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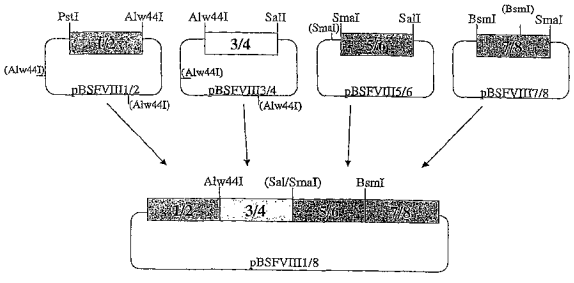
(54)发明名称

重组凝血因子在人类细胞系中的生产

(57)摘要

本发明涉及一种用于生产重组人凝血因子、详细地讲是因子VIII和因子IX的改良方法,该方法利用稳定表达病毒转录激活蛋白并携带含有与编码凝血因子的DNA序列功能性连接的启动子的载体的无限增殖化人类细胞系,条件是所述启动子不是由所述病毒转录激活蛋白刺激的病毒启动子;还涉及携带所述载体的无限增殖化人类细胞系;还涉及特别适用于上面所述生产方法的因子VIII突变蛋白;还涉及包含这类因子VIII突变蛋白的药用组合物以及这类因子VIII突变蛋白在制备用于治疗血友病的药物上的应用。



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1. 一种因子 VIII 突变蛋白，所述突变蛋白中在 Arg740 和 Glu1649 位置之间的 B- 结构域被 SEQ ID NO :9 的序列的富含 Arg 的接头肽所取代，其中所述因子 VIII 编号对应于在 SEQ ID NO :2 中显示的成熟野生型因子 VIII 序列。

2. 权利要求 1 所述的因子 VIII 突变蛋白，其中所述因子 VIII 突变蛋白至少具有下列额外突变 (a)、(b) 和 (c) 中之一 :

(a)162 位置上的 Val 由选自 Gly、Ala、Leu、Ile、Met 和 Pro 的一个中性氨基酸残基所取代 ;

(b)2011 位置上的 Ser 由选自 Asn、Thr 和 Gln 的一个亲水性氨基酸残基所取代 ;和

(c)2223 位置上的 Val 由选自 Glu 和 Asp 的一个酸性氨基酸所取代。

3. 权利要求 2 中所述的因子 VIII 突变蛋白，其中所述因子 VIII 突变蛋白至少具有突变 (a) 和突变 (b) 其中一种。

4. 权利要求 2 中所述的因子 VIII 突变蛋白，其中所述因子 VIII 突变蛋白具有全部三种突变 (a)、突变 (b) 和突变 (c)。

5. 依照权利要求 2-4 中任一项所述的因子 VIII 突变蛋白，其中在突变 (a) 中 162 位置上的 Val 被 Ala 所取代，在突变 (b) 中 2011 位置上的 Ser 被 Asn 所取代，和 / 或在突变 (c) 中 2223 位置上的 Val 被 Glu 所取代。

6. 一种编码权利要求 1-5 中任一项所限定的因子 VIII 突变蛋白的 DNA 序列。

7. 权利要求 6 中所述的 DNA 序列，它相对于在 SEQ ID NO :1 中显示的成熟野生型因子 VIII 的 DNA 序列至少具有 T485C 突变、G6032A 突变和 T6668A 突变中的一种。

8. 权利要求 7 中所述的 DNA 序列，其中所述 DNA 序列包含全部三种所述突变。

9. 一种包含权利要求 6-8 中任一项所限定的 DNA 的载体。

10. 权利要求 9 中所述的载体，它是分别显示于 SEQ ID NO :3、12 和 14 中的 pTGF8-1、pTGF8-2hyg-s 或 pTGF8-3。

11. 权利要求 9 中所述的载体，它是一种基因转移载体。

12. 一种权利要求 9-11 中任一项所限定的载体转化的和 / 或包含权利要求 6-8 中任一项所限定的 DNA 序列的宿主细胞。

13. 用于生产权利要求 1-5 中任一项所述的因子 VIII 突变蛋白的方法，所述方法包括 :

(a) 培养权利要求 12 中所限定的转化宿主细胞 ;并且

(b) 从所述液体培养基中分离所述因子 VIII 突变蛋白。

14. 一种药用组合物，所述药用组合物包含权利要求 1-5 中任一项所限定的因子 VIII 突变蛋白或权利要求 11 所限定的基因转移载体。

15. 权利要求 1-5 中任一项所限定的因子 VIII 突变蛋白或权利要求 11 所限定的基因转移载体在制备用于治疗血友病的药物上的应用。

16. 权利要求 15 的用途，其中所述血友病为 A 型血友病。

17. 权利要求 13 中所述的方法，所述方法包括 :

(a) 培养稳定表达猿猴病毒 T 抗原并且携带具有与编码所述因子 VIII 突变蛋白的 DNA 序列功能性连接的 CMV 启动子的载体的无限增殖化人类细胞系。

18. 权利要求 17 所述的方法，其中所述无限增殖化人类细胞系是无限增殖化肾细胞、

膀胱细胞、肝细胞、肺细胞、心肌细胞、平滑肌细胞、卵巢细胞或胃肠细胞。

19. 权利要求 18 所述的方法，其中所述无限增殖化人类细胞系来源于人胚肾细胞。

20. 权利要求 19 所述的方法，其中所述无限增殖化人类细胞系为 293T 细胞系。

21. 依照权利要求 17-20 中任一项所述的方法，其中所述载体还包含一个选择性标记和 / 或调节序列。

22. 依照权利要求 17-20 中任一项所述的方法，其中所述培养是在存在冯 •维勒布兰德氏因子 (vWF) 的情况下进行的。

23. 依照权利要求 13 和 17-22 中任一项所述的方法，所述方法还包括 ：

(c) 纯化在步骤 (b) 中分离的凝血因子，和 / 或

(d) 将在步骤 (b) 中分离的凝血因子或在步骤 (c) 中纯化的凝血因子进行病毒灭活处理。

24. 一种无限增殖化人类细胞系，所述细胞系稳定表达至少一种病毒转录激活蛋白并携带如权利要求 17-20 中任一项所定义的编码因子 VIII 突变蛋白的载体。

重组凝血因子在人类细胞系中的生产

[0001] 介绍

[0002] 本发明涉及一种用于生产重组人凝血因子，详细地讲是因子 VIII 和因子 IX 的改良方法，该方法利用稳定表达病毒转录激活蛋白并携带含有与编码凝血因子的 DNA 序列功能性连接的启动子的载体的无限增殖化人类细胞系，条件是所述启动子不是由所述病毒转录激活蛋白刺激的病毒启动 子；还涉及携带所述载体的无限增殖化人类细胞系；还涉及特别适合于上面所述生产方法的因子 VIII 突变蛋白；还涉及包含这类因子 VIII 突变蛋白的药用组合物以及这类因子 VIII 突变蛋白在制备用于治疗血友病的药物上的应用。

[0003] 相关技术概述

[0004] 血友病患者是由于凝血级联中的蛋白成分的功能受干扰引起的出血性病症而患病的。根据受影响的凝血因子可以区分为两种类型的血友病。它们的可溶性纤维蛋白原向不溶性纤维蛋白凝块的转化都被抑制。它们是主要影响男性人群的隐性 X- 染色体连锁遗传疾病。

[0005] A 型血友病在每 10,000 男性中影响 1-2 个个体。它是由于缺少或缺失一个非常大的糖蛋白（分子量大约 330kDa(Furie B.，Furie B.C.，Cell(1988)53,505-518)）、在凝血级联中作为一个重要组分的因子 VIII 引起的。所述多肽序列可以细分为 3 个区域，即由所谓的 A1 结构域和 A2 结构域组成的 N- 末端区域、中心 B- 结构域区域和由 A3 结构域、C1 结构域和 C2 结构域构成的 C- 末端区域。在血液凝固中因子 VIII 作为无活性前体存在。它被紧密地而非共价地结合到作为稳定载体蛋白的冯 •维勒布兰德因子 (vWF) 上。因子 VIII 在 3 个特定位置上 (740、372、1689) 被凝血酶蛋白酶切而导致它脱离 vWF，然后在所述级联中开启前凝血剂功能。因子 VIII 以它的活化形式作为因子 IXa 的辅因子发挥功能，从而将因子 X 的蛋白水解活化加快几个数量级。

[0006] B 型血友病在每 25,000 男性中发生大约 1 例。它的特征在于缺乏丝氨酸蛋白酶因子 IX（克里斯马斯氏因子）。这种 415 个氨基酸的多肽是在肝脏合成为 56KDa 的糖蛋白。为了获得它正确的功能，只在维生素 K 存在下才发生的转译后羧基化是需要的。

[0007] 对于这两种类型出血性疾病的疗法传统上包括输注来源于人血浆的因子 VIII 或因子 IX 蛋白浓缩物。虽然这个方法为血友病患者提供了一个有效的治疗，但它带来了传播各种传染性因子的风险，例如引起肝炎或 AIDS 的病毒，或者血栓栓塞因子。已有描述用于生产凝血因子的几种可替代的重组 DNA 技术。为此，野生型因子 VIII 和野生型因子 IX 的对应 cDNA 已被分离并克隆到合适的表达载体中 (EP-A-160457；WO-A-86/01961；美国专利号 4,770,999、5,521,070 和 5,521,070 号）。

[0008] 至于因子 VIII，用于生产显示凝血剂活性的复合体的亚单位的重组表达在本领域是已知的（例如，EP-A-150735、EP-A-232112、EP-A-0500734、WO-91/07490、WO-95/13300、美国专利号 :5,045,455 和 5,789,203）。此外，部分或全部缺失编码高糖基化 B- 结构域的序列的截短 cDNA 形式的表达已有描述（例如在 WO-86/06101、WO-87/04187、WO-87/07144、WO-88/00381、EP-A-251843、EP-A-253455、EP-A-254076、美 国 专 利 号 4,868,112 和 4,980,456、EP-A-294910、EP-A-265778、EP-A-303540 和 WO-91/09122 中）。最近，引入了

各种各样的选定的点突变，以抑制活化C蛋白对因子VIII的蛋白水解失活或者减弱导致治疗患者形成抑制性抗体的免疫原性（例如美国专利5,859,204、5,422,260和5,451,521、WO-97/49725和WO-99/29848）。

[0009] 重组凝血因子通常是从稳定转染的真核生物细胞系、优选哺乳动物细胞系的培养基中分离的。然而，普遍的作法是是在上文中提到的参考资料中所公开的生产方法中使用非人类细胞系，来排除共纯化某些可能由人类细胞包含和表达的传染性因子的风险。

[0010] 可是，尤其对于因子VIII，利用非人类细胞系遇到了一些缺陷。例如已有报道已表达蛋白分泌到培养基中的水平不能令人满意。这可能是由于不同类型的哺乳动物细胞在有可能对所述已表达多肽的生物学活性有影响的蛋白翻译和蛋白修饰的细胞内途径方面有细微差别。除开这点，还与从非人类表达系统中纯化得到的治疗性蛋白被能够在患者体内引发抗原反应的细胞成分所污染有关。

[0011] 此外，由非人类表达系统表达的蛋白可能具有在患者体内引发抗原反应的非人类糖基化模式。可是，凝血因子的生物稳定性和效力显著地受它们的N-糖基化模式所影响。特别是外周单糖和末端单糖是重要的，因为它们被来自负责其降解的细胞的特异性受体所探测到。凝血因子带有作为末端单糖的唾液酸残基。例如就所述凝血因子来说，在糖蛋白触角(tentacle)上的唾液酸组成的修饰可以导致异源糖基化模式。因而当修饰发生时，关键性地牵涉到生物稳定性和效力。因此，在生产重组凝血因子中评估相对于人类细胞系的来自非人类生产细胞系的糖基化影响是一个重要的考虑。总的来说，人类细胞系比非人类细胞系更胜任于生产重组凝血因子是似乎有道理的。这个假设的原因是因为在重组因子合成过程中可能没有外来寡糖会掺入到寡糖部分。

[0012] 另一方面，包括表达病毒转录激活蛋白的无限增殖化的稳定转染哺乳动物细胞系、用于所需基因高水平蛋白表达的通用方法在一段时间内已经成为可能（例如美国专利5,712,119）。另外，用一种载体构建物转化这些细胞系，在所述载体构建物中合适的病毒转录启动子与确定兴趣基因的DNA序列有效连接，所述转录激活蛋白激活所述病毒转录启动子从而启动兴趣基因的表达。这又与由这些细胞系表达的所述转录激活蛋白可能在靶治疗蛋白中造成污染是有关的。

[0013] 考虑到上述这些原因，仍然需要一个人凝血因子的有效生产方法。

[0014] 令人惊奇地，已经发现可以用上面提到的无限增殖化人类细胞系获得无污染的凝血因子。详细地讲，所述无限增殖化细胞系－如果携带含有与编码凝血因子的DNA序列功能性连接的启动子的载体，尽管所述启动子不是一个由所述病毒转录激活蛋白刺激的病毒启动子－能够表达所述凝血因子。与合适的蛋白纯化和病毒灭活方案联合，这个方法提供了一个生产可应用于人类治疗的安全和高活性重组凝血因子的有效系统。此外，发现了对蛋白水解失活异常稳定，因而可以承受强力病毒灭活方案的特定因子VIII突变蛋白。

[0015] 发明概述

[0016] 本发明提供：

[0017] (1)生产重组人凝血因子的方法，所述方法包括：

[0018] (a)培养稳定表达至少一种病毒转录激活蛋白，并且携带含有与编码所述人凝血因子的DNA序列功能性连接的启动子的载体的无限增殖化人类细胞系，条件是所述启动子不是由所述至少一种病毒转录激活蛋白刺激的病毒启动子，和

[0019] (b) 从液体培养基中分离所述凝血因子；

[0020] (2) 在上面 (1) 中限定的所述方法的优选实施方案，其中所述人凝血因子是因子 VIII 或其突变蛋白；

[0021] (3) 在上面 (2) 中限定所述方法的优选实施方案，其中所述因子 VIII 是至少具有下列突变之一的突变蛋白：

[0022] (a)162 位置上的 Val 被另外一个中性氨基酸残基所取代，

[0023] (b)2011 位置上的 Ser 被另外一个亲水氨基酸残基所取代，

[0024] (c)2223 位置上的 Val 被另外一个酸性氨基酸残基所取代，和

[0025] (d) 在 Arg740 和 Glu1649 之间的 B- 结构域被包含 10 至 25 个、优选为 14 到 20 个氨基酸残基的富含精氨酸的接头肽所取代，其中所述因子 VIII 编号对应于显示于 SEQ ID NO :2 中的成熟野生型因子 VIII 序列；

[0026] (4) 在上面 (1) 中限定的所述方法的优选实施方案，其中所述人凝血因子是因子 IX 或其突变蛋白；

[0027] (5) 携带编码在上面 (1) 至 (4) 中所限定的人凝血因子的载体的无限增殖化人类细胞系；

[0028] (6) 在上面 (3) 中所限定的因子 VIII 突变蛋白；

[0029] (7) 编码在上面 (6) 中限定的所述因子 VIII 突变蛋白的 DNA 序列；

[0030] (8) 包含上面 (7) 中限定的所述 DNA 的载体；

[0031] (9) 在上面 (8) 中限定的载体，它是一种基因转移载体；

[0032] (10) 用在上面 (8) 中所限定的载体转化的和 / 或包含在上面 (7) 中所限定的 DNA 序列的宿主细胞；

[0033] (11) 包含在上面 (6) 中限定的所述因子 VIII 突变蛋白或在上面 (9) 中所限定的基因转移载体的药用组合物；

[0034] (12) 在上面 (6) 中限定的所述因子 VIII 突变蛋白或在上面 (9) 中所限定的基因转移载体在制备用于治疗血友病的药物上的应用；和

[0035] (13) 治疗血友病的方法，所述方法包括予人血友病患者在上面 (6) 中限定的所述因子 VIII 突变蛋白或在上面 (9) 中所限定的基因转移载体。

[0036] 附图详述

[0037] 图 1 显示用于构建 B- 结构域缺失的因子 VIII 的所述片段 (实施例 1)。

[0038] 图 2 显示 pTGF8-1 载体，为 8720bp 环状 DNA，它的确切 DNA 序列在 SEQ ID NO :3 中给出 (所述 DNA 序列编码的因子 VIII 蛋白的序列参见 SEQ ID NO :4)。

[0039] 图 3 显示 pTGFG36 载体，为 5753bp 环状 DNA，它的确切 DNA 序列在 SEQ ID NO :6 中给出 (SEQ ID NO :6 中的碱基 689-2071 编码因子 IX 蛋白)。

[0040] 图 4 显示 pTG36hyg 载体，8124bp 环状 DNA。

[0041] 图 5A 描述本发明的优选接头序列 (SEQ ID NO :9)。

[0042] 图 5B 显示在实施例 6 中测定的重组 hFVIII 的凝固时间。

[0043] 图 6 显示 pTGF8-2hyg-s 和 pTGF8-3 的共同分子结构，它们均为 10698bp 环状 DNA，它们的确切 DNA 序列在 SEQ ID NO :12 中和 SEQID NO :14 中给出 (所述 DNA 序列编码的因子 VIII 蛋白的序列参见 SEQID NO :13 和 SEQ ID NO :15)。

[0044] 图 7A 显示实施例 5 中所描述的 FVIII ELISA 的校准曲线。

[0045] 图 7B 描述在实施例 5 中所述的不同培养滤液中重组 FVIII 浓度的测定结果。

[0046] 图 8 显示在实施例 9 中所描述的因子 VIII 特异性免疫荧光测定结果。上排：pTGF8-3 稳定转染的 293T 细胞，克隆 49/19。下排：阴性对照：未转染的 293T 细胞。A 和 C：白光、无滤光片；B 和 D：因子 VIII 的荧光检测，滤光片 550nm。

[0047] 图 9 显示在实施例 10 中所描述的热处理对培养滤液中 FIX 活性的影响。

[0048] 图 10 显示活性重组因子 IX 的表达对于在培养基中补加维生素 K 的依赖。

[0049] 发明详述

[0050] “功能性连接”是指其中所述启动子以它可以刺激编码人凝血因子的 DNA 序列的转录的方式位于所述载体中的所述载体的配置。“非功能性连接”是指所述启动子远离表达的凝血因子基因序列而不能够刺激它的转录的配置。

[0051] “基因”是指编码多肽、可选地包括前导序列、尾随序列、内含子和外显子的 DNA 序列。

[0052] “载体”是指当与适当的控制元件相连接时能够复制的任何基因构建物，例如质粒、噬菌体、粘粒等。该术语包括克隆载体和表达载体。“携带载体”包括在宿主细胞中稳定掺入和瞬时掺入功能性 DNA 区断。然而，优选稳定掺入。

[0053] 依照本发明的“基因转移载体”包括适用于基因治疗的载体。这种载体包含用于所需目的的本领域已知的功能性序列。

[0054] 术语“成熟”是指给定蛋白刚从细胞分泌出来后的分子结构（即，缺少它的 N- 末端输出信号的多肽）。

[0055] “启动子”是指用于控制 RNA 聚合酶所结合基因的转录的调节 DNA 序列区域。

[0056] 本发明所述药用组合物的“治疗有效剂量”是指对于治疗或预防有效的剂量，例如，产生有效治疗或减轻血友病症状的剂量。治疗有效剂量的确定是在本领域技术人员的技术范围之内。

[0057] “编码”是指核酸序列处于适当的调节序列控制之下时在体外或体内被转录（如果是 DNA）或被翻译（如果是 mRNA）成为多肽的特性。

[0058] 对于本申请的目的而言，“表达”是指编码蛋白的基因的转录和翻译。

[0059] 在上面 (1) 到 (13) 中所描述的本发明会在下文中更详细地描述。依照本申请发明的实施方案 (1)，与编码人凝血因子的 DNA 序列功能性连接的启动子不是由所述无限增殖化人类细胞系表达的至少一种病毒转录激活蛋白刺激的病毒启动子。

[0060] 所述无限增殖化人类细胞系优选是无限增殖化肾细胞、膀胱细胞、肝细胞、肺细胞、心肌细胞、平滑肌细胞、卵巢细胞或胃肠细胞。更优选所述无限增殖化细胞系来源于人胚胎肾细胞，最优选是 293T 细胞系 (ECACC：tsa201，ref.96121229；DSM ACC2494)。

[0061] 所述无限增殖化细胞系表达的至少一种转录激活蛋白包括：猿猴病毒 T 抗原、腺病毒 E1A 蛋白或腺病毒 E1B 蛋白、由牛乳头瘤病毒早期区 DNA 序列编码的蛋白和疱疹病毒 IE 蛋白。优选所述无限增殖化细胞系至少表达两种转录激活蛋白，例如，温度敏感型 SV40T 抗原和腺病毒 E1A 蛋白（例如上面的 293T 细胞系）。

[0062] 与编码人凝血因子的 DNA 序列功能性连接的所述启动子优选包括：

[0063] (i) 不被上面所限定的无限增殖化细胞所表达的激活蛋白刺激的病毒启动子（例

如 SV40 和 CMV）；

[0064] (ii) 宿主管家启动子（清蛋白）；和

[0065] (iii) 组织特异性启动子（例如对肝脏特异性的 α－抗胰蛋白酶）。依照本发明的最优选的启动子是 CMV 启动子（由所述无限增殖化细胞表达的转录激活蛋白不刺激所述启动子）。

[0066] 依照本发明，所述载体可以携带另外的由所述病毒转录激活蛋白刺激、但不与凝血因子功能性连接的病毒启动子。这类病毒启动子选自来源于腺病毒、劳斯肉瘤病毒和巨细胞病毒的启动子。所述载体还可以含有一个或多个下列功能性序列：选择标记、调节序列（例如，PRE）等。

[0067] 依照本发明实施方案 (1) 的所述人凝血因子包括但不限于因子 IX、因子 VIII、因子 VII、因子 V、冯·维勒布兰德氏因子 (vWF) 以及诸如此类。

[0068] 在本发明的优选实施方案 (2) 中，所述载体包含编码因子 VIII 或其突变蛋白的 DNA 序列。已经设计了多种修饰过的因子 VIII 表达构建物用于重组表达，而重组因子 IX 与从血浆中分离的野生型蛋白在总体结构上相同。考虑到功能性因子 VIII 多肽中与 vWF 相互作用的重要位点的域结构位于 A3 结构域（氨基酸 1680-1689）和 C2 结构域 (Kaufman 和 Pipe，Haemophilia(1998)4，第 370-379 页）。提出在 1689 后切割来从 vWF 释放因子 VIII 并使得因子 VIII 可以与带电荷磷脂相互作用。当缺少 vWF 结合位点的重组因子 VIII 构建物注射入因子 VIII 缺乏的小鼠时，它显示出非常易于被蛋白酶酶切。截短的因子 VIII 构建物在哺乳细胞培养物中的重组表达例证了所述 B 结构域的完全缺失不改变相应的因子 VIII 样蛋白的生物活性 (Eaton 等，Biochemistry(1986)25，8343-8347)。另外，由于细胞中 mRNA 水平增强了，因此 B 结构域缺失型构建物的可观察表达速度明显高于野生型因子 VIII(Pittman 等，Blood(1993)81，2925-2935)。目前有四种重组因子 VIII 制品（Recombinate® Baxter HealthCare；Kogenate® 和 Kogenate FS® Bayer Corporation 以及 Refacto® Wyeth，GeneticsInstitute) 上市。

[0069] 在本发明的优选实施方案 (3) 中，所述因子 VIII 突变蛋白至少具有下列 (a) 至 (d) 突变中的一个：

[0070] (a)162 位置上的 Val 被另外一个中性氨基酸残基所取代；

[0071] (b)2011 位置上的 Ser 被另外一个亲水性氨基酸残基所取代；

[0072] (c)2223 位置上的 Val 被一个酸性氨基酸残基所取代；和

[0073] (d) 在 Arg740 和 Glu1649 之间的 B- 结构域被包含 10 到 25、优选为 14 到 20 个氨基酸残基的富含精氨酸的接头肽所取代，其中所述因子 VIII 编号与显示于 SEQ ID NO：2 中的野生型因子 VIII 的氨基酸序列相对应的（所述成熟肽的氨基酸序列不包括 19 个氨基酸的信号肽，但包括完整的 B 结构域 (WO 99/29848)）。

[0074] 依照本发明的“另外一个中性氨基酸残基”包括甘氨酸、丙氨酸、亮氨酸、异亮氨酸、甲硫氨酸和脯氨酸，优选是丙氨酸。所述“另外一个亲水性氨基酸”包括天冬酰胺、苏氨酸和谷氨酰胺，优选是天冬酰胺。所述酸性氨基酸选自谷氨酸和天冬氨酸，优选是谷氨酸。

[0075] 在实施方案 (3) 的所述因子 VIII 突变蛋白中，优选所述因子 VIII 突变蛋白至少具有 (a)、(b) 和 (c) 突变中的一个，更优选至少具有 (a) 和 (b) 突变中的一个，最优选具有上面所限定的 (a) 至 (c) 全部三个突变。特别优选所述突变蛋白含有全部三个 V162A、

S2011N 和 V2223E 突变。

[0076] 基于同样的考虑，本发明的实施方案 (4) 中所述载体所包含的 DNA 序列与 SEQ ID NO :1 中显示的成熟野生型因子 VIII DNA 序列相比，具有 T485C、G6032A 和 T6668A 突变。在一个优选实施方案中，所述 DNA 序列也含有 T6816C 平静（即，沉默）突变（所述编号又是与成熟野生型因子 VIII 的 DNA 序列相对应）。

[0077] 在实施方案 (3) 中的所述因子 VIII 突变蛋白中，另一种优选方式是因子 VIII 突变蛋白具有上面所述 (d) 突变。

[0078] 本发明的优选表达系统利用独特因子 VIII 突变蛋白，它除了前文所述 (a) 至 (c) 点突变之外，还部分或全部缺少其 B 结构域 -，优选在 R740 和 E1649 位置间的 B 结构域被上面 (d) 中所述的特征性富含精氨酸的氨基酸间隔区所取代的突变蛋白。依照本发明的“富含精氨酸的”是指所述间隔区包含至少 3 个、优选至少 4 个精氨酸残基。在一个优选实施方案中，所述间隔区由野生型 B 结构域的 8 个氨基酸、然后是可变结构域的 8 个氨基酸组成（参见图 5a，SEQ ID NO :9）。在这种具有上文中所讨论的 B 结构域修饰的构建物中，提出的 vWF 结合位点保持不变，以阻止细胞培养基中或随后进入到被治疗患者的血液中的分泌型因子 VIII 立即被蛋白酶酶切消化。只有在通过凝血酶酶切特异性活化后，因子 VIII 才会从 vWF 释放。所述优选因子 VIII 的 cDNA 是通过装配 4 个 DNA 片段、例如实施例 1 中所描述的构建的。

[0079] 本发明实施方案 (3) 中的所述蛋白可以包含另外的 N- 末端序列或 C- 末端序列，它们包括但不限于天然输出信号肽（对应于 SEQ ID NO4、13 和 15 中所显示蛋白的氨基酸残基 -19 至 -1）或其片段或类似物、人工肽（例如用于高亲和纯化的寡组氨酸标记物）等等。

[0080] 用于表达因子 VIII 的优选载体是在图 2 中显示的 pTGF8-1 载体。所述载体的 DNA 序列显示于 SEQ ID NO :3 中，而且它包含上文所述的全部 5 个突变 (T485C、G6032A、T6668A 和 T6816C（这里 :T1217C、G4088A、T4724A 和 T4872C) 和编码 SEQ ID NO :9 中的 B 结构域接头的 DNA 序列）并编码在 SEQ ID NO :4 中描述的因子 VIII 突变蛋白。

[0081] 另外的最优选载体是 pTGF8-2hyg-s 和 pTGF8-3，它们共同的分子结构描述于图 6 中。

[0082] 显示于 SEQ ID NO :12 中的 pTGF8-2hyg-s 只包含 T6816C 沉默突变，产生一种由接头肽 SEQ ID NO.9 取代所述 B 结构域的因子 VIII 突变蛋白，但在参比野生型序列 SEQ ID NO.2 时一级蛋白结构上没有进一步的改变。

[0083] 显示于 SEQ ID NO :14 中的 pTGF8-3 含有 T485C、T6668A 和 T6816C 突变，产生一种除了显示出上面所述的 B 结构域取代外，还相对于 SEQ ID NO :2 显示出 V162A 和 V2223E 氨基酸取代的因子 VIII 突变蛋白。

[0084] 在生产因子 VIII 的情况下，所述培养是在冯·维勒布兰德氏因子存在下进行的。所述冯·维勒布兰德氏因子的用量优选为每 mol 因子 VIII10-100、更优选为 50-60mol vWF（在液体培养基中和 / 或纯化过程中在因子 VIII 溶液中）（参见下面）。

[0085] 在本发明的优选实施方案 (4) 中，所述人凝血因子是因子 IX 或其突变蛋白，优选是显示于 SEQ ID NO :5 中的野生型因子 IX。因子 IX 的合适突变蛋白包括所述因子 IX 的点突变形式和截短形式。用于表达因子 IX 的最优选载体是分别显示于图 3 和图 4 中的

pTGFG36 载体和 pTG36hyg 载体。

[0086] 在生产因子 IX 的情况下，所述培养优选在维生素 K 存在下进行，维生素 K 存在的量可以是 0.1-100μg/ml 液体培养基，更优选 1-20μg/ml 液体培养基。

[0087] 依照本发明实施方案 (1) 的所述方法还包含以下步骤：

[0088] (c) 纯化在步骤 (b) 中分离的凝血因子和 / 或

[0089] (d) 将在步骤 (b) 中分离的或在步骤 (c) 中纯化的凝血因子进行病毒灭活处理。

[0090] 适当的纯化步骤包括本领域所熟知的最大化地提高纯的、稳定的和高活性制品产量的方法，选自免疫亲和层析、阴离子交换层析、大小排阻层析等和它们联合应用。详细地讲，来自人血浆的凝结因子的详细纯化方法，例如在 WO93/15105、EP0813597、WO96/40883 和 WO96/15140/50 中有公开。它们很容易适合于分离重组因子 VIII 和重组因子 IX 所需要的特殊要求。对于因子 IX 已经采用了一个有效的方案，它包含硫酸铵沉淀步骤，然后是 DEAE 和 HIC 触手层析 (HIC tentaclechromatography) 以及肝素亲和层析 (US5919909)。在纯化过程中和纯化后纯化蛋白的质量和活性可以通过 ELISA 和凝固测定来监测。

[0091] 为了克服在纯化蛋白样品或从含有所选分泌型重组蛋白的细胞培养上清液中直接获得的制品中可能有传染性污染物的问题，所述样品和 / 或所述培养上清液要经过包括热处理（干燥的或液态，加入或不加入包括蛋白酶抑制剂在内的化学物质）的病毒灭活程序处理。在病毒灭活后，用于清除所述化学物质的另一纯化步骤可能是必要的。详细地讲，对于从血浆中分离的因子 VIII 来说，通过阴离子交换层析回收高纯度经病毒灭活的蛋白已有描述 (WO93/15105)。另外，已报道几种方法用于生产来自血浆或其他生物来源的高纯度、无感染性凝血因子。有脂包膜的病毒通过利用形成一个两相系统的疏水相处理潜在地传染性物质，随后除去水不溶部分而被有效灭活。已经证明，同时或随后利用非离子性生物相容去污剂和磷酸二烷基酯或磷酸三烷基酯的处理补充疏水相处理方法是另一个优点 (WO 9636369、EP0131740、US6,007,979)。无脂包膜的病毒要求由非离子性去污剂处理然后是数小时加热 (60-65℃) 组成的灭活方案 (WO94/17834)。

[0092] 鉴于以上结果，相信基于人类细胞系的有效蛋白表达系统与被批准的用于灭活潜在危险的感染性污染物的方法的结合可作为用于生产重组凝血因子的安全和简单的应用系统。

[0093] 而且，依照本发明的实施方案 (6)，提供了一种优良的因子 VIII 突变体。所述因子 VIII 突变体可以作为药用组合物的一部分，可以用于制备治疗血友病的药物以及可以应用于治疗血友病的方法中（本发明的实施方案 (11) 至实施方案 (13)）。上面所述的药用组合物和药物可以包含治疗有效剂量、例如 50-500μg(200ng 因子 VIII 相当于一个国际单位 (IU)) 的因子 VIII。根据血友病的类型，患者接受因子 VIII 的年剂量可达到 200,000IU，它通常是以每周一次剂量或每周两次剂量给予。

[0094] 应用于实施方案 (11) 至实施方案 (13) 的治疗血友病方法中的所述药用组合物、药物或制剂含有治疗有效剂量的实施方案 (6) 中所述因子 VIII 突变蛋白或实施方案 (9) 中所述基因转移载体。如果是前者，则它还可以包含药学上可接受的添加剂，包括人血清白蛋白 (HSA；优选为约 1mg/ml 溶液）；无机盐例如 $CaCl_2$（优选为 2-5mM）；氨基酸例如甘氨酸、赖氨酸和组氨酸（优选为每种氨基酸 0.1-1M）；双糖例如蔗糖和 / 或海藻糖（优选为 0.4-1M）；有机盐例如柠檬酸钠（优选为达到 50mM）；等。所述制剂可以是水性的或非水性

的。在后一种情况下，主要成分是甘油和 / 或聚乙二醇（例如，PEG-300）。所述制剂也可以是干燥形式（给药前溶解于所需的溶剂中）。

[0095] 如上面所阐述的，依照本发明实施方案 (9) 中的所述基因转移载体也可以作为药用组合物的一部分，可以用于制备治疗血友病的药物以及可以应用于治疗血友病的方法中（本发明的实施方案 (11) 至实施方案 (13)）。所述药用组合物和药物还可以包含适当的基质制剂，例如，在 WO 00/49147 中所论述的脂类或激素（其公开内容通过引用结合到本文中）。含有所述基因转移载体或本发明的基因转移载体的所述药用组合物或药物可以口服给药、静脉给药、肌内给药、皮下给药、局部给药、通过黏膜（包括口腔、鼻腔喷雾）或利用基因枪给药。口服给药（例如，微粉化激素分散体）是优选的。

[0096] 本发明实施方案 (6) 中所述因子 VIII 突变蛋白优选如上面实施方案 (3) 所限定的。所述 FVIII 突变蛋白还可以通过标准重组技术来制备，例如包含下面步骤的方法：

[0097] (a) 培养实施方案 (8) 中所述载体转化的和 / 或包含实施方案 (7) 中所述 DNA 的宿主细胞（也包括培养稳定表达至少一种病毒转录激活蛋白并携带含有与编码人凝血因子的 DNA 序列功能性连接的病毒转录启动子的载体的无限增殖化人类细胞系，其中所述病毒启动子被至少一种所述病毒转录激活蛋白刺激）；和

[0098] (b) 从液体培养基中分离所述凝血因子。适当的无限增殖化人类细胞系、转录激活蛋白和病毒启动子是在上文中提到的那些启动子。用于所述方法中的无限增殖化人类细胞系优选表达 2 种病毒转录激活蛋白，最优选表达温度敏感型 SV40T 抗原和腺病毒 E1A 蛋白。所述方法还可以包含上文中所描述的纯化和病毒灭活步骤 (c) 和 (d)。

[0099] 所述市售的 293T 细胞系 (ECACC：tsa201，ref.96121229) 在 2001 年 2 月 20 日，于 DMSZ（德意志微生物保藏中心 (Deutsche Sammlung vonMikroorganismen und Zellkulturen GmbH)，Mascheroder Weg 1b，38124Braunschweig，Germaany) 保藏，保藏号：DSM ACC2494。

[0100] 本发明通过下列实施例进一步举例说明。

[0101] 实施例

[0102] 实施例 1- 因子 VIII 的克隆：

[0103] 所述重组因子 VIII 的序列通过逆转录从完整人肝细胞 RNA 库中获得。然后利用设计的含有限制性位点的引物通过标准 PCR 扩增四个片段 (1/2，3/4，5/6，7/8)。为了将所述 3/4 片段和 5/6 片段安装在一起，将来自 pBSFVIII3/4 质粒的 SmaI/SalI 片段平端插入到 pBSFVII5/6 的 SalI 位点从而获得 pBSFVIII3/6。然后用 XhoI/BspHI 酶切和用 Alw44I 部分酶切 pBSFVIII3/6，获得 3/6 片段。这个片段和来自 pBSFVIII1/2 的 PstI/Alw44I 片段一起一步连接到用 PstI 和 XhoI 酶切的 pBSFVIII1/2 的载体骨架中，通过这种方法获得 pBSFVIII1/6。所述 7/8 片段通过用 SmaI 酶切和用 Mva1269I 部分酶切 pBSFVIII7/8 获得的，然后连接到用 XhoI 和 Mva1269I 切割的 pBSFVIII1/6 中，从而得到 pBSFVIII1/8。最后，来自 pBSFVIII1/8 的 SmaI/XhoI 片段平端插入到 Octagene Vector pTGFG67（所述载体的生产在 PCT/EP00/01368 中已经公开）的 SalI 位点，从而产生人因子 VIII 的真核表达载体 pTGF8-1（参见图 1 和图 2)。所述产生的载体编码具有 V162A、S2011N 和 V2223E 突变的因子 VIII 突变蛋白。

[0104] 实施例 2- 因子 IX 的克隆：

[0105] 载体pUC19(MBI Fermentas)用XbaI酶切,然后用克列诺酶处理并再连接。为了删除EcoRI位点,则这个XbaI缺失型载体用EcoRI酶切,然后用克列诺酶处理并再连接。为了将一个XbaI位点插入到这个载体的SacI位点,这个载体用SacI酶切,随后用T4DNA聚合酶处理,然后用碱性磷酸酶去磷酸化,再与XbaI接头CTCTAGAG(Biolabs#1032)连接。另外一个XbaI位点通过用HindIII酶切新产生的载体,用克列诺酶处理,碱性磷酸酶去磷酸化以及与XbaI接头CTCTAGAG(Biolabs#1032)连接来插入。这个载体被命名为pUC19/X。

[0106] 为了破坏存在于载体phGFP-S65T(Clontech)中的XbaI位点,这个载体用XbaI酶切,然后用克列诺酶处理并再连接,从而产生pGFP/0载体。用MluI酶切pGFP/0,随后用克列诺酶处理,再用BamHI酶切后,分离到包含GFP基因的2.3kb片段。这个片段插入到用SalI酶切、克列诺酶处理和BamHI酶切的pUC19/X载体的多克隆位点。产生的载体被命名为pTGFG1。

[0107] 寡核苷酸(Metabion)PRE-S(5'-GGG GTA CCA GCT TCG TAGCTA GAA CAT CAT GTT CTG GGA TAT CAG CTT CGT AGC TAG AAC ATCATG TTC TGG TAC CCC-3′ ;SEQ ID NO:10)和

[0108] PRE-AS(5′ -GGG GTA CCA GAA CAT GAT GTT CTA GCT ACG AAG CTG ATATCC CAG AAC ATG ATG TTC TAG CTA CGA AGC TGG TAC CCC-3′ ;SEQ IDNO:11)杂交,并通过激酶反应磷酸化形成插入片段PRE(ds)。

[0109] pTGFG1载体用Eco0109I酶切,用克列诺酶处理然后用碱性磷酸酶去磷酸化。然后与PRE(ds)插入片段连接,形成pTGFG5载体。pUC19载体(MBI Fermentas)用SalI酶切,用克列诺酶处理然后用碱性磷酸酶去磷酸化。它与NotI接头GCGGCCGC(Biolabs#1045)连接,形成pUC19/N载体。

[0110] 利用重叠因子IX开放读框的起始密码子和终止密码子的两种引物,从人肝脏cDNA(Clontech)中扩增因子IX cDNA,产生包含完整开放读框的1387bp片段。EcoRI(上游)和BamHI(下游)的限制性位点被包括在每个引物的末端来促进克隆。用Pwo DNA聚合酶(BoehringerMannheim)进行扩增,以50μl反应体积[10mM Tris HCl pH 8.85、25mMKCl、5mM$(NH_4)_2SO_4$、2mM $MgSO_4$]在96℃保持1分钟、60℃保持1分钟、72℃保持2分钟共30个温育循环,然后是最终的一个延伸步骤,在72℃保持10分钟。

[0111] 将反应产物连接到pUC19的EcoRI位点和BamHI位点,然后转化到大肠杆菌(E.coli)DH5-α中。选择阳性克隆。通过用标记引物(IR-700)从两端进行循环测序(Amersham)和在LiCor测序系统(MWG,Biotech)中进行自动分析来证实序列。

[0112] 使用下列引物:

[0113] GGAATTCCGCAAAGGTTATGCAGCGCGTGAACATGATCATGGC(上游;SEQ.ID NO:16)

[0114] CGCGGATCCATTAAGTGAGCTTTGTTTTTTCCTTAATCC(下游;SEQ.ID NO:17)

[0115] 将从人cDNA文库中分离的包含人凝血因子IX开放读框的1.4kb片段插入到上面通过用PstI酶切、T4聚合酶处理和碱性磷酸酶去磷酸化制备的pUC19/N载体的PstI位点中。通过用HindIII和NotI双酶切,从所得的pUC19/N-FIX载体切取包含人凝血因子IX开放读框的一个1.4kb片段。将这个片段连接到HindIII和NotI双酶切的pTGFG5载体的4.3kb片段上,产生显示于图3中的pTGFG36载体。这个载体是用于将编码因子IX的表达盒传递到所述细胞中的优选载体,它的DNA序列在SEQ ID NO:6中提供。

[0116] 实施例 3- 用于蛋白表达的人类细胞系

[0117] 一个优选的细胞系是 tsA201(ECACC Ref. :96121229),它是稳定表达 SV40 温度敏感 T 抗原的转化的人胚肾细胞系 (293, ECACC 保藏号 85120602)(J.Membrane Biol.1996 ;152 :39 ;Gene 1995 ;156 :235 ;PNAS USA 1994 ;91 :12785 ;Pflügers Arch.1994 ;427 :136 ;J.Gen.Physiol.1994 ;104 :507 ;BioTechniques 1993 ;15 :906)。这个细胞系的其他名称包括 293tsA1609neo(Mol.Cell.Biol.,1987,7 :379) 和 293T。这个上皮样细胞系已经被用于各种功能性表达检测,而且已有报道可以生产高水平的重组蛋白。它们可以在补充了 2mM 谷氨酰胺和 10% FCS 的 DMEM 中培养。对于因子 IX 的有效生产,可以通过加入达到 100μg/ml 的维生素 K 来改良所述培养基 (US4770999)。

[0118] 为了简化已表达多肽的纯化,细胞可以培养于含有适当补充成分的无血清培养基或无蛋白培养基中。出于稳定性的原因,分泌型因子 VIII 要求在培养基中存在 vWF(US5198349)。加入脂蛋白、磷脂、聚乙二醇、痕量金属、肝素、非离子表面活性剂或环糊精也已有报道 (EP0254076、US5679549、US5198349、US5250421、US5576194、EP0872487、WO94/11525、US5378612)。

[0119] 实施例 4- 用于瞬时生产因子 VIII 和因子 IX 的 293T 细胞的磷酸钙转染 :转染前一天,融合 293T 细胞以低密度接种到装有 6ml DMEM/10% FCS(对于 FIX 是 10μg/ml 维生素 K) 的 10cm 平皿中。转染大致按照 Chen 和 Okayama 的方法 (Mol.Cell Biol.,7 :2745(1987)) 进行。对于生产因子 VIII 用 12μg pTGF8-1 质粒转染,对于生产因子 IX 用 12μg pTGFG36 质粒转染。转染后 6 小时,培养基用新鲜的培养基更换,转染后 3 天收获上清液,然后或者进行进一步的纯化。或者不需要更进一步纯化而通过 ELISA 或凝固实验进行分析 (参见实施例 5 和实施例 6)。

[0120] 实施例 5- 通过 ELISA 对 FIX 和 FVIII 浓度的测定 :

[0121] 因子 IX :利用山羊多克隆抗 - 人 FIX(Enzyme Research Laboratories) 作为捕捉抗体,通过 ELISA 测定在转染 293T 细胞上清液中的人重组因子 IX 水平。所有的温育是在潮湿室中 22℃下进行 2 小时。用 100μl 的 8.8μg 抗体 /ml 包被缓冲夜包被平板 (Dynex, Immulon-4)。封闭不需要在所描述条件下进行。用 PBS-Tween® (0.1% v/v) 洗板 4 次 (Encore 2000, Merck) 足以封闭非特异性相互作用。

[0122] 每进行一步,都需要进行清洗以清除未结合蛋白。在每孔中加入 100μl 用 10μl 10mM PMSF 和 10μl 0.11M 柠檬酸钠处理的上清液。样品和标准品 (人因子 IX, 内标 (house standard), Octapharma) 用稀释缓冲液 (HBS-BSA-EDTA-Tween®) 稀释,并以 100μl/ 孔进行温育。检测抗体是浓度为 1μg 抗体 /ml 稀释缓冲液的过氧化物酶标记的山羊多克隆抗 -FIX(Enzyme Research Laboratories),并以 100μl/ 孔进行温育。在每孔中加入 150μl ABTS(Roche) 作为底物,1-2 小时后在 405nm 检测比色反应。通过标准品浓度对标准品吸光度的线性回归计算结果并总结于下表中 :

[0123]

细胞数量 [/ml]	因子 IX 浓度 [ng/ml]	凝固时间 [s]
2.1×10^5	36	45
8.7×10^5	20	79

[0124] 正常血浆： 37-39s

[0125] 因子 IX 缺乏血浆： 137-140s

[0126] 因子 VIII：利用亲和纯化的多克隆绵羊抗 FVIII :C 制剂 (F8C-EIA-C, Affinity Biologicals) 作为捕捉抗体，通过 ELISA 测定在转染 293T 细胞培养滤液中的人重组因子 VIII 水平。包被在潮湿室中 22℃下进行 2 小时。用 100μl 100 倍稀释到包被缓冲液 (50mM 碳酸钠 pH 9.6) 的抗体包被平板 (Dynex, Immulon-4)。用 PBS-Tween® (0.1% v/v) 洗板 4 次 (Encore 2000, Merck) 足以封闭非特异性相互作用。

[0127] 每进行一步，都需要进行清洗以清除未结合蛋白。100μl 取自 pTGF8-3 稳定转染的不同 293T 克隆的每种培养滤液样品在温育 48 小时后加入到每孔中。FVIII 标准品 (内标，Octapharma) 用稀释缓冲液 (HBS-BSA-EDTA-Tween®) 稀释，并以 100μl/ 孔进行温育。对于检测来说，过氧化物酶标记的山羊多克隆抗 -F IX(Enzyme ResearchLaboratories) 的现用现稀释液以 100μl/ 孔进行温育 60 分钟。对于比色反应来说，临使用之前将 5mg 的邻苯二胺 (P-6912, Sigma) 片剂溶于 12ml 底物缓冲液中，并补充 12μl 30% H_2O_2。在每孔中加入 150μl 这种底物溶液，室温下避光温育 10 分钟并通过在每孔中加入 50μl 2.5MH_2SO_4 终止反应后，利用 MRX Reader(Dynex) 在 490nm 处进行比色纪录。通过标准品浓度对标准品吸光度的线性回归 (图 7A) 计算结果并总结于图 7B 中。

[0128] 实施例 6：人凝血因子 VIII 和因子 IX 活性的检测

[0129] 在 293T 细胞 (如实施例 4 中所述，通过磷酸钙沉淀用 pTGF8-1 转染的) 的细胞培养物上清液中的人重组因子 VIII 的凝血活性测定如下：

[0130] 凝血活性的分析基于使用人工凝血仪器 (ML-2, InstrumentationLaboratories) 利用脑磷脂 (磷脂酰乙醇胺) 活化进行的部分凝血致活酶时间检测。对于这个研究来说，来自转染 293T 细胞的 100μl 未稀释上清液、100μl 缺乏型血浆 (Progen) 和 100μl 脑磷脂 (InstrumentationLaboratories) 在 37℃温育 5 分钟。通过加入 100μl $CaCl_2$ 起始凝固。样品凝固时间与正常血浆进行比较。所述结果总结于图 5B 中。从图 5B 中可以看出，来自 pTGF8-1 转染细胞的细胞上清液显示出与正常血浆相当凝固活性，而未转染细胞与缺乏因子 VIII 血浆的值相等。

[0131] 对于因子 IX 进行相同的检测。所述结果显示于实施例 5 的表中。对于表达对于维生素 K 存在依赖性的参见图 10。

[0132] 实施例 7- 病毒灭活：

[0133] 病毒灭活是按照美国专利号 6,007,979 的所述方法进行的。也就是，在潜在地感染性蛋白溶液中依次加入下列化合物，并搅拌：

[0134] 1. 0.2ml Tween® 80 和 0.06ml TNBP 加入到 19.74ml 的所述溶液中或

[0135] 2. 0.2ml Triton® X-100 和 0.2ml TNBP 加入到 19.6ml 的所述溶液中。

[0136] 在制剂 1 和制剂 2 中加入 1ml 蓖麻油，然后在室温下剧烈萃取一个小时。

[0137] 一旦分相就进行离心。对于感染性控制，重复地从水相部分每次取走 1ml 样品。

[0138] 实施例 8- 稳定表达因子 VIII 和因子 IX 的细胞系的建立：

[0139] 优选的 pTGF8-1 载体和 pTGFG36 载体分别包含用于在哺乳动物细胞中瞬时表达因子 VIII 和因子 IX 的构建物。为了能够获得稳定转染细胞克隆的选择方法，将潮霉素 B 磷酸转移酶盒（来自 TK-Hyg 的 HindIII-Mva 1260I 片段，Clontech）亚克隆到在两个载体中都存在的 SmaI 位点中。这样产生的构建物（pTGF8-1-hyg 和 pTG36hyg）包含带有一个 CMV 启动子和一个 SV40 聚腺苷酸化信号的人因子 VIII 或人因子 IX 的顺式表达盒，以及带有 HSV 胸苷激酶启动子和 HSV 胸苷激酶聚腺苷酸化信号的潮霉素 B 磷酸转移酶表达盒（参见图 4）。

[0140] pTGF8-2hyg-s 载体和 pTGF8-3 载体（图 6，SEQ ID NO：12 和 SEQID NO：14）是 pTGF8-1hyg 的衍生物，其中利用QuikChange®方案（Stratagene）通过依赖于 PCR 的方法，将 V162A、S2011N 和 V2223E(pTGF8-2hyg-s) 和 S2011N(pTGF8-3) 点突变回复为野生型序列。

[0141] 所述凝血因子的编码序列可以被另外的所选基因序列所取代。这些构建物可供通过磷酸钙转染以及随后的潮霉素抗性选择建立稳定表达的细胞系。此外，所述质粒含有一个孕酮效应元件（PRE）。在用 pTG36hyg 进行的瞬时转染实验中，约 40ng 活性因子 IX/ml 培养基的生产可以通过 ELISA 和凝固检测显示出来（参见实施例 5 和实施例 6）。

[0142] 对于因子 IX 的生产，293T 细胞培养于 10％ FCS 和 10μg/ml 维生素 K 补充的 DMEM 中（美国专利号：4,770,999；同样参见图 10）。首先，必须建立有效筛选稳定转染 293T 细胞的临界抗生素浓度。出于这个目的，所述细胞以低稀释度铺平板并在存在 10-800μg/ml 潮霉素 B 的情况下生长。两周后，在 200μg/ml 或更高的浓度下，没有细胞生长，因此选择这个浓度用于筛选稳定转染的细胞。

[0143] 293T 细胞在前一天以 1：15 传代后，在 10cm 的平皿中进行典型的转染。利用磷酸钙沉淀方法（Biotechniques 1998 6：7632-638）每平皿用 12μg 质粒转染，两天后，培养基更换为含有 200μg/ml 潮霉素 B 的新鲜培养基。选择 2-3 周后，通过 ELISA（参见实施例 5）检测所述培养基中因子 VIII 或因子 IX 的存在。分离阳性克隆并转移到 24 孔板上。经 ELISA 筛选和活性测定后，阳性克隆再进行两个循环的亚克隆，然后扩增并将等分部分冷冻以供进一步的应用和特性鉴定。

[0144] 实施例 9：通过原位免疫荧光检测因子 VIII 表达得到的稳定转染细胞表型一致性的证据：

[0145] 来自 DMEM+9.1％ FBS 中贴壁培养物的 pTGF8-3 转染的 $5\times10^7$293T 细胞（克隆 49/19）和未转染 293T 细胞（阴性对照）全部通过胰酶消化从所述培养平皿中脱离，洗涤多次，然后重新悬浮于 5ml PBS 缓冲液中。

[0146] 2μl 的这些细胞悬浮液转移到无菌显微镜载玻片上，在室温下孵育直到所有液体蒸发。将细胞在 70％的酒精中固定，然后在室温下干燥 5 分钟。通过于含有 10％ FBS 的 PBS 缓冲液中孵育，封闭玻片以防非特异性检测。第一抗体（羊（sh）抗 F VIII：C F8C-EIA-C，AffinityBiologicals）用含有 10％ FBS 和 0.1％皂苷的 PBS 缓冲液稀释 100 倍，然后在湿润的培养箱中室温孵育 60 分钟。用 PBS 充分洗涤后，制备 100 倍稀释的第二抗体（兔抗羊（rb 抗 sh）CY3 缀合物 313-165-003，Jackson ImmunoResearch）并根据上面所述方法进行

孵育。随后，充分洗涤所述显微镜制备物，然后用一层50％甘油和一个盖玻片覆盖。通过白光显微镜和荧光显微镜（570nm处发射）观察细胞。

[0147] 结果描述于图8中。

[0148] 实施例10：对于培养滤液中重组因子IX的热稳定性试验：

[0149] 将在100μg/ml维生素K存在下pTGFG36瞬时转染48小时后从293T细胞中收获并在-80℃下储存7天的培养滤液迅速解冻，分成7个500μl的等分部分，随后用等分部分进行下列的热孵育：

[0150]

样品	温度（℃）	时间（分钟）
1	0	240
2	20	30
3	20	60
4	20	240
5	37	30
6	37	60
7	37	240

[0151] 将样品于冰上冷却，按照实施例6中所标明的测FIX活性（双重测定）检。结果显示于图9中。在达到于37℃至多240分钟的孵育条件下，活性几乎保持稳定。

序列表

<110> 奥克塔金尼有限责任公司 (Octagene GmbH)

<120> 重组凝血因子在人类细胞系中的生产

<130>010613wo/JH/ml

<140>
<141>

<160>17

<170>PatentIn Ver.2.1

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<211>6996
<212>DNA
<213> 人 (Homo sapiens)

<220>
<221>CDS
<222>(1)..(6996)

<400>1

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  1               5                  10                  15

atg caa agt gat ctc ggt gag ctg cct gtg gac gca aga ttt cct cct   96
Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
             20                  25                  30

aga gtg cca aaa tct ttt cca ttc aac acc tca gtc gtg tac aaa aag  144
Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
         35                  40                  45

act ctg ttt gta gaa ttc acg gtt cac ctt ttc aac atc gct aag cca  192
Thr Leu Phe Val Glu Phe Thr Val His Leu Phe Asn Ile Ala Lys Pro
     50                  55                  60

agg cca ccc tgg atg ggt ctg cta ggt cct acc atc cag gct gag gtt  240
Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
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Tyr Asp Thr Val Val Ile Thr Leu Lys Ash Met Ala Ser His Pro Val
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agt ctt cat gct gtt ggt gta tcc tac tgg aaa gct tct gag gga gct 336
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

gaa tat gat gat cag acc agt caa agg gag aaa gaa gat gat aaa gtc 384
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

ttc cct ggt gga agc cat aca tat gtc tgg cag gtc ctg aaa gag aat 432
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
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ggt cca atg gcc tct gac cca ctg tgc ctt acc tac tca tat ctt tct 480
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
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His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
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cta gta tgt aga gaa ggg agt ctg gcc aag gaa aag aca cag acc ttg 576
Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

cac aaa ttt ata cta ctt ttt gct gta ttt gat gaa ggg aaa agt tgg 624
His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

cac tca gaa aca aag aac tcc ttg atg cag gat agg gat gct gca tct 672
His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
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Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
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Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
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Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
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Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile

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Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
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Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
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325 330 335

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Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

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Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
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Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
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Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
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Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
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ttg gga cct tta ctt tat ggg gaa gtt gga gac aca ctg ttg att ata 1392
Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

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Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
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His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
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Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
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Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

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Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
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Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
725 730 735

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755 760 765

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770 775 780

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785 790 795 800

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His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
805 810 815

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Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
915 920 925

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930 935 940

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980 985 990

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995 1000 1005

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Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp Lys Asn Ala
1045 1050 1055

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11701 175 1180

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1185 1190 1195 1200

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1235 1240 1245

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1285 1290 1295

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1300 1305 1310

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Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr Glu Leu Glu

1315 1320 1325

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Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn Met
1330 1335 1340

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Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys
1345 1350 1355 1360

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Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys
1380 1385 1390

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Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu
1395 1400 1405

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1410 1415 1420

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1475 1480 1485

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1490 1495 1500

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1570 1575 1580

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Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser
1585 1590 1595 1600

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1620 1625 1630

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1635 1640 1645

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1650 1655 1660

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1665 1670 1675 1680

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1685 1690 1695

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1700 1705 1710

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1715 1720 1725

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1765 1770 1775

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1795 1800 1805

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1810 1815 1820

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1825 1830 1835 1840

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1860 1865 1870

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Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
1875 1880 1885

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Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
1890 1895 1900

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1905 1910 1915 1920

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1925 1930 1935

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1940 1945 1950

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Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys

1955 1960 1965

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Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu

1970 1975 1980

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Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys

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2005 2010 2015

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Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile

2020 2025 2030

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Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro

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Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr

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2065 2070 2075 2080

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Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp

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Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly

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Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile

2130 2135 2140

att gct cga tac atc cgt ttg cac cca act cat tat agc att cgc agc 6480
Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser

2145 2150 2155 2160

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Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
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2260 2265 2270

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Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
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Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
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130 135 140
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
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His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
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Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
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His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
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His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
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Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
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Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
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Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
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Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
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Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
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Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
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Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser

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Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
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Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
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675 680 685
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700
Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
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725 730 735
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770 775 780
Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785 790 795 800
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
805 810 815
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820 825 830
Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
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Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
850 855 860
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865 870 875 880
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Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
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Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
980 985 990
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Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys

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Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg

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Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys

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Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu

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Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys

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Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys

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Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr

1460 1465 1470

Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr

1475 1480 1485

Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp

1490 1495 1500

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Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly
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Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr
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Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg
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His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
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Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
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Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
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Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
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Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
1795 1800 1805

Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
1810 1815 1820

Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
825 1830 1835 1840

Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
1845 1850 1855

Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr

1860 1865 1870

Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser

1875 1880 1885

Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn

1890 1895 1900

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala

905 1910 1915 1920

Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln

1925 1930 1935

Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn

1940 1945 1950

Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys

1955 1960 1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu

1970 1975 1980

Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys

985 1990 1995 2000

Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val

2005 2010 2015

Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile

2020 2025 2030

Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro

2035 2040 2045

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Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile

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2100 2105 2110

Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly

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Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile

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Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn
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acc tca gtc gtg tac aaa aag act ctg ttt gta gaa ttc acg gat cac   903
Thr Ser Val Val Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His
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cct acc atc cag gct gag gtt tat gat aca gtg gtc att aca ctt aag   999
Pro Thr Ile Gln Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys
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aac atg gct tcc cat cct gtc agt ctt cat gct gtt ggt gta tcc tac  1047
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380 385 390

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Lys Arg Asn Val Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp
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Gln Leu Glu Asp Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile
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Ser Val Phe Phe Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu
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Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn
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Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys

930 935 940

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser

945 950 955

Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn Pro

960 965 970

Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe Thr

975 980 985

Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu Arg

990 995 1000 1005

Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe Lys

1010 1015 1020

Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu

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Pro Gly Leu Val Met Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu

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Ser Met Gly Ser Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His

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Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn

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Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala

1090 1095 1100

Gly Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly

1105 1110 1115

Met Asn Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro Leu

1120 1125 1130

Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala Ser Gly

1135 1140 1145

Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His Tyr Ser Gly

150 1155 1160 1165

Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser Trp Ile Lys Val

1170 1175 1180

Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile Lys Thr Gln Gly Ala

1185 1190 1195

Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr
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Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly
1215 1220 1225
Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His
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Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro
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Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys
1265 1270 1275
Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile
1280 1285 1290
Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala
1295 1300 1305
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310 1315 1320 1325
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Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser
1360 1365 1370
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1375 1380 1385
Val Phc Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu
390 1395 1400 1405
Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
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Asp Leu Tyr
1440

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<212>PRT
<213> 人 (Homo sapiens)

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35 40 45
Ser Gly Lys Leu Glu Glu Phe Val Gln Gly Asn Leu Glu Arg Glu Cys
50 55 60
Met Glu Glu Lys Cys Ser Phe Glu Glu Ala Arg Glu Val Phe Glu Asn
65 70 75 80
Thr Glu Arg Thr Thr Glu Phe Trp Lys Gln Tyr Val Asp Gly Asp Gln
85 90 95
Cys Glu Ser Asn Pro Cys Leu Asn Gly Gly Ser Cys Lys Asp Asp Ile
100 105 110
Asn Ser Tyr Glu Cys Trp Cys Pro Phe Gly Phe Glu Gly Lys Asn Cys
115 120 125
Glu Leu Asp Val Thr Cys Asn Ile Lys Asn Gly Arg Cys Glu Gln Phe
130 135 140
Cys Lys Asn Ser Ala Asp Asn Lys Val Val Cys Ser Cys Thr Glu Gly
145 150 155 160
Tyr Arg Leu Ala Glu Asn Gln Lys Ser Cys Glu Pro Ala Val Pro Phe
165 170 175
Pro Cys Gly Arg Val Ser Val Ser Gln Thr Ser Lys Leu Thr Arg Ala
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Glu Ala Val Phe Pro Asp Val Asp Tyr Val Asn Ser Thr Glu Ala Glu
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Thr Ile Leu Asp Asn Ile Thr Gln Ser Thr Gln Ser Phe Asn Asp Phe
210 215 220
Thr Arg Val Val Gly Gly Glu Asp Ala Lys Pro Gly Gln Phe Pro Trp
225 230 235 240
Gln Val Val Leu Asn Gly Lys Val Asp Ala Phe Cys Gly Gly Ser Ile
245 250 255
Val Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Glu Thr Gly
260 265 270
Val Lys Ile Thr Val Val Ala Gly Glu His Asn Ile Glu Glu Thr Glu
275 280 285
His Thr Glu Gln Lys Arg Asn Val Ile Arg IleIle Pro His His Asn
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Tyr Asn Ala Ala Ile Asn Lys Tyr Asn His Asp Ile Ala Leu Leu Glu

305 310 315 320
Leu Asp Glu Pro Leu Val Leu Asn Ser Tyr Val Thr Pro Ile Cys Ile
325 330 335
Ala Asp Lys Glu Tyr Thr Asn Ile Phe Leu Lys Phe Gly Ser Gly Tyr
340 345 350
Val Ser Gly Trp Gly Arg Val Phe His Lys Gly Arg Ser Ala Leu Val
355 360 365
Leu Gln Tyr Leu Arg Val Pro Leu Val Asp Arg Ala Thr Cys Leu Arg
370 375 380
Ser Thr Lys Phe Thr Ile Tyr Asn Asn Met Phe Cys Ala Gly Phe His
385 390 395 400
Glu Gly Gly Arg Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro His Val
405 410 415
Thr Glu Val Glu Gly Thr Ser Phe Leu Thr Gly Ile Ile Ser Trp Gly
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<213> 人工序列

<220>
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<210>7
<211>8
<212>PRT
<213> 人工序列

<220>
<223> 人工序列的描述 :B- 结构域接头肽

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<210>8
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<212>PRT
<213> 人工序列

<220>
<223> 人工序列的描述 :B- 结构域接头肽

<400>8
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1 5

<210>9
<211>16
<212>PRT
<213> 人工序列

<220>
<223> 人工序列的描述 :B- 结构域接头肽

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<210>10
<211>78
<212>DNA
<213> 人 (Homo sapiens)

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atcatgttct ggtacccc 78

<210>11
<211>78
<212>DNA
<213> 人 (Homo sapiens)
<400>11
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ctacgaagct ggtacccc 78

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<213> 人工序列

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<220>
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ctagagaacc cactgcttac tggcttatcg aaattaatac gactcactat agggagaccc 660
aagcttgacc tcgag atg caa ata gag ctc tcc acc tgc ttc ttt ctg tgc  711
                Met Gln Ile Glu Leu Ser Thr Cys Phe Phe Leu Cys
                                -15                 -10
ctt ttg cga ttc tgc ttt agt gcc acc aga aga tac tac ctg ggt gca   759
Leu Leu Arg Phe Cys Phe Ser Ala Thr Arg Arg Tyr Tyr Leu Gly Ala
        -5              -1   1               5
gtg gaa ctg tca tgg gac tat atg caa agt gat ctc ggt gag ctg cct   807
Val Glu Leu Ser Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro
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gtg gac gca aga ttt cct cct aga gtg cca aaa tct ttt cca ttc aac   855
Val Asp Ala Arg Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn
                30                  35                  40
acc tca gtc gtg tac aaa aag act ctg ttt gta gaa ttc acg gat cac   903
Thr Ser Val Val Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His
            45                  50                  55
ctt ttc aac atc gct aag cca agg cca ccc tgg atg ggt ctg cta ggt   951
Leu Phe Asn Ile Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly
        60                  65                  70
cct acc atc cag gct gag gtt tat gat aca gtg gtc att aca ctt aag   999
Pro Thr Ile Gln Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys
    75                  80                  85
aac atg gct tcc cat cct gtc agt ctt cat gct gtt ggt gta tcc tac  1047
Asn Met Ala Ser His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr
 90                  95                  100                 105
tgg aaa gct tct gag gga gct gaa tat gat gat cag acc agt caa agg  1095
Trp Lys Ala Ser Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg
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gag aaa gaa gat gat aaa gtc ttc cct ggt gga agc cat aca tat gtc  1143
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ctt acc tac tca tat ctt tct cat gtg gac ctg gta aaa gac ttg aat  1239
Leu Thr Tyr Ser Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn
    155                 160                 165
tca ggc ctc att gga gcc cta cta gta tgt aga gaa ggg agt ctg gcc  1287
Ser Gly Leu Ile Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala
170                 175                 180                 185
aag gaa aag aca cag acc ttg cac aaa ttt ata cta ctt ttt gct gta  1335
Lys Glu Lys Thr Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val
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Phe Asp Glu Gly Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met
            205                 210                 215
cag gat agg gat gct gca tct gct cgg gcc tgg cct aaa atg cac aca  1431
Gln Asp Arg Asp Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr
        220                 225                 230
gtc aat ggt tat gta aac agg tct ctg cca ggt ctg att gga tgc cac  1479
Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His
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agg aaa tca gtc tat tgg cat gtg att gga atg ggc acc act cct gaa    1527
Arg Lys Ser Val Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu
250                 255                 260                 265
gtg cac tca ata ttc ctc gaa ggt cac aca ttt ctt gtg agg aac cat    1575
Val His Ser Ile Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His
                270                 275                 280
cgc cag gcg tcc ttg gaa atc tcg cca ata act ttc ctt act gct caa    1623
Arg Gln Ala Ser Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln
            285                 290                 295
aca ctc ttg atg gac ctt gga cag ttt cta ctg ttt tgt cat atc tct    1671
Thr Leu Leu Met Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser
        300                 305                 310
tcc cac caa cat gat ggc atg gaa gct tat gtc aaa gta gac agc tgt    1719
Ser His Gln His Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys
    315                 320                 325
cca gag gaa ccc caa cta cga atg aaa aat aat gaa gaa gcg gaa gac    1767
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Pro Glu Glu Pro Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp
330 335 340 345

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350 355 360

gat gac aac tct cct tcc ttt atc caa att cgc tca gtt gcc aag aag 1863
Asp Asp Asn Ser Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys
365 370 375

cat cct aaa act tgg gta cat tac att gct gct gaa gag gag gac tgg 1911
His Pro Lys Thr Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp
380 385 390

gac tat gct ccc tta gtc ctc gcc ccc gat gac aga agt tat aaa agt 1959
Asp Tyr Ala Pro Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser
395 400 405

caa tat ttg aac aat ggc cct cag cgg att ggt agg aag tac aaa aaa 2007
Gln Tyr Leu Asn Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys
410 415 420 425

gtc cga ttt atg gca tac aca gat gaa acc ttt aag act cgt gaa gct 2055
Val Arg Phe Met Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala
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1435 1440

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<223> 人工序列的描述 :pTGF8-3

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Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
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Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
50 55 60

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
65 70 75

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
80 85 90

His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
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Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
110 115 120 125

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
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Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
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Tyr Leu Ser His Ala Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
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175 180 185

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
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Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
210 215 220

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
225 230 235

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
240 245 250

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255 260 265
Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
270 275 280 285
Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
290 295 300
Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
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Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
320 325 330
Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp
335 340 345
Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
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415 420 425
Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
430 435 440 445
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450 455 460
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465 470 475
His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
480 485 490
Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
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Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
510 515 520 525
Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
530 535 540
Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
545 550 555
Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val

560 565 570

Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu

575 580 585

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp

590 595 600 605

Pro Glu Phe Gln Ala Ser Asn Ile Met His SerIle Asn Gly Tyr Val

610 615 620

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp

625 630 635

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe

640 645 650

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr

655 660 665

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro

670 675 680 685

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly

690 695 700

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp

705 710 715

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys

720 725 730

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Gln

735 740 745

Ala Tyr Arg Tyr Arg Arg Gly Glu Ile Thr Arg Thr Thr Leu Gln Ser

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Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile Ser Val Glu Met Lys

770 775 780

Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg

785 790 795

Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg

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Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg

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Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu

830 835 840 845

Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn

850 855 860

Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp

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930 935 940
Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His Ser
945 950 955
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1185 1190 1195

Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr

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Ser Leu Asp Gly Lys Lys Trp Gln Thr Tyr Arg Gly Asn Ser Thr Gly

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Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His

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Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro

1250 1255 1260

Thr His Tyr Ser Ile Arg Ser Thr Leu Arg Met Glu Leu Met Gly Cys

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1280 1285 1290

Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala

1295 1300 1305

Thr Trp Ser Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn

310 1315 1320 1325

Ala Trp Arg Pro Gln Glu Asn Asn Pro Lys Glu Trp Leu Gln Val Asp

1330 1335 1340

Phe Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys

1345 1350 1355

Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser

1360 1365 1370

Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys

1375 1380 1385

Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu

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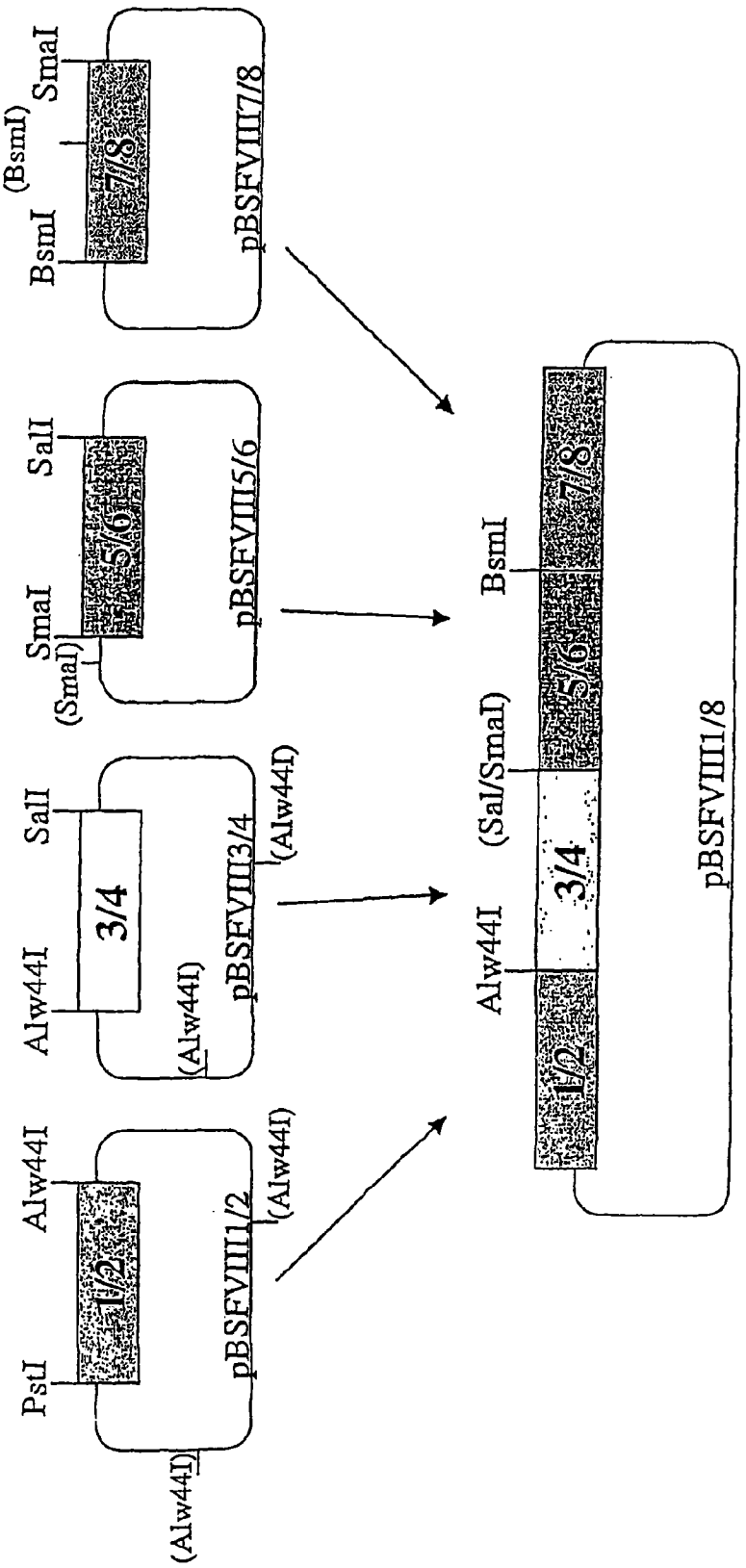
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pBSFVIII3/4
(Alw44I)
SmaI
(SmaI)
5/6
SalI
pBSFVIII5/6
BsmI
(BsmI)
SmaI
7/8
BsmI
pBSFVIII7/8
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3/4
(Sal/SmaI)
5/6
BsmI
7/8
pBSFVIII1/8

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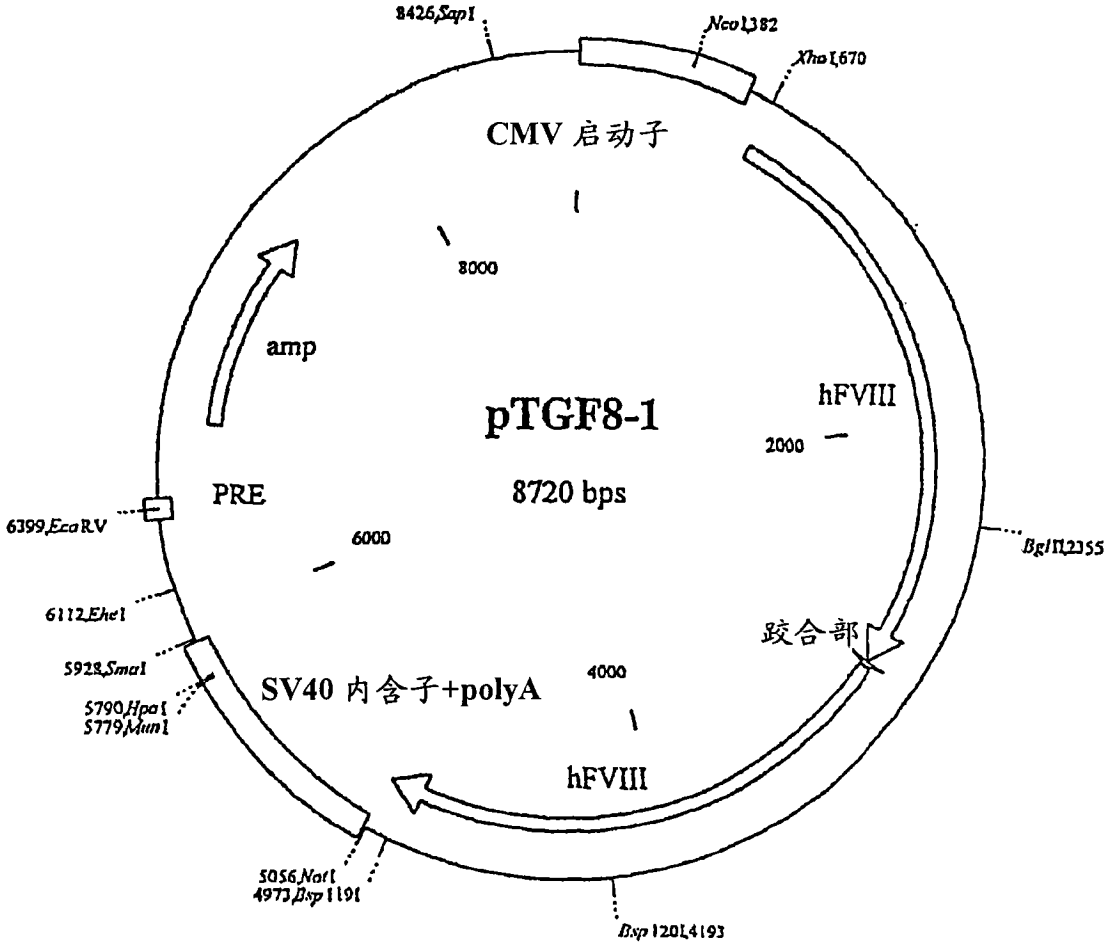
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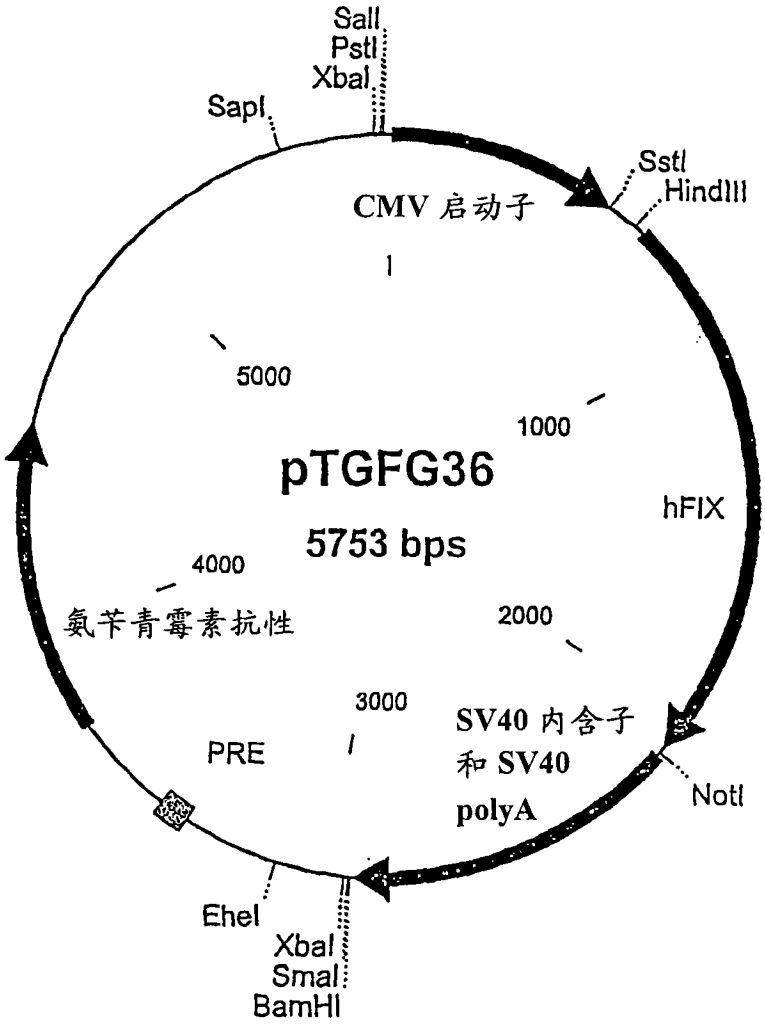
PRE
6399,EcoRV
6000
BglII,2355
6112,EheI
5928,SmaI
跤合部
SV40 内含子+polyA
4000
5790,HpaI
5779,MunI
hFVIII
5056,NotI
4973,BspI191
BspI201,4193

图 2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PstI
XbaI
SapI
SstI
HindIII
CMV 启动子
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hFIX
4000
氨苄青霉素抗性
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和 SV40
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EheI
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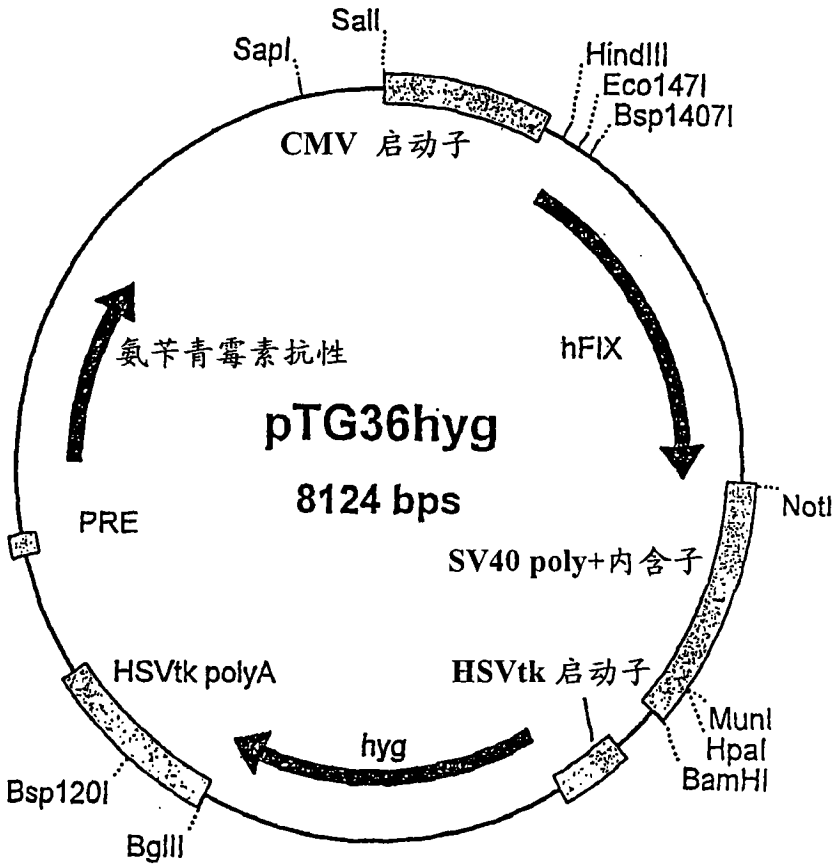
图 3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Bsp1407I
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氨苄青霉素抗性
hFIX
pTG36hyg
8124 bps
PRE
NotI
SV40 poly+内含子
HSVtk polyA
HSVtk 启动子
MunI
HpaI
BamHI
hyg
Bsp120I
BglII

图 4

图 5

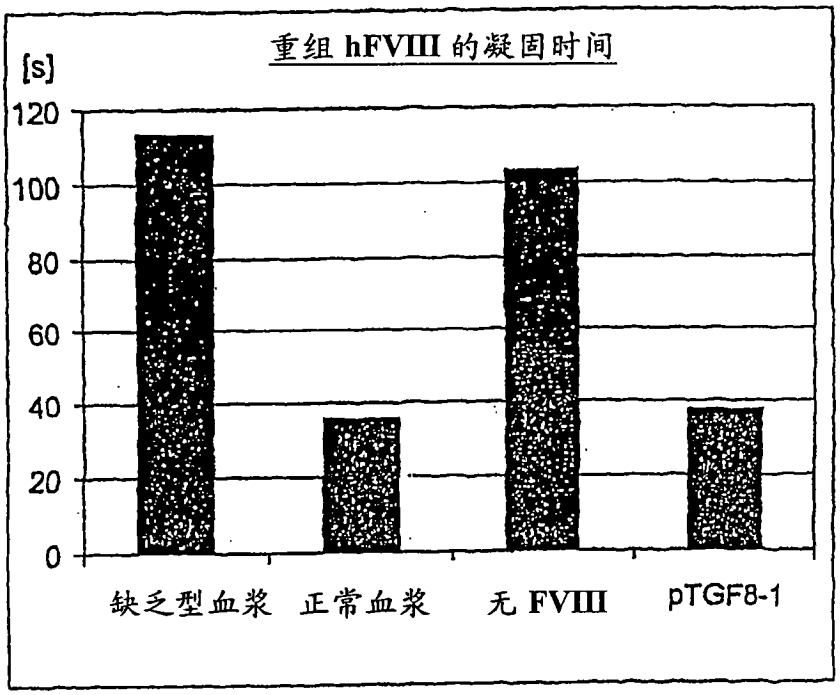
图 5A

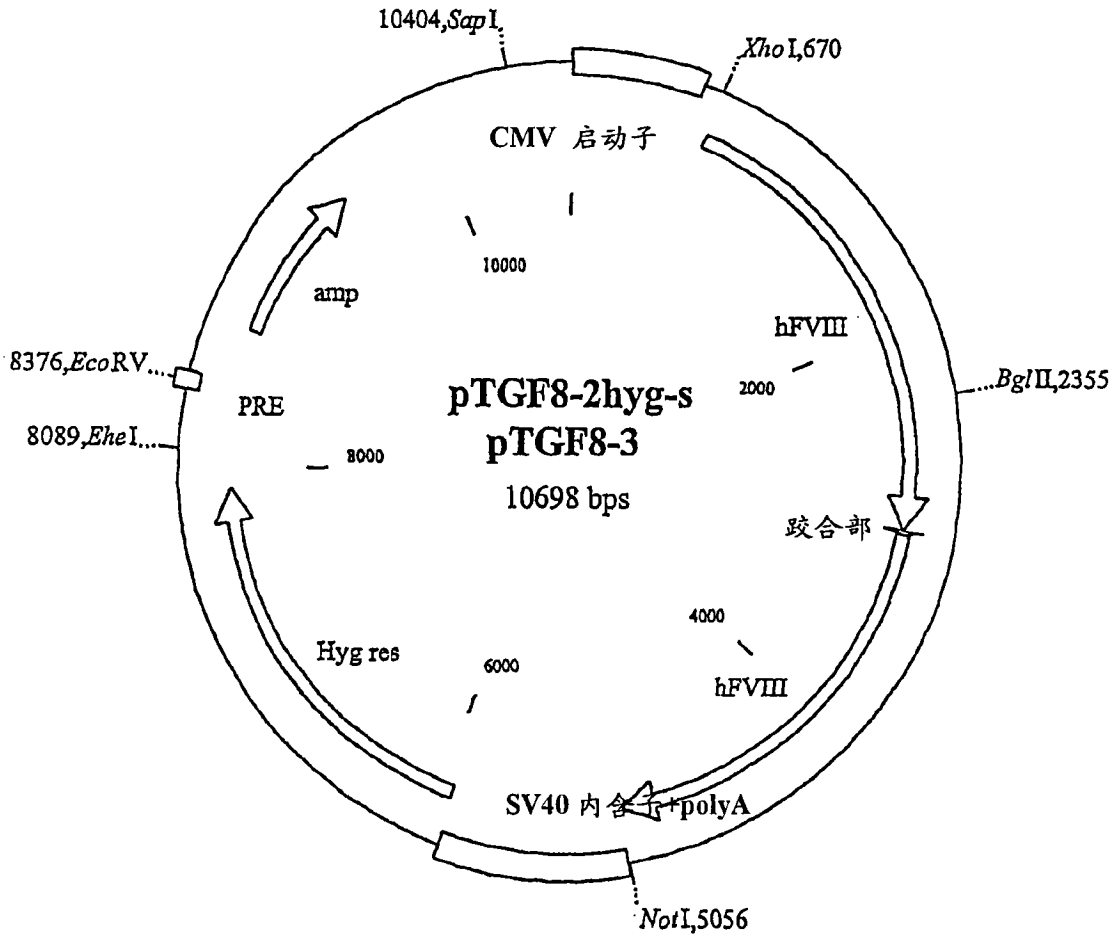
aga| agc ttc tcc cag aat tca aga cat caa gct tat cga tac cgt cga ggg gaa

R740 S F S Q N S R H Q A Y R Y R R G E1649

wt-B-结构域 可变区

图 5B

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BglII,2355
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Hyg res
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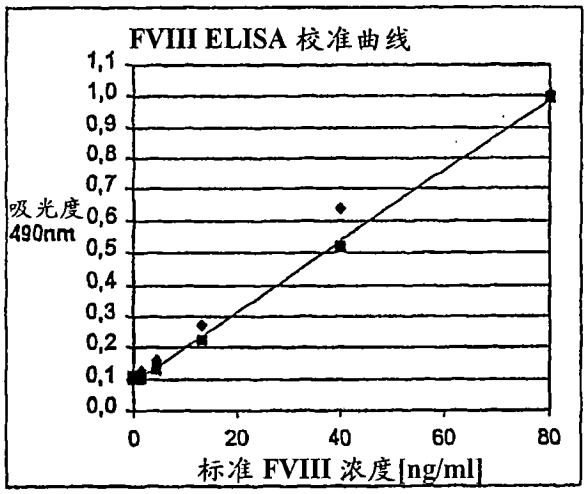
图 6

图 7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吸光度 490nm
1,1
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
20
40
60
80
标准 FVIII 浓度[ng/ml]

图 7A

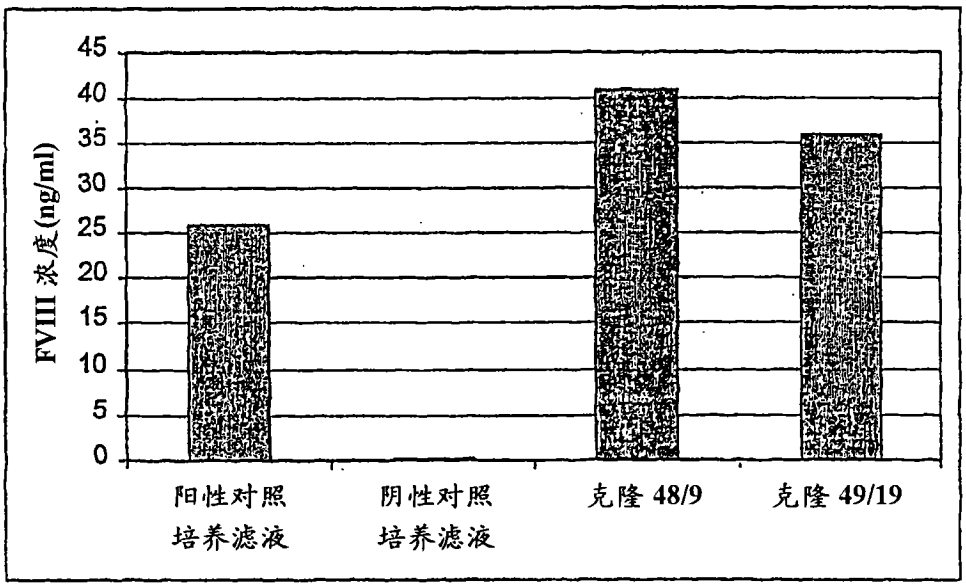
FVIII 浓度(ng/ml)
45
40
35
30
25
20
15
10
5
0
阳性对照 培养滤液
阴性对照 培养滤液
克隆 48/9
克隆 49/19

图 7B

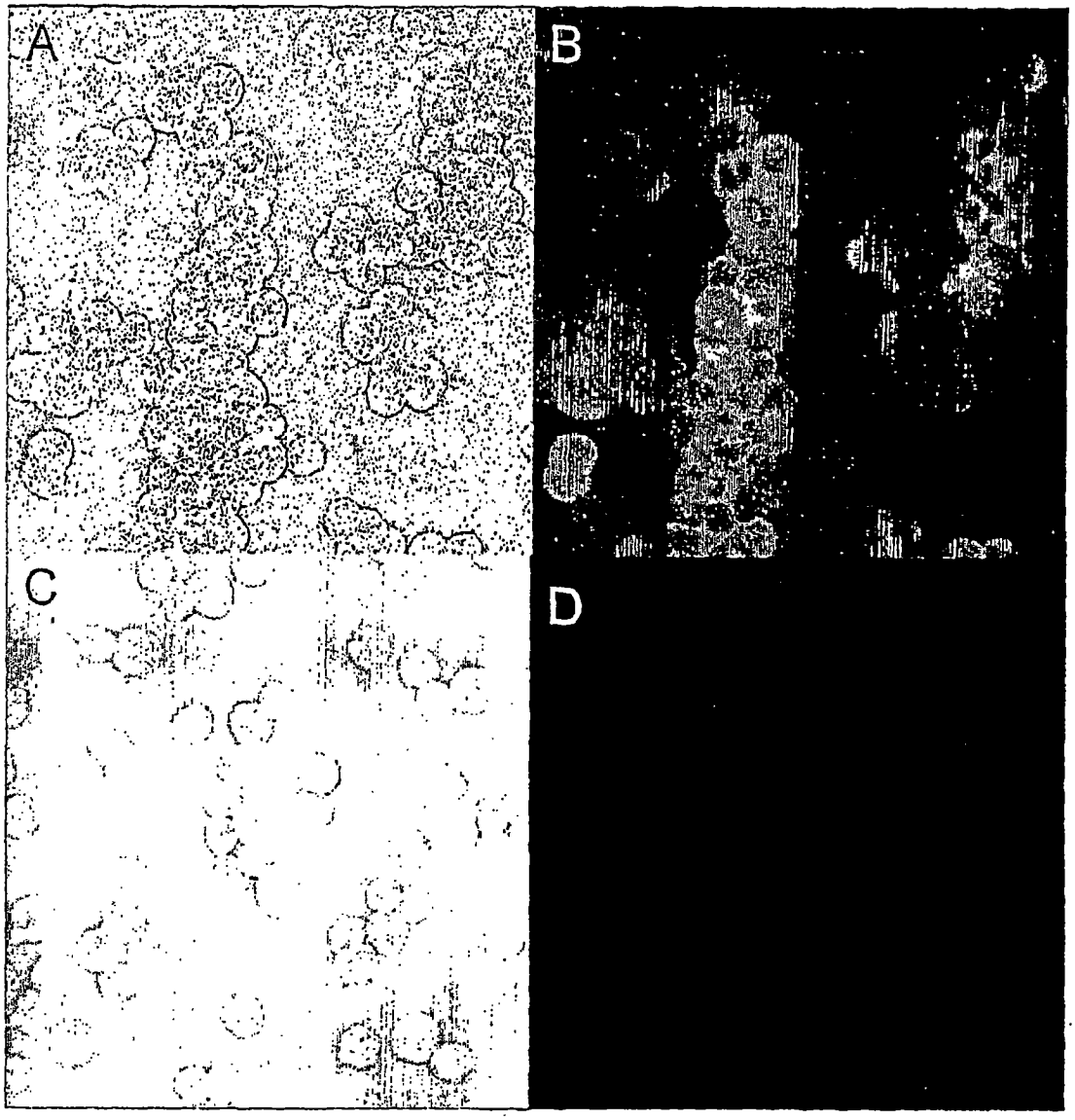

图 8

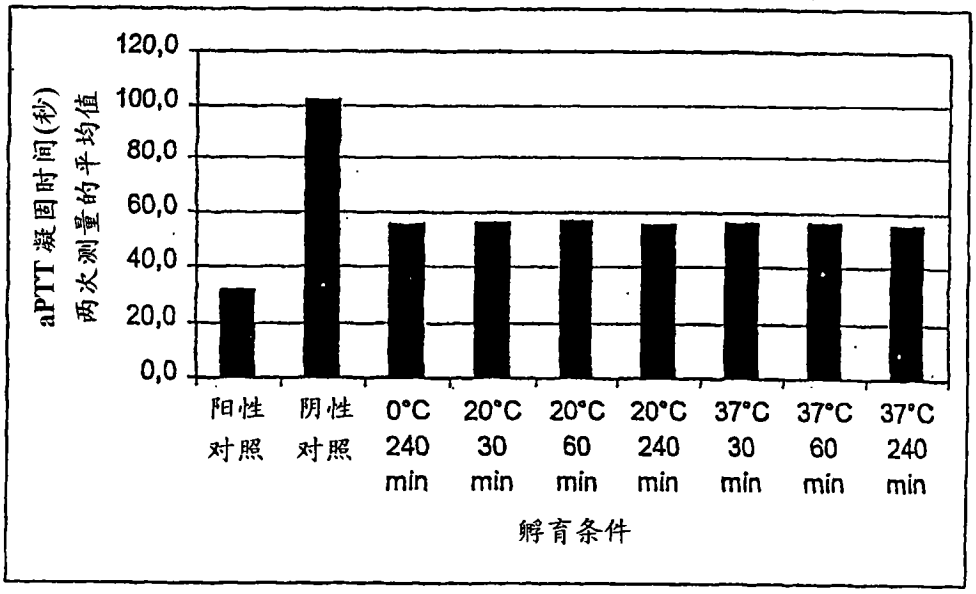
aPTT 凝固时间(秒)
两次测量的平均值
120,0
100,0
80,0
60,0
40,0
20,0
0,0
阳性对照
阴性对照
0°C 240 min
20°C 30 min
20°C 60 min
20°C 240 min
37°C 30 min
37°C 60 min
37°C 240 min
孵育条件

图 9

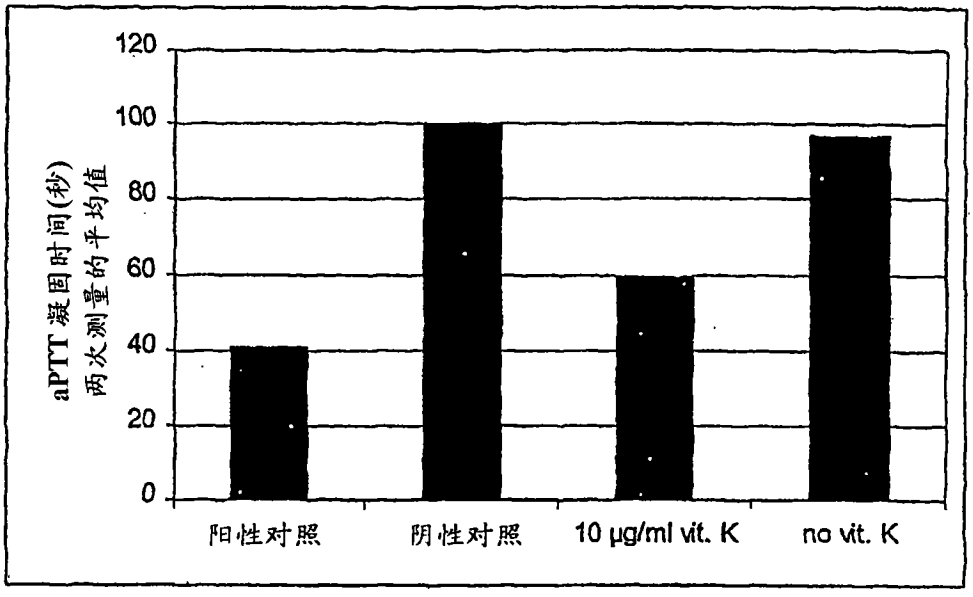
aPTT 凝固时间(秒)
两次测量的平均值
120
100
80
60
40
20
0
阳性对照
阴性对照
10 µg/ml vit. K
no vit. K

图 10