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(54)发明名称

一种构建TCR载体的方法

(57)摘要

本申请公开了一种构建TCR载体的方法,包括如下步骤:骨架载体的构建:将TRBJ基因和TCR β链TRBC基因与骨架载体进行整合,得到含有TRBJ-TRBC片段的骨架载体;TCR可变段的合成:合成α链可变区、α链恒定区以及β链可变区的基因。PCR扩增这三个片段,扩增产物与骨架载体进行酶切链接,得到TCR载体。该方法将TRBJ基因和TCR β链中的TRBC片段与骨架载体整合,构建10个骨架载体,以已经合成的α链可变区、α链恒定区、β链可变区为模板,扩增相应片段,然后进行组装即可完成TCR载体的构建,具有快速高效的优势。

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1.一种构建TCR载体的方法,其特征在于,包括如下步骤:

构建包含TRBC基因的骨架载体;

基因合成EF1A-TRAV基因片段、TRAC-连接子基因片段和连接子-TRBV基因片段:

通过引物分别扩增所述EF1A-TRAV基因片段、TRAC-连接子基因片段和连接子-TRBV基因片段,将扩增的基因片段与所述骨架载体进行组装得到TCR载体;

扩增的所述EF1A-TRAV基因片段中的第一TRAJ基因子片段与TRAC-连接子基因扩增片段中的第二TRAJ基因子片段可组成完整的TRAJ基因;连接子-TRBV基因扩增产物中的第一TRBJ基因子片段与骨架载体中的第二TRBJ基因子片段可组成完整的TRBJ基因片段;

所述引物的设计包括:引入内切酶识别位点,在EF1A-TRAV基因片段的下游引物的5'端引入第一TRAJ基因子片段,在连接子-TRBV基因片段的下游引物的5'端引入第一TRBJ基因子片段。

2.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,在进行组装时,采用Type IIS内切酶和DNA连接酶对骨架载体进行切割。

3.根据权利要求2所述的一种构建TCR载体的方法,其特征在于,所述Type IIS内切酶为BsmBI酶,DNA连接酶为T4 DNA连接酶。

4.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,所述骨架载体内含有CCDB自杀基因。

5.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,所述引物的5'端有Type IIS内切酶的识别序列。

6.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,所述连接子为自剪切肽的基因片段或IRES元件的基因片段。

7.根据权利要求6所述的一种构建TCR载体的方法,其特征在于,所述自剪切肽的基因片段为编码P2A、T2A、E2A或F2A的基因序列中的一种。

8.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,所述骨架载体包含以下序列的一种:SEQ ID Nos:48-57。

9.根据权利要求1所述的一种构建TCR载体的方法,其特征在于,通过包含SEQ ID No:62、SEQ ID No:65和SEQ ID No:66代表的固定序列中的引物合成TCR全长序列。

一种构建TCR载体的方法

技术领域

[0001]　本申请涉及TCR载体领域，尤其是涉及一种构建TCR载体的方法。

背景技术

[0002]　TCR指T细胞抗原受体，是可以赋予个体几乎是无限的抗原识别和应答能力的胚系基因，能够保证个体在多变环境中能和外来抗原(病原体)发生有效的免疫应答。

[0003]　TCR由两条不同肽链构成的异二聚体，由α、β两条肽链组成，每条肽链又可分为可变区(V区)，恒定区(C区)，跨膜区和胞质区等几部分。其中α链包含TRAV基因、TRAJ基因、TRAC基因，β链包含TRBV基因、TRBJ基因、TRBC基因。构建质粒载体的时，需要将α链和β链用2A肽串联在一起，这些结构需要无缝连接在一起，即连接的部分表达产物不能出现多余的氨基酸，连接的方式如附图1所示。

[0004]　目前常用的构建方法TCR载体的方法是基因合成TCR基因，再将基因片段通过同源重组或酶连接方法连接到载体上，合成费用高，时间也长。

发明内容

[0005]　本申请提供了一种构建TCR载体的方法、骨架载体以及引物序列。

[0006]　第一方面，一种构建TCR载体的方法，包括如下步骤：

[0007]　骨架载体的构建：构建包含TRBC基因的骨架载体；

[0008]　基因片段合成：基因合成EF1A-TRAV基因片段、TRAC-连接子基因片段和连接子-TRBV基因片段；

[0009]　通过引物分别扩增所述EF1A-TRAV基因片段、TRAC-连接子基因片段和连接子-TRBV基因片段，将所述扩增的基因片段与所述骨架载体进行组装得到TCR载体。

[0010]　在一些实施方案中，扩增的EF1A-TRAV基因片段中的第一TRAJ基因子片段与TRAC-连接子基因扩增片段中的第二TRAJ基因子片段可组成完整的TRAJ基因；连接子-TRBV基因扩增产物中的第一TRBJ基因子片段与骨架载体中的第一TRBJ基因子片段可组成完整的TRBJ基因。

[0011]　优选的，在进行组装时，采用TypeIIS内切酶和DNA连接酶对骨架载体进行切割及连接。在某些实施方案中，TypeIIS内切酶为BsmBI酶，连接酶为T4DNA连接酶。

[0012]　优选的，所述步骤包括引物设计步骤，所述引物的设计包括：引入内切酶识别位点，在EF1A-TRAV基因片段的下游引物的5’端引入第一TRAJ基因子片段，在连接子-TRBV基因片段的下游引物的5’端引入第一TRBJ基因子片段。

[0013]　优选的，所述骨架载体内含有CCDB自杀基因。

[0014]　优选的，所述引物的5’端有TypeIIS内切酶的识别序列。

[0015]　优选的，所述TRAC基因片段和TRBV基因片段之间存在连接片段，所述连接片段为自剪切肽的基因片段或IRES元件的基因片段。

[0016]　优选的，所述自剪切肽的基因片段为编码P2A、T2A、E2A或F2A的基因序列中的任意

一种。

[0017] 优选的,所述骨架载体包含以下序列的一种:SEQ ID Nos:48-57。

[0018] 优选的,所述TCR全长序列通过利用包含SEQ ID No:62、SEQ ID No:65和SEQ ID No:66的引物合成的。

[0019] 在某些实施方案中,如图3所示,Primer1代表SEQ ID NO:62,Primer2代表SEQ ID No:65,Primer3代表SEQ ID No:66,其中,TypeIIS内切酶为BsmBI酶,BsmBI酶切割位点可以前后移动,位置不同,设计的引物序列会有少许变化。采用如下方式,

[0020] Primer 1+Primer 2,Primer2 引物5撇端外挂第一TRAJ基因子片段序列,以EF1A-TRAV为模板,可以扩增出EF-1a+TRAV+TRAJ,片段包含完整的TRAV基因片段和第一TRAJ基因子片段。

[0021] Primer 3+Primer 4,Primer3引物5撇端外挂第二TRAJ基因子片段序列,以TRAC-P2A为模板,可以扩增出TRAJ+TRAC+P2A,扩增片段包含完整的TRAC基因片段和第二TRAJ基因子片段,与EF-1a+TRAV+TRAJ中的第一TRAJ基因子片段可拼接出完整的TRAJ基因。

[0022] Primer 5+Primer 6,Primer6 引物5撇端外挂第一TRBJ基因子片段序列,以P2A-TRBV为模板,可以扩增出P2A+TRBV+TRBJ。扩增片段包含完整的TRBC基因片段和第一TRBJ基因子片段,这第一TRBJ基因子片段与骨架载体上的第二TRBJ基因子片段可拼接成完整的TRBJ基因。

[0023] 这样设计将Primer 1、Primer4、Primer 5固定,根据目的TCR序列的TRAV、TRAJ、TRBJ的基因型,设计相应的Primer2、Primer3、Primer 6即可完成不同TCR载体的构建。最后在Primer 1-6五撇端加上酶切识别位点。

[0024] 第二方面,一种用于构建TCR载体的骨架载体,所述骨架载体包含以下序列的一种:SEQ ID Nos:48-57。

[0025] 第三方面,一种用于构建TCR载体的引物序列组,所述引物序列组包含SEQ ID No:62、SEQ ID No:65和SEQ ID No:66的序列。

[0026] 综上所述,本申请具有如下有益效果:

[0027] 1.本申请采用先将具有固定序列的TRBC片段与含有CCDB自杀基因的骨架载体整合,获得可以提前制备的含有BC片段和CCDB自杀基因片段的骨架载体,预先合成EF1A-TRAV基因,TRAC-P2A基因和P2A-TRBV基因;然后仅需3对引物,分别扩增得到TRAV到TRBV的基因片段,将三个扩增片段与骨架载体酶切连接,即可得到TCR载体。采用本申请的方法进行TCR载体的构建,连接部分不会出现多余的氨基酸;此外,本申请的TCR构建只需扩增三个基因片段,因此减少了合成的费用,节约了合成的时间。

附图说明

[0028] 图1 载体中TCR序列的示意图。

[0029] 图2 10个含有不同关键序列的骨架载体的部分结构示意图。

[0030] 图3 TCR全长序列中引物切割位点的结构示意图,其中,TypeIIS内切酶切割位点,可以前后移动,位置不同,设计的引物序列会有少许变化。

具体实施方式

[0031] 定义

[0032] T细胞受体(TCR)是存在于T细胞表面的分子,其负责识别肽-MHC复合物。TCR与肽-MHC复合物的特异性结合引发T细胞通过一系列由相关酶、共受体和辅助性分子介导的生化事件而活化。在95%的T细胞中,TCR异二聚体由α和β链组成,而在5%的T细胞中,TCR异二聚体由α和β链组成。TCR的每一条链均属于免疫球蛋白超家族的成员,具有一个N端免疫球蛋白(Ig)可变(V)结构域、一个Ig恒定(C)结构域、跨细胞膜区域(即,跨膜区)以及在C末端的短胞质尾。

[0033] CCDB基因位于大肠杆菌的F质粒上,是被CCD操纵子编码的毒性-抗毒性系统的一部分,确保含F质粒的细胞分裂后F质粒仍存在(分裂后含F质粒的细胞才能存活)。CCDB编码的毒性蛋白CCDB,作为DNA促旋酶抑制剂,锁定DNA促旋酶和断裂的双链DNA复合物,使DNA促旋酶不能发挥作用,最终导致细胞死亡。CCDA是CCD操纵子中的另一个基因,编码抗毒性蛋白CCDA,保护细胞免受CCDB的毒性作用。细胞因为丢失F质粒而丢失了CCDA基因,导致细胞屈服于CCDB的毒性。

[0034] 如本文所用,质粒载体是在天然质粒的基础上为满足实验需求而进行人为构建的。与天然质粒相比,质粒载体通常带有一个或一个以上的选择性标记基因(如抗生素抗性基因)和一个人工合成含有多个限制性内切酶识别位点的多克隆位点序列,并去掉了大部分非必需序列,使相对分子质量尽可能减少,以便于基因工程操作。

[0035] 其能够将一种或多种所关注的基因或序列递送入宿主细胞并且优选在宿主细胞中表达所述基因或序列。载体的实例包括但不限于病毒载体、质粒、粘粒或噬菌体载体。

[0036] TCR α链中TRAV与TRAJ基因之间会有重组,TCR β链中TRBV与TRBJ基因之间会有重组。即使相同的TRAV基因与TRAJ基因,TRAV基因与TRBJ基因重组后形成的TCR α链也可能不同。TCR β链TRAV基因与TRBJ重组连接后也是如此。本发明构建TCR质粒时,把这部分可变的区域序列放到扩增引物上,即第一TRAJ基因子片段、第二TRAJ基因子片段、第一TRBJ基因子片段和第二TRBJ基因子片段。

[0037] 第一TRAJ基因子片段:包含部分TRAJ序列的基因片段,与第二TRAJ基因子片段可组成完整的TRAJ基因。

[0038] 第二TRAJ基因子片段:包含部分TRAJ序列的基因片段,与第一TRAJ子基因片段可组成完整的TRAJ基因。

[0039] 第一TRBJ基因子片段:包含部分TRBJ序列的基因片段,与第二TRBJ子基因片段可组成完整的TRBJ基因。

[0040] 第二TRBJ基因子片段:包含部分TRBJ序列的基因片段,与第一TRBJ基因子片段可组成完整的TRBJ基因。

[0041] 以下结合实施例对本申请作进一步详细说明。

[0042] 实施例

[0043] 实施例1,含CCDB基因关键骨架质粒的构建

[0044] 如图1所示,在TCR基因中,TRBJ后面连接的是TRBC基因,将不同TRBJ末端氨基酸相同TRBJ分成同一组,预先将表达TRBJ末端相同氨基酸的基因片段连接到载体骨架上。从NCBI下载TRBJ氨基酸序列,共16个不同的TRBJ基因,通过对TRBJ氨基酸序列进行分析,将16

个TRBJ分成10组。将10组中的TRBJ的保守序列，与CCDB基因，TRBC连接到pKSH0350质粒载体上。10个骨架质粒的示意图如图2所示。

[0045] 16个TRBJ的氨基酸序列

[0046] >SEQ ID No.1:K02545|TRBJ1-1*01

[0047] NTEAFFGQGTRLTVV

[0048] >SEQ ID No.2:K02545|TRBJ1-2*01

[0049] NYGYTFGSGTRLTVV

[0050] >SEQ ID No.3:M14158|TRBJ1-3*01

[0051] SGNTIYFGEGSWLTVV

[0052] >SEQ ID No.4:M14158|TRBJ1-4*01

[0053] TNEKLFFGSGTQLSVL

[0054] >SEQ ID No.5:M14158|TRBJ1-5*01

[0055] SNQPQHFGDGTRLSIL

[0056] >SEQ ID No.6:M14158|TRBJ1-6*01

[0057] SYNSPLHFGNGTRLTVT

[0058] >SEQ ID No.7:L36092|TRBJ1-6*02

[0059] SYNSPLHFGNGTRLTVT

[0060] >SEQ ID No.8:X02987|TRBJ2-1*01

[0061] SYNEQFFGPGTRLTVL

[0062] >SEQ ID No.9:X02987|TRBJ2-2*01

[0063] NTGELFFGEGSRLTVL

[0064] >SEQ ID No.10:X02987|TRBJ2-3*01

[0065] STDTQYFGPGTRLTVL

[0066] >SEQ ID No.11:X02987|TRBJ2-4*01

[0067] AKNIQYFGAGTRLSVL

[0068] >SEQ ID No.12:X02987|TRBJ2-5*01

[0069] QETQYFGPGTRLLVL

[0070] >SEQ ID No.13:X02987|TRBJ2-6*01

[0071] SGANVLTFGAGSRLTVL

[0072] >SEQ ID No.14:M14159|TRBJ2-7*01

[0073] SYEQYFGPGTRLTVT

[0074] >SEQ ID No.15:X02987|TRBJ2-7*02

[0075] SYEQYVGPGTRLTVT

[0076] >SEQ ID No.16:X02987|TRBJ2-2P*01

[0077] LRGAAGRLGGGLLVL

[0078] 10组TRBJ

[0079] 表1

[0080]

相同氨基酸序列	TRBJ	TRBJ 基因表达的氨基酸
GTRLTVT	>TRBJ2-4*01	AKNIQYFGAGTRLSVL
	>TRBJ1-6*01	SYNSPLHFGNGTRLTVT
	>TRBJ1-6*02	SYNSPLHFGNGTRLTVT
	>TRBJ2-7*01	SYEQYFGPGTRLTVT
	>TRBJ2-7*02	SYEQYVGPGTRLTVT
PGTRLTVL	>TRBJ2-1*01	SYNEQFFGPGTRLTVL
	>TRBJ2-3*01	STDTQYFGPGTRLTVL
GSRLTVL	>TRBJ2-2*01	NTGELFFGEGSRLTVL
	>TRBJ2-6*01	SGANVLTFGAGSRLTVL
GTRLTVV	>TRBJ1-1*01	NTEAFFGQGTRLTVV
	>TRBJ1-2*01	NYGYTFGSGTRLTVV
GTRLSVL	>TRBJ2-4*01	AKNIQYFGAGTRLSVL
GTRLSIL	>TRBJ1-5*01	SNQPQHFGDGTRLSIL
GTRLLVL	>TRBJ2-5*01	QETQYFGPGTRLLVL
GTQLSVL	>TRBJ1-4*01	TNEKLFFGSGTQLSVL
GGGLLVL	>TRBJ2-2P*01	LRGAAGRLGGGLLVL
SWLTVV	>TRBJ1-3*01	SGNTIYFGEGSWLTVV

[0081] SEQ ID No.17: GTRLTVT

[0082] SEQ ID No.18: PGTRLTVL

[0083] SEQ ID No.19: GSRLTVL

[0084] SEQ ID No.20: GTRLTVV

[0085] SEQ ID No.21: GTRLSVL

[0086] SEQ ID No.22: GTRLSIL

[0087] SEQ ID No.23: GTRLLVL

[0088] SEQ ID No.24: GTQLSVL

[0089] SEQ ID No.25: GGGLLVL

[0090] SEQ ID No.26: SWLTVV

[0091] SEQ ID No.27: TRBJ2-4*01: AKNIQYFGAGTRLSVL

[0092] SEQ ID No.28: TRBJ1-6*01: SYNSPLHFGNGTRLTVT

[0093] SEQ ID No.29: TRBJ1-6*02: SYNSPLHFGNGTRLTVT

[0094] SEQ ID No.30: TRBJ2-7*01: SYEQYFGPGTRLTVT

[0095] SEQ ID No.31: TRBJ2-7*02: SYEQYVGPGTRLTVT

[0096] SEQ ID No.32: TRBJ2-1*01: SYNEQFFGPGTRLTVL

[0097] SEQ ID No.33: TRBJ2-3*01: STDTQYFGPGTRLTVL

[0098] SEQ ID No.34: TRBJ2-2*01: NTGELFFGEGSRLTVL

[0099] SEQ ID No.35: TRBJ2-6*01: SGANVLTFGAGSRLTVL

[0100] SEQ ID No.36: TRBJ1-1*01: NTEAFFGQGTRLTVV

[0101] SEQ ID No.37: TRBJ1-2*01: NYGYTFGSGTRLTVV

[0102] SEQ ID No.38: TRBJ2-4*01: AKNIQYFGAGTRLSVL

[0103] SEQ ID No.39: TRBJ1-5*01: SNQPQHFGDGTRLSIL

[0104] SEQ ID No.40: TRBJ2-5*01: QETQYFGPGTRLLVL

[0105] SEQ ID No.41: TRBJ1-4*01: TNEKLFFGSGTQLSVL

[0106] SEQ ID No.42: TRBJ2-2P*01: LRGAAGRLGGGLLVL

[0107] SEQ ID No.43: TRBJ1-3*01: SGNTIYFGEGSWLTVV

[0108] 以pKSH0350质粒(科士华(南京)生物技术有限公司 货号pZL0350)为骨架载体,构建10个CCDB的Backbone。在上海生工基因合成十个CCDB基因-第二TRBJ-TRBC的质粒,第二TRBJ基因子片段指按表1分组后表达相同氨基酸的DNA序列,十个质粒编号为pKSH0136-0145(科士华(南京)生物技术有限公司,货号pZL0136-pZL0145),在CCDB基因片段两侧有BsmBI酶切位点,CCDB基因在后续TCR基因质粒构建时被酶切,被TCR序列替换。

[0109] 设计引物如下:

[0110] SEQ ID No.44:356-FseI-CZ-F: GGGTAGTCTCAAGCTGGC

[0111] SEQ ID No.45:356-OV-R: TTAATTCTCACGTCTCGGGTCTCCCTATAGTGAGTCGTATTAG

[0112] SEQ ID No.46:356-OV-F:GAGACGTGAGAATTAATACGACTCACTATAGAG

[0113] SEQ ID No.47:356-SalI-CZ-R:AATCCAGAGGTTGATTGTCGACTCAGCTGTTCTTC

[0114] 1.1片段扩增

[0115] 以诺唯赞2 × Phanta Max Master Mix (Dye Plus)扩增片段,引物上海生工合成。

[0116] 表2

[0117]

组分	加量/μL	备注
2 × Phanta Max Master Mix (Dye Plus)	25	N/A
引物F(10uM)	1	引物模板见表3
引物R(10uM)	1	
模板	1ng	
PCR 用水	补水至50μL	N/A

[0118] 表3

[0119]

序号	模板	引物	大小（bp）	备注
1	pKSH0350	356-FseI-CZ-F、356-OV-R	553	355 片段 1
2	pKSH0136	356-OV-F、356-SalI-CZ-R	924	355 片段 2
3	pKSH0137	356-OV-F、356-SalI-CZ-R	924	356 片段 2
4	pKSH0138	356-OV-F、356-SalI-CZ-R	924	357 片段 2
5	pKSH0139	356-OV-F、356-SalI-CZ-R	924	358 片段 2
6	pKSH0140	356-OV-F、356-SalI-CZ-R	924	359 片段 2
7	pKSH0141	356-OV-F、356-SalI-CZ-R	924	360 片段 2
8	pKSH0142	356-OV-F、356-SalI-CZ-R	924	351 片段 2
9	pKSH0143	356-OV-F、356-SalI-CZ-R	924	362 片段 2
10	pKSH0144	356-OV-F、356-SalI-CZ-R	924	363 片段 2
11	pKSH0145	356-OV-F、356-SalI-CZ-R	924	364 片段 2

[0120] 扩增程序:预变性 95℃,3min;变性 95℃,15sec,退火 60℃,30sec,延伸 72℃ 30sec;扩增循环30;终延伸72℃,5min。

[0121] 1.2 pKSH0350载体酶切,回收大片段

[0122] 用NEB公司内切酶FseI(R0588V)和SalI(R0138V)酶切。

[0123] 体系 :50μL

[0124] 表4

[0125]

10×Cutsmart Buffer	5μL
pKSH0350	4ug
FseI	1μL
SalI	1μL
dH_2O	Up to 50 μL

[0126] 酶切条件:37℃,3h。

[0127] 酶切后理论大小：2383bp+7695bp，回收条带大小:7695bp。用1.5%琼脂糖凝胶电泳,切角回收目的片段。胶回收用南京诺唯赞生物胶回收试剂盒回收DNA片段(FastPure Gel DNA Extraction Mini Kit货号DC301-01),按试剂盒说明书操作。

[0128] 1.3、重组连接

[0129] 用南京诺唯赞ClonExpress Ultra One Step Cloning Kit V2试剂盒,将PCR片段分别连接到酶切后的载体上。加量见下表5

[0130] 表5

[0131]

名称 / 体积(μL)	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355	pKSH0355
重组酶MIX	5	5	5	5	5	5	5	5	5	5
酶切后质粒	30ng	30ng	30ng	30ng	30ng	30ng	30ng	30ng	30ng	30ng
350片段1	60ng	60ng	60ng	60ng	60ng	60ng	60ng	60ng	60ng	60ng
片段2	355片段2:60ng	356片段2:60ng	357片段2:60ng	358片段2:60ng	359片段2:60ng	360片段2:60ng	361片段2:60ng	362片段2:60ng	363片段2:60ng	364片段2:60ng
dH_2O	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10	Up to 10

[0132] 反应条件:50℃，30min。

[0133] 1.4转化

[0134] 将10μL连接后产物用上海唯地DB3.1 Chemically Competent Cell(CAT#:DL1040)感受态细胞热激转化,按感受态细胞说明书操作。转化后产物用含Kan抗性的LB固体培养基平板倒置放于37℃培养箱过夜培养。

[0135] 1.5 挑克隆送上海生工测序,将测序正确的10个CcdB的Backbone质粒依次命名为pKSH0355、pKSH0356、pKSH0357、pKSH0358、pKSH0359、pKSH0360、pKSH0361、pKSH0362、pKSH0363、pKSH0364。示意图如图2所示。

[0136] SEQ ID No.48 pKSH0355 PGTRLTVL ED ccdb骨架载体

[0137] GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCC

GCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTA
TTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCT
CCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCC
GCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGGG
GTCTCTCTGGTTAGACCAGATCTGAGCCTGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAA
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[0138] SEQ ID No.49 pKSH0356 GSRLTVL ED ccdb骨架载体

[0139] GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATG
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[0146] SEQ ID No.53 pKSH0360 GTRLSIL ED ccdb骨架载体

[0147] GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATG
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[0148] SEQ ID No.54 pKSH0361 GTRLLVL ED ccdb骨架载体

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[0154] SEQ ID No.57 pKSH0364 GSWLTVV ED ccdb骨架载体

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[0156] 实施例2 TCR质粒构建(以1G4 TCR为例)

[0157] SEQ ID No.58:1G4 TCR序列

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ga

[0159] 通过对序列分析，1G4序列由TRAV21*01，TRAJ6*01，TRAC，TRBV6-5*01，TRBJ2-2*01和TRBC组成。以预先基因合成EF1A-TRAV21*01基因（载体骨架上的EF1A序列和TRAV21*01基因序列），P2A-TRBV6-5*01基因（P2A序列和TRBV6-5*01）和TRAC-P2A作为后续PCR扩增模板。基因合成在上海生工完成，片段连接到PUC57质粒载体上。

[0160] SEQ ID No.59: EF1A-TRAV21*01基因（大写为EF1A和载体骨架序列，小写为TRAV21*01基因序列）

[0161] TTTTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTT
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tgacccagatccctgccgccctgtccgtgcctgagggcgaaaatctggtgctgaactgttcctttacagactccgc
catctacaacctgcagtggttcaggcaggatcccggcaagggcctgacctccctgctgctgatccagtcctcccag
agagagcagacaagcggcaggctgaatgcctccctggataagagcagcggcagaagcaccctgtacatcgccgcca
gccagcctggcgacagcgctacatacctgtgcgccgtgagacccaca

[0162] SEQ ID No.60: TRAC-P2A 的序列（小写为TRAC基因，大写为P2A序列）

[0163] tatatccagaaccctgaccctgccgtgtaccagctgagagactctaaatccagtgacaagtctgtctg
cctattcaccgattttgattctcaaacaaatgtgtcacaaagtaaggattctgatgtgtatatcacagacaaaact

gtgctagacatgaggtctatggacttcaagagcaacagtgctgtggcctggagcaacaaatctgactttgcatgtgcaaacgccttcaacaacagcattattccagaagacaccttcttccccagcccagaaagttcctgtgatgtcaagctggtcgagaaaagctttgaaacagatacgaacctaaactttcaaaacctgtcagtgattgggttccgaatcctcctcctgaaagtggccgggtttaatctgctcatgacgctgcggctgtggtccagcGCCACAAATTTCAGCCTGCTGAAGCAGGCCGGCGATGTGGAGGAGAACCCCGGCCCTAGA

[0164] SEQ ID No.61: P2A-TRBV基因序列(大写为P2A序列,小写为TRBV基因序列)

[0165] GCCACAAATTTCAGCCTGCTGAAGCAGGCCGGCGATGTGGAGGAGAACCCCGGCCCTAGAatgagcatcggcctgctgtgctgtgccgccctgagcctgctgtgggccggacctgtgaacgccggcgtgacacagacccctaagttccaggtgctgaagacaggccagtccatgaccctgcagtgcgcccaggatatgaaccacgagtacatgagctggtacagacaggatcctggcatgggcctgcggctgatccactacagcgtgggcgccggcatcaccgaccagggagaggtgcctaatggctacaacgtgagcaggtccacaacagaggattttcccctgagactgctgtccgccgccccctctcagacaagcgtgtacttctgcgccagctcctacgtgggc

[0166] 设计引物Primer1、Primer2、Primer3、Primer4、Primer5、Primer6扩增出所需片段,进行DNA电泳、胶回收。引物设计如下,引物与模板互补的部分退火温度50-60℃。引物Primer1引物结合区在质粒骨架上;引物Primer2在TRAV基因上,引物5撇端外挂第一TRAJ基因子片段的序列;引物Primer3引物结合区在TRAC基因,引物5撇端外挂第二TRAJ基因子片段的序列;引物Primer4和引物primer5引物结合区在P2A基因上;引物Primer6在TRBV基因上,引物5撇端外挂第一TRBJ基因子片段的序列;在所有引物5撇端,额外添加$(N)_{2-5}$CGTCTCN,N为随机碱基,5撇端的2至5个随机碱基为保护碱基。CGTCTC为内切酶BsmBI的酶切识别位点,酶切位置发生在CGTCTC三撇端后一个碱基。用其他TypeIIS内切酶时只需要将酶切识别序列换成对应的碱基序列即可。构建不同TCR基因中,TRAV,TRAJ,TRBV和TRBJ是变的,需要根据目标TCR基因本身,合成对应的EF1A-TCRAV,P2A-TCRBV基因,Primer2,Primer3和Primer6;因为TRAC,TRBC和P2A基因是固定不变的,所以Primer1,Primer4和Primer5引物是固定的。

[0167] SEQ ID No.62:Primer1:

[0168] TTA*CGTCTC*Tggtacctcactatagggagacccg

[0169] SEQ ID No.63:Primer2(5撇端CGTCTC为BsmBI酶切识别位点,酶切位置在酶切识别位点后第一个碱基与和第二个碱基之间。cgc为保护碱基。在3撇端大写碱基为与TRAV21*01基因互补配对的片段,这段TM在50-60℃,小写碱基为第一TRAJ基因子片段)

[0170] CGC*CGTCTC*TaaatgtagggatgtagctgccgccgcttgtgggTCTCACGGCGCACA

[0171] SEQ ID No.64:Primer3:大写为与TRAC互补配对的部分,这段TM在50-60℃。小写部分为第二TRAJ基因子片段。这第二TRAJ基因子片段与Primer2中的第一TRAJ基因子片段合并即为1G4 TCR基因中完整的TRAJ。

[0172] TTC*CGTCTC*CatttggcaggggcacatccctgatcgtgcaccccTATATCCAGAACCC

[0173] SEQ ID No.65:Primer4: 小写部分与P2A基因配对,用于扩增。5撇GCA为酶切保护碱基,斜体CCGTCT为BsmBI酶切识别位点,酶切发生在大写T和小写C之间。

[0174] GCA*CCGTCTC*Tcagcaggctgaaatttgtggc

[0175] SEQ ID No.66:Primer5:小写部分与P2A基因配对,用于扩增。5撇ACGT为酶切保护碱基,斜体CCGTCT为BsmBI酶切识别位点,酶切发生在大写T和小写g之间。

[0176] ACGT*CGTCTC*Tgctgaagcaggccggc

[0177] SEQ ID No.67:Primer6:3撇大写为与TRBV互补配对的部分,这段TM在50-60℃。小写部分为第一TRBJ基因子片段。1G4 TCR还有第二TRBJ基因子片段在载体骨架上。

[0178] ACGCT*CGTCTC*GagccctcgccaaagaacagctcgcctgtattGCCCACGTAGGAGCTG

[0179] 2.1 片段扩增

[0180] 片段1扩增

[0181] 以诺唯赞2 × Phanta Max Master Mix (Dye Plus)扩增片段,引物上海生工合成

[0182] 表6

[0183]

组分	加量μL
2 × Phanta Max Master Mix (Dye Plus)	25
Primer1 (10uM)	1
Primer2 (10uM)	1
EF1A-TRAV21*01 模板	1ng
PCR 用水	补水至 50μL

[0184] 片段2扩增

[0185] 以诺唯赞2 × Phanta Max Master Mix (Dye Plus)扩增片段,引物上海生工合成

[0186] 表7

[0187]

组分	加量/μL
2 × Phanta Max Master Mix (Dye Plus)	25
Primer3 (10uM)	1
Primer4 (10uM)	1
TRAC-P2A 模板	1ng
PCR 用水	补水至 50μL

[0188] 片段3扩增

[0189] 以诺唯赞2 × Phanta Max Master Mix (Dye Plus)扩增片段,引物上海生工合成

[0190] 表8

[0191]

组分	加量/μL
2 × Phanta Max Master Mix (Dye Plus)	25
Primer5 (10uM)	1
Primer6 (10uM)	1
P2A-TRBV6-5*01 基因	1ng
PCR 用水	补水至 50μL

[0192] 扩增程序:预变性 95℃,3min;变性 95℃,15sec,退火 60℃,30sec,延伸 72℃ 30sec;扩增循环30;终延伸72℃,5min。

[0193] 2.2 、PCR产物用1.5%琼脂糖凝胶,100V电泳1小时。DNA电泳结束后,在紫外灯下快速切下含目的DNA片段的凝胶。

[0194] 2.3、胶回收,用南京诺唯赞生物胶回收试剂盒回收DNA片段(FastPure Gel DNA Extraction Mini Kit货号DC301-01),按试剂盒说明书操作。

[0195] 2.4 将胶回收片段和TRBJ2-2*01对应的pKSH0356 GSRLTVL ED ccdb骨架载体参照如表9体系进行酶切连接反应

[0196] 表9

[0197]

名称	体积(μL)
pKSH0356 GSRLTVL ED ccdb 骨架载体	约 82ng
片段 1	约 12ng
片段 2	约 16ng
片段 3	约 12ng
BsmBI (NEB 公司 货号 R0580)	0.25μL
T4DNA Ligase (NEB 公司货号 M0202V)	0.25μL
ddH2O	Up to 10μL

[0198] 所有片段扩增引物5‘端均含BsmBI识别位点(*CCGTCTC*),酶切位置发生在识别位点

后第一个和第二个碱基之间，即酶切后5’端大写碱基均会去除。片段1的5‘端酶切后（由primer1引入的酶切识别位点）由四碱基粘性末端ggta与载体5撇端会互补连接；片段1的3撇端酶切（由下游引物primer2引入的酶切位点）后的四碱基粘性末端（aaat）与片段2的5撇端酶切（上游引物primer3引入的酶切位点）后的四碱基粘性末端（taaa）互补连接，片段2，片段3同理依次连接，最后片段3连接到载体3撇端。

[0199] 2.4 、转化

[0200] 将10μL连接后产物用上海唯地Stbl3 Chemically Competent Cell（CAT#:DL1046）感受态细胞热激转化，按感受态细胞说明书操作。转化后产物用含Kan抗性的LB固体培养基平板倒置放于37℃培养箱过夜培养。

[0201] 2.5 、将平板倒置放于37℃培养箱过夜培养。

[0202] 2.6 、挑十个克隆，37℃震荡过夜培养后，送上海生工测序。测序结果表明，十个克隆均为目的1G4 TCR质粒。可以看出此方法就有很高克隆阳性率。

[0203] 本发明将TCR基因质粒构建分为骨架载体质粒和三个PCR片段，通过预先构建骨架载体质粒和PCR扩增模板，比传统的TCR全基因合成速度更快，尤其是一次合成多个TCR时效率提高更为明显。NCBI数据库中，TRAV基因有功能的共有156中不同的型，TRBV基因有功能的共有127种。通过预先构建十个骨架载体和合成156个EF1A-TRAV和127个P2A-TRBV，预先合成Primer1，Primer4和Primer5，根据目的TCR序列，设计、订购Primer2，Primer3和Primer6，扩增三个片段和酶切连接反应，就可以快速构建出TCR载体。

[0204] 本具体实施例仅仅是对本申请的解释，其并不是对本申请的限制，本领域技术人员在阅读完本说明书后可以根据需要对本实施例做出没有创造性贡献的修改，但只要在本申请的权利要求范围内都受到专利法的保护。

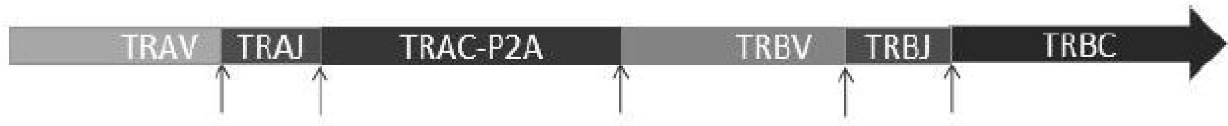

图 1

BsmBI　BsmBI

EF1α　ccdb　PGTRLTVL　TRBC

EF1α　ccdb　GSRLTVL　TRBC

EF1α　ccdb　GTRLTVT　TRBC

EF1α　ccdb　GTRLTVV　TRBC

EF1α　ccdb　GTRLSVL　TRBC

EF1α　ccdb　GTRLSIL　TRBC

EF1α　ccdb　GTRLLVL　TRBC

EF1α　ccdb　GTQLSVL　TRBC

EF1α　ccdb　GGGLLVL　TRBC

EF1α　ccdb　GSWLTVV　TRBC

图 2

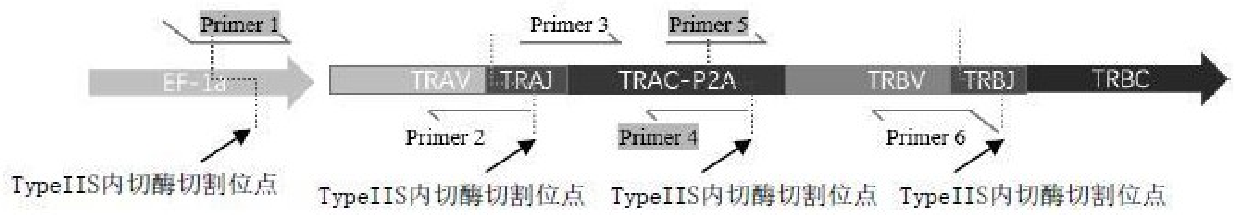

图 3