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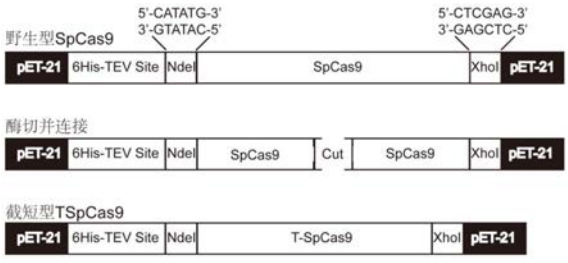
(54)发明名称

酿脓链球菌的CRISPR核酸酶SpCas9 的截短变异体及其应用

(57)摘要

本发明属于蛋白质工程技术领域，具体为来源于酿脓链球菌的CRISPR核酸酶SpCas9的截短变异体及其应用。本发明中，CRISPR-Cas9(TSpCas9)核酸酶属于CRISPR-Cas9系统，具有野生型CRISPR-Cas9核酸酶相当的剪切活性，所述CRISPR-Cas9(TSpCas9)核酸酶是将野生型CRISPR-Cas9核酸的氨基酸序列截掉120个即第180位到299位的氨基酸后重组所得；所述野生型CRISPR-Cas9核酸酶的氨基酸序列如SEQ ID NO.7所示；或者所述CRISPR-Cas9核酸酶含有如SEQ ID NO.7所示90%的氨基酸序列，即SEQ ID NO.15。该截短变异体可用于基因组编辑、基因打靶、基因组工程、表观基因组工程、基因治疗与体外诊断。



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1.一种CRISPR-Cas9核酸酶,属于CRISPR-Cas9系统,具有与野生型CRISPR-Cas9核酸酶相当的剪切活性,所述CRISPR-Cas9核酸酶是将野生型CRISPR-Cas9核酸酶的第180位到299位的氨基酸截掉后所得;所述野生型CRISPR-Cas9核酸酶来源于酿脓链球菌,其氨基酸序列如SEQ ID NO.7所示;或者所述CRISPR-Cas9核酸酶的氨基酸如SEQ ID NO.16所示。

2.一种多核苷酸序列,其编码权利要求1中所述的CRISPR-Cas9核酸酶。

3.一种表达载体,其特征在于含有如权利要求2所述的多核苷酸序列。

4.一种宿主细胞,其是经过权利要求3所述的表达载体转化后得到。

5.一种制备权利要求1所述的CRISPR-Cas9核酸酶的方法,其特征在于,包括以下步骤:首先,构建含有权利要求2所述的多核苷酸序列的表达载体;将所述表达载体转化宿主细胞,筛选并挑出单克隆;将筛选得到的单克隆细胞诱导表达,并通过亲和层析或/和离子交换方法从表达产物中分离出所述的CRISPR-Cas9核酸酶。

6.权利要求1所述的CRISPR-Cas9核酸酶、权利要求2中所述的多核苷酸序列或权利要求3中所述的表达载体作为基因编辑工具的用途,所述的用途不包括疾病的治疗与诊断。

7.根据权利要求6所述的用途,其特征在于,所述编辑是单点编辑,或者是编辑位点大于等于两个的多点编辑;所述编辑方式选自基因的删除、突变、插入、倒位、移位、重复或易位。

8.根据权利要求6所述的用途,其特征在于,所述基因编辑工具还包括与靶标DNA片段匹配的引导sgRNA。

9.根据权利要求6所述的用途,其特征在于,利用权利要求1所述的CRISPR-Cas9核酸酶与能够介导它的sgRNA组合,对基因进行编辑。

10.根据权利要求6所述的用途,其特性在于,将权利要求3所述的载体和与之匹配的引导sgRNA一同转入宿主细胞,对基因进行编辑。

11.根据权利要求7所述的用途,其特性在于,所述的单点或多点的基因编辑是利用权利要求1所述CRISPR-Cas9核酸酶对双链DNA进行剪切,并通过宿主细胞的修复系统对断裂的缺口进行修复。

酿脓链球菌的CRISPR核酸酶SpCas9 的截短变异体及其应用

技术领域

[0001] 本发明属于蛋白质工程技术领域，具体涉及来源于酿脓链球菌(Streptococcus pyogenes) 的CRISPR核酸酶SpCas9的蛋白质工程，及其在基因组编辑、基因组打靶、表观基因组工程和体外诊断中的应用。

背景技术

[0002] 由细菌免疫系统经漫长进化而产生的CRISPR-Cas9系统是一种革命性的基因编辑技术，被称为“基因魔剪”，可方便地对基因组特定基因的DNA链进行高效切割编辑，在生物医学领域有巨大的应用潜力，如进行基因组编辑、基因组打靶、表观基因组工程和体外诊断等[1-11]。

[0003] 来源于酿脓链球菌(Streptococcus pyogenes)的CRISPR-Cas9核酸酶SpCas9最目前最为广泛使用的CRISPR核酸酶[12]。截至目前，该技术也被广泛应用到多个领域，包括医学研究以及生物技术等，比如细胞或动物模型的构建快速、功能基因的筛选便捷以及部分遗传疾病的治疗彻底[13-24]。

[0004] 该系统的出现，大大克服了传统编辑技术耗时长的缺点，仅需数周就可以实现过去利用传统技术耗时一年之久才能完成的编辑[8]。除此之外，该技术在体内编辑不仅可以大规模、高特异地进行，而且还可以减少花费，节约成本。

[0005] 虽然，CRISPR-Cas9系统的优势促使其能够广泛应用于许多领域，并最近在医学领域的研究上也异军突起，但是，许多挑战仍然悬而未决，尤其是寻找一个适合并高效将CRISPR-Cas9 靶向特异性疾病组织的运载系统，至今成为临床应用的瓶颈问题。

[0006] 以现在的科学技术和认知，欲将感兴趣的基因靶向运载到体内或细胞当中，需要借助一些病毒载体，如腺病毒和慢病毒(具有致癌性)等。不过，腺病毒存在的不足(如缺少高效的包装细胞，制备复杂，滴度低的缺陷)，尤其装载量较小(小于4.7kb)的自身特性限制了将编码区大于4kp的SpCas9+sgRNA靶向运载到体内和细胞的靶标位置[25]。因此，基于这个问题，考虑将SpCas9缩小到适合的大小，以实现腺病毒将小型的SpCas9装载，并高效靶向运载到靶位点，从而以期作为临床医学治疗上的优势工具，具有极大潜在医学价值。

发明内容

[0007] 本发明的目的是提供一种来源于酿脓链球菌的CRISPR-Cas9核酸酶SpCas9的截短变异体及其用途。

[0008] 为实现上述目的，本发明采用以下技术方案。

[0009] 本发明提供一种体积比野生型小的CRISPR-Cas9核酸酶(TSpCas9)，其是将野生型的 SpCas9第180位到299位氨基酸截掉所得，属于CRISPR-Cas9系统，具有野生型CRISPR-Cas9 (SpCas9)相当的剪切活性。

[0010] 所述野生型SpCas9核酸酶的核苷酸序列和氨基酸序列分别为SEQ ID NO.6和SEQ ID NO.7 所示。

[0011] 所述CRISPR-Cas9核酸酶(TSpCas9)的核苷酸序列和氨基酸序列分别为SEQ ID NO.15和SEQ ID NO.16所示,与野生型SpCas9的相似度达90%以上。

[0012] 本发明还提供一种多核苷酸序列,可以转录和翻译所述的CRISPR-Cas9核酸酶(TSpCas9)。

[0013] 本发明还提供一种表达载体,其含有上述多核苷酸序列。

[0014] 本发明还提供一种宿主细胞,可以用于转化上述表达载体。

[0015] 本发明还提供一种制备所述CRISPR-Cas9核酸酶(TSpCas9)的方法,具体步骤包括:首先,构建所述CRISPR-Cas9核酸酶的多核苷酸序列表达载体;然后,将所述表达载体转化至宿主细胞,筛选并挑出单克隆;最后,将所述单克隆诱导表达,并通过亲和层析、离子交换等方法从表达产物中分离出所述的CRISPR-Cas9核酸酶。

[0016] 本发明还提供上述CRISPR-Cas9核酸酶、多核苷酸序列以及表达载体均可作为基因组编辑工具用途,用于基因组DNA片段的相关编辑。

[0017] 本发明中所述的编辑可以是单点编辑、也可以是编辑位点大于等于两个的多点编辑。

[0018] 所述编辑手段包括删除、突变、插入、倒位、移位、重复或易位。

[0019] 所述CRISPR-Cas9编辑工具包括与靶标DNA片段匹配的引导sgRNA。

[0020] 所述的CRISPR-Cas9核酸酶与能够介导它的sgRNA组合,能够对基因进行编辑。

[0021] 与现有技术相比,本发明的CRISPR-Cas9(TSpCas9),属于CRISPR-Cas9免疫系统,含有如:SEQ ID NO.15和SEQ ID NO.16的核苷酸序列和氨基酸序列,其体积比野生型CRISPR-Cas9 (SpCas9)小120个氨基酸,剪切DNA的活性与野生型CRISPR-Cas9(SpCas9)相当,有望方便腺病毒将其运载到细胞和动物体的靶位点,具有潜在的生物医学应用价值。本发明截短变异体可用于基因组编辑、基因打靶、基因组工程、表观基因组工程、基因治疗与体外诊断。

附图说明

[0022] 图1.含有TSpCas9质粒的构建策略。

[0023] 图2.含有TSpCas9的质粒转化及单克隆筛选培养。

[0024] 图3.含有TSpCas9的质粒筛选。

[0025] 图4.含有TSpCas9的质粒测序。

[0026] 图5.TSpCas9目标蛋白的纯化方法。

[0027] 图6.TSpCas9目标蛋白纯化获取的过程。

[0028] 图7.TSpCas9目标蛋白的电泳鉴定。

[0029] 图8.引导sgRNA的在靶和脱靶序列。

[0030] 图9.野生型SpCas9和截短型TSpCas9体外剪切活性的检测。

[0031] 图10.野生型SpCas9体外脱靶效应的检测。

[0032] 图11.截短型TSpCas9体外脱靶效应的检测。

具体实施方式

[0033] 下述实施例中所用的实验方法,如无特定说明,均为常规方法。

[0034] 下述实施例中所用的材料、试剂等，如无特定说明，均为从商业途径获得。

[0035] 一、CRISPR-Cas9核酸酶

[0036] 本发明的CRISPR-Cas9核酸酶(TSpCas9)，其是将野生型的SpCas9第180位到299位氨基酸截掉所得，属于CRISPR-Cas9系统，具有野生型CRISPR-Cas9(SpCas9)相当的剪切活性；

[0037] 所述CRISPR-Cas9核酸酶(TSpCas9)含有如SEQ ID NO.16所示的氨基酸序列。

[0038] 二、编码CRISPR-Cas9核酸酶的多核苷酸

[0039] 转录和翻译所述CRISPR-Cas9(TSpCas9)的多核苷酸序列，包括DNA或RNA。DNA还可以细分为质粒DNA、基因组DNA或人工合成的DNA；

[0040] 编码所述CRISPR-Cas9(TSpCas9)的多核苷酸序列，可以利用该领域科研或技术人员所熟悉的相关分子生物学技术来制备，其不局限于重组DNA技术和化学合成方法。

[0041] 三、表达载体

[0042] 所述表达载体含有编码所述CRISPR-Cas9核酸酶(TSpCas9)的多核苷酸序列。该表达载体可以通过科研或技术人员所熟悉的分子生物学方法来构建，包括DNA重组技术和DNA合成技术等，主要将CRISPR-Cas9核酸酶(TSpCas9)的DNA有效连接到载体上的多克隆位点中，然后通过转录翻译等过程表达目的蛋白TSpCas9。

[0043] 四、宿主细胞

[0044] 所述宿主细胞可以用于表达CRISPR-Cas9核酸酶的重组质粒所转化。宿主细胞主要包括原核细胞(如细菌)，低等真核细胞(如酵母)，高等真核细胞(如哺乳动物细胞)等。常用的宿主细胞如大肠杆菌DH5α、毕赤酵母、HEK293、CHO、Hela细胞等。

[0045] 五、CRISPR-Cas9核酸酶(TSpCas9)及其编码该酶的核苷酸序列和所述表达载体的用途

[0046] 本发明的CRISPR-Cas9核酸酶(TSpCas9)及其编码该酶的多核苷酸序列和所述的表达载体能够用于基因组DNA片段的编辑或用于制备基因编辑工具。CRISPR-Cas9核酸酶(TSpCas9) 编辑包括单位点和多位点编辑，其编辑手段包括删除、突变、插入、倒位、移位、重复或易位等。

[0047] 六、基因编辑工具及其方法

[0048] 本发明的基因编辑工具属于CRISPR-Cas9系统，CRISPR-Cas9(TSpCas9)在特定的sgRNA 的引导下可以在目的基因DNA片段PAM(NGG)位点上游3到4位间剪切底物DNA片段。该编辑过程可以在体内或体内进行。当sgRNA是单个的时候可以进行单点编辑，当sgRNA是两个或两个以上时可以进行多位点编辑。

[0049] 如本发明的一些实施方式中所列举的，CRISPR-Cas9核酸酶(TSpCas9)在sgRNA的引导下，可以在体外对底物DNA(920bp)进行剪切，其产物为760bp和260bp。

[0050] 在本发明中，Cas9可作为CRISPR-Cas9核酸酶的简称使用，其含义与CRISPR-Cas9核酸酶相同。本发明中的截短突变体蛋白为TSpCas9，即对野生型SpCas9第180位氨基酸到299 位氨基酸对应的质粒片段去掉，将剩余部分氨基酸的质粒重组连接并表达得到的蛋白。

[0051] 在进一步描述本发明具体实施方式之前，应理解，本发明的保护范围并不局限为下述特定的具体实施方案，还应理解为，本发明实施例中的术语是为了描述特定的具体实

施方案,而不是为了限制本发明的保护范围。下例实施例中未注明具体条件的试验方法,tongchang按照常规条件操作,或者按照各生产厂商所建议的条件操作。

[0052] 当实施例给出数值范围时,应理解,除非本发明另有说明,每个数值范围的两个端点以及两个端点之间任何一个数值均可选用。除非另外定义,本发明中使用的所有技术和科学术语与本技术领域技术人员通常理解的意义相同。除实施例中使用的具体方法、设备、材料外,根据本技术领域的技术人员对现有技术的掌握及本发明的记载,还可以使用与本发明实施例中所述的方法、设备、材料相似或等同的现有技术的任何方法、设备和材料来实现本发明。

[0053] 除非另外说明,本发明中所公开的实验方法、检测方法、制备方法均采用本技术领域常规的分子生物学、生物化学、重组DNA技术及相关领域的常规技术。

[0054] 实施例1,构建CRISPR-Cas9核酸酶(TSpCas9)的质粒。

[0055] 1.突变体的设计。

[0056] 以pet21-6His-TEV-SpCas9质粒,即SEQ ID NO.5为模板,截掉180aa(5822bp)～299aa (6181bp),相当于碱基序列SEQ ID NO.8和氨基酸序列SEQ ID NO.9,把剩下部分重组成截短的SpCas9,将其称为TSpCas9。其改造设计思路如图1所示,其详细步骤简述如下:

[0057] 首先,利用引物F-F和F-R,S-F和S-R分别在质粒Pet21-6His-TEV-SpCas9(相当于SEQ ID NO.5)上扩增5267～5821bp(相当于SEQ ID NO.10的1-538bp)和6182～9391bp(相当于 SEQ ID NO.12的898-4104bp),并通过AxyPrep™ DNA Gel Extraction Kit(购于Axygen) 纯化回收,将它们称为扩增片段F和S;

[0058] 其次,使用限制性内切酶NdeI和XhoI(购于NEB)分别酶切pet21-6His-TEV-SpCas9和F扩增片段,并通过AxyPrep™ DNA Gel Extraction Kit纯化回收;

[0059] 再次,使用T4ligase(购于Takara)将上一步纯化回收的两个片段连接,将此连接产物称为pet21-6His-TEV-F;

[0060] 最后,用XhoI和NotI(购于NEB)分别酶切pet21-6His-TEV-F和S扩增片段,并通过AxyPrep™ DNA Gel Extraction Kit纯化回收,T4ligase连接,即可获得目的产物TSpCas9。

[0061] (1)采购扩增试剂盒

[0062] 所用扩增试剂盒Fast HiFidelity PCR Kit从天根生化科技(北京)有限公司订购。

[0063] (2)采购引物

[0064] 所用引物均从上海生工生物工程有限公司订购,引物的5'端均加有所需的酶切位点和该酶切位点的保护碱基。它们的序列如下:

[0065] 扩增5267-5821bp的正反引物:

[0066] F:GGAATTCCATATGGAAAATCTCTACTTCCAAG(SEQ ID NO.1)

[0067] R:CCGCTCGAGCGGCCGCTGTTGTCTGGGTTCAGGT(SEQ ID NO.2)

[0068] 扩增6182-9391bp的正反引物:

[0069] F:ATAAGAATGCGGCCGCATTCTGCTGAGTGATATTCTGCG(SEQ ID NO.3)

[0070] R:CCGCTCGAGTCAGTCTCCACCGAGCTGAG(SEQ ID NO.4)。

[0071] 扩增体系如下:

[0072]

扩增Pet21-SpCas9（SEQ ID NO.10）第5267到5821 bp	
组分	体积（μl）
模板Pet21-SpCas9（SEQ ID NO.5）	0.5 （~ 50 ng）
正向引物F（SEQ ID NO.1）	2.5
反向引物R（SEQ ID NO.2）	2.5
缓冲液5×Fast HiFidelity PCR Buffer	10
20×Fast PCR Enhancer	2.5
Fast HiFidelity Polymerase	1
ddH2O	31

。

[0073] PCR反应条件：

[0074]

扩增Pet21-SpCas9（SEQ ID NO.10）第5267到5821 bp			
过程	温度（℃）	时间（sec）	循环数（cycles）
预变性	94	120	1

[0075]

变性	94	15	38
退火	60	10	
延伸	68	30	
延伸修复	68	300	1

。

[0076] 扩增体系如下：

[0077]

扩增Pet21-SpCas9（SEQ ID NO.12）第6182到9391 bp	
组分	体积（μl）
模板Pet21-SpCas9（SEQ ID NO.5）	0.5 （~ 50 ng）
正向引物F（SEQ ID NO.1）	2.5
反向引物R（SEQ ID NO.2）	2.5
缓冲液5×Fast HiFidelity PCR Buffer	10
20×Fast PCR Enhancer	2.5
Fast HiFidelity Polymerase	1
ddH2O	31

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[0078] PCR反应条件：

[0079]

扩增Pet21-SpCas9（SEQ ID NO.12）第6182到9391 bp			
过程	温度（℃）	时间（sec）	循环数（cycles）
预变性	94	120	1
变性	94	15	38
退火	60	10	
延伸	68	120	
延伸修复	68	300	1

。

[0080] (3)割胶回收试剂盒采购

[0081] 所用割胶回收试剂盒AxyPrep™ DNA Gel Extraction Kit从Axygen公司(公司链接)订购,割胶回收操作均按其说明书进行。

[0082] (4)限制内切酶及T4连接酶

[0083] 所用限制内切酶NdeI、XhoI、NotI和T4连接酶均从NEB公司订购(公司链接)订购。

[0084] 酶切反应体系

[0085]

F segment （SEQ ID NO.10）的酶切		Pet21-SpCAS9 （SEQ ID NO. 5）的酶切	
组分	体积（μl）	组分	体积（μl）
NEB Buffer 3.1	5	NEB Buffer 3.1	5
NdeI	2	NdeI	2
XhoI	2	XhoI	2
片段DNA	40 （~ 1μg）	质粒DNA	40 （~ 1μg）
ddH2O	1	ddH2O	1

[0086] 按该反应体系37℃孵育至少2小时,并电泳割胶纯化回收。

[0087] 链接反应体系

[0088]

酶切的F segment （SEQ ID NO.10）与Pet21-SpCAS9 （SEQ ID NO. 5）链接重组	
组分	体积（μl）
T4连接酶	1
已酶切的F segment （SEQ ID NO.10）	1.5（200 ng）
已酶切的Pet21-SpCAS9 （SEQ ID NO.5）	6.5 （150 ng）
10×T4 DNA Ligase Buffer	1

。

[0089] 该反应体系16℃孵育至少2小时,并转化大肠杆菌DH5α筛选单克隆pet21-6His-TEV-F,如图2所示,其核苷酸序列为SEQ ID NO.14,培养细菌,抽提质粒,抽提方法见Qiagen质粒小提试剂盒使用说明。

[0090]

S segment （SEQ ID NO.12）的酶切		pet21-6His-TEV-F （SEQ ID NO.14）的酶切	
组分	体积（μl）	组分	体积（μl）
NEB Buffer 3.1	5	NEB Buffer 3.1	5
NdeI	2	NdeI	2
NotI	2	NotI	2
片段DNA	40 （~ 1μg）	质粒DNA	40 （~ 1μg）
ddH2O	1	ddH2O	1

。

[0091] 链接反应体系

[0092]

酶切的S segment （SEQ ID NO.12）与pet21-6His-TEV-F （SEQ ID NO.14）链接重组	
组分	体积（μl）

[0093]

T4连接酶	1
已酶切的S segment （SEQ ID NO.12）	1.5 （200 ng）
已酶切的pet21-6His-TEV-F （SEQ ID NO.14）	6.5 （150 ng）
10×T4 DNA Ligase Buffer	1

。

[0094] 该反应体系16℃孵育至少2小时，并转化大肠杆菌DH5α筛选单克隆TSpCas9，如图2所示，其核苷酸序列和氨基酸分别为序列为SEQ ID NO.15和SEQ ID NO.16。

[0095] 其筛选结果如图3示，通过常规质粒抽提方法获取12个样品质粒(1到12号)，其大小应该在参照质粒1(SEQ ID NO.5)和2(SEQ ID NO.14)之间，利用琼脂糖凝胶电泳检测，我们发现样品质粒8和10符合要求；随后，通过一代测序，发现样品质粒10构建正确，其结果如图4所示。

[0096] 实施例2，制备CRISPR-Cas9(TSpCas9)核酸酶。

[0097] 2.蛋白表达与纯化。

[0098] 2.1蛋白表达

[0099] (1)打开超净台，用含75％酒精的棉球擦拭桌面以及各种器具，开紫外灯照射20min，启动风机备用；

[0100] (2)移液枪吸10μl表达Pet21-6His-TEV-TSpCas9的Rosetta(DE3)(购于TIANGEN)菌液转至6ml含有双抗(Amp与Cm)的LB液体培养基中，37℃，200r/min振荡培养过夜；

[0101] (3)将过夜培养的菌液按照体积比为1:100转至500ml含双抗的LB(购于生工)液体培养基中，37℃，200r/min振荡培养。在培养过程中，随时检测菌液的OD值；

[0102] (4)当菌液的OD值接近0.4～0.8时，加入蛋白诱导剂IPTG，使其终浓度为0.1mM，然后16℃，200r/min振荡培养20h；

[0103] (5)收集菌液，5000r/min离心5min使菌体沉淀，弃上清，并称重 Pet21-6His-TEV-

TSpCas9菌体。

[0104] 2.2蛋白纯化

[0105] 所述蛋白纯化主要通过镍柱亲和层析技术,如图5所示;其纯化过程包括菌体破碎、蛋白样品离心收集、蛋白样品与镍柱介质共孵育以及目的蛋白的洗脱等,如图6所示。其详细步骤简述如下:

[0106] (1)向菌体中加入预先冰浴且PMSF终浓度为0.1mM的裂解液(20mM HEPES,500mMKCl,pH7.5;1g菌体加入5ml),涡旋仪重悬使菌块分散混匀,细胞超声破碎仪破碎细胞,超声3 sec停3sec,一次10min,超声两次,超声过程均在冰浴中进行;

[0107] (2)向破碎的菌液中加入终浓度为10μg/ml RNase(生工),5μg/ml DNase I(生工),冰浴处理30min后,4℃10000r/min离心45～60min,收集上清;

[0108] (3)将上清与预先用平衡液(20mM HEPES,500mMKCl,1％蔗糖,pH7.5)处理的Qiagen Ni-NTA介质孵育,此过程在冰浴上进行,并加以振荡(150r/min),1.5h后静置,待Qiagen Ni-NTA 沉淀;

[0109] (4)将Qiagen Ni-NTA装载到重力柱中,BioLogic LP系统的监测下,分别以流速为2 ml/min的平衡液和洗脱液(20mM HEPES,500mMKCl,500mM咪唑,1％蔗糖,pH7.5), 20、30、40、50、100、250、500Mm洗脱液冲洗Qiagen Ni-NTA,并收集蛋白;

[0110] (5)将不同咪唑浓度下的蛋白溶液跑SDS-PAGE(购于EpiZyme Scientific)电泳,考马斯亮蓝染色,脱色剂脱色,观察目的蛋白的表达和挂柱效果。

[0111] 所述蛋白TSpCas9的纯化结果如图7所示,其显示该目的蛋白TSpCas9的表达和纯化情况,从上清液观察,目的蛋白TSpCas9表达较多,从穿流液观察,Ni-NTA吸附的能力适中,从洗脱液观察,50和100mM咪唑洗脱液下的目的蛋白TSpCas9相对较纯,可以收集浓缩做活性检测。

[0112] 实施例3,检验CRISPR-Cas9(TSpCas9)核酸酶剪切活性。

[0113] 3.突变体活性检测。

[0114] 所用底物DNA(SEQ ID NO.20),主要利用引物QG-F:TAGTCCTGTCGGGTTTCG(SEQ ID NO.17) 和QG-R:TTCCATTCGCCATTCAGG(SEQ ID NO.18)通过常规PCR扩增获取。其反应体系和扩增条件如下:

[0115] 扩增体系如下:

[0116]

扩增Psk-sgRNA（SEQ ID NO.19）第1167到2586 bp	
组分	体积（μl）
模板Pet21-SpCas9（SEQ ID NO.19）	0.5 （~ 50 ng）
正向引物F（SEQ ID NO.17）	2.5
反向引物R（SEQ ID NO.18）	2.5
缓冲液5×Fast HiFidelity PCR Buffer	10
20×Fast PCR Enhancer	2.5
Fast HiFidelity Polymerase	1
ddH2O	31

。

[0117] PCR反应条件：

[0118]

扩增Psk-sgRNA（SEQ ID NO.19）第1167到2586 bp			
过程	温度（℃）	时间（sec）	循环数（cycles）
预变性	94	120	1
变性	94	15	38
退火	60	10	
延伸	68	30	
延伸修复	68	300	1

。

[0119] (3)割胶回收试剂盒采购

[0120] 所用割胶回收试剂盒AxyPrep™ DNA Gel Extraction Kit从Axygen公司订购，割胶回收操作均按其说明书进行，可以获得较纯的底物DNA(SEQ ID NO.20)。

[0121] Cas9与sgRNA以等摩尔混合，而根据实验需要，底物DNA可调为Cas9摩尔质量的0.2～1 倍。其反应体系如下

[0122]

10 μl体系		
成分	体积（μl）	终浓度（nM）
2 × 体外测活液	5	
各Cas9相关蛋白	2 （约300 ng）	200
sgRNA （图8中的0号）	1 （约75 ng）	200
底物DNA （SEQ ID NO.20）	1 （约150 ng）	30
RNase Inhabitor	0.25（10 U）	
去核酶ddH20	补至10 μl	

。

[0123] 将反应体系置37℃孵育，1h后70℃作用10min，最后琼脂糖凝胶电泳检测评价目的蛋白TSpCas9的体外切割活性。其结果如图9所示，从图9可以发现，与野生型SpCas9相比，截短型TSpCas9在体外能够剪切底物DNA(SEQ ID NO.20)(泳道4)，生成产物1和产物2，其剪切情况与野生型SpCas9基本一致，由此说明截短型TSpCas9的剪切活性和野生型SpCas9 的剪切活性相当。虽然TSpCas9在体外的DNA剪切活性并不比野生型SpCas9的强，但是其体积在小型化上却比野生型SpCas9有优势，即我们的TSpCas9体积比野生型SpCas9小，对方便腺病毒AAV运输而言，比野生型SpCas9有较大优势。

[0124] 实施例4，CRISPR-Cas9(TSpCas9)体外脱靶检测的评价方法

[0125] 4.脱靶效应检测

[0126] 利用不同的sgRNA，如图8中1到8号所示，引导TSpCas9的体外切割活性来评价TSpCas9 的脱靶效应，其反应体系如下：

[0127]

10 μl体系		
成分	体积（μl）	终浓度（nM）
2 × 体外测活液	5	
各Cas9相关蛋白	2 （约300 ng）	200
sgRNA （图8中的1-8号）	1 （约75 ng）	200
底物DNA （SEQ ID NO.20）	1 （约150 ng）	30
RNase Inhabitor	0.25（10 U）	
去核酶ddH20	补至10 μl	

。

[0128] 首先，评价野生型SpCas9在体外的脱靶效应，如图10所示，与0号sgRNA引导的SpCas9 剪切活性相比(泳道3)，1到8号sgRNA引导的SpCas9均能够在体外剪切底物DNA(SEQ ID NO.20)(泳道4到11)，生成产物1和产物2。尽管只有部分sgRNA的引导活性强，即1到4 号sgRNA引导的SpCas9体外剪切活性比较强(泳道4到7)，5到8号sgRNA引导的SpCas9 体外剪切活性却依次减弱(8号到11号)，但是，该结果依然反应野生型SpCas9在体外的脱靶效应比

较严重。由此说明，野生型SpCas9有较强的脱靶效应，尤其在1到4号sgRNA的引导下更为突出。

[0129] 其次，评价截短型TSpCas9在体外的脱靶效应，如图11所示，与0号sgRNA引导的TSpCas9 剪切活性相比(泳道3)，1到8号sgRNA引导的截短型TSpCas9均能够在体外剪切底物DNA(SEQ ID NO.20)(泳道4到11)，生成产物1和产物2。虽然1到8号引导sgRNA整体上引导截短型TSpCas9的体外剪切活性比0号sgRNA引导的弱，但是该结果也不能排除截短型TSpCas9的脱靶效应减弱。由此说明截短型TSpCas9依然存在脱靶效应。

[0130] 虽然，在脱靶效应上，截短型TSpCas9并不比野生型SpCas9优，但是，就小型化而言，我们获得的截短型TSpCas9的体积比野生型SpCas9小，方便腺病毒AAV运输，比野生型SpCas9 有较大优势。这对将来CRISPR-Cas9系统方便应用到临床医学上提供了潜在的价值。

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[0001] 序列表

[0002] <110> 复旦大学

[0003] <120> 酿脓链球菌的CRISPR核酸酶SpCas9 的截短变异体及其应

[0004] <130> 001

[0005] <160> 20

[0006] <170> SIPOSequenceListing 1.0

[0007] <210> 1

[0008] <211> 32

[0009] <212> DNA

[0010] <213> 人工序列(Artificial Sequence)

[0011] <400> 1

[0012] ggaattccat atggaaaatc tctacttcca ag 32

[0013] <210> 2

[0014] <211> 34

[0015] <212> DNA

[0016] <213> 人工序列(Artificial Sequence)

[0017] <400> 2

[0018] ccgctcgagc ggccgctgtt gtctgggttc aggt 34

[0019] <210> 3

[0020] <211> 39

[0021] <212> DNA

[0022] <213> 人工序列(Artificial Sequence)

[0023] <400> 3

[0024] ataagaatgc ggccgcattc tgctgagtga tattctgcg 39

[0025] <210> 4

[0026] <211> 29

[0027] <212> DNA

[0028] <213> 人工序列(Artificial Sequence)

[0029] <400> 4

[0030] ccgctcgagt cagtctccac cgagctgag 29

[0031] <210> 5

[0032] <211> 9553

[0033] <212> DNA

[0034] <213> 人工序列(Artificial Sequence)

[0035] <400> 5

[0036] tggcgaatgg gacgcgccct gtagcggcgc attaagcgcg gcgggtgtgg tggttacgcg 60

[0037] cagcgtgacc gctacacttg ccagcgccct agcgcccgct cctttcgctt tcttcccttc 120

[0038] ctttctcgcc acgttcgccg gctttccccg tcaagctcta aatcgggggc tccctttagg 180

[0039] gttccgattt agtgctttac ggcacctcga ccccaaaaaa cttgattagg gtgatggttc 240

[0040] acgtagtggg ccatcgccct gatagacggt ttttcgccct ttgacgttgg agtccacgtt 300

[0041] ctttaatagt ggactcttgt tccaaactgg aacaacactc aaccctatct cggtctattc 360

[0042] ttttgattta taagggattt tgccgatttc ggcctattgg ttaaaaaatg agctgattta 420
[0043] acaaaaattt aacgcgaatt ttaacaaaat attaacgttt acaatttcag gtggcacttt 480
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[0046] gagtattcaa catttccgtg tcgcccttat tccctttttt gcggcatttt gccttcctgt 660
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[0057] ggcccttccg gctggctggt ttattgctga taaatctgga gccggtgagc gtgggtctcg 1320
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[0277] Gly Trp Ala Val Ile Thr Asp Glu Tyr Lys Val Pro Ser Lys Lys Phe
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[0279] Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile
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[0281] Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
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[0295] Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro

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[0303] Leu Ile Ala Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn

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[0305] Leu Ile Ala Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe

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[0307] Asp Leu Ala Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp

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[0309] Asp Asp Leu Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp

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[0313] Ile Leu Arg Val Asn Thr Glu Ile Thr Lys Ala Pro Leu Ser Ala Ser

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[0327] Gly Glu Leu His Ala Ile Leu Arg Arg Gln Glu Asp Phe Tyr Pro Phe

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[0329] Leu Lys Asp Asn Arg Glu Lys Ile Glu Lys Ile Leu Thr Phe Arg Ile

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[0335] Val Val Asp Lys Gly Ala Ser Ala Gln Ser Phe Ile Glu Arg Met Thr

[0336] 485 490 495

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[0369] Arg His Lys Pro Glu Asn Ile Val Ile Glu Met Ala Arg Glu Asn Gln

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[0377] Gln Asn Gly Arg Asp Met Tyr Val Asp Gln Glu Leu Asp Ile Asn Arg

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[0379] Leu Ser Asp Tyr Asp Val Asp His Ile Val Pro Gln Ser Phe Leu Lys

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[0409] Val Trp Asp Lys Gly Arg Asp Phe Ala Thr Val Arg Lys Val Leu Ser

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[0412] 1090 1095 1100

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[0415] Ala Arg Lys Lys Asp Trp Asp Pro Lys Lys Tyr Gly Gly Phe Asp Ser

[0416] 1125 1130 1135

[0417] Pro Thr Val Ala Tyr Ser Val Leu Val Val Ala Lys Val Glu Lys Gly

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[0419] Lys Ser Lys Lys Leu Lys Ser Val Lys Glu Leu Leu Gly Ile Thr Ile

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[0423] Lys Gly Tyr Lys Glu Val Lys Lys Asp Leu Ile Ile Lys Leu Pro Lys

[0424] 1185 1190 1195 1200

[0425] Tyr Ser Leu Phe Glu Leu Glu Asn Gly Arg Lys Arg Met Leu Ala Ser

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[0431] Pro Glu Asp Asn Glu Gln Lys Gln Leu Phe Val Glu Gln His Lys His

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[0433] Tyr Leu Asp Glu Ile Ile Glu Gln Ile Ser Glu Phe Ser Lys Arg Val

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[0439] Phe Thr Leu Thr Asn Leu Gly Ala Pro Ala Ala Phe Lys Tyr Phe Asp

[0440] 1315 1320 1325

[0441] Thr Thr Ile Asp Arg Lys Arg Tyr Thr Ser Thr Lys Glu Val Leu Asp

[0442] 1330 1335 1340

[0443] Ala Thr Leu Ile His Gln Ser Ile Thr Gly Leu Tyr Glu Thr Arg Ile

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[0445] Asp Leu Ser Gln Leu Gly Gly Asp

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[0448] <211> 360

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[0453] ccgatcaacg catccggagt tgacgccaaa gcaatcctga gcgctaggct gtccaaatcc 120

[0454] cggcggctcg aaaacctcat cgcacagctc cctggggaga agaagaacgg cctgtttggt 180

[0455] aatcttatcg ccctgtcact cgggctgacc cccaacttta aatctaactt cgacctggcc 240

[0456] gaagatgcca agcttcaact gagcaaagac acctacgatg atgatctcga caatctgctg 300

[0457] gcccagatcg gcgaccagta cgcagacctt tttttggcgg caaagaacct gtcagacgcc 360

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[0466] 20 25 30

[0467] Leu Ser Ala Arg Leu Ser Lys Ser Arg Arg Leu Glu Asn Leu Ile Ala

[0468] 35 40 45

[0469] Gln Leu Pro Gly Glu Lys Lys Asn Gly Leu Phe Gly Asn Leu Ile Ala

[0470] 50 55 60

[0471] Leu Ser Leu Gly Leu Thr Pro Asn Phe Lys Ser Asn Phe Asp Leu Ala

[0472] 65 70 75 80

[0473] Glu Asp Ala Lys Leu Gln Leu Ser Lys Asp Thr Tyr Asp Asp Asp Leu

[0474] 85 90 95

[0475] Asp Asn Leu Leu Ala Gln Ile Gly Asp Gln Tyr Ala Asp Leu Phe Leu

[0476] 100 105 110

[0477] Ala Ala Lys Asn Leu Ser Asp Ala

[0478] 115 120

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[0487] gccacgcggc tcaaaagaac agcacggcgc agatataccc gcagaaagaa tcggatctgc 240

[0488] tacctgcagg agatctttag taatgagatg gctaaggtgg atgactcttt cttccatagg 300

[0489] ctggaggagt ccttttggt ggaggaggat aaaaagcacg agcgccaccc aatctttggc 360

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[0491] aagcttgtag acagtactga taaggctgac ttgcggttga tctatctcgc gctggcgcat 480

[0492] atgatcaaat ttcggggaca cttcctcatc gaggggacc tgaacccaga caacagc 537

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[0502] Lys Val Leu Gly Asn Thr Asp Arg His Ser Ile Lys Lys Asn Leu Ile

[0503] 35 40 45

[0504] Gly Ala Leu Leu Phe Asp Ser Gly Glu Thr Ala Glu Ala Thr Arg Leu
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[0506] Lys Arg Thr Ala Arg Arg Arg Tyr Thr Arg Arg Lys Asn Arg Ile Cys
[0507] 65 70 75 80
[0508] Tyr Leu Gln Glu Ile Phe Ser Asn Glu Met Ala Lys Val Asp Asp Ser
[0509] 85 90 95
[0510] Phe Phe His Arg Leu Glu Glu Ser Phe Leu Val Glu Glu Asp Lys Lys
[0511] 100 105 110
[0512] His Glu Arg His Pro Ile Phe Gly Asn Ile Val Asp Glu Val Ala Tyr
[0513] 115 120 125
[0514] His Glu Lys Tyr Pro Thr Ile Tyr His Leu Arg Lys Lys Leu Val Asp
[0515] 130 135 140
[0516] Ser Thr Asp Lys Ala Asp Leu Arg Leu Ile Tyr Leu Ala Leu Ala His
[0517] 145 150 155 160
[0518] Met Ile Lys Phe Arg Gly His Phe Leu Ile Glu Gly Asp Leu Asn Pro
[0519] 165 170 175
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[1200] cgcaattaat gtgagttagc tcactcatta ggcaccccag gctttacact ttatgcttcc 420
[1201] ggctcgtatg ttgtgtggaa ttgtgagcgg ataacaattt cacacaggaa acagctatga 480
[1202] ccatgattac gccaagctcg aaattaaccc tcactaaagg gaacaaaagc tggagctcca 540
[1203] ccgcggtggc ggccgctcta gaactagtgg atcccccggg ctgcaggaat tcgatatcaa 600
[1204] gcttatcgat taccgctcca gtcgttcatg aggttagagc tagaaatagc aagttaaaat 660
[1205] aaggctagtc cgttatcaac ttgaaaaagt ggcaccgagt cggtgctctc gagggggggc 720
[1206] ccggtaccca attcgcccta tagtgagtcg tattacaatt cactggccgt cgttttacaa 780
[1207] cgtcgtgact gggaaaaccc tggcgttacc caacttaatc gccttgcagc acatcccccc 840
[1208] ttcgccagct ggcgtaatag cgaagaggcc cgcaccgatc gcccttccca acagttgcgc 900
[1209] agcctgaatg gcgaatggaa 920

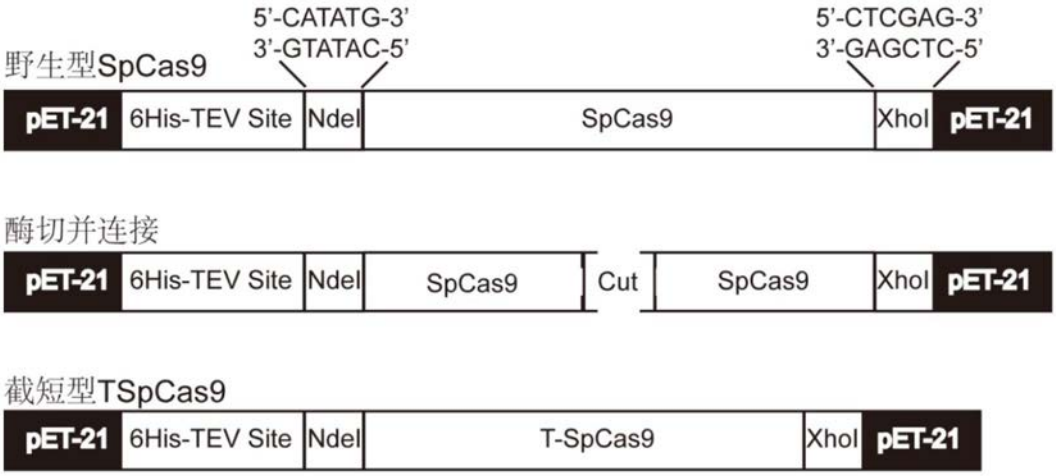
野生型SpCas9
5'-CATATG-3'
3'-GTATAC-5'
5'-CTCGAG-3'
3'-GAGCTC-5'
pET-21
6His-TEV Site
NdeI
SpCas9
XhoI
pET-21
酶切并连接
pET-21
6His-TEV Site
NdeI
SpCas9
Cut
SpCas9
XhoI
pET-21
截短型TSpCas9
pET-21
6His-TEV Site
NdeI
T-SpCas9
XhoI
pET-21

图1

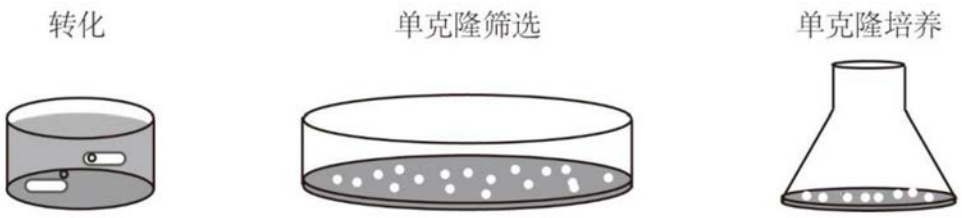
转化
单克隆筛选
单克隆培养

图2

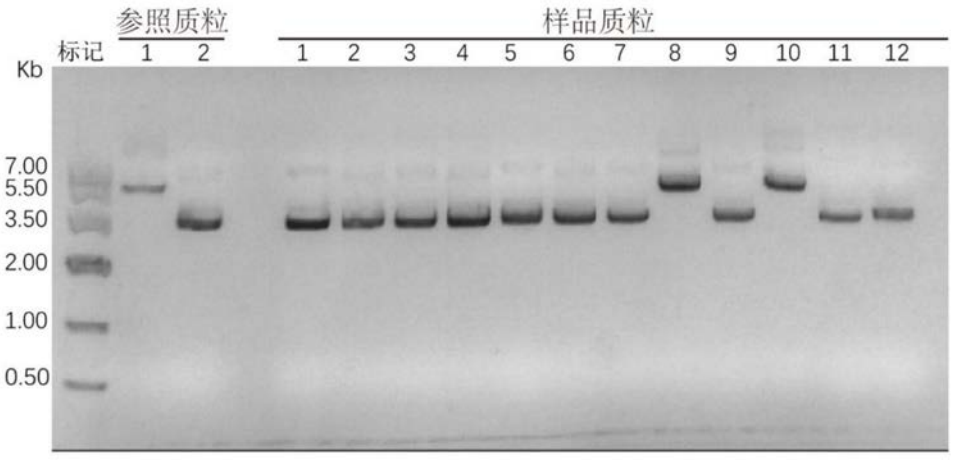
参照质粒
样品质粒
标记
1
2
1
2
3
4
5
6
7
8
9
10
11
12
Kb
7.00
5.50
3.50
2.00
1.00
0.50

图3

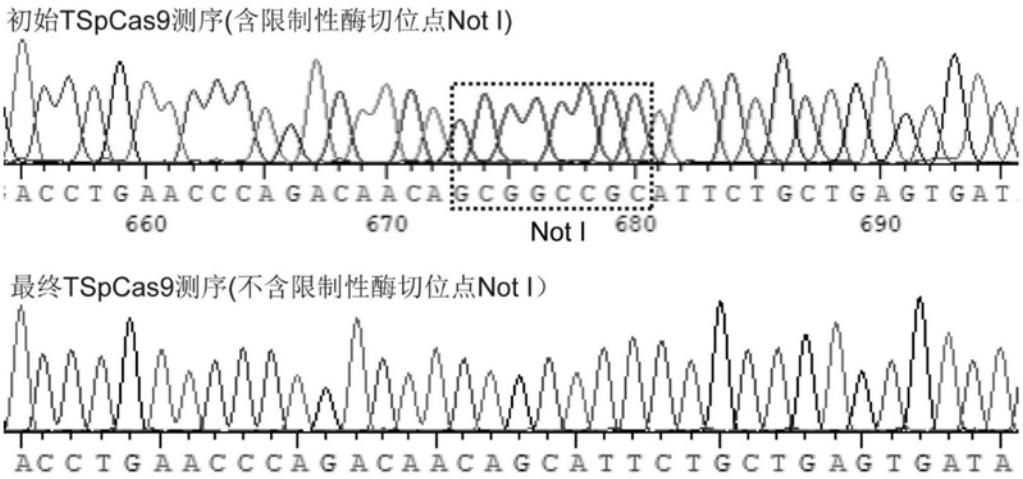
初始TSpCas9测序(含限制性酶切位点Not I)
ACCTGAACCCAGACAACAGCGGCCGCATTCTGCTGAGTGAT
660
670
Not I
680
690
最终TSpCas9测序(不含限制性酶切位点Not I)
ACCTGAACCCAGACAACAGCATTCTGCTGAGTGATA

图4

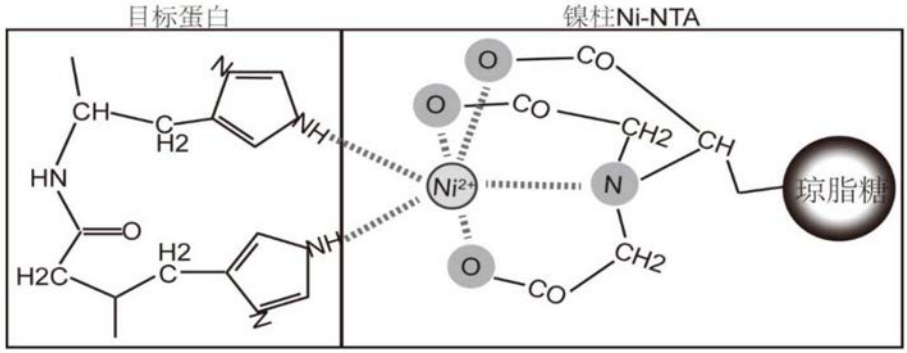
目标蛋白
镍柱Ni-NTA
Ni2+
琼脂糖

图5

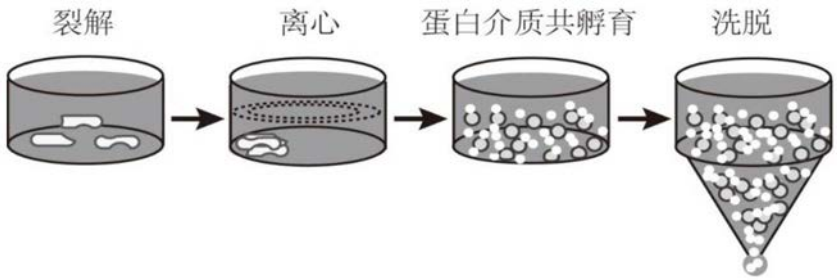
裂解
离心
蛋白介质共孵育
洗脱

图6

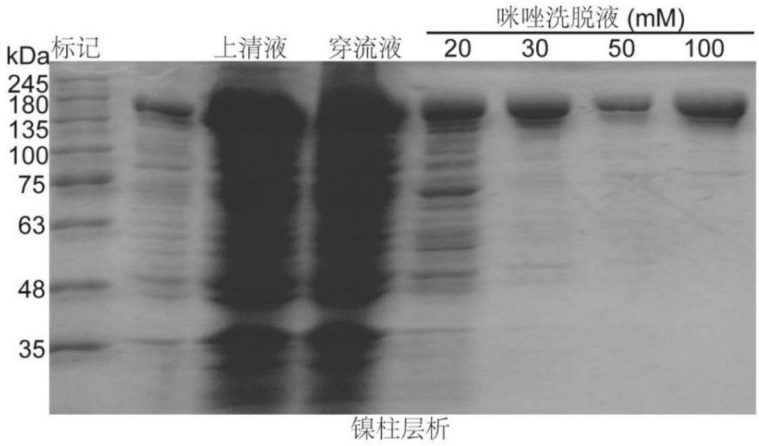
咪唑洗脱液 (mM)
kDa
标记
上清液
穿流液
20
30
50
100
245
180
135
100
75
63
48
35
镍柱层析

图7

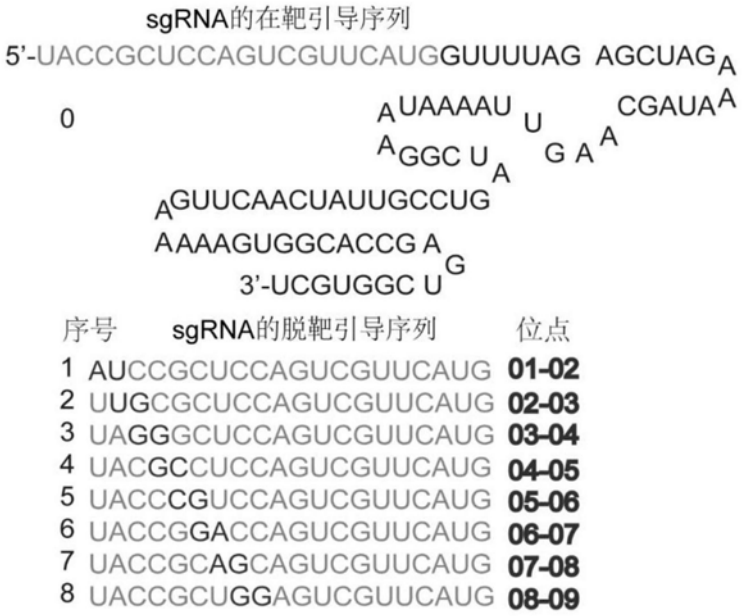
sgRNA的在靶引导序列
5'-UACCGCUCCAGUCGUUCAUGGUUUUAG AGCUAGA
0
AUAAAAU U CGAUAA
AGGC U A G A A
AGUUCAACUAUUGCCUG
AAAAGUGGCACCG A G
3'-UCGUGGC U
序号 sgRNA的脱靶引导序列 位点
1 AUCCGCUCCAGUCGUUCAUG 01-02
2 UUGCGCUCCAGUCGUUCAUG 02-03
3 UAGGGCUCCAGUCGUUCAUG 03-04
4 UACGCCUCCAGUCGUUCAUG 04-05
5 UACCCGUCCAGUCGUUCAUG 05-06
6 UACCGGACCAGUCGUUCAUG 06-07
7 UACCGCAGCAGUCGUUCAUG 07-08
8 UACCGCUGGAGUCGUUCAUG 08-09

图8

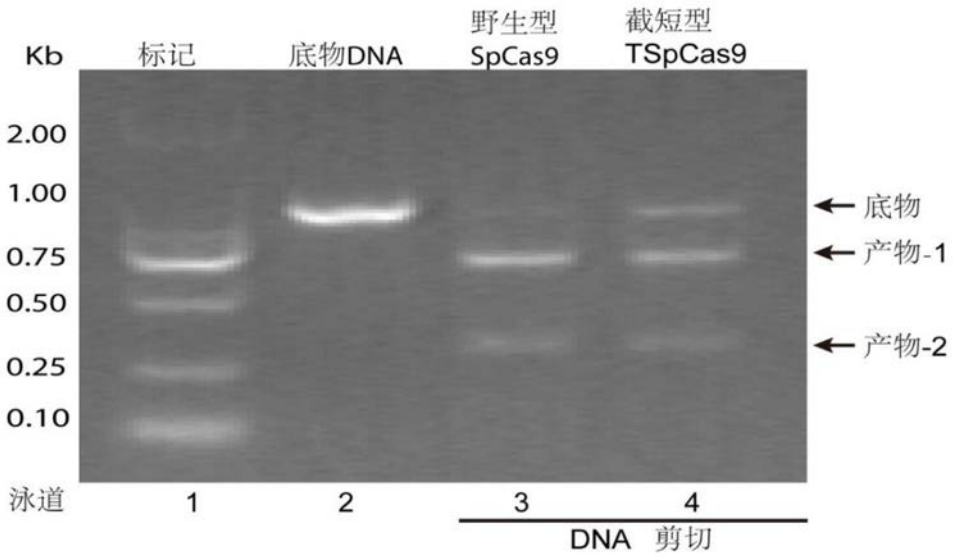
Kb
标记
底物DNA
野生型
SpCas9
截短型
TSpCas9
2.00
1.00
0.75
0.50
0.25
0.10
底物
产物-1
产物-2
泳道
1
2
3
4
DNA 剪切

图9

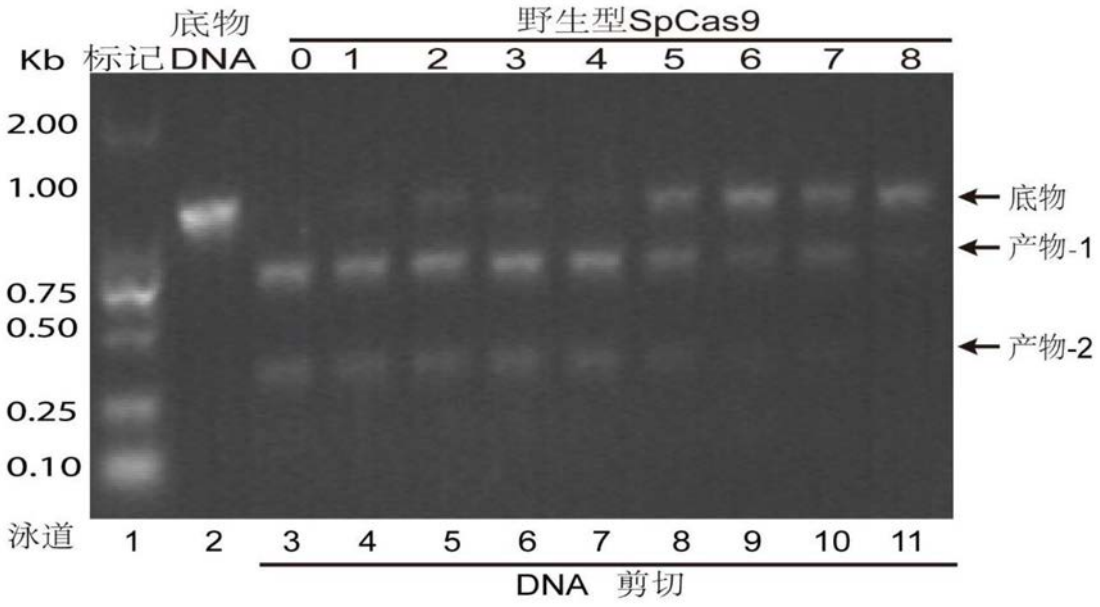
底物
野生型SpCas9
Kb
标记
DNA
0 1 2 3 4 5 6 7 8
2.00
1.00
0.75
0.50
0.25
0.10
底物
产物-1
产物-2
泳道
1 2 3 4 5 6 7 8 9 10 11
DNA 剪切

图10

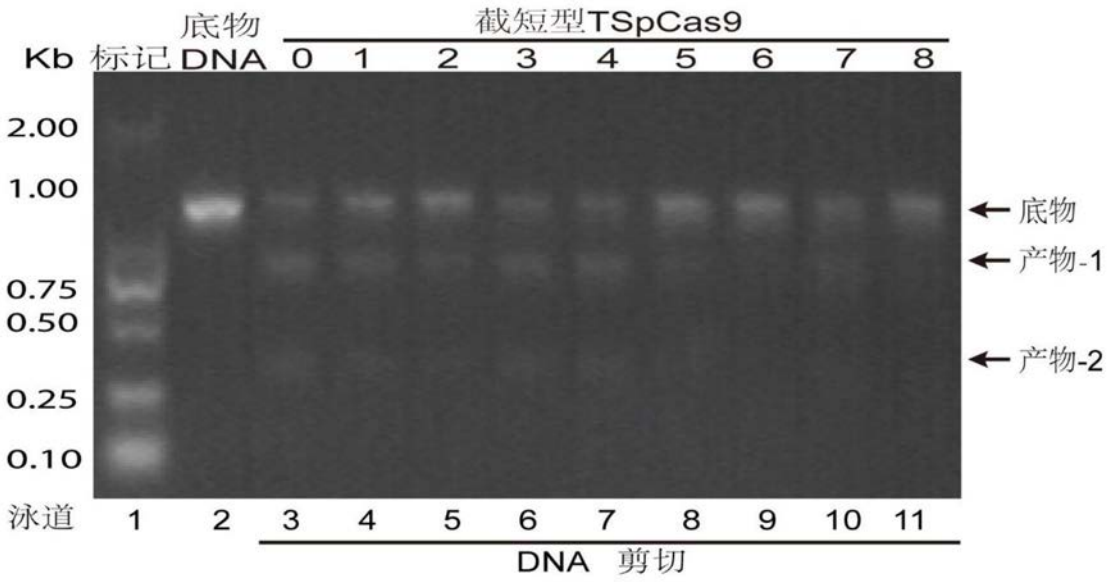
底物
截短型TSpCas9
Kb 标记
DNA
0 1 2 3 4 5 6 7 8
2.00
1.00
0.75
0.50
0.25
0.10
底物
产物-1
产物-2
泳道
1 2 3 4 5 6 7 8 9 10 11
DNA 剪切

图11