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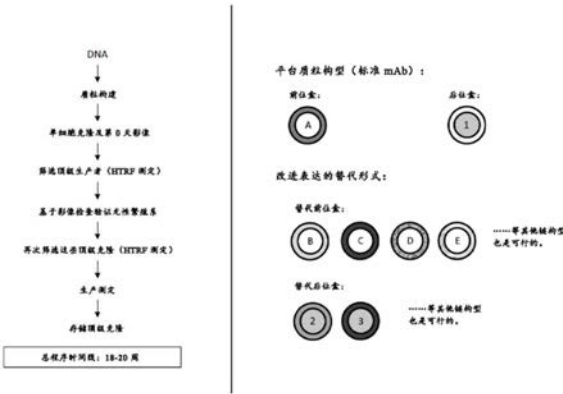
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(54) 发明名称

核酸的随机化构型靶向整合

(57) 摘要

本公开的主题涉及“随机化构型靶向整合”(本文也称为“随机化链靶向整合”(RCTI)策略,用于产生和鉴定宿主细胞,所述宿主细胞能够表达重组蛋白例如单克隆抗体,以及由其衍生的组合物例如双特异性抗体、及其他复杂形式的蛋白质例如膜蛋白复合物和其他难以表达的分子。



1. 一种用于产生和高通量筛选表达至少一个目的序列(SOI)的靶向整合(TI)宿主细胞文库的方法,其包括:

a) 通过如下产生TI宿主细胞文库,其中所述文库包含表达一个或多个SOI的多个TI宿主细胞:

i) 提供多个TI宿主细胞;

ii) 使所述多个TI宿主细胞与多个载体接触,其中所述载体包含一个或多个SOI;

iii) 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个;

b) 将所述文库分离成单个克隆;以及

c) 针对特定细胞属性或特定产物属性筛选所述克隆。

2. 根据权利要求1所述的方法,其中通过重组酶介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

3. 根据权利要求1所述的方法,其中通过基因编辑介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

4. 根据权利要求1所述的方法,其中所述一个或多个SOI可操作地连接至一个或多个可调型启动子。

5. 根据权利要求4所述的方法,其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

6. 根据权利要求1所述的方法,其中所述TI宿主细胞为哺乳动物宿主细胞。

7. 根据权利要求6所述的方法,其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

8. 根据权利要求7所述的方法,其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

9. 根据权利要求1所述的方法,其中所述SOI编码多亚基蛋白质的多肽亚基或其片段。

10. 根据权利要求1所述的方法,其中所述SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白。

11. 根据权利要求1所述的方法,其中所述特定细胞属性选自:细胞生长、细胞效价、比生产力、体积生产力、克隆稳定性。

12. 根据权利要求1所述的方法,其中所述特定产物属性选自:糖基化水平、电荷变化水平、错配减少、蛋白质/肽聚集减少、蛋白质序列异质性。

13. 根据权利要求1所述的方法,其中将所述一个或多个SOI在一个或多个基因座处引入所述多个TI宿主细胞中的一个或多个,所述一个或多个基因座与选自下列的序列至少约90%同源:SEQ ID NO.1-12;NW_006874047.1;NW_006884592.1;NW_006881296.1;NW_003616412.1;NW_003615063.1;NW_006882936.1;以及NW_003615411.1。

14. 一种产生包含多个外源核苷酸SOI的TI宿主细胞文库的方法,其包括:

a) 提供多个TI宿主细胞;

b) 使所述多个TI宿主细胞与多个载体接触,其中所述载体包含一个或多个SOI;

c) 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

15. 根据权利要求14所述的方法,其中所述多个TI宿主细胞中的一个或多个包含整合

在所述TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列,并且其中所述外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个RRS。

16. 根据权利要求14所述的方法,其中所述载体包括:

- a) 至少两个RRS,其匹配经整合的外源核苷酸序列上的所述至少两个RRS;以及
- b) 一个或多个外源SOI和侧接有所述RRS的至少一个第二选择标记物。

17. 根据权利要求16所述的方法,其中通过重组酶介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

18. 根据权利要求14所述的方法,其中通过基因编辑介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

19. 根据权利要求14所述的方法,其中所述一个或多个SOI可操作地连接至一个或多个可调型启动子。

20. 根据权利要求19所述的方法,其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

21. 根据权利要求14所述的方法,其中所述SOI编码多亚基蛋白质的多肽亚基或其片段。

22. 根据权利要求14所述的方法,其中所述SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白。

23. 根据权利要求14所述的方法,其中(a)的所述一个或多个外源核苷酸序列整合在一个或多个基因座处,所述一个或多个基因座与选自下列的序列至少约90%同源:SEQ ID NO.1-12;NW_006874047.1;NW_006884592.1;NW_006881296.1;NW_003616412.1;NW_003615063.1;NW_006882936.1;以及NW_003615411.1。

24. 根据权利要求14所述的方法,其中至少一个目的序列包含2、3、4、5、6、7、8、9、10个目的序列。

25. 根据权利要求14所述的方法,其中所述TI宿主细胞为哺乳动物宿主细胞。

26. 根据权利要求25所述的方法,其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

27. 根据权利要求26所述的方法,其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

28. 一种制备表达一个或多个SOI的TI宿主细胞的方法,其包括:

- a) 提供多个TI宿主细胞;
- b) 使所述多个TI宿主细胞与多个载体接触,其中所述载体包含一个或多个SOI;
- c) 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个;以及
- d) 选择表达所述一个或多个SOI的所述TI宿主细胞。

29. 根据权利要求28所述的方法,其中通过重组酶介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

30. 根据权利要求28所述的方法,其中通过基因编辑介导的整合,将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

31. 根据权利要求28所述的方法,其中所述一个或多个SOI可操作地连接至一个或多个

可调型启动子。

32. 根据权利要求31所述的方法, 其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

33. 根据权利要求28所述的方法, 其中所述TI宿主细胞为哺乳动物宿主细胞。

34. 根据权利要求33所述的方法, 其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

35. 根据权利要求34所述的方法, 其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

36. 根据权利要求28所述的方法, 其中所述S0I编码多亚基蛋白质的多肽亚基或其片段。

37. 根据权利要求28所述的方法, 其中所述S0I编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白。

38. 根据权利要求28所述的方法, 其中将所述一个或多个S0I在一个或多个基因座处引入所述多个TI宿主细胞中的一个或多个, 所述一个或多个基因座与选自下列的序列至少约90%同源: SEQ ID NO.1-12; NW_006874047.1; NW_006884592.1; NW_006881296.1; NW_003616412.1; NW_003615063.1; NW_006882936.1; 以及NW_003615411.1。

39. 根据权利要求28所述的方法, 其中所述S0I编码多亚基蛋白质的多肽亚基或其片段。

40. 根据权利要求28所述的方法, 其中所述S0I编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白, 及其片段。

41. 根据权利要求28所述的方法, 其中:

a) 所述多个TI宿主细胞中的一个或多个包含整合在所述TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列, 并且其中所述外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个RRS;

b) 所述载体包含:

a. 至少两个RRS, 其匹配经整合的外源核苷酸序列上的所述至少两个RRS; 以及

b. 一个或多个外源S0I和侧接有所述RRS的至少一个第二选择标志物;

c) 将一种或多种重组酶或编码重组酶的核酸引入所述多个TI宿主细胞中的一个或多个, 其中所述一种或多种重组酶识别所述RRS; 以及

d) 选择表达所述第二选择标志物的TI细胞, 从而分离出表达目的序列的TI宿主细胞。

42. 根据权利要求41所述的方法, 其中所述S0I编码多亚基蛋白质的多肽亚基或其片段。

43. 根据权利要求41所述的方法, 其中所述S0I编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白, 及其片段。

44. 根据权利要求41所述的方法, 其中(a)的所述一个或多个外源核苷酸序列整合在一个或多个基因座处, 所述一个或多个基因座与选自下列的序列至少约90%同源: SEQ ID NO.1-12; NW_006874047.1; NW_006884592.1; NW_006881296.1; NW_003616412.1; NW_003615063.1; NW_006882936.1; 以及NW_003615411.1。

45. 根据权利要求41所述的方法, 其中:

a. 所述外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的第一RRS和第二RRS, 以及位于所述第一RRS与所述第二RRS之间的第三RRS, 并且所有RRS都是异种特异性的; 并且

b. 所述多个载体包含:

i. 第一载体, 其包含两个RRS, 所述两个RRS匹配经整合的外源核苷酸序列上的第一RRS和第三RRS并且位于至少一个第一外源SOI和至少一个第二选择标志物的侧翼;

ii. 第二载体, 其包含两个RRS, 所述两个RRS匹配经整合的外源核苷酸序列上的第二RRS和第三RRS并且位于至少一个第二外源SOI的侧翼; 以及

c. 选择表达所述第二选择标志物的TI细胞, 从而分离出表达所述第一目的序列和所述第二目的序列的TI宿主细胞。

46. 根据权利要求28、41和45中任一项所述的方法, 其中包含至少一个目的序列的所述载体包含2、3、4、5、6、7、8、9、10个目的序列。

47. 根据权利要求28、41和45中任一项所述的方法, 其中所述TI宿主细胞为哺乳动物宿主细胞。

48. 根据权利要求47所述的方法, 其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

49. 根据权利要求48所述的方法, 其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

50. 一种表达SOI的方法, 其包括:

a) 提供多个TI宿主细胞;

b) 使所述多个TI宿主细胞与多个载体接触, 其中所述载体包含一个或多个SOI;

c) 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个; 以及

d) 选择表达目的序列的TI宿主细胞;

e) 在适合于表达来自其中的所述目的序列的条件下培养d) 中的所述细胞。

51. 根据权利要求50所述的方法, 其中通过重组酶介导的整合, 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

52. 根据权利要求50所述的方法, 其中通过基因编辑介导的整合, 将所述一个或多个SOI引入所述多个TI宿主细胞中的一个或多个。

53. 根据权利要求50所述的方法, 其中所述一个或多个SOI可操作地连接至一个或多个可调型启动子。

54. 根据权利要求53所述的方法, 其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

55. 根据权利要求50所述的方法, 其中所述TI宿主细胞为哺乳动物宿主细胞。

56. 根据权利要求55所述的方法, 其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

57. 根据权利要求56所述的方法, 其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主

细胞。

58. 根据权利要求50所述的方法, 其中所述SOI编码多亚基蛋白质的多肽亚基或其片段。

59. 根据权利要求50所述的方法, 其中所述SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白。

60. 根据权利要求50所述的方法, 其中将所述一个或多个SOI在一个或多个基因座处引入所述多个TI宿主细胞中的一个或多个, 所述一个或多个基因座与选自下列的序列至少约90%同源: SEQ ID NO.1-12; NW_006874047.1; NW_006884592.1; NW_006881296.1; NW_003616412.1; NW_003615063.1; NW_006882936.1; 以及NW_003615411.1。

61. 根据权利要求50所述的方法, 其中至少一个目的序列包含2、3、4、5、6、7、8、9、10个目的序列。

62. 一种表达SOI的方法, 其包括:

a) 提供多个TI宿主细胞, 其中各TI宿主细胞包含整合在所述TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列, 并且其中所述外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个RRS;

b) 使所述多个TI宿主细胞与多个载体接触, 其中所述载体包含:

a. 至少两个RRS, 其匹配经整合的外源核苷酸序列上的所述至少两个RRS; 以及

b. 一个或多个外源SOI和侧接有所述RRS的至少一个第二选择标志物;

c) 引入一种或多种重组酶或编码重组酶的核酸, 其中所述一种或多种重组酶识别所述RRS;

d) 选择表达所述第二选择标志物的TI细胞, 从而分离出表达目的序列的TI宿主细胞; 以及

e) 在适合于表达所述目的序列的条件下培养d)中的所述细胞, 并且从其中回收所述目的序列的产物。

63. 根据权利要求62所述的方法, 其中所述SOI编码多亚基蛋白质的多肽亚基或其片段。

64. 根据权利要求62所述的方法, 其中所述一个或多个SOI可操作地连接至一个或多个可调型启动子。

65. 根据权利要求64所述的方法, 其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

66. 根据权利要求62所述的方法, 其中所述SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)或Fc融合蛋白, 及其片段。

67. 根据权利要求62所述的方法, 其中(a)的所述一个或多个外源核苷酸序列整合在一个或多个基因座处, 所述一个或多个基因座与选自下列的序列至少约90%同源: SEQ ID NO.1-12; NW_006874047.1; NW_006884592.1; NW_006881296.1; NW_003616412.1; NW_003615063.1; NW_006882936.1; 以及

NW_003615411.1。

68. 根据权利要求62所述的方法, 其中:

a. 所述外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的第一RRS和第二

RRS,以及位于所述第一RRS与所述第二RRS之间的第三RRS,并且所有RRS都是异种特异性的;并且

b.所述多个载体包含:

i.第一载体,其包含两个RRS,所述两个RRS匹配经整合的外源核苷酸序列上的第一RRS和第三RRS并且位于至少一个第一外源SOI和至少一个第二选择标志物的侧翼;

ii.第二载体,其包含两个RRS,所述两个RRS匹配经整合的外源核苷酸序列上的第二RRS和第三RRS并且位于至少一个第二外源SOI的侧翼;以及

c.选择表达所述第二选择标志物的TI细胞,从而分离出表达所述第一目的序列和所述第二目的序列的TI宿主细胞。

69.根据权利要求62或68所述的方法,其中所述至少一个目的序列包含2、3、4、5、6、7、8、9、10个目的序列。

70.根据权利要求62所述的方法,其中至少一个第一SOI和至少一个第二SOI可操作地连接至一个或多个可调型启动子。

71.根据权利要求70所述的方法,其中所述一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

72.根据权利要求62或68所述的方法,其中所述TI宿主细胞为哺乳动物宿主细胞。

73.根据权利要求72所述的方法,其中所述TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。

74.根据权利要求73所述的方法,其中所述TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

核酸的随机化构型靶向整合

[0001] 相关申请的交叉引用

[0002] 本申请要求2019年6月26日提交的美国专利临时申请号62/866,893和2020年3月19日提交的美国专利临时申请号62/991,708的优先权,各申请的内容以引用方式整体并入本文,并要求各自的优先权。

技术领域

[0003] 本公开的主题涉及“随机化构型靶向整合”(本文也称为“随机化链靶向整合(RCTI)策略”),用于产生和鉴定宿主细胞,所述宿主细胞能够表达重组蛋白例如单克隆抗体,以及由其衍生的组合物例如双特异性抗体、及其他复杂形式的蛋白质例如膜蛋白复合物和其他难以表达的分子。

[0004] 序列表

[0005] 本说明书参照序列表(2020年6月26日以电子形式提交,文件名“00B206_0926_SL.txt”)。00B208_0926_SL.txt文件于2020年6月24日生成且大小为1,296,777位字节。序列表的全部内容以引用方式并入本文。

背景技术

[0006] 由于细胞生物学及免疫学的快速发展,对开发用于多种疾病(包括癌症、心血管疾病、及代谢性疾病)的新型治疗性重组蛋白(例如单克隆抗体、双特异性抗体及复合物形式蛋白质)的需求日益增加。此类生物医药候选物通常由能够表达目的蛋白的商业细胞系制造而成。举例而言,近年来,中国仓鼠卵巢(CHO)细胞已广泛适用于制造单克隆抗体或双特异性抗体以及更多复合物形式蛋白质。

[0007] 用于开发商业细胞系的传统策略通常涉及不断致力于整合关于编码目的多肽的核苷酸序列(随机或特定(“靶向”)位置),然后选择并分离产生多肽的细胞系。然而,该方法具有若干缺点。首先,虽然随机整合方法提供获得具有不同组成及比例的待表达转基因的不同克隆的可能性,但在转染后需要筛选数百、甚至数千个克隆以分离展现所期望的表达水平、产物品质属性、及生产培养效能的细胞系,是一项耗时且占用大量资源的工作。此外,由于不稳定或未能筛选足够数量的克隆,具有各种转基因最佳比例的克隆有可能不存在或无法分离出来。此外,当生产多链多肽(例如单克隆抗体)时,可能需要进一步筛选以解决编码此类多肽的核酸的数量和排列。在使用随机整合方法时,很难确定引入基因组的所有转基因的数量和位置及其排列方式,而这是在转基因排列/拷贝数与所期望产物属性之间进行合理关联的重要步骤。

[0008] 相比之下,靶向整合方法可对各特定转基因的排列和拷贝数提供更多的控制,鉴于转基因是插入到基因组内预定的特定位置。然而,这种靶向整合方法旨在将产生不同转基因的随机组合及比例的可能性最小化。因此,如果设计的转基因排列和拷贝数恰好是次优的,那么所有衍生的克隆同样会有次优的产物品质和/或效价。为了增加使用靶向整合方法表达复杂或难以表达的分子的机会,对多种不同的转基因排列和拷贝数配置单独测试,

这导致细胞系开发 (CLD) 的工作量和资源需求量增加。因此,本技术领域需要新的细胞系开发策略,该策略在节省资源的同时,产生的细胞系表达出与传统方法相媲美的表达水平、产物品质属性、及生产培养效能。

发明内容

[0009] 本公开的主题涉及“随机化构型靶向整合”(本文也称为“随机化链靶向整合(RCTI)策略”),用于生成和鉴定宿主细胞,所述宿主细胞能够表达重组蛋白(例如单克隆抗体)及由其衍生的组合物(例如双特异性抗体)、和其他复杂形式的蛋白(例如膜蛋白复合物)、以及其他难以表达的分子。本公开所述RCTI方法可用于筛选与标准细胞系开发方法相同数量(如果不是更多)的载体构型,同时使用的资源更少。通过使用本公开所述RCTI方法,也可筛选较少的单个克隆。

[0010] 在某些实施例中,本公开提供了一种用于产生和高通量筛选表达至少一个目的序列(SOI)的靶向整合(TI)宿主细胞文库的方法,其包括:a)通过下列步骤产生TI宿主细胞文库,其中该文库包含表达一个或多个SOI的多个TI宿主细胞;i)提供多个TI宿主细胞;ii)将多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI;iii)将一个或多个SOI引入多个TI宿主细胞中的一个或多个;b)将该文库分离成单一克隆;以及c)针对特定细胞属性或特定产物属性来筛选该克隆。

[0011] 在某些实施例中,本公开提供了一种产生TI宿主细胞文库的方法,该宿主细胞包含多个外源性核苷酸SOI,该方法包括:a)提供多个TI宿主细胞;b)将该多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI;c)将一个或多个SOI引入多个TI宿主细胞中的一个或多个。在某些实施例中,多个TI宿主细胞的一个或多个包含一个或多个外源核苷酸序列,其整合在该TI宿主细胞的基因组的一个或多个基因座处,并且外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个重组酶识别序列(RRS)。在某些实施例中,该载体包含:a)至少两个RRS,其匹配经整合的外源性核苷酸序列上的至少两个RRS;以及b)一个或多个外源SOI和侧接有该RRS的至少一个第二选择标志物。

[0012] 本公开的主题也提供用于将一或多种外源性核酸RCTI至宿主细胞中以促进目的序列的表达的方法。在某些实施例中,该方法包含经由重组酶介导的整合或经由基因编辑介导的整合将一或多种外源性核酸靶向整合至宿主细胞中。在某些实施例中,该方法涉及一种细胞,其包含整合在该TI宿主细胞的基因组的基因座内的一个或多个外源核苷酸序列,其中该基因座包含与选自SEQ ID NO:1-12的序列至少约90%同源的核苷酸序列。

[0013] 在某些实施例中,本公开提供一种制备表达一个或多个SOI的TI宿主细胞的方法,其包括:a)提供多个TI宿主细胞;b)使该多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI;c)将该一个或多个SOI引入该多个TI宿主细胞中的一个或多个;以及d)选择表达该一个或多个SOI的该TI宿主细胞。在某些实施例中,该多个TI宿主细胞中的一个或多个包含整合在该等TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列,并且其中该外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个RRS。在某些实施例中,该载体包含:a.至少两个RRS,其匹配经整合的外源性核苷酸序列上的至少两个RRS;以及b.一个或多个外源SOI和侧接有RRS的至少一个第二选择标志物。在某些实施例中,该方法包括将一种或多种重组酶或编码重组酶的核酸引入多个TI宿主细胞中

的一个或多个,其中所述一种或多种重组酶识别所述RRS;以及d)选择表达该第二选择标志物的TI细胞,从而分离出表达目的序列的TI宿主细胞。在某些实施例中,该外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的第一RRS和第二RRS,以及位于该第一RRS与该第二RRS之间的第三RRS,并且所有RRS都是异种特异性的;并且该多个载体包含:i.第一载体,其包含两个RRS,该两个RRS匹配经整合的外源核苷酸序列上的第一RRS和第三RRS并且位于至少一个第一外源SOI和至少一个第二选择标志物的侧翼;ii.第二载体,其包含两个RRS,该两个RRS匹配经整合的外源核苷酸序列上的第二RRS和第三RRS并且位于至少一个第二外源SOI的侧翼;以及选择表达该第二选择标志物的TI细胞,从而分离出表达该第一目的序列和该第二目的序列的TI宿主细胞。

[0014] 在某些实施例中,本公开提供了一种表达SOI的方法,其包括:a)提供多个TI宿主细胞;b)使多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI;c)将该一个或多个SOI引入多个TI宿主细胞中的一个或多个;以及d)选择表达目的序列的TI宿主细胞;e)在适合于表达来自其中的细胞的该目的序列的条件下培养d)中的该细胞。

[0015] 在某些实施例中,本公开提供了一种表达SOI的方法,其包括:a)提供多个TI宿主细胞,其中各TI宿主细胞包含整合在TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列,并且其中该外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的至少两个RRS;b)使该多个TI宿主细胞与多个载体接触,其中该载体包含:a.至少两个RRS,其匹配经整合的外源核苷酸序列上的至少两个RRS;以及b.一个或多个外源SOI和侧接有该RRS的至少一个第二选择标志物;c)引入一种或多种重组酶或编码重组酶的核酸,其中该一种或多种重组酶识别该RRS;d)选择表达该第二选择标志物的TI细胞,从而分离出表达目的序列的TI宿主细胞;以及e)在适合于表达该目的序列的条件下培养d)中的细胞,并且从其中回收该目的序列的产物。在某些实施例中,该外源核苷酸序列包含位于至少一个第一选择标志物的侧翼的第一RRS和第二RRS,以及位于该第一RRS与该第二RRS之间的第三RRS,并且所有RRS都是异种特异性的;并且该多个载体包含:i.第一载体,其包含两个RRS,该两个RRS匹配经整合的外源核苷酸序列上的第一RRS和第三RRS并且位于至少一个第一外源SOI和至少一个第二选择标志物的侧翼;ii.第二载体,其包含两个RRS,该两个RRS匹配经整合的外源核苷酸序列上的第二RRS和第三RRS并且位于至少一个第二外源SOI的侧翼;以及选择表达该第二选择标志物的TI细胞,从而分离出表达该第一目的序列和该第二目的序列的TI宿主细胞。

[0016] 在某些上述实施例中,通过重组酶介导的整合,将一个或多个SOI引入多个TI宿主细胞中的一个或多个。在某些上述实施例中,通过基因编辑介导的整合,将一个或多个SOI引入多个TI宿主细胞中的一个或多个。

[0017] 在某些上述实施例中,一个或多个SOI可操作地连接至一个或多个可调型启动子。在某些上述实施例中,该一个或多个可调型启动子选自由SV40和CMV启动子组成的组。

[0018] 在某些上述实施例中,该TI宿主细胞为哺乳动物宿主细胞。在某些上述实施例中,该TI宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞或小鼠宿主细胞。在某些上述实施例中,该TI宿主细胞为CHO宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞或CHO K1M宿主细胞。

[0019] 在某些上述实施例中,SOI编码多亚基蛋白质的多肽亚基或其片段。在某些上述实

施例中,该SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)、或Fc融合蛋白。

[0020] 在某些上述实施例中,该特定细胞属性选自:细胞生长、细胞效价(cell titer)、比生产力(specific productivity)、体积生产力、克隆稳定性。在某些上述实施例中,该特定产物属性选自:糖基化水平、电荷变化水平、错配减少、蛋白质/肽聚集减少、蛋白质序列异质性。

[0021] 在某些上述实施例中,将一个或多个SOI在一个或多个基因座处引入多个TI宿主细胞中的一个或多个,该一个或多个基因座与选自下列的序列至少约90%同源:SEQ ID NO.1-12;NW_006874047.1;NW_006884592.1;NW_006881296.1;NW_003616412.1;NW_003615063.1;NW_006882936.1;以及NW_003615411.1。

[0022] 在某些上述实施例中,该至少一个目的序列包含2、3、4、5、6、7、8、9、10个目的序列。

附图说明

[0023] 图1A至图1B是说明示例性靶向整合(TI)细胞系开发(CLD)工作流程的示意图。

[0024] 图2A至图2B是说明标准TI和“随机化构型靶向整合”(本文也称为“随机化链靶向整合”(RCTI))工作流程的比較的示意图。

[0025] 图3A至图3B描述针对在生产培养中所评价的克隆的效价和HMWS%的分布以及所鉴定的构型。

[0026] 图4显示标准TI CLD与RCTI方法之间的其他产物品质属性是相当的。

[0027] 图5描绘标准CLD与RCTI方法之间的产物品质属性是相当的。

[0028] 图6A至图6C说明RCTI方法在保持克隆效能的同时,提供经改善的时间线弹性。

[0029] 图7A至图7B描绘针对分子-Y的标准CLD与RCTI方法之间的效价是相当的,但是RCTI方法的克隆的比生产力高于标准CLD。

[0030] 图8A至图8G描绘针对分子-Y的标准CLD与RCTI方法之间的产物品质属性是相当的。

[0031] 图9A至图9B描绘针对分子-Z的标准CLD与RCTI方法之间的效价是相当的,但是RCTI方法的克隆的比生产力高于标准CLD。

[0032] 图10A至图10F描绘针对分子-Z的标准CLD与RCTI方法之间的产物品质属性是相当的。

[0033] 图11A至图11C是说明通过本公开的方法所表达的示例性分子的示意图。

具体实施方式

[0034] 在某些实施例中,本文所述的宿主细胞、基因构建体(例如,载体)、组成物、以及方法可用于开发和/或使用“随机化构型靶向整合”(本文也称为“随机化链靶向整合”(RCTI)策略),以更有效(例如较少时间和/或资源密集)识别表现出与传统方法相当的表达水平、产物品质属性、以及生产培养效能的宿主细胞。

[0035] 为使公开更清晰但不作为限制,详细描述分为以下小节:

[0036] 1. 定义

[0037] 2. 宿主细胞制备&筛选策略

[0038] 3. 外源核苷酸序列

[0039] 4. 宿主细胞

[0040] 5. 靶向整合

[0041] 6. 产物

[0042] 1. 定义

[0043] 除非另外定义, 否则本文中使用的所有技术和科学术语所具有的含义与本领域普通技术人员通常理解的含义相同。如有冲突, 以本文件包括定义为准。下文描述了优选方法和材料, 尽管与本文描述的那些方法或材料相似或等同的方法和材料可以用于实践或测试当前公开的主题。本文提及的所有出版物、专利申请、专利和其他参考文献通过引用以其全文合并于本文。本文公开的材料、方法和实例仅是说明性的, 而不旨在限制。

[0044] 如本文所用, 术语“包含”、“包括”、“具有”、“有”、“可以”、“含有”及其变体旨在作为开放式过渡短语、术语或词语, 并不排除另外的行为或结构的可能性。除非另有明确规定, 否则单数形式“一”、“一种”及“该/所述”包括复数指代。本公开还考虑了无论是否明确提出地“包括”本文中呈现的实施例或元件、“由其组成”和“基本上由其组成”的其他实施例。

[0045] 为了列举本文的数值范围, 明确考虑了它们之间具有相同精确度的每个中间数字。例如, 对于6-9的范围, 除了6和9外, 还考虑了数字7和8; 并且对于6.0-7.0的范围, 明确考虑了数字6.0、6.1、6.2、6.3、6.4、6.5、6.6、6.7、6.8、6.9、和7.0。

[0046] 如本文所用, 术语“约”或“大致”意指在如本领域普通技术人员所确定的特定值的可接受误差范围内, 所述可接受误差范围将部分地取决于如何测量或确定该值, 即测量系统的局限性。例如, 按照本领域中的实践, “约”可以意指3个之内或多于3个标准偏差。可替代地, “约”可以意指给定值的至多20%, 优选地至多10%, 更优选地至多5%, 并且更优选地还是至多1%的范围。可替代地, 特别是关于生物学系统或过程, 该术语可以意指在某一值的某一数量级内, 优选地在5倍以内, 更优选地在2倍以内。

[0047] 如本文所用, 术语“选择标志物”可以是一种基因, 其允许携带该基因的细胞在相应的选择剂的存在下被特异性地选择或排除。例如, 但并非限制性地, 选择标志物可允许在存在该基因的情况下, 阳性筛选用选择标志物基因转化的宿主细胞; 未转化的宿主细胞将不能在选择条件下生长或存活。选择标志物可为阳性、阴性或双功能选择标志物。阳性选择标志物可允许选择带有标志物的细胞, 而阴性选择标志物则可允许选择性排除带有标志物的细胞。选择标志物可导致药物抗性或补偿宿主细胞中的代谢或分解代谢缺陷。在原核细胞中, 可使用导致对氨苄青霉素、四环素、卡那霉素或氯霉素抗性的基因。在真核细胞中用作选择标志物的抗性基因包括但不限于氨基糖苷磷酸转移酶 (APH) (例如, 潮霉素磷酸转移酶 (HYG)、新霉素和G418APH)、二氢叶酸还原酶 (DHFR)、胸苷激酶 (TK)、谷氨酰氨合成酶 (GS)、天冬酰胺合成酶、色氨酸合成酶 (吡啶)、组氨醇脱氢酶 (组氨醇D) 的基因以及编码对嘌呤霉素、杀稻瘟素、博莱霉素、腐草霉素、氯霉素、吉欧霉素和霉酚酸的抗性的基因。更多标记基因如W092/08796和W0 94/28143中所描述。

[0048] 除在相应选择剂存在下促进选择之外, 选择标志物还可提供编码细胞中通常不存在的分子的基因, 例如绿色荧光蛋白 (GFP)、增强型GFP (eGFP)、合成GFP、黄色荧光蛋白 (YFP)、增强型YFP (eYFP)、青色荧光蛋白 (CFP)、mPlum、mCherry、tdTomato、mStrawberry、J-

red、DsRed单体、mOrange、mKO、mCitrine、Venus、YPet、Emerald、CyPet、mCFPm、Cerulean和T-Sapphire。可通过检测经编码多肽发出的荧光来区分含有该基因的细胞与不含该基因的细胞。

[0049] 如本文所用,术语“可操作地连接”是指两个或更多个组分的并置,其中组分处于允许它们以其预期方式起作用的关系。例如,如果启动子和/或增强子起到调节编码序列转录的作用,则该启动子和/或该增强子与编码序列可操作地连接。在某些实施例中,“可操作地连接”的DNA序列在一条染色体上是连续且相邻的。在某些实施例中,例如,当需要连接两个蛋白质编码区(例如分泌前导序列和多肽)时,序列是连续、相邻的,并且在同一读框中。在某些实施例中,可操作连接的启动子位于编码序列的上游,并且可以与其相邻。在某些实施例中,例如,关于调节编码序列的表达的增强子序列,两种组分尽管不相邻,但是可操作地连接。如果增强子增加了编码序列的转录,则该增强子可操作地连接至该编码序列。可操作地连接的增强子可位于编码序列的上游、内部或下游,并且可位于距编码序列的启动子相当远的距离。可操作地连接可通过本领域中已知的重组方法来完成,例如,使用PCR方法和/或在方便的限制位点处连接。如果不存在方便的限制位点,则可以按照常规做法使用合成的寡核苷酸衔接子或连接基。如果内部核糖体进入位点(IRES)允许以5'末端独立的方式在内部位置启动ORF的翻译,则认为其与开放读框(ORF)可操作地连接。

[0050] 如本文所用,术语“表达”是指转录和/或翻译。在某些实施例中,所需产物的转录水平可根据存在的相应mRNA的量来确定。例如,可通过PCR或Northern杂交对由目的序列转录的mRNA进行定量。在某些实施例中,由目的序列编码的蛋白质可通过多种方法定量,例如通过ELISA、通过测定蛋白质的生物学活性、或通过使用识别并结合蛋白质的抗体采用与该等活性无关的测定(如蛋白质印迹或放射免疫测定)来定量。

[0051] 本文所用的术语“目的序列”是指多肽序列(或在某些情况下,是指编码多肽序列的核酸),其中该多肽序列的表达是目的。在某些实施例中,该多肽序列可包含多亚基蛋白质复合物的亚基。在某些实施例中,该多肽序列可包含该亚基的片段。在某些实施例中,该多肽序列可包含抗体序列,例如抗体重链或轻链序列。在某些实施例中,该多肽序列可包含该抗体序列的片段。

[0052] 本文的术语“抗体”以最广泛的含义使用,并且包括各种抗体结构,包括但不限于单克隆抗体、多克隆抗体、多特异性抗体(例如,双特异性抗体)、半抗体和抗体片段,只要它们表现出所需的抗原结合活性即可。

[0053] 如本文所用,“标准抗体”和“标准单克隆抗体”是具有单一结合特异性的抗体或抗体片段。在某些实施例中,标准抗体的单一结合特异性是重链序列或其片段与轻链序列或其片段配对的结果。

[0054] 如本文所用,“双特异性抗体”或“BsAb”是可以同时结合两个不同表位的抗体,例如两个不同抗原上的两个不同表位或单个抗原上的两个不同表位。BsAbs包括许多不同的结构,包括那些包含两个不同的亲代单克隆抗体的变异重链(V_H)和轻链(V_L)结构域的配对结构,从而导致BsAb的一个“臂”(即,一对 V_H 和 V_L)具有第一亲代抗体的结合特异性,并且BsAb的第二“臂”具有第二亲代抗体的结合特异性。BsAbs是多特异性抗体的子集,其中多特异性抗体具有至少两种结合特异性(即BsAbs),但也包括三特异性抗体以及具有更多重特异性的抗体。

[0055] 如本文所用,术语“抗体片段”是指除了完整抗体以外的分子,其包括完整抗体的一部分且结合完整抗体结合的抗原。抗体片段的实例包括但不限于Fv、Fab、Fab'、Fab'-SH、F(ab')₂;双体抗体;线性抗体;单链抗体分子(例如,scFv);以及由抗体片段形成的多特异性抗体。

[0056] 如本文所用,术语“可变区”或“可变结构域”是指抗体重链或轻链的参与抗体与抗原结合的结构域。天然抗体的重链和轻链的可变结构域(分别为V_H和V_L)通常具有相似的结构,其中每个结构域包含四个保守框架区(FR)和三个高变区(HVR)。参见例如Kindt等人,Kuby Immunology,第6版,W.H.Freeman and Co.,第91页(2007)。单个V_H或V_L结构域可足以赋予抗原结合特异性。此外,结合特定抗原的抗体可分别使用来自结合该抗原的抗体的V_H或V_L结构域来进行分离,以筛选互补V_L或V_H结构域的文库。参见例如Portolano等人,J.Immunol.150:880-887(1993);Clarkson等人,Nature 352:624-628(1991)。

[0057] 如本文所用,术语“载体”是指一种核酸分子,其能够传送与其连接的另一种核酸。该术语包括作为自我复制核酸结构的载体以及掺入已引入该宿主细胞的基因组中的载体。在某些实施例中,载体指导与其可操作地连接的核酸的表达。该载体在本文中称为“表达载体”。

[0058] 如本文所用,术语“同源序列”是指具有通过序列比对确定的显著序列相似性的序列。例如,两个序列可具有约50%、60%、70%、80%、90%、95%、99%、或99.9%的同源性。比对是通过算法和计算机程序(包括但不限于BLAST、FASTA和HMME)执行的,其可以比较序列并基于因素诸如序列长度、序列同一性和相似性以及序列不匹配和缺口的存在与长度来计算匹配的统计显著性。同源序列可以是指DNA和蛋白序列两者。

[0059] 如本文所用,术语“侧翼”是指位于第二核苷酸序列的5'或3'末端或两端的第一核苷酸序列。侧翼核苷酸序列可与第二核苷酸序列相邻或相距给定的距离。侧翼核苷酸序列的长度无具体限制。例如,侧翼序列可包含几个碱基对或几千个碱基对。

[0060] 如本文所用,术语“外源”表示核苷酸序列并非来源于宿主细胞,而是通过传统的DNA递送方法,例如通过转染、电穿孔或转化方法引入宿主细胞中。术语“内源”是指核苷酸序列来源于宿主细胞。“外源”核苷酸序列可具有在碱基组成上相同的“内源”对应物,但是其中“外源”序列通过例如重组DNA技术被引入宿主细胞中。

[0061] 如本文所用,“整合位点”包括在宿主细胞基因组内插入外源核苷酸序列的核酸序列。在某些实施例中,整合位点在宿主细胞基因组的两个相邻核苷酸之间。在某些实施例中,整合位点包含一段核苷酸序列。在某些实施例中,整合位点位于TI宿主细胞基因组的特定基因座内。在某些实施例中,整合位点在TI宿主细胞的内源基因内。

[0062] 如本文所用,术语“TI宿主细胞”是指包含用于表达目的序列的一个或多个基因座即整合位点的细胞。在某些实施例中,在包含两个或更多个RRS的一个或多个整合位点的外源核苷酸序列有利于将SOI整合到TI宿主细胞中。在某些实施例中,能够在一个或多个整合位点编辑TI宿主细胞基因组的基因组编辑系统有利于将SOI整合到TI宿主细胞中。

[0063] 2. 宿主细胞制备&筛选策略

[0064] 标准CLD策略通常涉及通过编码一个或多个目的序列的一个或多个特定的外源核酸进行多轮宿主细胞转染。如图1A所示,制备该外源核酸,例如包含抗体重链和轻链编码序列的质粒,然后在靶向经整合的背景下通过例如重组酶介导的盒式交换(RMCE)进行转染。

无论整合策略如何(例如,随机或靶向整合),均可在选择和单细胞克隆(SCC)之前恢复转染的宿主细胞库。然后,通过均匀时间分辨荧光("HTRF")效价测定法进行多轮克隆分析,以缩小克隆数量,从而进行更详细的生产率和产物质量测定,例如高分子量种类含量(HMWS%)、大小变化、酸性变化和糖基化变化。

[0065] 图2A示出了基于标准靶向经整合的CLD策略,其中采用多个CLD循环来识别表现出所需的表达水平、产物质量属性和生产培养性能的细胞。在该实例中,通过使多个宿主细胞与编码目的序列的两种外源核酸的特定组合接触来制备每个转染细胞库。尽管图2A示出了一种策略,其中涉及将目的序列从第一个"前"质粒和第二个"后"质粒插入到宿主基因组的特定基因座中(有关双载体RCME策略的一般说明,请参见下文第5.1节),应当理解,靶向整合方法可能涉及单个目的序列或两个以上的目的序列的整合。此外,如本文所概述的,靶向整合策略不仅可以采用如图2A和2B所示的重组酶介导的S0I整合,而且可以采用其他基因座特异性策略整合S0I,例如基因编辑介导的S0I整合。但是,如上所述,靶向整合方法的一种限定特征是它们被设计成导致特定序列以特定排列方式整合,即基因组内固定构型的转基因。根据该设计特征,如图2A所概述,有必要执行多个CLD循环,以确保测定数量足够多的目的序列的拷贝数和排列方式。

[0066] 与图2A所示的标准资源密集型多循环靶向整合CLD策略相比,本专利申请的主题涉及"随机化构型靶向整合"(本文也称为"随机化链靶向整合"(RCTI)策略),该策略允许单周期分析目的序列的拷贝数和排列的变化,但保留靶向经整合的基因座特异性整合优势。如图2B所示,可通过产生单个转染池来评估编码目的序列的所有相关的外源核酸。通过将编码目的序列的相关外源核酸的完整互补序列与多个宿主细胞相结合,可创建单个转染池,从中可以识别表现出所需的表达水平、产物质量属性和生产培养性能的克隆。

[0067] 尽管示例性图2B示出采用三个"前"和三个"后"质粒(各自包含一个或多个目的序列)的实施例,其中质粒被整合到存在于宿主细胞基因组内特定基因座处的外源核苷酸序列的前或后盒中(关于该"双载体RMCE"策略的完整说明,参见下文第5.1节),但是应当理解,本公开的主题涵盖该靶向整合策略的各种变型。例如,除使用包括前盒的第一质粒和包括后盒的第二质粒外,本公开还涵盖涉及单盒经整合的方法。此外,本公开还包括涉及将三个、四个、五个、六个、七个、八个、九个、十个或更多个盒整合到存在于宿主细胞基因组内特定基因座处的单个外源核苷酸序列中的方法。而且,每个盒不仅可包含一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个单独目的序列,而且每个宿主基因组可包含存在于宿主细胞基因组内的特定基因座处的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个外源核苷酸序列,可将包含目的序列的盒整合到所述序列中。因此,在示例性图2B示出了可以使用三个前盒和三个后盒来产生九个可能的目的序列转基因构型的策略的同时,本公开涵盖了用于产生更少和更多的转基因构型的策略,包括在宿主基因组中的一个以上的位点产生这些构型。

[0068] 采用本文所概述的RCTI策略可显著节省资源,包括但不限于节省成本和时间、节省人工以及缓解自动化的瓶颈。如图3-5所示,与标准CLD策略相比,这种资源节省不会对期望的表达水平的分布、产物质量属性或观察到的生产培养效能产生不利影响。

[0069] 如图6A-6C所示,RCTI方法还允许在早期、中期或完全恢复阶段进行单细胞克隆。由于标准CLD策略通常涉及在进行单细胞克隆之前需等到转染池完全恢复(例如,>90%的

细胞存活率),因此采用RCTI策略可节省大量时间,而不影响克隆的性能(具有高效价和所需产物质量的克隆的百分比)或异质性(载体构型、效价和产物质量的范围)。

[0070] 在某些实施例中,本公开涉及筛选表达至少一种SOI的TI宿主细胞文库的基于RCTI的高通量方法。在某些实施例中,该方法包括生成TI宿主细胞文库,其中所述文库包含表达一个或多个SOI的多个TI宿主细胞,该方法包括以下步骤:将文库分为单个克隆,并筛选克隆中的特定细胞和/或特定产物属性。在某些实施例中,生成TI宿主细胞文库包括以下步骤:提供多个TI宿主细胞,使多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI,以及将一个或多个SOI引入多个TI宿主细胞中的一个或多个。

[0071] 在某些实施例中,可通过重组酶介导的整合将目的序列引入本公开的宿主细胞中。在某些实施例中,可通过基因编辑介导的整合将目的序列引入本公开的宿主细胞。

[0072] 在某些实施例中,本公开的筛选方法可包括筛选细胞中的细胞属性,这些属性可以是但不限于细胞生长、细胞效价、比生产力、体积生产力、克隆稳定性。这些细胞属性的筛选可通过本领域已知的任何技术来进行。

[0073] 在某些实施例中,本公开的筛选方法可包括筛选细胞的产物属性,这些产物属性可以是但不限于糖基化水平、电荷变化水平、错配减少、蛋白质/肽聚集减少、蛋白质序列异质性。可以用本领域已知的任何技术筛选这些产物属性,这些技术例如但不限于体积排阻色谱(SEC)、cDNA测序、肽图分析、CE-SDS或SDS-PAGE、HPLC、基于CE的聚糖测定、IEF及离子交换色谱。

[0074] 在某些实施例中,本公开提供了一种基于RCTI的方法,该方法用于生成包含多个外源核苷酸SOI的TI宿主细胞库,包括使多个TI宿主细胞与具有一个或多个SOI的载体接触,将一个或多个SOI引入TI宿主细胞,然后选择表达一个或多个SOI的TI细胞。在某些实施例中,通过本公开的方法生成的TI宿主细胞将包含整合在TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列。在某些实施例中,外源核苷酸序列可包含可位于一个或多个选择标志物的侧翼的至少两个RRS。

[0075] 在某些实施例中,载体可包含一个或多个选择标志物基因,以提供表型性状,用于选择转化的宿主细胞,例如用于真核细胞培养基的二氢叶酸还原酶或新霉素抗性。在某些实施例中,宿主细胞可为高等真核细胞,例如哺乳动物细胞,也可以是较低等的真核细胞,例如酵母细胞,或者宿主细胞可为原核细胞,例如细菌细胞。在某些实施例中,可筛选文库中特定的目的序列,例如但不限于多肽序列。在某些实施例中,可筛选所得文库中的克隆,其在表型分析中对目的多肽表现出活性。在某些实施例中,可通过本领域已知的方法筛选文库中特定蛋白质(例如酶)的活性。

[0076] 在某些实施例中,本公开涉及一种选择表达目的序列的宿主细胞的基于RCTI的方法,其包括:提供多个TI宿主细胞;使多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI及至少一个标志物;将该一个或多个SOI引入该多个TI宿主细胞中的一个或多个;以及选择表达该选择标志物的TI宿主细胞,从而分离出表达目的序列的TI宿主细胞。在某些实施例中,SOI可编码多亚基蛋白质的多肽亚基或其片段。在某些实施例中,SOI可编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)、或Fc融合蛋白。在某些实施例中,外源核苷酸整合到其中的基因座与选自SEQ ID NO.1-7的序列具有至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%的同源性。

[0077] 在某些实施例中,一个或多个外源核苷酸序列可整合到一个或多个基因座,其与选自以下的序列具有至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%的同源性:SEQ ID Nos.1-12;NW_006874047.1;NW_006884592.1;NW_006881296.1;NW_003616412.1;NW_003615063.1;NW_006882936.1;以及NW_003615411.1。

[0078] 在某些实施例中,外源核苷酸所整合到的基因座与选自以下的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源:美国专利号9816110的序列SEQ ID NO:1-4,对应于本公开的SEQ ID NO:8-11;或国际专利申请号PCT/US2017/028555的序列SEQ ID NO:1,对应于本公开的SEQ ID NO:12。在某些实施例中,可通过重组酶介导的整合将一个或多个目的序列引入本公开的宿主细胞中。在某些实施例中,可通过基因编辑介导的整合将一个或多个目的序列引入本公开的宿主细胞中。

[0079] 在某些实施例中,本公开所述的选择表达目的序列的宿主细胞的基于RCTI的方法中所用的TI宿主细胞可各自具有整合到TI宿主细胞的基因组的一个或多个基因座处的一个或多个外源核苷酸序列。在某些实施例中,外源核苷酸序列可包含至少两个RRS,其位于至少一个第一选择标志物的侧翼。在某些实施例中,用于选择宿主细胞的基于RCTI的方法中的载体可包含:至少两个RRS,其匹配经整合的外源核苷酸上的至少两个RRS;以及一个或多个外源SOI以及侧接有RRS的至少一个第二选择标志物。在某些实施例中,外源核苷酸序列可包含位于至少一个第一选择标志物的侧翼的第一RRS和第二RRS,以及位于该第一RRS与该第二RRS之间的第三RRS,且所有RRS都是异种特异性的。在某些实施例中,多个载体可包含第一及第二载体。在某些实施例中,第一载体可包含:两个RRS,其匹配经整合的外源核苷酸序列上的第一RRS和第三RRS并位于至少一个第一外源SOI的侧翼,以及至少一个第二选择标志物;并且第二载体可包含两个RRS,其匹配经整合的外源核苷酸序列上的第二RRS和第三RRS并位于至少一个第二外源SOI的侧翼。在某些实施例中,多个载体可包含1、2、3、4、5、6、7、8、9、10个或更多个载体。

[0080] 在某些实施例中,目的序列包含多亚基蛋白质复合物的一个或多个亚基的序列。在某些实施例中,该多肽序列可包含此类亚基序列的片段。在某些实施例中,目的序列可包含此类亚基序列的组合。例如,但并非限制性地,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个序列。此外,在某些实施例中,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显变化,和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显变化。

[0081] 在某些实施例中,多个目的序列包含一个或多个重链序列(H)和/或一个或多个抗体轻链序列(L)。如本文所用,该H和L序列可以是全长重链或轻链序列以及重链或轻链片段,包括但不限于变异区片段和互补决定区片段。在某些实施例中,目的序列可包含该H和L序列的组合。例如,但并非限制性地,该组合可包含一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个H序列和一个、两个、三个、四个、五个、六个、七个、八个、九个、

十个或更多个L序列。此外,在某些实施例中,该组合可包含一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个相同或不同的明显H'序列和/或一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个相同或不同的明显L'序列。本发明所公开的主题还包括一至十个(或更多)附加的H和L序列,例如H''、H'''、L''和L'''。示例性实施例包括但不限于包含各种不同的H序列及各种不同的L序列的任何组合的序列,例如:HL;LH;H'L;LH';H'L';L'H';HLL;HHL;LLH;LHH;H'LL;H'HL;L'HH;L'LH;H'H'L;LH'H';HLLL;LLLH;HLHL;LHLH;H'LLL;L'LLH;H'LHL;L'HLH;等。

[0082] 在某些实施例中,本公开涉及一种表达SOI的方法,其包括:提供多个TI宿主细胞;使该多个TI宿主细胞与多个载体接触,其中该载体包含一个或多个SOI和至少一个标志物;将一个或多个SOI引入一个或多个该多个TI宿主细胞;选择表达该目的序列的TI细胞;以及在适合于表达目的序列的条件下培养细胞并且从其中回收目的序列。

[0083] 在某些实施例中,转染的宿主细胞将包含一个或多个目的序列,其中目的序列包含多亚基蛋白质复合物的一个或多个亚基的序列。在某些实施例中,该多肽序列可包含此类亚基序列的片段。在某些实施例中,目的序列可包含此类亚基序列的组合。例如,但并非限制性地,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个序列。此外,在某些实施例中,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个相同或不同的明显变化,和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个相同或不同的明显变化。

[0084] 在某些实施例中,转染的宿主细胞将包含一个或多个目的序列,其中目的序列包含H及L序列的组合。例如,但并非限制性地,该组合可包含一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个H序列和一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个L序列。此外,在某些实施例中,该组合可包含一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显的H'序列和/或一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显的L'序列。本发明所公开的主题还包括一至十个(或更多)附加的H和L序列,例如H''、H'''、L''和L'''。示例性实施例包括但不限于包含各种不同的H序列及各种不同的L序列的任何组合的序列,例如:HL;LH;H'L;LH';H'L';L'H';HLL;HHL;LLH;LHH;H'LL;H'HL;L'HH;L'LH;H'H'L;LH'H';HLLL;LLLH;HLHL;LHLH;H'LLL;L'LLH;H'LHL;L'HLH;等。

[0085] 3. 外源核苷酸序列

[0086] 本发明所公开的主题提供了适合整合外源核苷酸序列的宿主细胞。在某些实施例中,外源核苷酸序列用作整合位点,例如通过包含一个或多个重组酶识别序列来实现。在某些实施例中,外源核苷酸序列编码目的序列。因此,在某些实施例中,宿主细胞将包含一个或多个外源核苷酸序列,该序列将有利于编码一个或多个目的序列的一个或多个外源核苷酸序列的靶向整合。在某些实施例中,包含整合在宿主细胞基因组的整合位点上的外源核苷酸序列的宿主细胞被称为TI宿主细胞。然后可以将编码一个或多个目的序列的外源核苷酸序列引入TI宿主细胞中,并将整合靶向到整合位点。如下文所概述,TI宿主细胞可包含多

个整合位点,这些整合位点由存在的外源核苷酸序列定义,该外源核苷酸序列包含有助于整合编码一个或多个目的序列的外源核苷酸序列的元素(例如重组酶识别序列)。

[0087] 在某些实施例中,整合位点和/或位于整合位点的侧翼的核苷酸序列可通过实验进行识别。在某些实施例中,可通过全基因组筛选方法来识别整合位点和/或位于整合位点的侧翼的核苷酸序列,以分离出以期望的水平表达由整合到一个或多个外源核苷酸序列的一个或多个S0I编码的目标多肽,其中外源序列本身被整合到宿主细胞基因组中的一个或多个基因座中。在某些实施例中,在基于转位酶的盒整合事件后,可通过全基因组筛选方法识别整合位点和/或位于整合位点的侧翼的核苷酸序列。在某些实施例中,通过强力随机整合筛选来识别整合位点和/或位于整合位点的侧翼的核苷酸序列。在某些实施例中,整合位点和/或位于整合位点的侧翼的核苷酸序列可通过常规的测序方法确定,例如靶基因座扩增(TLA),然后是新一代测序(NGS)和全基因组NGS。在某些实施例中,整合位点在染色体上的位置可通过常规的细胞生物学方法诸如荧光原位杂交(FISH)分析来确定。

[0088] 在某些实施例中,宿主细胞包含整合在宿主细胞基因组中特定第一基因座内的第一整合位点处的第一外源核苷酸序列以及整合在基因组中特定第二基因座内的第二整合位点处的第二外源核苷酸序列。在某些实施例中,宿主细胞包含整合在宿主细胞基因组中的多个整合位点处的多个外源核苷酸序列。

[0089] 3.1包含重组酶识别序列的外源序列

[0090] 在某些实施例中,经整合的外源核苷酸序列包含一个或多个重组酶识别序列(RRS),其中RRS可由重组酶进行识别。在某些实施例中,经整合的外源核苷酸序列包含至少两个RRS。在某些实施例中,经整合的外源核苷酸序列包含两个RRS,并且这两个RRS相同。在某些实施例中,经整合的外源核苷酸序列包含两个RRS,并且这两个RRS不同。在某些实施例中,经整合的外源核苷酸序列包含三个RRS,其中第三RRS位于第一RRS与第二RRS之间。在某些实施例中,第一RRS与第二RRS相同,并且第三RRS不同于第一RRS或第二RRS。在某些实施例中,所有三个RRS均不同。在某些实施例中,经整合的外源核苷酸序列包含四个、五个、六个、七个或八个RRS。在某些实施例中,经整合的外源核苷酸序列包含多个RRS。在某些实施例中,所述两个或更多个RRS相同。在某些实施例中,所述两个或更多个RRS不同。在某些实施例中,RRS总数的子集相同,或RRS总数的子集不同。在某些实施例中,一个或多个RRS可选自由以下序列所组成的群组:LoxP序列、LoxP L3序列、LoxP 2L序列、LoxFas序列、Lox511序列、Lox2272序列、Lox2372序列、Lox5171序列、Loxm2序列、Lox71序列、Lox66序列、FRT序列、Bxb1 attP序列、Bxb1 attB序列、 ϕ C31 attP序列以及 ϕ C31 attB序列。

[0091] 在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物。在某些实施例中,经整合的外源核苷酸序列包含一个RRS和至少一个选择标志物。在某些实施例中,经整合的外源核苷酸序列包含第一RRS和第二RRS以及至少一个选择标志物。在某些实施例中,选择标志物位于第一RRS与第二RRS之间。在某些实施例中,两个RRS位于至少一个选择标志物的侧翼,即第一RRS位于上游5'端处,并且第二RRS位于选择标志物的下游3'端处。在某些实施例中,第一RRS与选择标志物的5'端相邻,并且第二RRS与选择标志物的3'端相邻。

[0092] 在某些实施例中,选择标志物位于第一RRS与第二RRS之间,并且这两个侧翼RRS相同。在某些实施例中,位于选择标志物的侧翼的两个RRS均为LoxP序列。在某些实施例中,位于选择标志物的侧翼的两个RRS均为FRT序列。在某些实施例中,选择标志物位于第一RRS与

第二RRS之间,并且这两个侧翼RRS不同。在某些实施例中,第一侧翼RRS为LoxP L3序列,并且第二侧翼RRS为LoxP 2L序列。在某些实施例中,LoxP L3序列位于选择标志物的5'端,并且LoxP 2L序列位于选择标志物的3'端。在某些实施例中,第一侧翼RRS为野生型FRT序列,并且第二侧翼RRS为突变型FRT序列。在某些实施例中,第一侧翼RRS为Bxb1attP序列,并且第二侧翼RRS为Bxb1 attB序列。在某些实施例中,第一侧翼RRS为 ϕ C31 attP序列,并且第二侧翼RRS为 ϕ C31 attB序列。在某些实施例中,两个RRS处于相同的方向。在某些实施例中,两个RRS均处于正向或反向。在某些实施例中,两个RRS处于相反的方向。

[0093] 在某些实施例中,选择标志物可为氨基糖苷磷酸转移酶 (APH) (例如,潮霉素磷酸转移酶 (HYG)、新霉素和G418APH)、二氢叶酸还原酶 (DHFR)、胸苷激酶 (TK)、谷氨酰氨合成酶 (GS)、天冬酰胺合成酶、色氨酸合成酶 (吡啶)、组氨酸脱氢酶 (组氨酸D) 以及编码对嘌呤霉素、杀稻瘟素、博莱霉素、腐草霉素、氯霉素、吉欧霉素或霉酚酸的抗性的基因。在某些实施例中,选择标志物可为GFP、eGFP、合成GFP、YFP、eYFP、CFP、mPlum、mCherry、tdTomato、mStrawberry、J-red、DsRed单体、mOrange、mKO、mCitrine、Venus、YPet、Emerald、CyPet、mCFPm、Cerulean、或T-Sapphire标志物。

[0094] 在某些实施例中,经整合的外源核苷酸序列包含侧接有两个RRS的两个选择标志物,其中第一选择标志物不同于第二选择标志物。在某些实施例中,两个选择标志物均选自以下所组成的组:谷氨酰氨合成酶选择标志物、胸苷激酶选择标志物、HYG选择标志物及嘌呤霉素抗性选择标志物。在某些实施例中,经整合的外源核苷酸序列包含胸苷激酶选择标志物和HYG选择标志物。在某些实施例中,第一选择标志物选自以下所组成的组:氨基糖苷磷酸转移酶 (APH) (例如,潮霉素磷酸转移酶 (HYG)、新霉素和G418 APH)、二氢叶酸还原酶 (DHFR)、胸苷激酶 (TK)、谷氨酰氨合成酶 (GS)、天冬酰胺合成酶、色氨酸合成酶 (吡啶)、组氨酸脱氢酶 (组氨酸D) 以及编码对嘌呤霉素、杀稻瘟素、博莱霉素、腐草霉素、氯霉素、吉欧霉素或霉酚酸的抗性的基因,并且第二选择标志物选自以下所组成的组:GFP、eGFP、合成GFP、YFP、eYFP、CFP、mPlum、mCherry、tdTomato、mStrawberry、J-red、DsRed单体、mOrange、mKO、mCitrine、Venus、YPet、Emerald、CyPet、mCFPm、Cerulean、以及T-Sapphire标志物。在某些实施例中,第一选择标志物为谷氨酰氨合成酶选择标志物,并且第二选择标志物为GFP标志物。在某些实施例中,位于两个选择标志物的侧翼的两个RRS相同。在某些实施例中,位于两个选择标志物的侧翼的两个RRS不同。

[0095] 在某些实施例中,选择标志物可操作地连接至启动子序列。在某些实施例中,选择标志物可操作地连接至SV40启动子。在某些实施例中,选择标志物可操作地连接至巨细胞病毒 (CMV) 启动子。

[0096] 在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物和IRES,其中IRES可操作地连接至该选择标志物。在某些实施例中,可操作地连接至IRES的选择标志物选自以下所组成的组:GFP、eGFP、合成GFP、YFP、eYFP、CFP、mPlum、mCherry、tdTomato、mStrawberry、J-red、DsRed单体、mOrange、mKO、mCitrine、Venus、YPet、Emerald、CyPet、mCFPm、Cerulean、以及T-Sapphire标志物。在某些实施例中,可操作地连接至IRES的选择标志物为GFP标志物。在某些实施例中,经整合的外源核苷酸序列包含IRES以及侧接有两个RRS的两个选择标志物,其中IRES可操作地连接至第二选择标志物。在某些实施例中,经整

合的外源核苷酸序列包含IRES以及侧接有两个RRS的三个选择标志物,其中IRES可操作地连接至第三选择标志物。在某些实施例中,经整合的外源核苷酸序列包含IRES以及侧接有两个RRS的三个选择标志物,其中IRES可操作地连接至第三选择标志物。在某些实施例中,第三选择标志物不同于第一选择标志物或第二选择标志物。在某些实施例中,经整合的外源核苷酸序列包含可操作地连接至启动子的第一选择标志物以及可操作地连接至IRES的第二选择标志物。在某些实施例中,经整合的外源核苷酸序列包含可操作地连接至SV40启动子的谷氨酰氨合成酶选择标志物以及可操作地连接至IRES的GFP选择标志物。在某些实施例中,经整合的外源核苷酸序列包含可操作地连接至CMV启动子的胸苷激酶选择标志物和HYG选择标志物以及可操作地连接至IRES的GFP选择标志物。

[0097] 在某些实施例中,经整合的外源核苷酸序列包含三个RRS。在某些实施例中,第三RRS位于第一RRS与第二RRS之间。在某些实施例中,所有三个RRS均相同。在某些实施例中,第一RRS与第二RRS相同,并且第三RRS不同于第一RRS或第二RRS。在某些实施例中,所有三个RRS均不同。

[0098] 在某些实施例中,用作整合位点的外源核苷酸序列将存在于TI宿主细胞的基因组的特定基因座内的位点。在某些实施例中,外源核苷酸所整合到的基因座与选自SEQ ID NO.1-7的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。在某些实施例中,外源核苷酸所整合到的基因座与选自以下的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源:美国专利号9816110的序列SEQ ID NO:1-4,对应于本公开的SEQ ID NO:8-11;或国际专利申请号PCT/US2017/028555的序列SEQ ID NO:1,对应于本公开的SEQ ID NO:12。

[0099] 在某些实施例中,外源核苷酸序列整合在TI宿主细胞的基因组特定基因座内的位点处。在某些实施例中,外源核苷酸所整合到的基因座与选自下列的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源:ContigsNW_006874047.1、NW_006884592.1、NW_006881296.1、NW_003616412.1、NW_003615063.1、NW_006882936.1、以及NW_003615411.1。

[0100] 在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.1的数量为1-1,000bp;1,000-2,000bp;2,000-3,000bp;3,000-4,000bp;以及4,000-4,301bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.2的数量为1-100,000bp;100,000-200,000bp;200,000-300,000bp;300,000-400,000bp;400,000-500,000bp;500,000-600,000bp;600,000-700,000bp;以及700,000-728785bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.3的数量为1-100,000bp;100,000-200,000bp;200,000-300,000bp;300,000-400,000bp;以及400,000-413,983bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.4的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;以及30,000-30,757bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.5的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;30,000-40,000bp;40,000-50,000bp;50,000-60,000bp;以及60,000-68,962bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整

合位点位于选自SEQ ID NO.6的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;30,000-40,000bp;40,000-50,000bp;以及50,000-51,326bp的核苷酸内。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点位于选自SEQ ID NO.7的数量为1-10,000bp;10,000-20,000bp;以及20,000-22,904bp的核苷酸内。

[0101] 在某些实施例中,紧邻经整合的外源序列5'端的核苷酸序列选自由以下所组成的组:NW_006874047.1的核苷酸41190-45269、NW_006884592.1的核苷酸63590-207911、NW_006881296.1的核苷酸253831-491909、NW_003616412.1的核苷酸69303-79768、NW_003615063.1的核苷酸293481-315265、NW_006882936.1的核苷酸2650443-2662054、或NW_003615411.1的核苷酸82214-97705,以及与其至少50%同源的序列。在某些实施例中,紧邻经整合的外源序列5'端的核苷酸序列与NW_006874047.1的核苷酸41190-45269、NW_006884592.1的核苷酸63590-207911、NW_006881296.1的核苷酸253831-491909、NW_003616412.1的核苷酸69303-79768、NW_003615063.1的核苷酸293481-315265、NW_006882936.1的核苷酸2650443-2662054、或NW_003615411.1的核苷酸82214-97705至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。

[0102] 在某些实施例中,紧邻经整合的外源序列3'端的核苷酸序列选自由以下所组成的组:NW_006874047.1的核苷酸45270-45490、NW_006884592.1的核苷酸207912-792374、NW_006881296.1的核苷酸491910-667813、NW_003616412.1的核苷酸79769-100059、NW_003615063.1的核苷酸315266-362442、NW_006882936.1的核苷酸2662055-2701768、或NW_003615411.1的核苷酸97706-105117,以及与其至少50%同源的序列。在某些实施例中,紧邻经整合的外源序列3'端的核苷酸序列与NW_006874047.1的核苷酸45270-45490、NW_006884592.1的核苷酸207912-792374、NW_006881296.1的核苷酸491910-667813、NW_003616412.1的核苷酸79769-100059、NW_003615063.1的核苷酸315266-362442、NW_006882936.1的核苷酸2662055-2701768、或NW_003615411.1的核苷酸97706-105117至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。

[0103] 在某些实施例中,经整合的外源核苷酸序列可操作地连接至选自由SEQ ID.NO.1-7所组成的组的核苷酸序列,以及与其至少50%同源的序列。在某些实施例中,可操作地连接至外源核苷酸序列的核苷酸序列与选自SEQ ID.NO.1-7的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。在某些实施例中,经整合的外源核苷酸序列包含至少一个S0I。在某些实施例中,与随机经整合的S0I相比,可操作地连接的核苷酸序列增加了S0I的表达水平。在某些实施例中,经整合的外源S0I比随机经整合的S0I的表达高约20%、30%、40%、50%、100%、2倍、3倍、5倍或10倍。

[0104] 在某些实施例中,经整合的外源序列的5'端侧接有选自由以下所组成的组的核苷酸序列:NW_006874047.1的核苷酸41190-45269、NW_006884592.1的核苷酸63590-207911、NW_006881296.1的核苷酸253831-491909、NW_003616412.1的核苷酸69303-79768、NW_003615063.1的核苷酸293481-315265、NW_006882936.1的核苷酸2650443-2662054、和NW_003615411.1的核苷酸82214-97705,以及与其至少50%同源的序列;并且3'端侧接有选自

由以下所组成的组的核苷酸序列: NW_006874047.1的核苷酸45270-45490、NW_006884592.1的核苷酸207912-792374、NW_006881296.1的核苷酸491910-667813、NW_003616412.1的核苷酸79769-100059、NW_003615063.1的核苷酸315266-362442、NW_006882936.1的核苷酸2662055-2701768、和NW_003615411.1的核苷酸97706-105117,以及与其至少50%同源的序列。在某些实施例中,位于经整合的外源核苷酸序列的5'端的侧翼的核苷酸序列与NW_006874047.1的核苷酸41190-45269、NW_006884592.1的核苷酸63590-207911、NW_006881296.1的核苷酸253831-491909、NW_003616412.1的核苷酸69303-79768、NW_003615063.1的核苷酸293481-315265、NW_006882936.1的核苷酸2650443-2662054、以及NW_003615411.1的核苷酸82214-97705至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源,并且位于经整合的外源核苷酸序列的3'端的侧翼的核苷酸序列与NW_006874047.1的核苷酸45270-45490、NW_006884592.1的核苷酸207912-792374、NW_006881296.1的核苷酸491910-667813、NW_003616412.1的核苷酸79769-100059、NW_003615063.1的核苷酸315266-362442、NW_006882936.1的核苷酸2662055-2701768、以及NW_003615411.1的核苷酸97706-105117至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。

[0105] 在某些实施例中,经整合的外源核苷酸整合至紧邻选自由以下序列所组成的组的序列的全部或一部分的基因座中,该序列与选自SEQ ID NO.1-7的序列至少约90%同源。

[0106] 在某些实施例中,经整合的外源核苷酸序列邻近选自由SEQ ID.NO.1-7以及与其至少50%同源的序列所组成的组的核苷酸序列。在某些实施例中,经整合的外源核苷酸序列与选自由SEQ ID.NO.1-7以及与其至少50%同源的序列所组成的组的序列相距约100bp、约200bp、约500bp、约1kb。在某些实施例中,邻近外源核苷酸序列的核苷酸序列与选自SEQ ID.NO.1-7的序列至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。

[0107] 在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.1的数量为1-1,000bp;1,000-2,000bp;2,000-3,000bp;3,000-4,000bp;以及4,000-4,301bp的核苷酸。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.2的数量为1-100,000bp;100,000-200,000bp;200,000-300,000bp;300,000-400,000bp;400,000-500,000bp;500,000-600,000bp;600,000-700,000bp;以及700,000-728785bp的核苷酸。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.3的数量为1-100,000bp;100,000-200,000bp;200,000-300,000bp;300,000-400,000bp;以及400,000-413,983bp的核苷酸。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.4的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;以及30,000-30,757bp的核苷酸。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.5的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;30,000-40,000bp;40,000-50,000bp;50,000-60,000bp;以及60,000-68,962bp的核苷酸。在某些实施例中,外源核苷酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.6的数量为1-10,000bp;10,000-20,000bp;20,000-30,000bp;30,000-40,000bp;40,000-50,000bp;以及50,000-51,326bp的核苷酸。在某些实施例中,外源核苷

酸序列整合在整合位点处,该整合位点邻近选自SEQ ID NO.7的数量为1-10,000bp;10,000-20,000bp;以及20,000-22,904bp的核苷酸。

[0108] 在某些实施例中,包含外源核苷酸序列的整合位点的基因座不编码开放读框(ORF)。在某些实施例中,包含外源核苷酸序列的整合位点的基因座包括顺式作用元件,例如启动子和增强子。在某些实施例中,包含外源核苷酸序列的整合位点的基因座不含任何增强基因表达的顺式作用元件,例如启动子和增强子。

[0109] 在某些实施例中,外源核苷酸序列整合在外源基因内的整合位点处,该外源基因选自由LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2所组成的组。内源LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因包括LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因的野生型和所有同源性序列。在某些实施例中,LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、及XP_003512331.2基因的同源性序列可与野生型LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、及XP_003512331.2基因至少约50%、至少约60%、至少约70%、至少约80%、至少约90%、至少约95%、至少约99%、或至少约99.9%同源。在某些实施例中,LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因为野生型哺乳动物LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因。在某些实施例中,LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因为野生型人LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因。在某些实施例中,LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因为野生型仓鼠LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、以及XP_003512331.2基因。

[0110] 在某些实施例中,整合位点可操作地连接至选自由LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、XP_003512331.2及其至少约90%同源的序列所组成的组的内源基因。在某些实施例中,整合位点侧接有选自由LOC107977062、LOC100768845、ITPR2、ERE67000.1、UBAP2、MTMR2、XP_003512331.2及其至少约90%同源的序列所组成的组的内源基因。

[0111] 表1提供了示例性TI宿主细胞整合位点:

[0112] 表1-TI宿主细胞整合位点

[0113]	宿主	Contig	Contig 大小 (kb)	整合位点 (bp)	基因 (SEQ ID NO.)
	1	NW_006874047.1	727	45269	LOC107977062 (SEQ ID NO. 1)
	2	NW_006884592.1	931	207911	LOC100768845 (SEQ ID NO. 2)
	3	NW_006881296.1	1016	491909	ITPR2 (SEQ ID NO. 3)
	4	NW_003616412.1	127	79768	ERE67000.1 (SEQ ID NO. 4)
	5	NW_003615063.1	372	315265	UBAP2 (SEQ ID NO. 5)
	6	NW_006882936.1	3042	2662054	MTMR2 (SEQ ID NO. 6)
	7	NW_003615411.1	277	97706	XP_003512331.2 (SEQ ID NO. 7)

[0114] 3.2包含目的序列的外源核苷酸序列

[0115] 在某些实施例中,经整合的外源核苷酸序列包含至少一个外源SOI。在某些实施例

中,经整合的外源核苷酸序列包含至少一个选择标志物以及至少一个外源SOI。在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物、至少一个外源SOI、以及至少一个RRS。在某些实施例中,经整合的外源核苷酸序列包含至少一个、至少两个、至少三个、至少四个、至少五个、至少六个、至少七个、至少八个、至少九个、至少十个或更多SOI。在某些实施例中,SOI相同。在某些实施例中,SOI不同。

[0116] 如上所述,在某些实施例中,SOI编码多亚基蛋白质复合物的一个或多个亚基。在某些实施例中,该多肽序列可包含此类亚基序列的片段。在某些实施例中,目的序列可包含此类亚基序列的组合。例如,但并非限制性地,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个序列。此外,在某些实施例中,该组合可包含第一亚基序列中的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显变化,和/或第二、第三、第四、第五、第六、第七、第八、第九、第十或更多亚基序列的一个、两个、三个、四个、五个、六个、七个、八个、九个、十个或更多个明显变化。

[0117] 在某些实施例中,SOI编码单链抗体或其片段。在某些实施例中,SOI编码抗体重链序列或其片段。在某些实施例中,SOI编码抗体轻链序列或其片段。在某些实施例中,经整合的外源核苷酸序列包含编码抗体重链序列或其片段的SOI以及编码抗体轻链序列或其片段的SOI。在某些实施例中,经整合的外源核苷酸序列包含编码第一抗体重链序列或其片段的SOI、编码第二抗体重链序列或其片段的SOI以及编码抗体轻链序列或其片段的SOI。在某些实施例中,经整合的外源核苷酸序列包含编码第一抗体重链序列或其片段的SOI、编码第二抗体重链序列或其片段的SOI、编码抗体第一轻链序列或其片段的SOI以及编码抗体轻链序列或其片段的第二SOI。在某些实施例中,可选择编码重链和轻链序列的SOI的数目,以实现所需的重链和轻链多肽的表达水平,例如,以实现所需量的双特异性抗体产生。在某些实施例中,可以将编码重链和轻链序列的单个SOI整合到,例如,存在于单个整合位点处的单个外源核酸序列、存在于单个整合位点处的多个外源核酸序列、或整合在TI宿主细胞内不同整合位点处的多个外源核酸序列。

[0118] 在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物、至少一个外源SOI、以及一个RRS。在某些实施例中,RRS邻近至少一个选择标志物或至少一个外源SOI。在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物、至少一个外源SOI、以及两个RRS。在某些实施例中,经整合的外源核苷酸序列包含至少一个选择标志物以及位于第一RRS与第二RRS之间的至少一个外源SOI。在某些实施例中,位于两个选择标志物的侧翼的两个RRS与外源SOI相同。在某些实施例中,位于两个选择标志物的侧翼的两个RRS与外源SOI不同。在某些实施例中,第一侧翼RRS为LoxP L3序列,并且第二侧翼RRS为LoxP 2L序列。在某些实施例中,L3 LoxP序列位于选择标志物和外源SOI的5'端,并且LoxP 2L序列位于选择标志物和外源SOI的3'端。

[0119] 在某些实施例中,经整合的外源核苷酸序列包含三个RRS以及两个外源SOI,并且第三RRS位于第一RRS与第二RRS之间。在某些实施例中,第一SOI位于第一RRS与第三RRS之间,并且第二SOI位于第三RRS与第三RRS之间。在某些实施例中,第一SOI和第二SOI不同。在某些实施例中,第一RRS与第二RRS相同,并且第三RRS不同于第一RRS或第二RRS。在某些实

施例中,所有三个RRS均不同。在某些实施例中,第一RRS为LoxP L3位点,第二RRS为LoxP 2L位点,第三RRS为LoxFas位点。在某些实施例中,经整合的外源核苷酸序列包含三个RRS、一个外源SOI以及一个选择标志物。在某些实施例中,该SOI位于第一RRS与第三RRS之间,并且选择标志物位于第三RRS与第三RRS之间。在某些实施例中,经整合的外源核苷酸序列包含三个RRS、两个外源SOI以及一个选择标志物。在某些实施例中,第一SOI和选择标志物位于第一RRS与第三RRS之间,并且第二SOI位于第三RRS与第三RRS之间。

[0120] 在某些实施例中,外源SOI编码目的多肽,包括但不限于抗体、酶、细胞因子、生长因子、激素、病毒蛋白、细菌蛋白、疫苗蛋白、或具有治疗功能的蛋白质。在某些实施例中,外源SOI编码抗体或其抗原结合片段。在某些实施例中,外源SOI编码单链抗体、抗体轻链、抗体重链、单链Fv片段(scFv)、或Fc融合蛋白。在某些实施例中,外源SOI可操作地连接至至少一个顺式作用元件,例如启动子或增强子。在某些实施例中,外源SOI可操作地连接至CMV启动子。

[0121] 在某些实施例中,经整合的外源核苷酸序列包含两个RRS和位于两个RRS之间的至少两个外源SOI。在某些实施例中,编码抗体的一条重链和一条轻链的SOI位于两个RRS之间。在某些实施例中,编码抗体的一条重链和两条轻链的SOI位于两个RRS之间。在某些实施例中,编码抗体的重链和轻链的拷贝的不同组合的SOI位于两个RRS之间。

[0122] 在某些实施例中,经整合的外源核苷酸序列包含三个RRS和至少两个外源SOI,并且第三RRS位于第一RRS与第二RRS之间。在某些实施例中,至少一个SOI位于第一RRS与第三RRS之间,并且至少一个SOI位于第三RRS与第三RRS之间。在某些实施例中,第一RRS与第二RRS相同,并且第三RRS不同于第一RRS或第二RRS。在某些实施例中,所有三个RRS均不同。在某些实施例中,编码第一抗体的一条重链和一条轻链的SOI位于第一RRS与第三RRS之间,并且编码第二抗体的一条重链和一条轻链的SOI位于第三RRS与第二RRS之间。在某些实施例中,编码第一抗体的一条重链和两条轻链的SOI位于第一RRS与第三RRS之间,并且编码第二抗体的一条重链和一条轻链的SOI位于第三RRS与第二RRS之间。在某些实施例中,编码第一抗体的一条重链和三条轻链的SOI位于第一RRS与第三RRS之间,并且编码第一抗体的一条轻链和第二抗体的一条重链和一条轻链的SOI位于第三RRS与第二RRS之间。在某些实施例中,编码第一抗体的一条重链和三条轻链的SOI位于第一RRS与第三RRS之间,并且编码第一抗体的两条轻链和第二抗体的一条重链和一条轻链的SOI位于第三RRS与第二RRS之间。在某些实施例中,编码多个抗体的重链和轻链拷贝的不同组合的SOI位于第一RRS与第三RRS之间以及第三RRS与第二RRS之间。

[0123] 4. 宿主细胞

[0124] 在某些实施例中,宿主细胞为真核宿主细胞。在某些实施例中,宿主细胞为哺乳动物宿主细胞。在某些实施例中,宿主细胞为仓鼠宿主细胞、人宿主细胞、大鼠宿主细胞、或小鼠宿主细胞。在某些实施例中,宿主细胞为中国仓鼠卵巢(CHO)宿主细胞、CHO K1宿主细胞、CHO K1SV宿主细胞、DG44宿主细胞、DUKXB-11宿主细胞、CHOK1S宿主细胞、或CHO K1M宿主细胞。

[0125] 在某些实施例中,宿主细胞选自以下所组成的组:由SV40(COS-7)转化的猴肾CV1系;人胚胎肾系(如Graham等人,J.Gen Virol.36:59(1977)中所述的293或293细胞);幼仓鼠肾细胞(BHK);小鼠睾丸支持细胞(如Mather,Biol.Reprod.23:243-251(1980)中所述

的TM4细胞);猴肾细胞(CV1);非洲绿猴肾细胞(VERO-76);人宫颈癌细胞(HELA);犬肾细胞(MDCK);水牛鼠肝细胞(BRL 3A);人肺细胞(W138);人肝细胞(Hep G2);小鼠乳腺肿瘤细胞(MMT 060562);TRI细胞(如Mather等人,Annals N.Y.Acad.Sci.383:44-68 (1982)所述)、MRC 5细胞、FS4细胞、Y0细胞、NS0细胞、Sp2/0细胞、以及PER.C6®细胞。

[0126] 在某些实施例中,宿主细胞是细胞系。在某些实施例中,宿主细胞是已经培养了多代的细胞系。在某些实施例中,宿主细胞是原代细胞。

[0127] 在某些实施例中,如果在10、20、30、50、100、200或300代内表达水平维持在一定水平,提升或降低小于20%,则认为目的多肽的表达是稳定的。在某些实施例中,如果无需进行任何选择即可维持培养,则目的多肽的表达是稳定的。在某些实施例中,如果目的基因的多肽产物达到约1g/L、约2g/L、约3g/L、约4g/L、约5g/L、约10g/L、约12g/L、约14g/L、或约16g/L,则表明目的多肽的表达水平高。

[0128] 在某些实施例中,产生目的多肽并将其分泌到细胞培养基中。在某些实施例中,目的多肽得到表达并保留在宿主细胞内。在某些实施例中,目的多肽得到表达,被插入并保留在宿主细胞膜内。

[0129] 可利用常规细胞生物学方法将目的外源核苷酸或载体引入宿主细胞,这些方法包括但不限于转染、转导、电穿孔或注射。在某些实施例中,利用基于化学的转染方法将目的外源核苷酸或载体引入宿主细胞,这些方法包括基于脂质的转染方法、基于磷酸钙的转染方法、基于阳离子聚合物的转染方法或基于纳米颗粒的转染。在某些实施例中,利用病毒介导的转导方法将目的外源核苷酸引入宿主细胞,该方法包括但不限于慢病毒、逆转录病毒、腺病毒或腺相关病毒介导的转导。在某些实施例中,利用基因枪介导的注射将目的外源核苷酸引入宿主细胞。在某些实施例中,使用本文所述的方法将DNA和RNA分子均引入宿主细胞。

[0130] 5. 靶向整合

[0131] 靶向整合方法可以将外源核苷酸序列整合到宿主细胞基因组的一个或多个预定位点。在某些实施例中,靶向整合由识别一个或多个RRS的重组酶介导。在某些实施例中,靶向整合由同源重组介导。在某些实施例中,靶向整合由外源位点特异性核酸酶介导,然后由HDR和/或NHEJ介导。

[0132] 5.1. 通过重组酶介导的重组进行靶向整合

[0133] “重组酶识别序列”(RRS)是由重组酶识别的核苷酸序列,并且对于重组酶介导的重组事件是必要和充分的。RRS可用于定义核苷酸序列中将发生重组事件的位置。

[0134] 在某些实施例中,RRS选自由以下序列所组成的群组:LoxP序列、LoxP L3序列、LoxP 2L序列、LoxFas序列、Lox511序列、Lox2272序列、Lox2372序列、Lox5171序列、Loxm2序列、Lox71序列、Lox66序列、FRT序列、Bxb1 attP序列、Bxb1 attB序列、 ϕ C31 attP序列以及 ϕ C31 attB序列。

[0135] 在某些实施例中,RRS可由Cre重组酶识别。在某些实施例中,RRS可由FLP重组酶识别。在某些实施例中,RRS可由Bxb1整合酶识别。在某些实施例中,RRS可由 ϕ C31整合酶识别。

[0136] 在某些实施例中,当RRS为LoxP位点时,宿主细胞需要Cre重组酶进行重组。在某些

实施例中,当RRS为FRT位点时,宿主细胞需要FLP重组酶进行重组。在某些实施例中,当RRS为Bxb1 attP或Bxb1 attB位点时,宿主细胞需要Bxb1整合酶进行重组。在某些实施例中,当RRS为 ϕ C31 attP或a ϕ C31 attB位点时,宿主细胞需要 ϕ C31整合酶进行重组。可使用包含酶的编码序列的表达载体将重组酶引入宿主细胞。

[0137] Cre-LoxP位点特异性重组体系已广泛用于许多生物学实验系统中。Cre是一种38kDa位点特异性DNA重组酶,可识别34bp LoxP序列。Cre来源于噬菌体P1,并且属于酪氨酸家族位点特异性重组酶。Cre重组酶可以介导LoxP序列之间的分子内和分子间重组。LoxP序列由8bp的非回文核心区组成,该非回文核心区侧接有两个13bp的反向重复序列。Cre重组酶与13bp重复序列结合,从而介导8bp核心区内的重组。Cre-LoxP介导的重组效率高,且无需任何其他宿主因子。如果将两个LoxP序列以相同的方向置于同一核苷酸序列上,则Cre介导的重组将切除位于两个LoxP序列之间的DNA序列,使其成为共价闭合环。如果两个LoxP序列位于同一核苷酸序列的反向位置,则Cre介导的重组将颠倒位于两个序列之间的DNA序列的方向。还可以将LoxP序列置于不同的染色体上,以促进不同染色体之间的重组。如果两个LoxP序列位于两个不同DNA分子上,并且一个DNA分子是环状分子,则Cre介导的重组将导致环状DNA序列的整合。

[0138] 在某些实施例中,LoxP序列为野生型LoxP序列。在某些实施例中,LoxP序列为突变型LoxP序列。已开发出突变型LoxP序列以提高Cre介导的整合或替换的效率。在某些实施例中,突变型LoxP序列选自由以下序列所组成的组:LoxP L3序列、LoxP 2L序列、LoxFas序列、Lox511序列、Lox2272序列、Lox2372序列、Lox5171序列、Loxm2序列、Lox71序列、以及Lox66序列。例如,Lox71序列在左侧13bp重复序列中具有5bp的突变。Lox66序列在右侧13bp重复序列中具有5bp的突变。野生型和突变型LoxP序列均可介导Cre依赖性重组。

[0139] FLP-FRT位点特异性重组系统类似于Cre-Lox系统。其中涉及翻转酶(FLP)重组酶,该酶来源于酿酒酵母的2 μ m质粒。FLP也属于酪氨酸家族位点特异性重组酶。FRT序列是一种34bp序列,由各自位于8bp间隔区的侧翼的两个13bp回文序列组成。FLP与13bp回文序列结合,并在8bp间隔区内介导DNA断裂、交换和连接。与Cre重组酶类似,两个FRT序列的位置和方向决定了FLP介导的重组的结果。在某些实施例中,FRT序列为野生型FRT序列。在某些实施例中,FRT序列为突变型FRT序列。野生型和突变型FRT序列均可介导FLP依赖性重组。在某些实施例中,FRT序列与应答性受体结构域序列诸如但不限于他莫昔芬应答性受体结构域序列融合。

[0140] Bxb1和 ϕ C31属于丝氨酸重组酶家族。它们均来源于噬菌体,并被这些噬菌体用于确定溶原性,以促进噬菌体基因组到细菌基因组的位点特异性整合。这些整合酶催化短(40-60bp)DNA基质(称为attP和attB序列)之间的位点特异性重组事件,这些基质最初分别为位于噬菌体DNA和细菌DNA上的附着位点。重组后,形成两个新序列,分别称为attL和attR序列,并且每个序列包含来源于attP和attB的一半序列。重组也可能发生在attL与attR之间,以将经整合的噬菌体从细菌DNA中切除。两种整合酶均可催化重组,而无需任何其他宿主因子的辅助。在不存在任何辅助因子的情况下,这些整合酶介导的attP与attB之间的单向重组效率超过80%。由于可由这些整合酶识别的短DNA序列及单向重组,因此将这些重组系统开发为对广泛用于基因工程目的的Cre-LoxP和FRT-FLP系统的补充。

[0141] 术语“匹配RRS”表示两个RRS之间发生重组。在某些实施例中，两个匹配RRS相同。在某些实施例中，两个RRS均为野生型LoxP序列。在某些实施例中，两个RRS均为突变型LoxP序列。在某些实施例中，两个RRS均为野生型FRT序列。在某些实施例中，两个RRS均为突变型FRT序列。在某些实施例中，两个匹配RRS为不同序列，但是可通过相同的重组酶识别。在某些实施例中，第一匹配RRS是Bxb1 attP序列，并且第二匹配RRS是Bxb1 attB序列。在某些实施例中，第一匹配RRS是 ϕ C31 attB序列，并且第二匹配RRS是 ϕ C31 attB序列。

[0142] 在某些实施例中，采用“单载体RMCE”策略。例如，在某些实施例中，经整合的外源核苷酸序列包含两个RRS，并且载体包含两个RRS，该两个RRS匹配经整合的外源核苷酸序列上的两个RRS，即经整合的外源核苷酸序列上的第一RRS匹配载体上的第一RRS，并且经整合的外源核苷酸序列上的第二RRS匹配载体上的第二RRS。在某些实施例中，经整合的外源核苷酸序列上的第一RRS和载体上的第一RRS与经整合的外源核苷酸序列上的第二RRS和载体上的第二RRS相同。在某些实施例中，经整合的外源核苷酸序列上的第一RRS和载体上的第一RRS与经整合的外源核苷酸序列上的第二RRS和载体上的第二RRS不同。在某些实施例中，经整合的外源核苷酸序列上的第一RRS和载体上的第一RRS均为LoxP L3序列，并且经整合的外源核苷酸序列上的第二RRS和载体上的第二RRS均为LoxP 2L序列。

[0143] 在某些实施例中，采用“双载体RMCE”策略。例如，但并非限制性地，经整合的外源核苷酸序列可包含三个RRS，例如其排列方式为第三RRS (RRS3) 存在于第一RRS (RRS1) 与第二RRS (RRS2) 之间，而第一载体包含两个RRS，该两个RRS匹配经整合的外源核苷酸序列上的第一RRS和第三RRS，并且第二载体包含两个RRS，该两个RRS匹配经整合的外源核苷酸序列上的第三RRS和第二RRS。通过在每对RRS之间整合适当数量的SOI，这两种载体RMCE策略均允许引入十个或更多个SOI。

[0144] 单载体和双载体RMCE均允许将一个或多个供体DNA分子单向整合到宿主细胞基因组的预定位点中，并使供体DNA上存在的DNA盒与整合位点所在的宿主基因组上的DNA盒精确交换。DNA盒的特征在于，位于至少一个选择标志物的侧翼的两个异种特异RRS (尽管在某些双载体RMCE实例中，可使用如本文所概述的“分离选择标志物”) 和/或至少一个外源SOI。RMCE参与靶基因组基因座内两个异种特异RRS与供体DNA分子之间的重组酶催化的双重重组交换事件。RMCE旨在将SOI或选择标志物的拷贝引入宿主细胞基因组的预定位点。与仅涉及一个交换事件的重组不同，RMCE可实施为不将原核载体序列引入宿主细胞基因组中，从而减少和/或防止不希望的宿主免疫或防御机制触发。可使用多个DNA盒重复执行RMCE程序，例如，在图2B中，采用RMCE程序方便地引入三个不同的“前位”盒和三个不同的“后位”盒。但是，如上所述，可以采用RMCE (和其他整合策略) 引入少至一个盒以及多达十个或更多个盒。

[0145] 在某些实施例中，通过一次交换重组事件实现靶向整合，其中将包含与至少一个外源SOI或至少一个选择标志物相邻的一个RRS的一个外源核苷酸序列整合到宿主细胞基因组的预定位点。在某些实施例中，通过一次RMCE实现靶向整合，其中将包含至少一个外源SOI或至少一个选择标志物 (侧接有两个异种特异RRS) 的DNA盒整合到宿主细胞基因组的预定位点。在某些实施例中，靶向整合通过两次RMCE实现，其中将各自包含至少一个外源SOI或至少一个选择标志物 (侧接有两个异种特异RRS) 的两个不同的DNA盒整合到宿主细胞基因组的预定位点。在某些实施例中，靶向整合通过多次RMCE实现，其中将来自多个载体的

DNA盒(各自包含至少一个外源SOI或至少一个选择标志物(侧接有两个异种特异RRS))全部整合到宿主细胞基因组的预定位点。在某些实施例中,选择标志物可部分地编码在第一载体上,并且部分地编码在第二载体上,使得两次RMCE的整合均允许表达选择标志物。

[0146] 在某些实施例中,通过重组酶介导的重组实现的靶向整合导致选择标志物或一个或多个外源SOI与来自原核载体的序列一起整合到宿主细胞基因组的一个或多个预定整合位点。在某些实施例中,通过重组酶介导的重组实现的靶向整合导致选择标志物或一个或多个外源SOI整合到宿主细胞基因组的一个或多个预定整合位点,而不包含来自原核载体的序列。

[0147] 5.2通过同源重组(HDR或NHEJ)进行靶向整合

[0148] 本发明所公开的主题还涉及通过同源重组或通过外源位点特异性核酸酶以及HDR或NHEJ介导的靶向整合。在某些实施例中,该整合在本文中称为“基因编辑介导的整合”。

[0149] 同源重组是具有广泛序列同源性的DNA分子之间的重组。它可用于引导双链DNA断裂的无错误修复,并在减数分裂过程中在配子中产生序列变异。由于同源重组涉及两个同源DNA分子之间的遗传信息交换,因此不改变基因在染色体上的整体排列。在同源重组过程中,双链DNA(dsDNA)形成缺口或断裂,然后单链DNA末端侵入同源dsDNA分子,同源序列发生配对,分支迁移以形成霍利迪连结体,并最终分离霍利迪连结体。

[0150] 双链断裂(DSB)是最严重的DNA损伤形式,此类DNA损伤的修复对于维持所有生物体的基因组的完整性至关重要。DSB修复途径主要有两种。第一种修复途径是同源定向修复(HDR)途径,并且同源重组是HDR中最常用的形式。由于HDR需要细胞中存在同源DNA,因此该修复途径通常用于细胞周期的S和G2期,其中新复制的姐妹染色单体可作为同源模板。HDR也是修复DNA复制过程中折叠的复制叉的主要修复途径。HDR被视为一种相对无错误的修复途径。针对DSB的第二修复途径是非同源末端连接(NHEJ)。NHEJ作为一种修复途径,其中将断裂DNA的末端连接在一起而无需同源DNA模板。

[0151] 外源位点特异性核酸酶,例如基因编辑核酸酶,然后是HDR,可以促进靶向整合。这是因为可通过在特定靶基因组位点处引入DSB来增加同源重组的频率。在某些实施例中,外源核酸酶可选自由以下各项所组成的组:锌指核酸酶(ZFN)、ZFN二聚体、转录激活因子样效应物核酸酶(TALEN)、TAL效应子结构域融合蛋白、RNA引导的DNA核酸内切酶、工程化大范围核酸酶以及成簇的规律间隔的短回文重复序列(CRISPR)关联(Cas)的核酸内切酶。

[0152] CRISPR/Cas和TALEN系统是两种基因组编辑工具,可提供最佳的构建简便性和高效率。CRISPR/Cas被认为是细菌对入侵噬菌体的免疫防御机制。Cas是一种核酸酶,当由合成导向RNA(gRNA)引导时,能够与细胞中的特定核苷酸序列相关联,并且通过例如形成单链断裂、DSB和/或点突变中的一种或多种来编辑核苷酸序列内或周围的DNA。TALEN是一种工程化位点特异性核酸酶,由TALE(转录激活因子样效应物)的DNA结合结构域和限制性核酸内切酶FokI的催化结构域组成。通过改变DNA结合结构域单体的高度可变的残基区域中存在的氨基酸,可创建不同的人工TALEN,以靶向各种核苷酸序列。DNA结合结构域随后将核酸酶引导至靶序列,并创建DSB。

[0153] 通过同源重组或HDR进行的靶向整合涉及使同源序列存在于整合位点。在某些实施例中,同源序列存在于载体上。在某些实施例中,同源序列存在于多核苷酸上。

[0154] 在某些实施例中,同源重组在不含任何辅助因子的情况下进行。在某些实施例中,

由于能够经整合的载体的存在而促进同源重组。在某些实施例中，整合载体选自以下各项所组成的组：腺相关病毒载体、慢病毒载体、逆转录病毒载体及噬菌体整合载体。

[0155] 5.3调控系统

[0156] 本公开的主题进一步涉及在RCTI (称为“随机化构型靶向整合”(在本文中也称为“随机链调控靶向整合”(RCRTI))中使用的调控系统。例如，在许多情况下，蛋白质表达水平并非最佳，主要是由于编码蛋白难以表达。难以表达的蛋白质的低表达水平可能具有不同的难以确定的原因。一种可能是宿主细胞中表达的蛋白质的毒性。在此等情况下，可使用调控表达系统来表达有毒蛋白质，其中编码蛋白质的目的序列在诱导型启动子的控制下。在这些系统中，仅当将调节剂诸如但不限于四环素或其类似物强力霉素(DOX)等小分子加入培养基时，才引起难以表达的蛋白质的表达。调节有毒蛋白质的表达可减轻其毒性作用，使培养物在生产前达到所需的细胞生长。在某些实施例中，“随机化构型调控靶向整合”(本文中也称为“随机化链调控靶向整合”(RCRTI)系统包含整合到特定基因座(例如包含一个或多个RRS的外源核酸序列)的SOI，并在与其可操作连接的调控启动子下转录。在某些实施例中，RCRTI系统可用于确定难于表达的分子(例如但不限于抗体)的蛋白质表达水平低的原因。在某些实施例中，可通过选择性关闭RCRTI系统中SOI的表达的能力将SOI的表达与观察到的不良反应联系起来。

[0157] 在某些实施例中，为了在难以表达的分子的根本原因分析过程中最小化转录和细胞系变异性的影响，可使用“随机化构型调控靶向整合”(本文中也称为“随机化链调控靶向整合”(RCRTI)系统。例如，但并非限制性地，可通过向培养物中添加调节剂例如多西环素来触发TI宿主中SOI的表达。在某些实施例中，RCRTI载体利用四环素调节的启动子表达SOI，该SOI可整合到例如包含RRS的外源核酸序列中，而RRS本身可整合到宿主细胞基因组的整合位点中，以实现SOI的调控表达。

[0158] 在某些实施例中，与对照细胞系相比，本公开中描述的RCRTI系统可用于成功确定SOI例如治疗性抗体的低蛋白表达水平的根本原因。在某些实施例中，一旦确认SOI(例如治疗性抗体)在RCRTI细胞系中的相对表达水平较低，便可利用蛋白质翻译抑制剂治疗(例如，Dox及放线菌酮)来评估SOI的细胞内积累和分泌水平。

[0159] 例如，但并非限制性地，该调控可基于通过调节转录抑制因子或激活因子与组成或最小启动子的偶联来阻断或激活mRNA合成的基因开关。在某些非限制性实施例中，可通过结合抑制蛋白来实现抑制，例如，该蛋白在空间上阻断转录起始，或通过抑制转录沉默子主动抑制转录。在某些非限制性实施例中，哺乳动物或无病毒增强子的最小启动子的激活可通过与激活结构域的调节式偶联来实现。

[0160] 在某些实施例中，转录抑制因子或激活因子的条件式偶联可通过使用变构蛋白质来实现，该变构蛋白质能结合启动子以响应于外部刺激。在某些实施例中，转录抑制因子或激活因子的条件式偶联可通过使用从整合蛋白质释放的细胞内受体来实现，从而能够结合靶启动子。在某些实施例中，转录抑制因子或激活因子的条件式偶联可通过使用化学诱导的二聚体来实现。

[0161] 在某些实施例中，本公开的TI系统中所用的变构蛋白质可为响应于抗生素、细菌群体感应信使、分解代谢物或培养参数诸如温度(冷或热)而调节转录活性的蛋白质。在某些实施例中，该RCRTI系统可基于分解代谢物，例如，将控制其他碳源分解代谢基因的细菌

抑制因子转移到哺乳动物细胞中。在某些实施例中,可通过抑制因子CymR的累积应答结合来抑制靶启动子。在某些实施例中,基于分解代谢物的系统可通过与单纯疱疹VP16反式激活结构域融合的原核抑制因子HdnoR的6-羟基烟碱反应性结合,依赖于嵌合启动子的激活。

[0162] 在某些实施例中, TI系统可为基于群体感应的表达系统, 该系统来源于原核生物, 该原核生物通过群体感应分子来管理群体内和群体间交流。该群体感应分子与靶细胞中的受体结合, 调节受体与同源启动子的亲和性, 从而引发特定调节子开关的启动。在某些实施例中, 群体感应分子可为N-(3-氧代-辛酰基)-高丝氨酸内酯, 在存在该分子的情况下, TraR-p65融合蛋白激活融合到TraR特异性操纵子序列的最小启动子的表达。在某些实施例中, 在基于天蓝色链霉菌A3(2) ScbR抑制因子(在不存在SCB1的情况下结合其同源操纵子OScbR)的系统中, 群体感应分子可以为丁内酯SCB1(外消旋2-(1'-羟基-6-甲基庚基)-3-(羟甲基)-丁醇化物)。在某些实施例中, 群体感应分子可以为RTI系统中所用的高丝氨酸衍生诱导剂, 其中铜绿假单胞菌群体感应抑制因子RhIR和LasR与SV40 T抗原核定位序列和单纯疱疹VP16结构域融合并可激活包含特定操纵子序列的启动子(las框)。

[0163] 在某些实施例中, 调节本公开的RCRTI系统中所用的变构蛋白质的诱导分子可以为但不限于cumate、异丙基- β -D-吡喃半乳糖苷(IPTG)、大环内酯类、6-羟基烟碱、强力霉素、链阳菌素、NADH、四环素。

[0164] 在某些实施例中, 本公开的RCRTI系统中所用的细胞内受体可以为细胞质或核受体。在某些实施例中, 本公开的RCRTI系统可利用通过小分子从螯合和抑制蛋白质中释放出转录因子。在某些实施例中, 本公开的RCRTI系统可依赖类固醇调节, 其中激素受体与天然或人工转录因子融合, 该天然或人工转录因子可从HSP90在细胞质中释放出来, 迁移至细胞核中并激活选定的启动子。在某些实施例中, 可使用由合成类固醇类似物调节的突变型受体, 以免内生类固醇激素引起串扰。在某些实施例中, 受体可以是对4-羟基他莫昔芬有反应的雌激素受体变体或可经RU486诱导的孕激素受体突变体。在某些实施例中, 基于人核过氧化物酶体增殖物激活受体 γ (PPAR γ)的核受体衍生的罗格列酮响应转录开关可用于本发明的RCRTI系统中。在某些实施例中, RheoSwitch可为类固醇反应性受体的变体, 其基于修饰后的云杉色卷蛾蜕皮激素受体和融合至Gal4 DNA结合结构域和VP 16反式激活因子的小鼠类视色素X受体(RXR)。在存在合成蜕皮激素的情况下, RheoSwitch变体可结合并激活与Gal4反应元件的多个重复序列融合的最小启动子。

[0165] 在某些实施例中, 本文所公开的RCRTI系统可利用化学诱导的DNA结合蛋白和转录激活因子的二聚作用来激活与同源操纵子融合的最小核心启动子。在某些实施例中, 本文所公开的RCRTI系统可利用雷帕霉素调节的FRBP与FRB的二聚作用。在该系统中, FRB与p65反式激活因子融合, 并且FKBP与锌指结构域融合, 该锌指结构域特异于置于工程化最小白细胞介素12启动子上游的同源操纵子位点。在某些实施例中, FKBP可发生突变。某些实施例中, 本文所公开的RCRTI系统可利用细菌促旋酶B亚基(GyrB), 其中在存在抗生素库马霉素的情况下GyrB发生二聚作用并与新生霉素解离。

[0166] 在某些实施例中, 本公开的RCRTI系统可用于受调控的siRNA表达。在某些实施例中, 受调控的siRNA表达系统可以是四环素、大环内酯、或OFF和ON型QuoRex系统。在某些实施例中, RTI系统可利用Xenopus末端低聚嘧啶元件(TOP), 该元件通过在5'非翻译区形成发夹结构来阻止翻译启动。

[0167] 在某些实施例中,本公开所述的RCRTI系统可利用气相控制表达例如乙醛诱导的调控(AIR)系统。AIR系统可采用构巢曲霉的AlcR转录因子,其在无毒浓度的气态或液态乙醛的存在下,特异性激活由AlcR特异性操纵子组装的PAIR启动子,该操纵子融合至最小的人类巨细胞病毒启动子。

[0168] 在某些实施例中,本公开的RCRTI系统可利用Tet-On或Tet-Off系统。在该系统中,一种或多种SOI的表达可由四环素或其类似物强力霉素进行调节。

[0169] 在某些实施例中,本公开的RCRTI系统可利用PIP-on或PIP-off系统。在该系统中,SOI的表达可由例如原始霉素、四环素和/或红霉素进行调节。

[0170] 6.产物

[0171] 本公开的宿主细胞可用于表达任何目的分子。在某些实施例中,本公开的宿主细胞可用于表达多肽,例如哺乳动物多肽。此类多肽的非限制性实例包括激素、受体、融合蛋白、调节因子、生长因子、补体系统因子、酶、凝血因子、抗凝血因子、激酶、细胞因子、CD蛋白、白细胞介素、治疗性蛋白质、诊断性蛋白质及抗体。在某些实施例中,本公开的宿主细胞可用于表达分子伴侣、蛋白质修饰酶、shRNA、gRNA或其他蛋白质或肽,同时组成性或调控地表达目的的治疗性蛋白质或分子。

[0172] 在某些实施例中,目的多肽是双特异性、三特异性或多特异性多肽,例如双特异性抗体。

[0173] 与常规细胞培养方法中所用的细胞例如非TI细胞相比,本公开的宿主细胞可在较短的时间内用于产生大量目的分子。在某些实施例中,与常规细胞培养方法中所用的细胞例如非TI细胞相比,本公开的宿主细胞可用于提高目的分子的质量。在某些实施例中,本公开的宿主细胞可用于通过预防慢性毒性来增强种子序列的稳定性,该慢性毒性可由引起细胞应激和克隆随时间推移的不稳定性的产物而引起。在某些实施例中,本公开的宿主细胞可用于实现急性毒性产物的最佳表达。

[0174] 在某些实施例中,本公开的宿主细胞和系统可用于细胞培养过程的优化和/或流程开发。

[0175] 在某些实施例中,本公开的宿主细胞可用于治疗性分子的所选亚基的组成性表达以及同一治疗性分子的其他不同亚基的调控表达。在某些实施例中,治疗性分子可为融合蛋白。在某些实施例中,本公开的宿主细胞可用于理解每个抗体亚基在完全组装的抗体分子的表达及分泌中的作用和效应。

[0176] 在某些实施例中,本公开的宿主细胞可用作研究工具。在某些实施例中,本公开的宿主细胞可用作诊断工具,以针对各种细胞中存在问题的分子找出低蛋白表达水平的根本原因。在某些实施例中,本公开的宿主细胞可用于将观察到的现象或细胞行为与细胞中的转基因表达直接联系起来。本公开的宿主细胞还可用于证明观察到的行为在细胞中是否可逆。在某些实施例中,本公开的宿主细胞可用于识别和缓解有关转基因在细胞中转录和表达的问题。

[0177] 在某些实施例中,本公开的宿主细胞可用于将难以表达的分子的转基因亚基(例如但不限于抗体的HC和LC亚基)与系统中的平均分子的亚基交换,以识别有问题的亚基。在某些实施例中,然后可使用氨基酸序列分析来缩小范围并集中于可能导致低蛋白表达水平的氨基酸残基或区域。

[0178] 实例

[0179] 材料与方法

[0180] 细胞培养基

[0181] 通过将编码抗体的盒靶向整合至来源于CHO-K1细胞系的宿主细胞系中来执行稳定的细胞系开发(Crawford Y.等人,Biotechnol Prog 2013,29,1307-1315)。将细胞置于125mL摇瓶中(转速为150rpm)或50mL管式生物反应器中(转速为225rpm),在37℃和5%CO₂下在专有培养基中进行培养。每3-4天以4x10⁵个细胞/mL的接种密度传代培养的细胞。

[0182] 通过PCR反应确定表达RCTI克隆的分子X的载体构型

[0183] 使用DNeasy血液和组织试剂盒(目录号69506, Qiagen)从2x10⁶个细胞中提取基因组DNA,并使用LongAmp Taq预混液(目录号M0287, New England Biolabs)进行PCR测定。热循环参数:在94℃下持续3分钟,40X(在94℃下持续30秒,在60℃下持续1分钟,并在65℃下持续9.75分钟),以及在65℃下持续20分钟。使用来自New England Biolabs的酶对PCR产物进行诊断消化。使用以下引物进行PCR测定:正向引物:GGTTCTCCTTGACCAATACCTCGTAAG;反向引物:GCGGGACTATGGTTGCTGACTAAT。

[0184] 摇瓶流加培养及AmbrTM生物反应器生产测定

[0185] 在摇瓶中使用专有的化学组成确定的培养基在14天的生产过程中,对单克隆抗体A(mAb A)和表达Molecule-Z的克隆进行流加培养评估。在第0天以1x10⁶个细胞/mL接种细胞,在第3天将培养物的温度由37℃改为35℃,并在第3、7和10天添加由专有的化学组成确定的混合物组成的大剂量进料。用Vi-Cell XR(Beckman Coulter)测量活力和活细胞计数,并使用Nova BioProfile 400(Nova Biomedical)测定乳酸和葡萄糖水平。

[0186] 为评估表达Molecule-Y的克隆,在ambrTM系统(Sartorius)中进行14天的生产培养(Hsu,W.T.等人Cytotechnology 2012,64,667-678),进行以下修改:在摇瓶中于37℃和150rpm下以1x10⁶个细胞/mL的密度接种4天。将生产培养物以2x10⁶个细胞/mL的密度接种,最初保持在36℃,然后在第6天将温度改为35℃。在第3、6、8和10天添加由专有的化学组成确定的混合物组成的大剂量进料。在BioProfile Flex2(Nova Biomedical)上测量活力和细胞计数。在BioProfile Flex2(Nova Biomedical)或ABL90 Flex(Radiometer)上测定pH、pO₂、pCO₂、葡萄糖、乳酸盐以及其他代谢物。

[0187] 效价和产物质量的分析测定

[0188] 使用protein-A亲和色谱和UV检测测定效价。大小和电荷变化分别通过protein A PhyTip纯化(PhyNexus)、体积排阻色谱和成像毛细管等电聚焦(icIEF)进行测量。经icIEF分析之前,用羧肽酶B处理经过Phytip纯化的样品。对于Molecule-Y,使用液相色谱-质谱(LC-MS)来定量分析正确组装的异二聚体、同二聚体、半抗体和轻链错配物种的相对量,如Williams,A.J.等人所述(Ind Eng Chem Res 2017,56,1713-1722)。

[0189] 当前转染(TFX)策略与RCTI的TFX策略的比较

[0190] 使用“随机化构型靶向整合”(本文也称为“随机化链靶向整合”)方法产生稳定细胞系,该稳定细胞系生成单克隆抗体A(mAb A)(图11A)。在该方法中,使用三个“前”和三个“后”表达载体的文库转染宿主细胞,每个表达载体包含mAb A重链或轻链序列重复序列的三种可能的组合之一。如本实例中所用,对“前”和“后”表达载体的引用是指在基于两个载体RCME的外源目的序列引入的背景下的上游(前)和下游(后)盒位点。有关两种基于RCME的策

略的更多细节,请参见例如上文第5.1节。顶级克隆的效价为3.4-4.7g/L,并根据效价、生产率、细胞培养性能、产物质量和流式细胞仪群体异质性进行选择。

[0191] 与之前针对mAb A的细胞系开发(CLD)所做的工作相比,本文所述的RCTI CLD方法生产的克隆的效价、产物质量和生产培养性能与标准CLD方法生产的克隆相当,同时以更少的CLD循环筛选更少的克隆。

[0192] 先前针对mAb A的CLD工作已在独立的标准CLD方法工作流程中测试了前后载体的单独组合(见图2A)。总共转染了7种前后载体构型组合,并通过池生产对其进行评估。在这7种载体组合中,将4种向前推进至基于池生产效价进行SCC和克隆评估。因此,执行四次独立的CLD循环以及三个附加的部分CLD循环,其中转染三个附加的载体组合并通过池生产对其进行评估。

[0193] 相比之下,RCTI CLD方法仅执行一次CLD循环。表1总结了针对各种方法评估的克隆的数量,图2A和2B示出用于mAb A克隆评估的RCTI和标准CLD工作流程的比较。

[0194] 表1. 针对标准方法和RCTI CLD筛选的克隆总结

[0195]	标准CLD	RCTI CLD
执行的CLD循环次数	4 ^a	1
从SCC中挑选的克隆	4,224	704
在生产测定中评估的克隆	120	24

[0196] ^a对于标准CLD方法,同时执行四次独立的CLD循环以及三个附加的部分CLD循环,其中转染三个附加的载体组合并通过池生产对其进行评估。

[0197] 对于RCTI CLD方法,对三个实验组进行评估,它们分别对应于三个回收阶段的转染池:(i) 早期(转染后第5天,归入选择培养基中),(ii) 中期(选择后,克隆恢复大约35%的活力),或(iii) 完全(选择后,克隆恢复>90%的活力)(见图6A)。为比较RCTI CLD方法与标准CLD方法,本节仅讨论来自第三组的克隆(选择后,克隆恢复>90%的活力),因为标准CLD规定池的SCC活力>90%。对于标准CLD方法和RCTI方法,一次CLD循环被定义为从转染到克隆生产培养评估的整个过程。

[0198] 对于之前的mAb A CLD工作,大部分具有最高效价的克隆也表达出高聚集度,即HMWS高于10-15%。因此,在比较标准CLD方法和RCTI方法生产的克隆时,在选择顶部RCTI CLD克隆时同时考虑到效价和HMWS%。对于通过标准CLD方法生产的克隆,某些构型的HMWS%分布与前后载体构型大致成簇(图3B)。例如,具有C-2构型的克隆(图3A)通常产生较低的HMWS%,而具有B-2构型的克隆(图3A)产生较高的HMWS%。

[0199] 对于RCTI克隆,效价和HMWS%的范围与标准CLD克隆产生的效价范围相当(图3B),尽管筛选的克隆要少得多。由于RCTI方法涉及转染前后DNA载体的随机文库,因此使用长程PCR来确定生产培养中评估的克隆的前载体构型。尽管24个克隆中的3个克隆的长程PCR结果不确定,但与标准CLD克隆相比,其余克隆在前载体构型和HMWS%成簇方面相似。具有C前载体构型的大多数克隆表达出的HMWS%低于具有B构型的克隆,而具有A前构型的克隆涵盖HMWS%的范围。此处不需确定后载体构型,因为HMWS%的水平仅与前载体构型直接相关。RCTI克隆的第14天最高效价也与标准CLD克隆(≥ 4.5 g/L)相当。需注意,如果仅以标准CLD方法测试了B-2(图3A)或A-1(图3A)构型,则可以获得具有高效价和低聚集体的组合的少数克隆(如有)。

[0200] 标准CLD和RCTI CLD方法之间的增长、生产率及其他产物质量属性也相当。图5总结了两种CLD方法在生产培养基中评估的所有克隆中各种属性的分布。同样地,由RCTI CLD方法产生的顶级克隆在生产培养基中的表达与通过标准CLD方法产生的顶级克隆相当。在标准CLD方法中测试的构型中,仅A-2和C-2构型具有高效价($\geq 3.6\text{g/L}$),而产生的聚集度较低($\text{HMWS}\% \leq 15.4\%$)。通过RCTI方法获得的克隆具有与标准CLD方法相似的聚集度和效价范围。尽管由RCTI方法得到的代表性高产克隆似乎在表2中呈C构型,如图3B所示,但也观察到来自构型A的高效价和低聚集度的克隆,尽管它们并未排在产量最高的克隆之列。表2比较了根据标准方法和RCTI CLD方法得到的顶级克隆的效价、生长和产物质量属性。

[0201] 表2. 使用标准CLD和RCTI CLD生产的顶级mAb A克隆的比较

构型	D14 效价 (g/L)	D14 IVCC	HMWS (%)	Acidics (%)	Man5 (%)	D14 活力 (%)
标准 CLD 方法克隆						
A-2	4.5	994	15.4	33.7	0.9	96.2
A-2	4.2	939	14.1	32.0	0.6	96.0
A-2	3.9	1007	5.7	30.7	0.4	96.3
A-2	3.9	1386	7.4	29.8	0.4	97.7
A-2	3.6	1302	6.8	31.3	0.5	96.1
C-2	4.0	1262	7.1	34.6	0.7	93.2
RCTI CLD 方法克隆 ^a						
C	4.0	1782.4	10.9	29.6	0.4	94.4
C	4.0	1595.8	8.6	35.0	0.4	93.1
C	4.7	1875.0	11.4	34.7	0.5	93.8
C	3.4	1463.1	5.9	31.6	0.3	91.7

[0203] ^a对于RCTI克隆,在该比较中仅考虑来自第三实验组(库选择后,回收率高于90%的SCC)的顶级克隆。

[0204] 与之前mAb A的CLD工作(使用标准CLD方法)相比,RCTI方法具有许多优势。首先,单个RCTI CLD循环可用于筛选与标准CLD循环相比具有相同数量(如果不是更多)的载体构型,同时使用的资源较少。对于mAb A,标准CLD方法需要4次以上的CLD循环来筛选9种可能的载体构型组合中的7种,而RCTI方法在一次CLD循环中即可筛选全部9种组合。也可以筛选较少的单个克隆。在针对mAb A的所有标准CLD循环中,从SCC中总共挑选了4,224个克隆,并缩小为120个克隆以在生产培养基中进行筛选。相比之下,从SCC中总共选择了704个克隆用于RCTI方法,并在生产培养基中筛选了24个克隆。由于减少了CLD循环并减少了要筛选的克隆的数量,RCTI方法能够减少CLD的人工需求,包括减少人工成本和执行SCC、成像及匹配结果挑选的自动化资源负担。最后,尽管减少了所需的CLD循环及筛选的克隆数量,但通过RCTI方法产生的克隆的效价、细胞培养基性能和产物质量的分布与标准CLD方法相当。通过两种方法产生的顶级克隆也相当。

[0205] RCTI CLD表达双特异性抗体的评估

[0206] 评估了RCTI方法对具有高临床需求的疾病适应症的双特异性抗体 (Molecule-Y) 的表达,因此需要在克隆筛选过程中分离出高效价的细胞系。双特异性抗体在单个宿主中的表达可导致不想要的副产物的组装,例如具有常见或错配轻链物的同二聚体和异二聚体 (Dillon M. 等人, mAbs 2017, 9, 213-230; Carter, P. J., Experimental cell research 2011, 317, 1261-1269)。因此,双特异性抗体克隆评估中的关键步骤是分离克隆,其不仅具有较高的总效价,而且具有高效的效价(所需的双特异性抗体种类),并且副产物的水平最低,难以从目标双特异性抗体中纯化出来。对于Molecule-Y (图11B),要纯化的最具挑战性的副产物是具有常见轻链(本文称为“常见LC BsAb”)的异二聚体重链。因此,在标准TI CLD过程中,尝试并行CLD时使用四种不同的载体构型,并且在SCC之前进行池生产评估期间,使用质谱法测量常见LC BsAb以及有效效价水平。针对SLC选择含量最低的常见LC BsAb池,然后在针对标准TI CLD方法的生产测定中(图7A,正方形)评估具有最高效价和最低LC-BsAb水平的排名前62个克隆。

[0207] 由于之前已经确定,在RCTI CLD方法中,回收的池仍保持载体构型异质性(图6B和图6C),对于Molecule-Y的RCTI CLD,所有四个载体构型均用于转染TI宿主,并且仅在池完全回收后才进行SCC。此外,在早期筛选步骤中,仅根据通过快速火焰质谱法获得的有效效价对克隆进行排名。在此过程中,在克隆选择中不考虑常见LC BsAb或其他单个副产物的水平。基于这些标准,然后在生产测定中评估排名前36个RCTI克隆(图7A,圆圈)。尽管在生产测定中仅评估了一半克隆,但相对于标准TI CLD克隆,通过RCTI CLD方法可以分离出总效价(约6g/L)和有效效价(4-5.5g/L)的克隆(图7A)。此外,与标准TI克隆相比,RCTI克隆总体上具有更出色的比生产力(图7B)、更高的活力(图8A)以及略低的生长(图8B)特征。由于通过流程优化可以更轻松地改善培养生长,因此相比标准TI克隆对应物,表达Molecule-Y的RCTI克隆可能具有更高的总有效效价。RCTI克隆还具有非常低的(约5%或更少)的常见LC BsAb副产物,大约与标准TI克隆所观察到的结果一样低(图8C)。但是,预计标准TI克隆具有总体上较低的常见LC BsAb副产物,因为他们在生产培养基中进行评估之前已经筛选出这一特质。RCTI克隆的产物质量与标准TI同类产品相当或更出色。相对于标准TI克隆,表达Molecule-Y的RCTI克隆也具有相对较低的聚集度(图8D)、较低的酸性物种(图8E)和较高的主要物种%(图8F),这些都是更理想的产物质量参数。在从这两种方法分离出的克隆之间,碱性物种%的水平相当(图8G)。

[0208] RCTI CLD对复合嵌合抗体/配体分子表达的评估

[0209] 许多即将出现的用于各种疾病适应症的治疗性分子不再仅仅是常规抗体,而是具有更复杂的结构,因此更难以表达。在标准TI方法中表达一种该等复杂分子(Molecule-Z),需要使用六种不同的载体构型进行并行CLD,然后在生产培养基中筛选96种不同的克隆,以获得具有高效效价的克隆。(图9A,正方形)。因此,包含与Fc融合的配体复合的半抗体的Molecule-Z(图11C)被视为难以表达的分子,并且我们决定使用RCTICLD方法测试其表达。使用所有六种不同载体构型的混合物转染TI宿主细胞,并在一个池中选择它们,然后进行SCC筛选,在生产测定中仅评估23种RCTI克隆,可分离出与通过标准TI CLD方法分离的顶级克隆具有相当的效价和产物质量的克隆(图9A)。与标准TI克隆相比,RCTI克隆具有更高的总体比生产力(图9B)、相当的活力(图10A)、略低的生长速度(图10B)和较低的总体聚集度(图10C)。由于具有较高的比生产力(约高2倍,图9B),如果通过流程优化进一步提高细胞的

生长,RCTI克隆可实现更高的有效效价。最后,RCTI克隆与标准TI克隆具有相当的酸性电荷变异体(图10D)、主电荷变异体%(图10E)和碱性电荷变异体%(图10F)。总而言之,这些数据支持以下观点:RCTI方法可用于分离克隆并实现与标准TI方法相当的效价和产物质量属性,但是所需的资源和工作量大大减少。

[0210] ****

[0211] 前述实例仅是对当前公开的主题的说明,而不应以任何方式视为限制。

[0212] 除了所描绘和要求保护的各种实施例之外,所公开的主题还涉及具有本文所公开和要求保护的特征的其他组合的其他实施例。这样,本文所呈现的特征可以在所公开的主题的范围内以其他方式彼此组合,使得所公开的主题包括本文所公开的特征的任何合适的组合。为了说明和描述的目的,已经呈现了所公开的主题的特定实施例的前述描述。并不旨在穷举或将所公开的主题限制为所公开的那些实施例。

[0213] 对于本领域技术人员显而易见的是,在不脱离所公开主题的精神或范围的情况下,可以对所公开主题的组合物和方法进行各种修改和变化。因此,旨在所公开的主题包括在所附权利要求及其等同物的范围内的修改和变化。

[0214] 本文引用了各种出版物、专利和专利申请,其内容通过引用以其全文并入本文。

序列表

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<212> DNA

<213> 灰仓鼠

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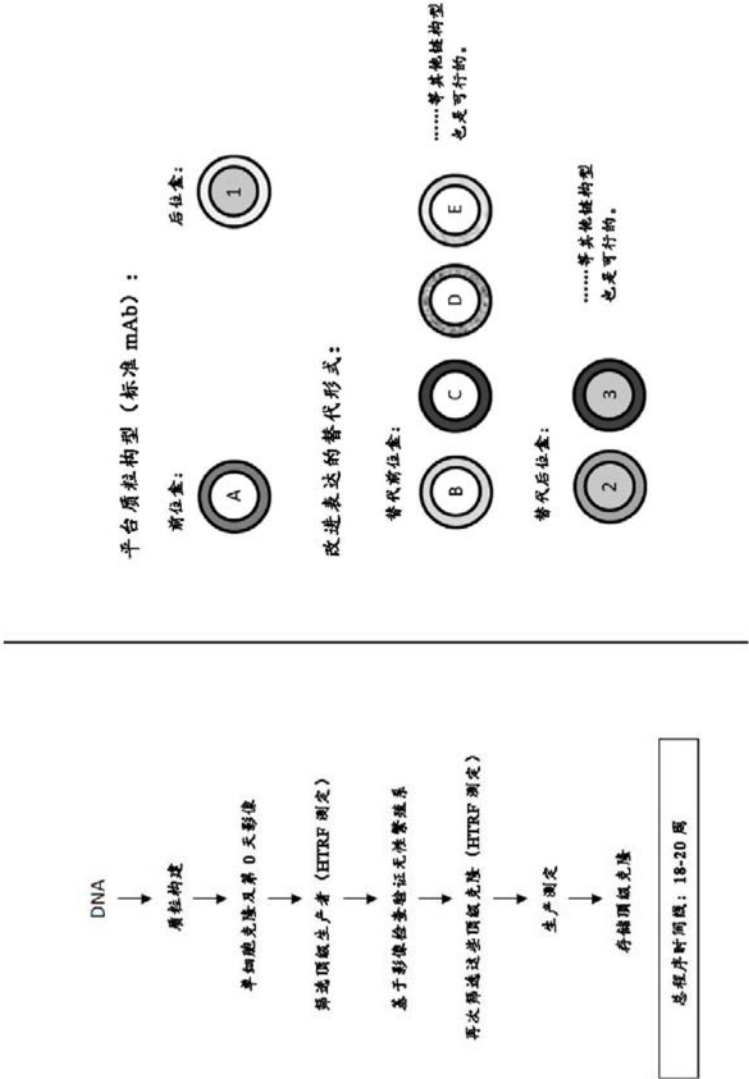


图 1A

图 1B

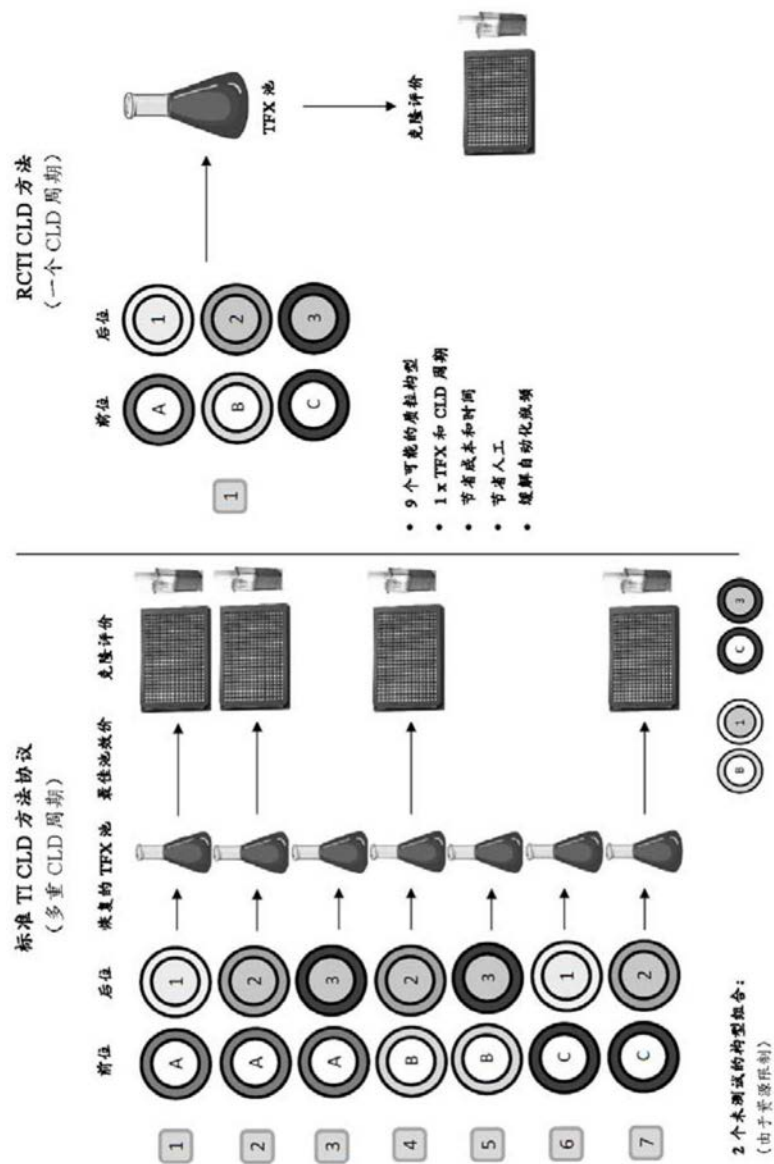
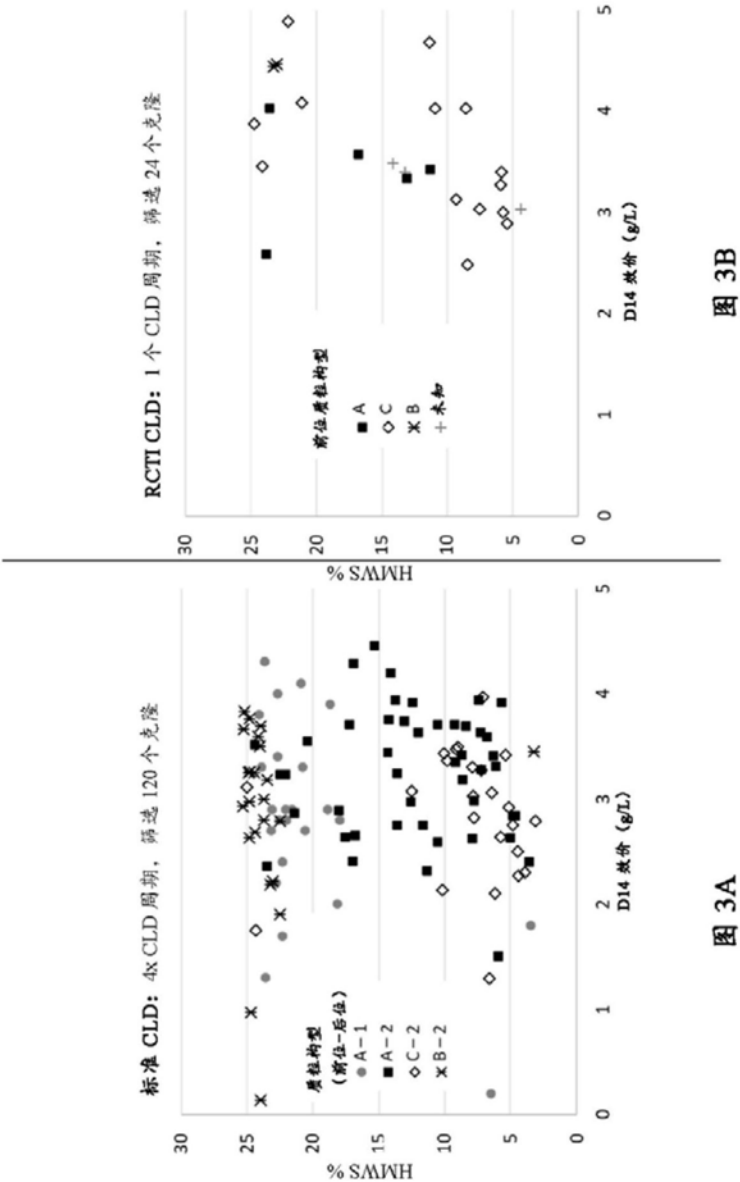


图 2A

图 2B



	生产和生产力			电荷密度			主要参数种类			
	D14 IVCC [10 ³ μm ² /d/L]	D14 潜力 %	D14 Qp [deg·mm ² /d]	酸洗 %	主峰 %	碱洗 %	Man5	G1	G0	G0F
Std CLD: 最小值	640.6	15.0	1.9	28.2	51.4	0.7	0.3	0.3	0.5	42.9
Std CLD: 最大值	2168.0	98.5	53.8	47.0	68.8	7.9	2.4	1.7	3.2	72.3
RCTI: 最小值	668.3	46.2	10.8	26.6	53.3	0.0	0.3	0.6	0.5	48.4
RCTI: 最大值	2846.4	97.8	45.6	42.8	69.4	6.0	2.8	3.8	4.5	69.2

图4

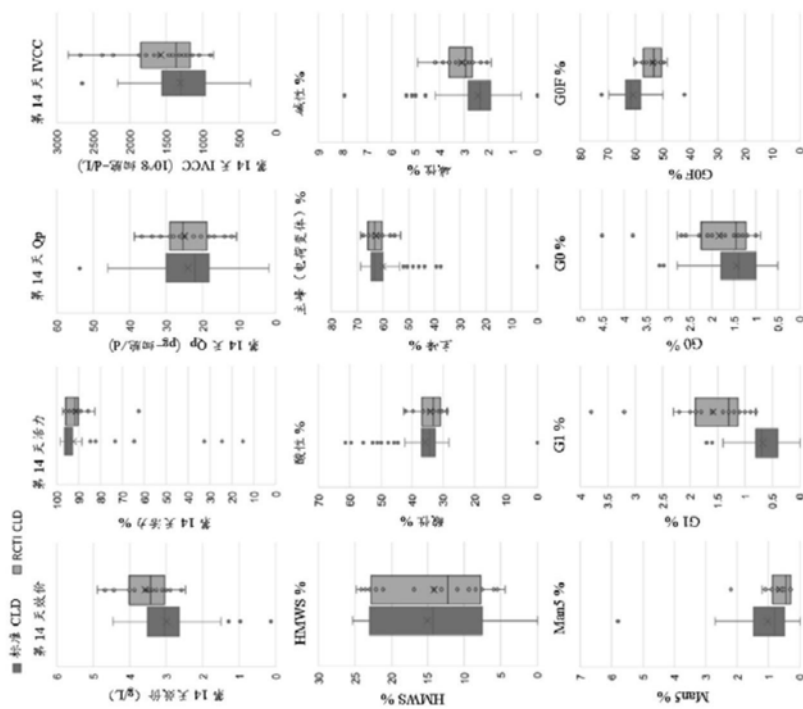


图5

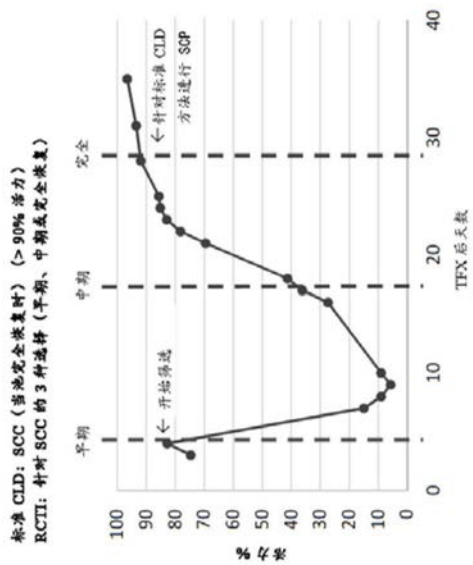


图6A

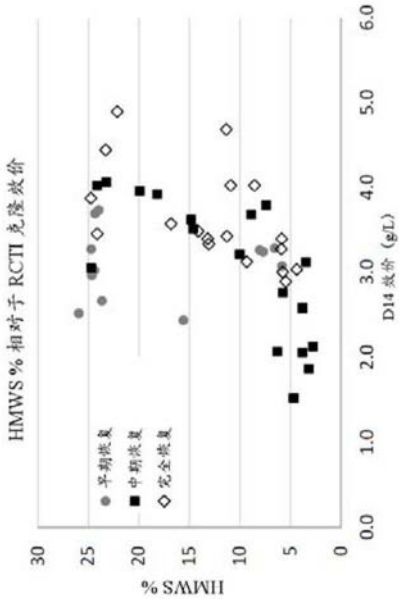


图6B

恢复	时间缓节省	克隆效能 ^a	克隆异质性 ^b
早期	+++	++	+++
中期	++	++	+++
完全	+	+++	+++

^a 具有 ≥ 3g/L D14 的克隆的百分比
^b 克隆所产生的 HMWS % 和 D14 效率范围

图6C

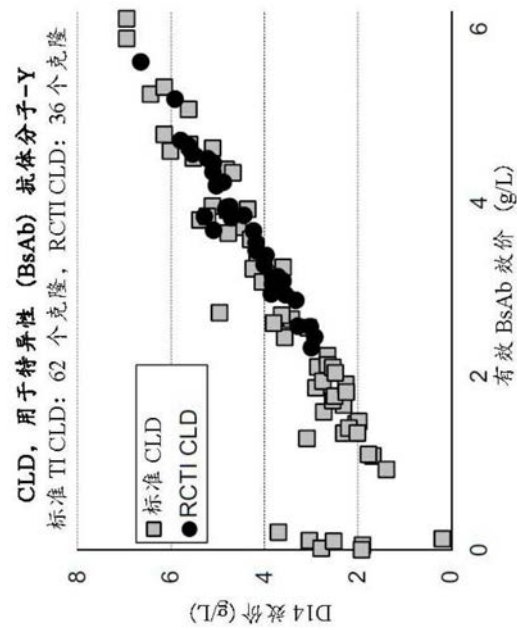


图7A

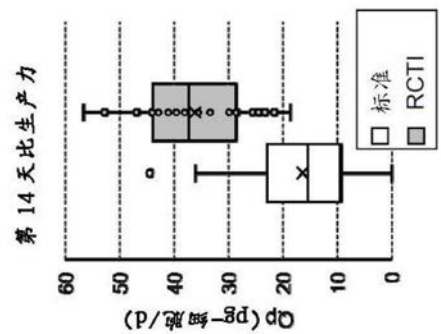


图7B

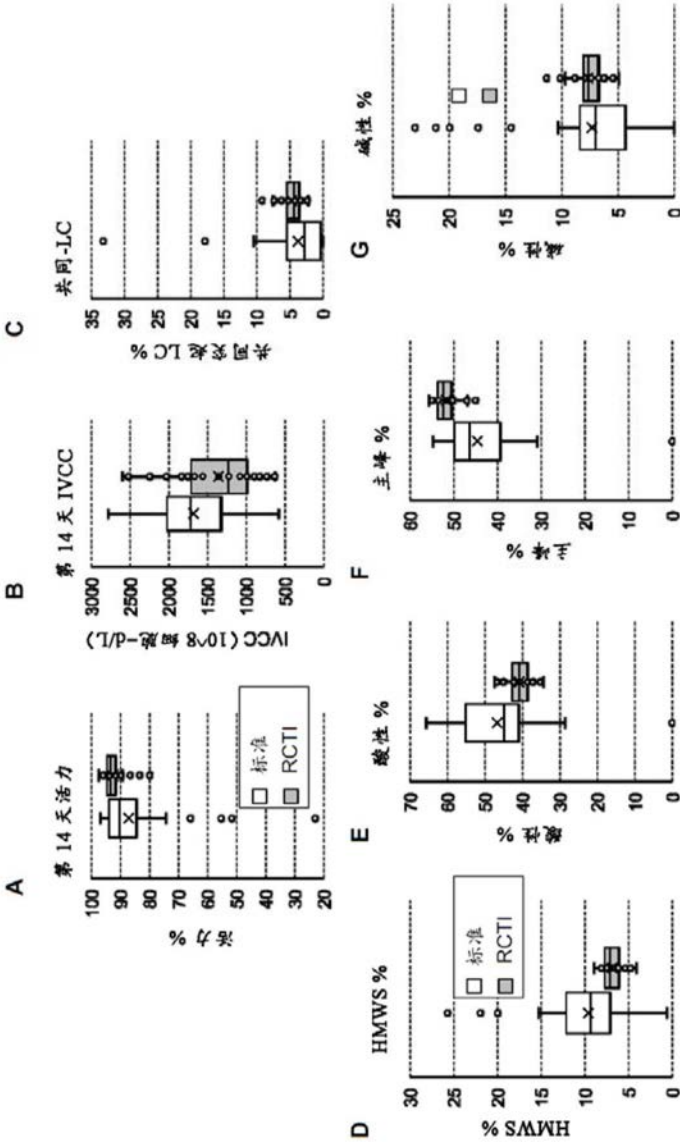


图8

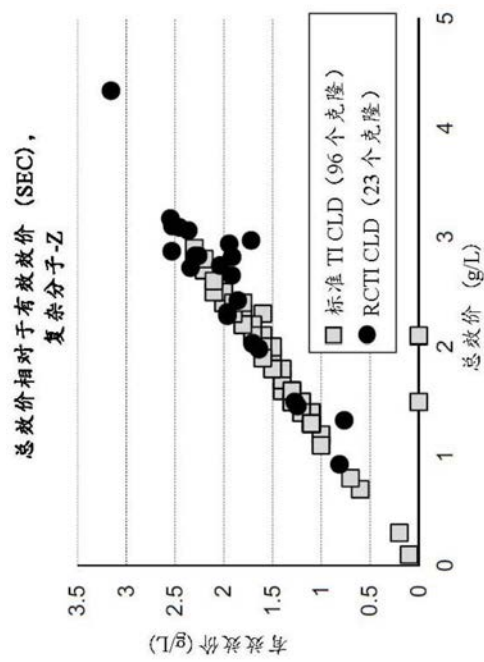


图9A

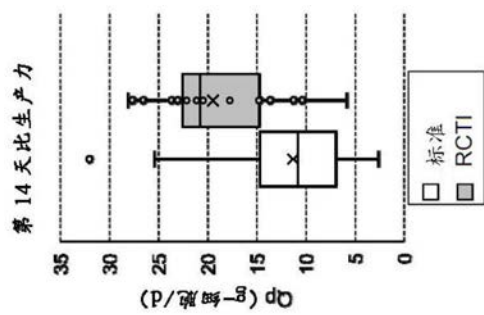


图9B

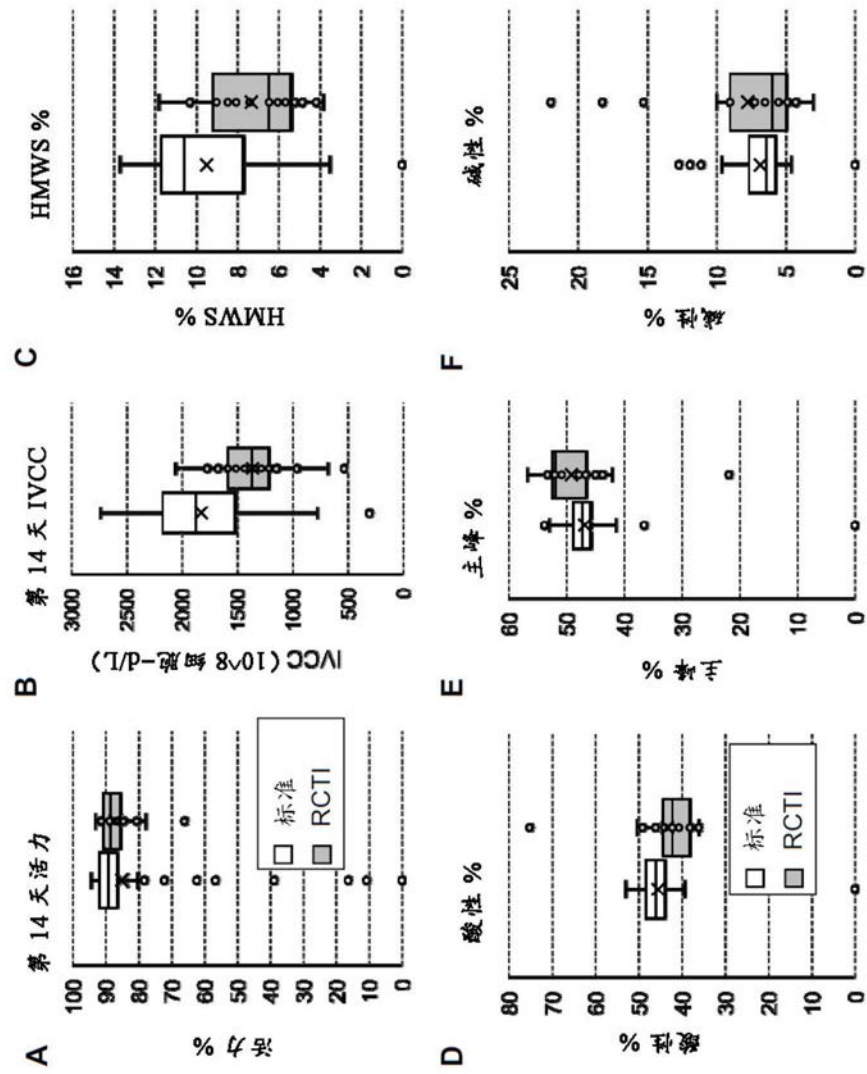


图10

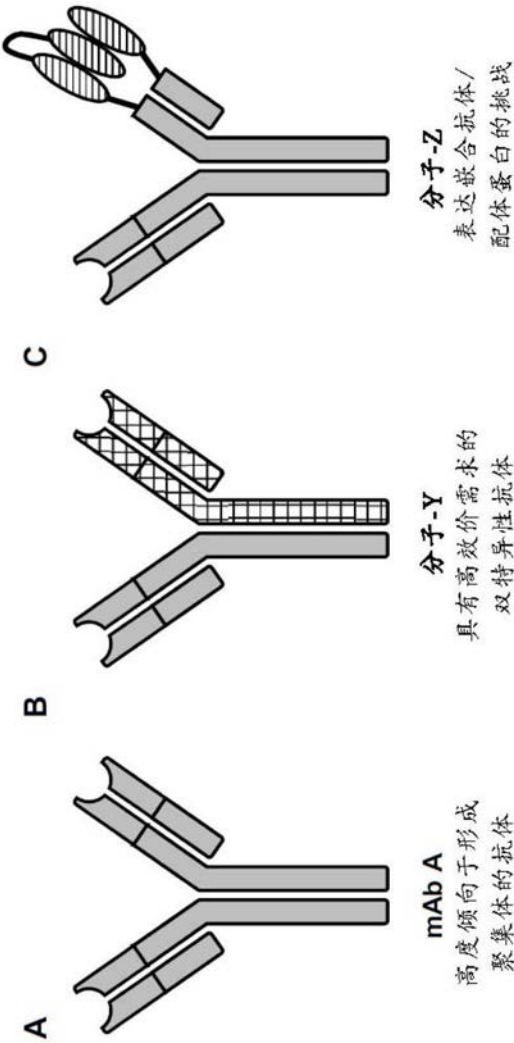


图11