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CRISPR-Cas系统在医学诊断中的研究进展

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摘　要

簇状规则间隔短回文重复序列(CRISPR)和CRISPR相关蛋白(Cas)系统对核酸具有特异的识别、顺式切割和非特异性的反式切割能力，已经成为分子诊断领域的关键工具。凭借其卓越的特异性和灵敏度，CRISPR-Cas系统结合生物传感技术，能够高效检测核酸、蛋白质、小分子等多种靶标，近年来在医学诊断领域展现巨大潜力。本文首先对CRISPR-Cas系统的组成及分类进行介绍，然后简述了CRISPR/Cas系统在核酸和非核酸靶标医学诊断领域的应用，最后讨论了CRISPR/Cas系统当前的挑战及未来的发展前景。

关键词

CRISPR-Cas系统，Cas蛋白，医学诊断

Research Progress of CRISPR-Cas System in Medical Diagnosis

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Abstract

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) systems, characterized by their specific nucleic acid recognition, cis-cleavage, and nonspecific trans-cleavage activities, have emerged as pivotal tools in molecular diagnostics. With high specificity and sensitivity, CRISPR-Cas systems integrated with biosensing technologies enable efficient detection of diverse targets, including nucleic acids, proteins, and small molecules, demonstrating significant potential in medical diagnostics in recent years. In this review, we first introduce

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the components and classification of CRISPR-Cas systems. Then, we briefly describe the application of the CRISPR-Cas system in the medical diagnosis of nucleic acid and non-nucleic acid targets. Finally, we discuss the current challenges and future prospects for CRISPR-Cas systems.

Keywords

CRISPR-Cas System, Cas Protein, Medical Diagnosis

1. 引言

通过对生物样本中 DNA、RNA 和蛋白质等生物标志物的检测在医学诊断中起着至关重要的作用，可以为疾病的预防、早期诊断、治疗提供关键信息[1] [2]。目前已开发了多种检测生物标志物的方法，包括聚合酶链反应(polymerase chain reaction, PCR)、RNA 印迹法(Northern blotting)、下一代测序技术、流式细胞仪和酶联免疫吸附测定(enzyme-linked immunosorbent assay, ELISA) [3]等，虽然在特异性和灵敏度上表现出色，但其检测耗时长、成本高、依赖专业设备和专业人员等不足，限制了其在临床中的即时检测(point-of-care testing, POCT)的广泛应用[3] [4]。因此，开发亟需一种新的便捷、快速、低成本且高效的一种分子诊断技术。

CRISPR-Cas 系统最初是从细菌和古菌中发现的一种适应性免疫机制，依靠 CRISPR RNA (crRNA)对靶区域的引导以及 Cas 蛋白酶的核酸切割能力来抵御外源基因(如噬菌体)入侵[5]-[7]。成簇规律间隔短回文重复序列(Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR)/CRISPR 相关蛋白(CRISPR-associated protein, Cas)系统一般由 Cas 蛋白和 CRISPRRNA (crRNA)组成，其中 CRISPR 序列包含重复序列和间隔序列，间隔序列与目标序列配对，形成核糖核蛋白(ribonucleoprotein, RNP)复合体，Cas 蛋白识别和切割外源核酸。近年来，基于 CRISPR/Cas 系统的特异性识别和高效切割能力，将其与电化学、电化学发光、荧光等生物传感技术相结合，已经成功实现了核酸和蛋白质、金属离子等多种非核酸靶标的高特异、高灵敏检测[8] [9]。与传统技术相比，CRISPR-Cas 系统不仅表现出了更加出色的高灵敏度和高特异度，而且具有反应条件温和、操作简单、无需复杂的设备、成本低的特点，尤其适用于快速的即时检测(point-of-care testing, POCT) [10] [11]，在病原体检测、遗传疾病诊断和癌症标志物检测等医学诊断领域展现出广阔的应用前景[12]。本文介绍了不同类型的 CRISPR-Cas 系统，重点介绍了近年来基于 CRISPR-Cas 系统在核酸和非核酸靶标检测上的研究及应用，并讨论了其目前面临的挑战和未来的发展方向，以期为该技术的推广应用提供参考。

2. 基于 CRISPR-Cas 系统的检测原理和方法

根据其进化关系，CRISPR 系统被分为两大类六个亚型，I 类不太常用，II 类是目前研究最多、应用最广泛的系统[13]。I 类 CRISPR/Cas 系统由多个 Cas 蛋白组成的效应模块，I、III 和 IV 型属于经典的一类 CRISPR-Cas 系统；II 类 CRISPR/Cas 系统是由 RNA 介导的单一多结构域的 Cas 蛋白系统，主要包括 Cas9 (II 型)、Cas13 (VI 型)、Cas12 (V 型)和 Cas14 (V 型) [14]。Cas 蛋白被激活后具有顺式切割与反式切割两种能力。顺式切割是指 Cas 蛋白特异性识别并切割目标核酸序列的过程，通常依赖于目标序列与引

导 RNA 的互补配对，以及某些 Cas 蛋白所需的原间隔邻近基序(protospacer-adjacent motif, PAM)。反式切割是指 Cas 蛋白在激活后，非特异性地无差别切割任意单链 DNA 或 RNA 的过程。在诊断应用领域，常常通过设计一系列反应使靶标去激活 Cas 蛋白，从而反向切割荧光猝灭报告探针，最后释放荧光信号与靶标的浓度产生联系[15]。本文重点介绍 CRISPR/Cas9、CRISPR/Cas12、CRISPR/Cas13 和 CRISPR/Cas14 系统。

2.1. CRISPR-Cas 系统分类和检测原理

2.1.1. CRISPR-Cas9 系统

Cas9 是 II 型 CRISPR 系统的核心蛋白，是第一个用于基因编辑的 CRISPR/Cas 系统，Cas9 蛋白包含识别结构域和两个酶切割结构域(HNH 和 RuvC) [16]。Cas9 核酸酶在反式激活 CRISPR RNA (tracrRNA)和 CRISPR RNA (crRNA)的帮助下切割靶 DNA。Cas9 蛋白酶切割活性是由 sgRNA 调控的，通过与 sgRNA 结合，Cas9 能够特异性识别并切割目标双链 DNA (double-stranded DNA, dsDNA)，其切割活性依赖于 PAM 序列[17] [18]。此外，失活形式的死亡 Cas9 (dead Cas9, dCas9)保留了结合能力但失去切割活性[19]，可用于开发多种生物传感器。Cas9 系统的优势在于其对 dsDNA 的精准切割能力，使其在基因检测和基因编辑领域得到广泛应用，尤其适合检测基因型等应用场景。

2.1.2. CRISPR-Cas12 系统

Cas12 是 V 型 CRISPR 系统的效应蛋白，具有独特的反式切割活性。不同于 Cas9，Cas12a (也称为 Cpf1)和 Cas12b (也称为 C2c1)蛋白通常更小[20]，Cas12 仅包含识别双链 DNA (dsDNA)或单链 DNA (ssDNA)的 RuvC 结构域[20] [21]，Cas12a 的各种亚型也拓宽了其使用前景[21]。在 crRNA (CRISPR RNA)引导下，Cas12 靶向 PAM 附近的 DNA 序列进行识别，并在特定位置切割目标链，同时激活其对非特异性单链 DNA 的无差别切割能力[20]-[22]。这种反式切割特性使其可通过荧光报告分子输出检测信号，极大地简化了检测流程。在低浓度核酸诊断和现场快速检测等医学诊断中非常重要[22]。

2.1.3. CRISPR-Cas13 系统

Cas13 是 VI 型 CRISPR 系统的代表蛋白，是一种 RNA 引导的 RNA 靶向蛋白，即 Cas13 对单链 RNA 表现出特异性，能够特异性识别并切割 RNA 靶标[23]。与 Cas9 和 Cas12 不同，Cas13 不依赖 PAM 序列，大多数报道的 Cas13 家族的变体依赖原间隔子侧翼序列(protospacer flanking sequence, PFS)，且切割目标 RNA 链的同时具有非特异性反式切割单链 RNA (single-stranded RNA, ssRNA)的能力[24] [25]。这种特性使其在低浓度 RNA 检测和多路检测中表现出色。Cas13 系统的优势在于其对 RNA 的高效切割能力和灵活的应用设计，为基础研究与临床应用提供了强大的工具与平台[26] [27]，比如乙型肝炎病毒、埃博拉病毒和甲型流感病毒等检测[28]-[30]。

2.1.4. CRISPR-Cas14 系统

作为一种紧凑型 V 型核酸酶，Cas14 与 Cas12 类似，Cas14 既能特异性识别和切割双链 DNA，也能特异性识别并切割单链 DNA (ssDNA)，也具有高效切割 ssDNA 报告分子的反式切割活性[31] [32]，但对靶标分子的识别并不受限于双链 DNA 靶点的特定序列(即原间隔区邻近基序，PAM) [31]-[33]，使其在核酸适配子传感器的研发中更具潜力。因此，Cas14 的优势在于对 ssDNA 的高特异性和高灵敏性，尤其适用于单核苷酸多态性(SNP)检测[34] [35]。Zhao 等[36]使用 CRISPR/Cas14a 系统、G-四链体 DNA 酶和基于微流体的分析设备的组合开发了一种级联比色检测，可以检测低至 5 拷贝/μL 的 ASFV (African swine fever virus，非洲猪瘟病毒)，并以 2-nt 的差异区分野生型和突变型 ASFV DNA。上述几种 CRISPR/Cas 系统的主要特征见表 1。

Table 1. Main features of class II CRISPR/Cas systems
表 1. II 类 CRISPR/Cas 系统的主要特征

	Cas9	Cas12	Cas13	Cas14
靶标类型	dsDNA	dsDNA/ssDNA	ssRNA	dsDNA/ssDNA
向导 RNA	sgRNA	crRNA/sgRNA	crRNA	sgRNA
PAM	5'-NGG-3'	5'-TTTN-3'	PFS 序列 A/U/C-3'	—
顺式切割	有	有	有	有
反式切割	—	ssDNA	ssRNA	ssDNA

3. 医学诊断中的 CRISPR-Cas 系统

由于特异性识别、顺式切割和非特异性反式切割能力，CRISPR/Cas 系统已经实现了核酸靶标(DNA 和 RNA)和非核酸靶标(例如蛋白质、外泌体、细胞和小分子)的检测[37]。下面重点介绍了 CRISPR/Cas 系统在检测核酸和非核酸靶标方面的各种类型的应用。

3.1. 用于核酸靶标检测的 CRISPR-Cas 系统

CRISPR 系统不仅能够特异性识别并结合目标核酸序列，Cas12、Cas13 和 Cas14 等还具有反式切割活性，可以切割周围的单链核酸(ssDNA 或 ssRNA)报告分子从而产生并放大可检测的信号(如荧光信号)，使得 CRISPR 技术在核酸检测中表现出极高的灵敏度和特异性。传统的核酸诊断技术与 CRISPR/Cas 相结合，可用于实验室检测以及现场快速诊断(POCT)等医学诊断[33]。例如在耐药基因检测中，Lai 等[33]人研发了超快速 PCR 与 CRISPR/Cas14 结合的 Cas14VIDet (Cas14-based Visual Instant Detection)的平台，10 min 内完成检测幽门螺杆菌左氧氟沙星耐药基因(GyrA)，灵敏度接近单个细菌集落的水平(100 CFU/mL)。此外，在病毒基因检测中，Cas12a-DETECTR (DNA endonuclease targeted CRISPR trans reporter，DNA 核酸内切酶靶向 CRISPR 反式报告基因)系统结合重组酶聚合酶扩增(RPA)被用于检测 HPV16、HPV18 [38]以及 ASFV (African swine fever virus，非洲猪瘟病毒) [39]，检测限达 aM 水平；在此基础上结合了逆转录(RT)检测 H1N1 和 SARS-CoV-2 的病毒核酸[40]，检测限为 1~2.5 拷贝/μL，均可在不到 1 小时的时间完成检测。Cai 等人[41]研究了一种基于液滴配对－合并的数字 RPA-CRISPR/Cas12a (DIMERIC)检测方法，通过微流控芯片实现 RPA 和 CRISPR/Cas12a 反应的空间分离和时间优化，能够在 20 分钟内完成临床血清样本中乙型肝炎病毒 DNA 的定量检测。

此外，近年来已经提出了许多基于 CRISPR 的生物传感器用于 RNA 诊断的检测方法。MicroRNA (miRNA)和信使 RNA (mRNA)因其异常表达与癌症[42]、神经系统和心血管疾病密切相关而受到广泛关注[43]。Zhang 等人[44]提出了一种基于一锅法 Cas13a 的超灵敏微流控检测系统，用于包括 miR-21、miR-141、miR-196a 和 miR-1246 等 microRNA 的多重检测，实现对乳腺癌与肺癌的临床分析。Pei 等人[45]设计了一种称为哑铃探针(DP)桥接 Cas13a/NDCR 的新方法，在靶标 miRNA 存在的情况下，激活的 Cas13a 裂解了哑铃探针(DP)，导致被暴露的中间链与电极表面的亚甲基蓝标记发夹探针(MB-HP)杂交，MB-HP 从电极表面分离，从而引发电化学信号的变化。通过使用机器学习(ML)分析来自四种结直肠癌相关 miRNA (miRNA-17、miRNA-21、miRNA-182 和 miRNA-223)的电化学信号。

3.2. 用于非核酸靶标检测的 CRISPR-Cas 系统

基于 CRISPR 的诊断方法已被广泛用于检测多种分析物，包括蛋白质、抗生素、金属离子等非核酸

靶标的检测[37] [46]。在许多情况下，CRISPR 系统充当报告器或放大器，而不能直接识别感知非核酸靶标。因此，在非核酸靶标检测中，往往需要设计 ssDNA 或 RNA 分子探针，作为适配体与靶蛋白相互作用[47] [48]，进行生物转导。例如，Yue 等人[49]研发了 CRISPR/Cas14a 结合 DNA walker 的纳米生物传感器，借助核酸适体与蛋白的高亲和力，打开发夹，从而引发后面的信号扩增反应，实现对 HPV16 E7 蛋白的超灵敏检测，最低检测限为 67.17 fg/mL。Jia 等人[50]提出了一种简单、经济、便携的基于 CRISPR 技术的生物传感平台，通过设计 AFP 与 AFP 适配体结合后释放被适配体封闭的激活剂，从而促进下游酶促反应来实现血清样本中肿瘤标志物甲胎蛋白(AFP)的定量检测，最低检测限为 10 ng/mL。此外，依赖金属离子的 DNAzyme [51]-[53]、与 ELISA [54]结合的各种夹心策略等也被设计参与 CRISPR 生物传感器的识别环节，用于检测金属离子[51] [52]、碱性磷酸酶[53]、尿趋化因子配体 9 (CXCL9) [54]抗原、抗体的免疫测定也承担了生物转导功能。由于生物转导和 Cas 相关处理的复杂性和耗时，基于 CRISPR 的生物传感的非核酸靶标检测仍存在挑战，需要在分析设计和优化中进一步探索和完善。CRISPR/Cas 系统在检测核酸和非核酸靶标方面的最新进展见表 2。

Table 2. Overview of recent CRISPR-based biosensors in medical diagnosis
表 2. 医学诊断中基于 CRISPR 的最新生物传感器的概览

医学诊断	靶标	Cas 蛋白	检测限	读出检测器	参考文献
幽门螺杆菌	DNA	Cas14	1 CFU/mL	荧光分光光度计	[33]
早期癌症筛查	miRNA-21	Cas13a	4.34 aM	荧光分光光度计	[44]
早期癌症筛查	miRNA-21	Cas13a	8.26 fM	电化学工作站	[45]
人乳头瘤病毒	E7 蛋白	Cas14a	67.17 fg/mL	电化学工作站	[49]
早期癌症筛查	miRNA-21	Cas12a	3.43 aM	电化学工作站	[55]
人乳头瘤病毒	DNA	Cas12a	200 aM	肉眼观察	[56]
结核分枝杆菌	DNA	Cas12a	2.42 aM	场效应晶体管	[57]
病原菌	DNA	dCas9a	1 CFU/mL	拉曼光谱仪	[58]
急性心肌梗死	ATP	Cas12a	20 nM	荧光分光光度计	[59]
人腺病毒	DNA	Cas13a	2.5 拷贝/μL	肉眼观察	[60]
肺炎克雷伯菌	DNA	Cas12a	10 CFU/μL	肉眼观察	[61]
金黄色葡萄球菌	DNA	Cas12a	5 CFU/mL	荧光分光光度计	[62]
人乳头瘤病毒	DNA	Cas12a	1 aM	肉眼观察	[63]
汞中毒	汞离子	Cas12a	0.44 nM	荧光分光光度计	[64]

4. 结论与展望

综上所述，CRISPR 系统是医学诊断中重要的工具之一，在 DNA、RNA、蛋白质、金属离子等核酸靶标与非核酸靶标检测中被广泛应用，在肿瘤早筛、病毒检测、细菌检测等医学诊断中快速发展。CRISPR 系统克服了传统技术的缺点，提供了一种高度灵敏、经济高效且快速简便分子诊断方法，在临床即时检测展现出巨大潜力。

尽管 CRISPR 技术在生物标志物检测领域具有巨大的应用潜力，但它的广泛应用仍面临一些挑战，包括 PAM 序列识别的限制、对预扩增的依赖以及实现多重检测的困难。为应对这些问题，研究人员已经采取了多种策略：首先，例如分裂式 CRISPR-Cas12a、crRNA 的延长等工程化设计与研究使得开发不受 PAM 序列限制的 Cas 核酸酶成为可能。其次，CRISPR 技术在试管平台、微流体系统和纸质平台上的进一步研究有望简化分析流程，实现无需预扩增的快速即时检测。此外，通过将 CRISPR-Cas 系统与微流控技术、横向流动技术、信号逻辑门、多重 crRNA 设计等技术结合，有望实现单次检测中的多重指标检测。因此，尽管存在挑战，CRISPR 技术仍有望在即时检和多重检测等方面取得突破，从而迅速推动其在医学诊断领域的普及和发展。

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