免疫吸附实验 (phage enzyme-

linked immunosorbent assay, phage-ELISA) 鉴定阳性克隆^[43]。用 0.01 mol/L 磷酸盐缓冲液 (phosphate buffered saline, PBS) 将抗原稀释至 5 $\mu\text{g/mL}$, 4 °C 过夜孵育。使用含 0.05% 吐温-20 的磷酸盐缓冲液 (phosphate buffered saline with tween-20, PBST) 洗板 3 次后加入 5% 脱脂乳封闭 2 h。加入 100 μL 救援的噬菌体, 37 °C 孵育 1 h。PBST 洗板 6 次后加入辣根过氧化物酶 (horseradish peroxidase, HRP) 偶联的抗 M13 二抗 (1:7 500 稀释) 孵育 1 h。洗板 8 次后加入 100 μL 的 TMB 显色液, 孵育 6–8 min。加入 50 μL H_2SO_4 (2 mol/L) 终止反应, 多功能读数仪测定 OD_{450} 值, 将信噪比 (signal-to-noise ratio, S/N) 即实验组 OD_{450}

值/阴性对照组 OD_{450} 值 >2 视为阳性克隆。

1.3.4 纳米抗体的表达纯化与活性鉴定

每种抗原分别挑选 1 个阳性序列克隆至表达载体 pET-25b, 钙转化 *E. coli* BL21(DE3)。挑取单菌落接种于 5 mL LB 液体培养基(含 100 $\mu\text{g/mL}$ Amp), 37 $^{\circ}\text{C}$ 过夜培养, 将培养物以 1% 的接种量转接至 50 mL LB 液体培养基(含 100 $\mu\text{g/mL}$ Amp), 37 $^{\circ}\text{C}$ 培养至 $OD_{600}=0.6-0.8$ 。加入终浓度为 0.1 mmol/L 的 IPTG, 16 $^{\circ}\text{C}$ 诱导 14 h。离心后, 使用 1/5 体积预冷的 0.01 mol/L PBS (pH 7.4) 重悬菌体, 超声破碎后按照说明书使用 Ni-NTA 亲和层析柱纯化目标蛋白, 聚丙烯酰胺凝胶电泳(sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE)分析蛋白表达以及纯化情况, 使用软件 Quantity One 进行灰度定量分析。通过间接酶联免疫吸附实验(enzyme-linked immunosorbent assay, ELISA)鉴定蛋白活性。使用生物膜干涉技术(bio-layer interferometry, BLI)对抗原与其对应的纳米抗体之间的相互作用进行测定。纯化的纳米抗体分别被固定在第二代氨基偶联生物传感器(amine reactive second-generation, AR2G)生物传感器上, 使用乙醇胺(1 mol/L, pH 8.5)对传感器上未结合纳米抗体的羧基进行封闭, 梯度浓度的抗原在 AR2G 上进行结合和解离步骤, 根据 1:1 的摩尔比拟合曲线并计算亲和力 KD 值。

2 结果与分析

2.1 VHH 全长片段的获取

通过 3 轮 PCR 获得完整的 VHH 全长片段。如图 2A 所示, PCR1 和 PCR2 均获得了与理论大小相符的条带(120 bp 和 270 bp)。以纯化后的 PCR1 和 PCR2 产物作为模板进行第 3 轮 PCR 反应, 在 VHH 的 3' 和 5' 端引入酶切位点 *Sfi* I, 结果如图 2B 所示, PCR 产物与理论条带大小(440 bp)一致, 表明 VHH 全长片段的 3 个互补决定区 (complementarity determining region,

CDR)均成功多样化。

2.2 SS-Library 的构建及质量鉴定

如图 3A 所示, pComb3XSS 经 *Sfi* I 酶切后出现 2 条明显条带, 胶回收其中大小为 3 319 bp 的条带用于后续实验。图 3B 表明 VHH 全长片段经酶切后得到了大小约 400 bp 的条带, 与理论值相符。使用胶回收试剂盒对目的条带进行回收, 结果如图 3C、3D 所示, 胶回收条带单一、大小正确, 表明可以将其用于后续的酶连实验。将连接产物进行电转化后获得了包含 6×10^9 个克隆的 SS-Library (表 3)。

菌落 PCR 结果如图 4 所示, 在随机挑取的 48 个克隆中, 46 个能扩增出与理论大小 640 bp 相符的条带, 插入率达 95.8%, 初步证明文库质量良好。Sanger 测序结果如图 5 所示, 其中

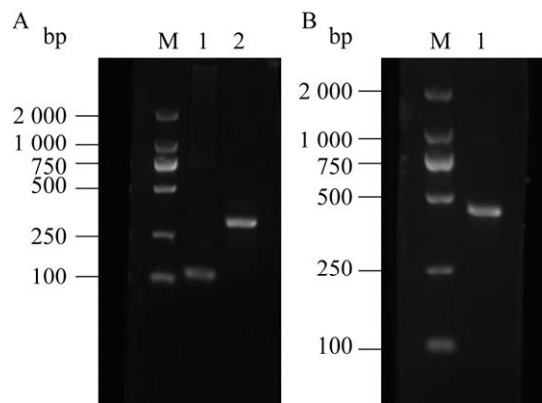


图2 Overlap PCR 扩增 VHH 全长片段琼脂糖凝胶电泳图 A: 第 1、2 轮 PCR 产物琼脂糖凝胶电泳图。泳道 M: DL2000 DNA marker; 泳道 1: PCR1 产物; 泳道 2: PCR2 产物。B: Overlap PCR 产物琼脂糖凝胶电泳图。泳道 M: DL2000 DNA marker; 泳道 1: VHH 全长片段。

Figure 2 Agarose gel electrophoresis of overlap PCR amplified VHH full-length fragment. A: Agarose gel electropherograms of the first two rounds of PCR products. Lane M: DL2000 DNA marker; Lane 1: PCR1 product; Lane 2: PCR2 product. B: Agarose gel electrophoresis of overlap PCR. Lane M: DL2000 DNA marker; Lane 1: VHH full-length fragment.

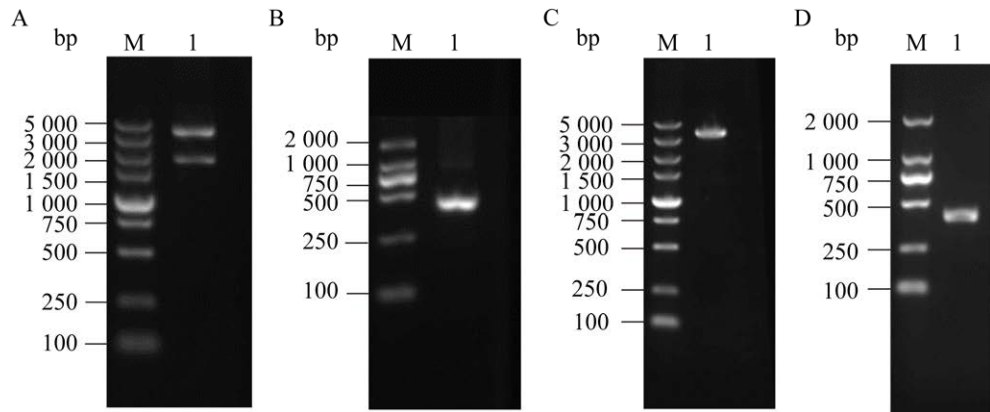


图 3 文库构建的琼脂糖凝胶电泳图 A: pComb3XSS 酶切琼脂糖凝胶电泳图。泳道 M: DL5000 DNA marker; 泳道 1: pComb3XSS 酶切产物。B: VHH 酶切琼脂糖凝胶电泳图。泳道 M: DL2000 DNA marker; 泳道 1: VHH 酶切产物。C: 胶回收载体琼脂糖凝胶电泳图。泳道 M: DL5000 DNA marker; 泳道 1: 噬菌粒胶回收产物。D: 胶回收片段琼脂糖凝胶电泳图。泳道 M: DL2000 DNA marker; 泳道 1: VHH 胶回收产物。

Figure 3 Agarose gel electrophoresis of library construction. A: Agarose gel electrophoresis of pComb3XSS digestion. Lane M: DL5000 DNA marker; Lane 1: pComb3XSS digest product. B: Agarose gel electrophoresis of VHH. Lane M: DL2000 DNA marker; Lane 1: VHH digest product. C: Agarose gel electrophoresis of carrier recovery. Lane M: DL5000 DNA marker; Lane 1: Phage gel recovery product. D: Agarose gel electrophoresis of target fragment recovery. Lane M: DL2000 DNA marker; Lane 1: VHH gel recovery product.

表 3 梯度稀释法测定文库库容
Table 3 Gradient dilution method for library capacity determination

Dilution factor	10 ²	10 ³	10 ⁴	10 ⁵
Colony count	—	—	4 000	500

“—”代表菌落太多，无法计数。

“—” means too many colonies to count.

38 个克隆序列完整，每个位置的氨基酸分布与预期相符，Sanger 测序序列与设计序列之间的皮尔森相关系数(Pearson correlation)为 0.979 9，文库的正确插入率为 75.8%，表明所合成的序列与设计高度吻合。

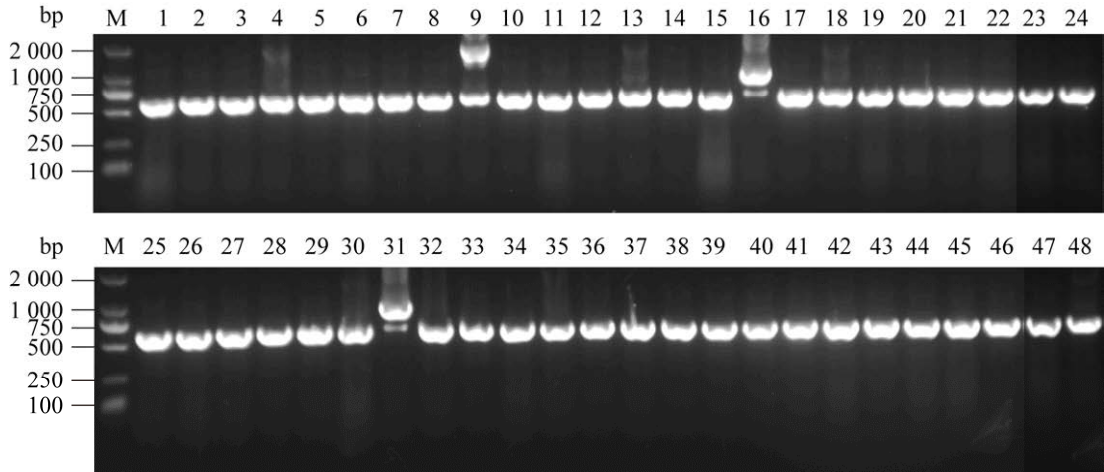


图 4 菌落 PCR 琼脂糖凝胶电泳图 泳道 M: DL2000 DNA marker; 泳道 1–48: 48 个单克隆菌落 PCR 结果。
Figure 4 PCR agarose gel electrophoresis of colonies. Lane M: DL2000 DNA marker; Lane 1–48: PCR results of 48 monoclonal colonies.

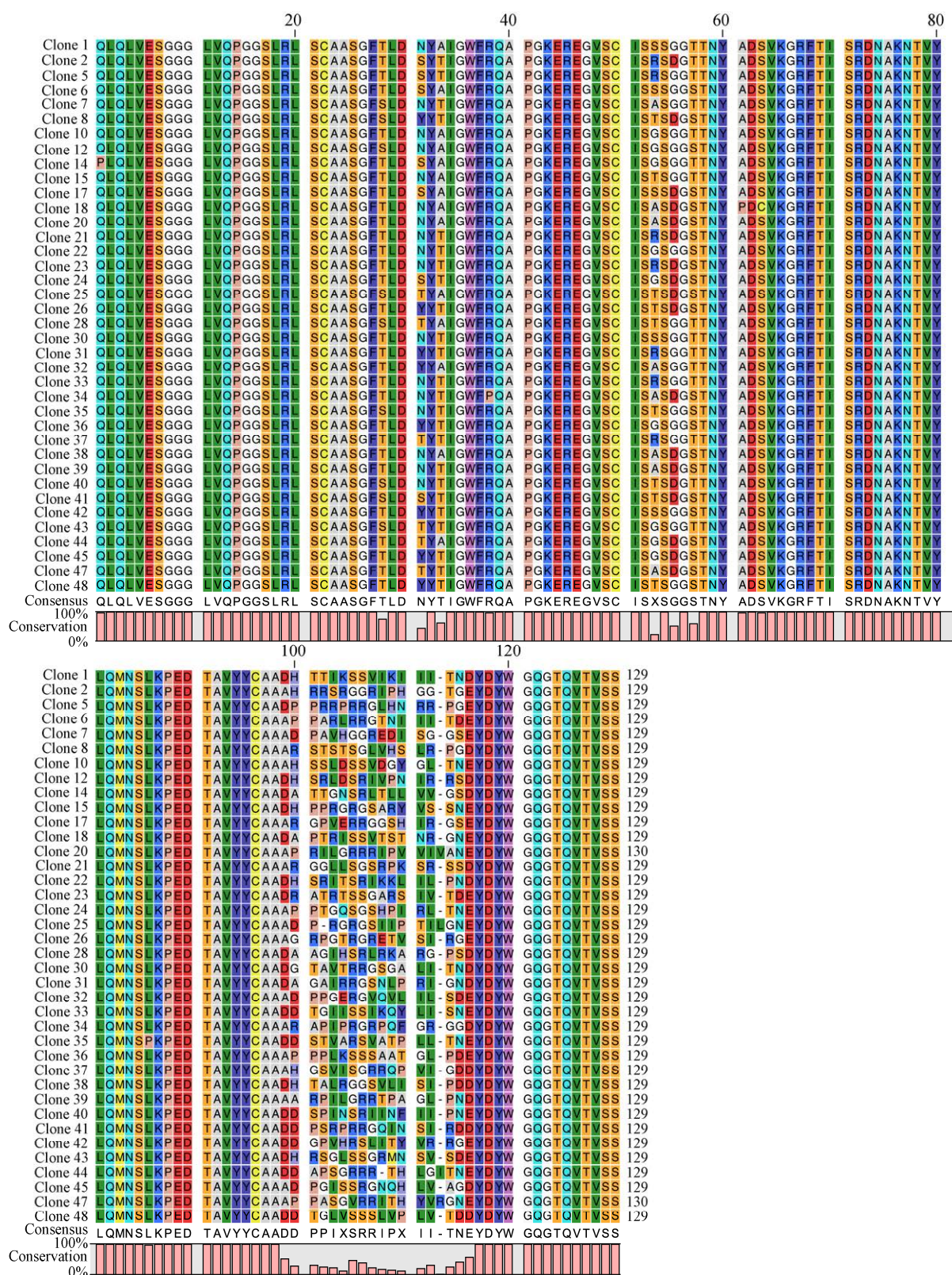


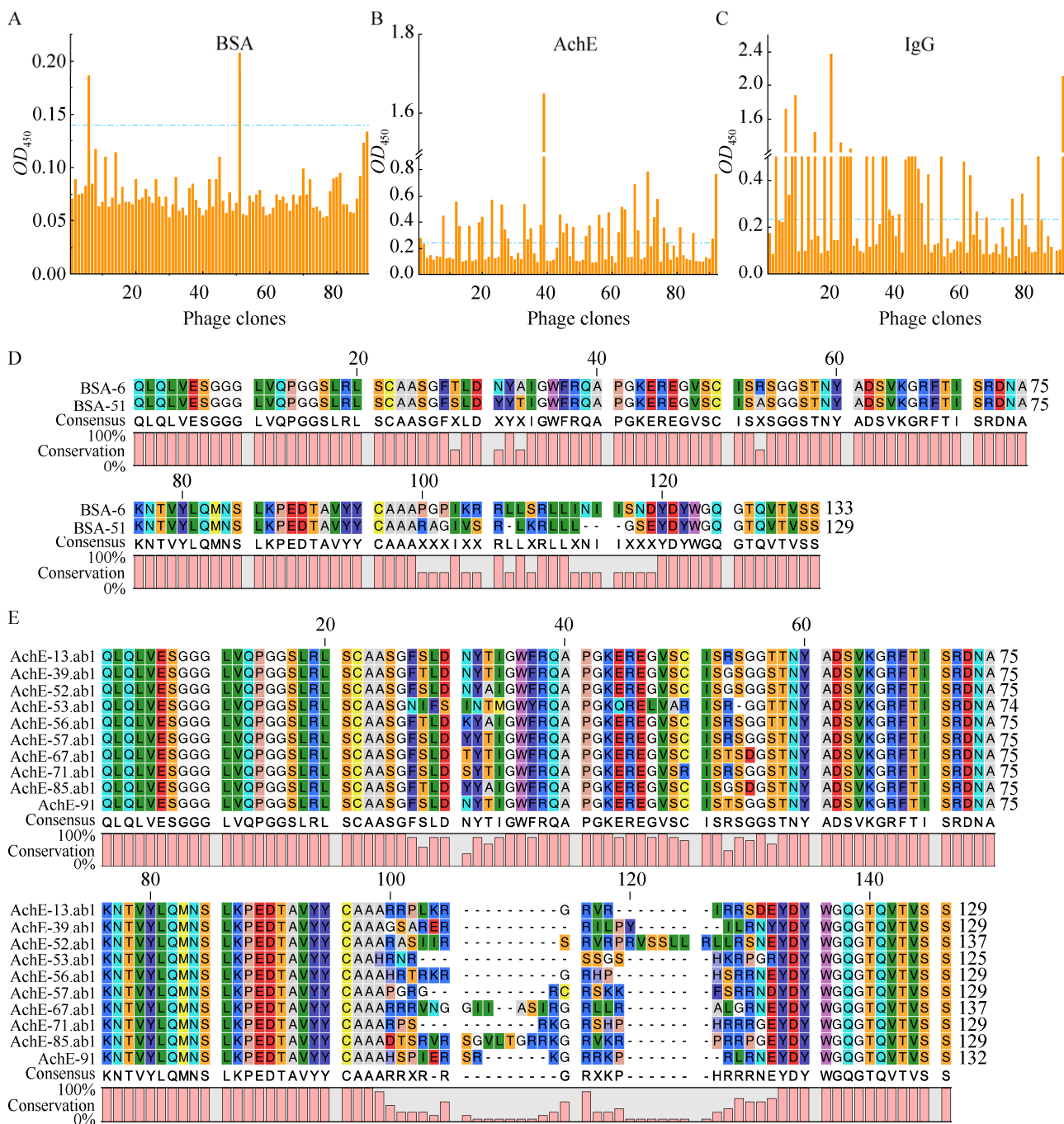
图5 Sanger 测序鉴定 48 个克隆的氨基酸组成及多样性

Figure 5 Amino acid composition and diversity of 48 clones identified by Sanger sequencing.

2.3 噬菌体文库的筛选

初始文库经辅助噬菌体 M13K07 救援后, 展示文库滴度达到 10^{13} PFU/mL, 满足后续筛选要求。经过 4 轮固相筛选, 最终分别获得 2 个针对 BSA、38 个针对 AchE 和 38 个针对 IgG 的阳性克

隆(图 6A–6C)。筛选过程中, 噬菌体洗脱量和回收率逐轮呈现递增趋势, 表明阳性噬菌体得到了有效的富集。Sanger 测序结果显示, 共筛选得到了 2 种针对 BSA、10 种针对 AchE 以及 15 种针对 IgG 的纳米抗体, 且其序列差异性较大(图 6D–6F)。



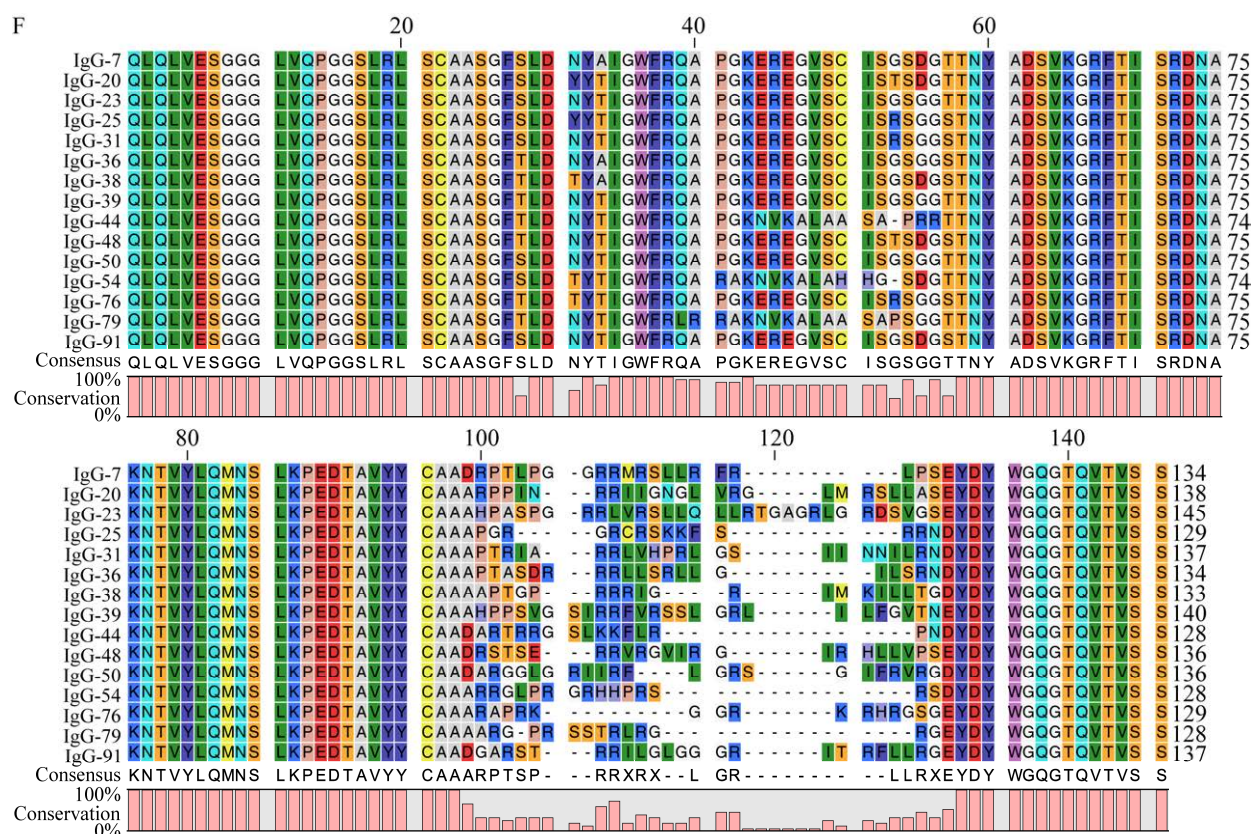


图 6 合成文库噬菌体展示筛选结果 A–C: 间接 phage-ELISA 验证。蓝色虚线(S/N=2)为阳性序列鉴定线。D–F: Sanger 测序鉴定阳性克隆的氨基酸序列。

Figure 6 Results of phage display screening of synthesized libraries. A–C: Indirect phage-ELISA validation. Blue dashed line (S/N=2) is the line of positive sequence identification. D–F: Amino acid sequences of positive clones identified by Sanger sequencing.

2.4 表达纯化以及生物活性表征

为了进一步对所得纳米抗体的活性进行表征, 针对每种抗原随机挑选了 1 个阳性序列进行克隆表达。使用 0.1 mmol/L 的 IPTG 在 16 °C 下过夜诱导, SDS-PAGE 鉴定结果(图 7A)表明 3 种纳米抗体均为可溶性表达。所制备的纳米抗体纯度达 80%, 满足后续实验的需求。间接 ELISA 结果如图 7B 所示, 表明抗 AchE 和 IgG 的纳米抗体可以特异性结合相应抗原。抗原与纳米抗体的亲和力常数(K_D 值)测定结果详见表 4, anti-AchE-39 和 anti-IgG-36 的 K_D 值分别为 294 nmol/L 和 250 nmol/L (图 8A、8B)。

3 讨论

构建纳米抗体文库的核心包括 2 个至关重要的因素: 框架区的选择以及 CDRs 区的设计。性能优异的框架通常具有较高的溶解性、稳定性以及表达水平等特性^[44]。通用框架的开发可以追溯到 2005 年 Saerens 等^[40]提出的首个经典的人源化框架 cAbBCII10, 该框架具有较高的稳定性以及表达水平, 且在缺少经典二硫键的情况下亦能发挥其正常功能, 故该框架已在多项研究中被用作合成文库的框架^[34,45]。除此之外, 新的框架也可以源自天然文库的共识序列,

Sevy 等^[46]通过 2 个已经公开的天然文库的高通量测序(next generation sequencing, NGS)数据集确定了 1 个框架 IGHV3S53*01, 并构建了 4 个

不同大小的合成文库。在 CDR 区的设计过程中, 通常通过固定每个 CDR 区的长度来实现结构的稳定性^[45]。但近些年为了增加一定的可变

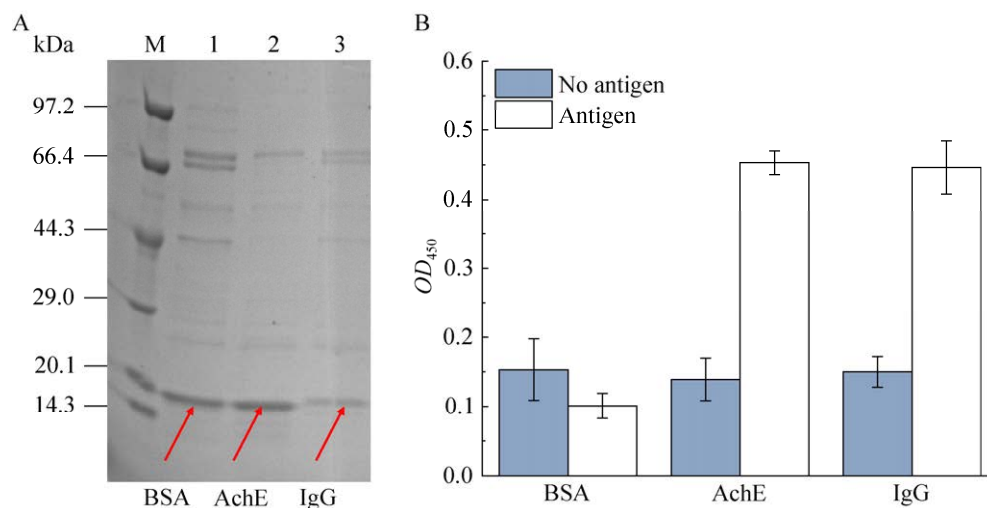


图 7 SDS-PAGE 验证纳米抗体表达纯化情况及蛋白活性鉴定 A: SDS-PAGE 验证纳米抗体表达纯化情况。泳道 M: 低分子量蛋白 marker; 泳道 1-3: 针对 3 种抗原的纳米抗体(BSA、AchE 和 IgG)。B: 间接 ELISA 鉴定 3 种抗体活性, 2 种不同颜色的柱子分别表示未包被抗原以及包被抗原时 ELISA 的显色值。

Figure 7 SDS-PAGE verification of nanobody expression purification and protein activity identification A: SDS-PAGE to verify the purification of nanobody expression. Lane M: Low molecular weight protein marker; Lane 1-3: Nanobodies against three antigens (BSA, AchE, and IgG). B: Indirect ELISA to identify the activity of the three antibodies, the two different colors of the columns indicate the color values of ELISA for the unencapsulated and encapsulated antigens.

表 4 纳米抗体与抗原的亲和力常数测定

Table 4 Determination of affinity constants of nanobodies to antigens

Binders	K _D (nmol/L)	K _a ($\times 10^3$ mol/(L·s))	K _d ($\times 10^{-4}$ s ⁻¹)
anti-AchE-39	294	2.917	8.603
anti-IgG-36	250	2.274	5.695

性, 拥有不同 CDR 区长度(主要是 CDR3 区)的合成文库应运而生^[31-32,35]。Zimmermann 等^[32]设计了 3 种不同的 CDR3 长度(6、12、16 个氨基酸)以保证抗体与抗原结合时结合位点的空间结构。Nie 等^[47]对已报道的一些纳米抗体分析后将 CDR3 区长度设计为出现频率最高的

13、14、15 个氨基酸。为了充分覆盖自然界中纳米抗体 CDR3 区的长度多样性, Moutel 等^[31]在设计中引入了 4 种特定长度的 CDR3 区, 分别为 9、12、15 和 18 个氨基酸。

本研究选用 IGHV3S65*01-IGHJ4*01 作为一个新的框架用于文库的设计, 并对 3 个 CDR 区均进行了多样化, 所选用的框架及 3 个 CDR 区的长度和氨基酸分布均是基于对天然纳米抗体文库的 NGS 结果分析而设计^[30,41]。该策略显著减少了设计文库的序列多样性, 进而优化了文库的合成流程。针对 3 种不同抗原的筛选结果表明在天然纳米抗体文库中发现的特征对于

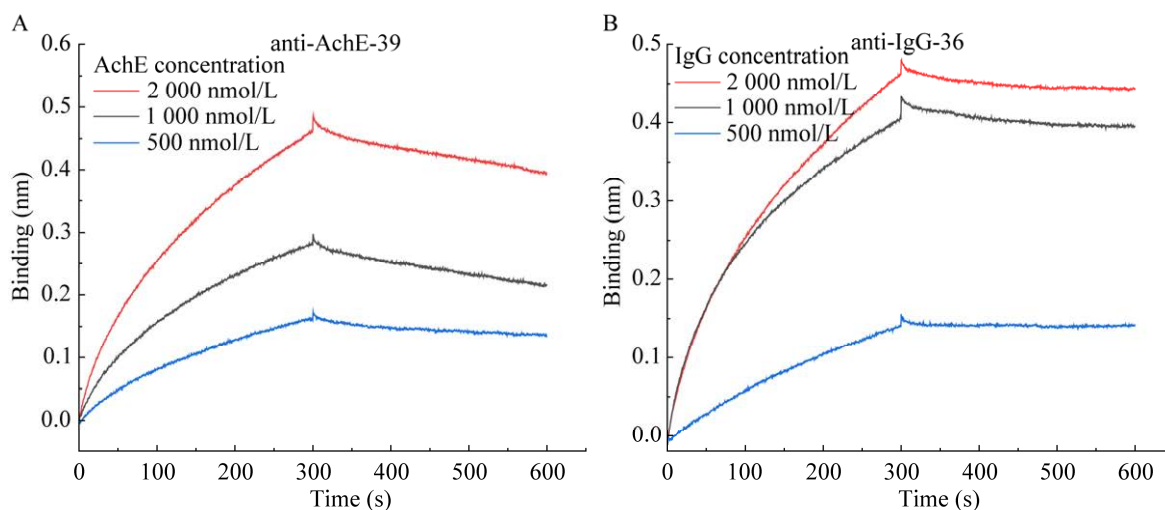


图8 纳米抗体动力学和亲和力测定
Figure 8 Nanobody kinetics and affinity assay.

合成文库的设计具有重要的指导意义。尽管在ELISA活性鉴定中,最终筛选得到的蛋白表现出较弱的信号(图7B),提示其亲和力可能较低,该文库仍具有进一步改进的空间。可通过一些途径提高文库的性能。例如:(1)使用多种框架进行文库构建,或者使用不同长度的CDR3区,便于更加全面地涵盖整个天然抗体文库的基因,使其具有更好的代表性。(2)可参考Zimmermann等^[32]报道的将噬菌体展示与核糖体展示平台联用以提高筛选效率。噬菌体展示技术因受到转化效率的限制,往往需要增加电转化次数以提高文库的库容。本研究合成的SS-Library有效库容为 4.5×10^9 ,比设计的理论多样性 3.8×10^{15} 低6~7个数量级,使用核糖体展示进行初筛可大大提高初始文库的库容以及多样性(10^{12}),经历1~2轮筛选的富集,后续凭借噬菌体展示足以涵盖文库的多样性。(3)使用人工智能(artificial intelligence, AI)辅助设计文库。Li等^[48]将通过AI辅助设计的一个单链抗体(single chain antibody fragment, scFv)库与定向进化方法进行对比,结果发现使用AI辅助设计产生

的最佳scFv的结合力是定向进化的28.7倍。AI的兴起有望为纳米抗体合成文库设计开发复杂的模型。

4 结论

本研究提出的基于NGS数据为指导的合成文库设计与构建策略显著减少了设计文库的序列多样性,进而优化了文库的合成流程,整个过程无需特殊且昂贵的试剂,适用于普通的分子生物学实验室,设计的文库与合成获得的纳米抗体序列高度吻合。对3种抗原进行筛选均获得了阳性克隆,且随机挑选的阳性克隆重组表达均为可溶性表达,提示选择框架IGHV3S65*01-IGHJ4*01合成的文库具有通用性强、偏好性低等特点,有望成为一种通用的纳米抗体发现平台,用于先导特异性纳米抗体的制备与开发。

作者贡献声明

罗颖:方案设计、实验操作、初稿写作、数据管理;李燕萍、何庆华:提供材料、经费支持、监督指导、数据管理;涂追:经费支持、提供材

料、监督指导、稿件润色修改、数据管理。

作者利益冲突公开声明

作者声明没有任何可能会影响本文所报告工作的已知经济利益或个人关系。

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