SOP: SP043. .

Recombinant Plasmid Map Design – Vector NTI

Materials and Reagents:
1. Dell Dimension XPS T450 Room C210
2. Vector NTI 9 application, on desktop
3. Tuberculist database open in Internet Explorer window at http://genolist.pasteur.fr/TubercuList/ (note 1)
4. VectorNTI 9 Online Help

Protocol:
1. _____ Find sequence for gene of interest in Tuberculist (note 1).

2. _____ IMPORTANT: check whether the sequence to be cloned requires removal of signal peptide sequence, stop codon, and change of a gtg or ttg start codon to ATG.

3. _____ Open Vector NTI 9 (note 2) from desktop. A three pane window appears.

4. _____ Open Local Database. Path: pulldown File → select Local Database. An additional two-pane window titled Exploring Local Vector NTI Database appears (note 2).

5. _____ Verify that the upper left control window reads DNA/RNA Molecules (the DNA/RNA molecule table used for all local database processes in this SOP).

6. _____ Hold both the Vector NTI window and the Exploring Local Vector NTI Database windows open throughout processing.

7. _____ Set up a project folder in Exploring window, DNA/RNA Molecules. Path: Top toolbar → pull down DNA/RNA → New → Subset for Selection → Group 1 folder.

8. _____ Using right-click, rename Group 1 folder (usually, name of designer).

9. _____ Drag vector(s) of choice from Cloning Vectors list (Exploring window, DNA/RNA Molecules MAIN) to project subset (note 4).

10. _____ Create a gene sequence file in either VNTI or in Exploring windows. (note 3).

11. _____ In Vector NTI window, pull down File → Create new sequence → Using sequence editor.

12. _____ In New DNA/RNA molecule window fill in tabs as detailed in steps 13 – 20.

13. _____ General – name the new sequence with its Rv designation (eg Rv.nnnn)

14. _____ DNA/RNA molecule – select linear.

15. _____ Fill in Description: include Rv number, whether stop codon is removed, whether a signal sequence has been removed.

16. _____ Fill in Comments–copy and paste description from step 15 into comments. Include date/designer.

17. _____ Sequence and maps – Edit Sequence – copy and paste coding sequence from Tuberculist DNA pane.

18. _____ Remove nucleotides that code a signal sequence if cloning into an E. *coli* vector (typically, but not always done).

19. _____ Remove stop codon if cloning into pET23(b) or pVV16, to permit fusion to c-terminal 6 his tag.

20. _____ Change gtg or ttg to ATG (also typically, but not always done)

21. _____ Click OK.

22. _____ Save as: Rv. nnnn in project subset.

23. _____ Select PCR primers (note 5) (steps 24 – 46).

24. _____ Open file of sequence to be cloned (note 6).

25. _____ In text pane, highlight all sequence.

26. _____ Top toolbar, pull down Analyses → Primer Design → Find PCR Primer.

27. _____ Primer tab – Important – product length must be set so that min and max length are same. This ensures that sequences at start and end of file are included in primer search (note 5).

28. _____ Pairs tab – Tm difference = 5.0 (can be changed if no pairs of this Tm difference are found). GC difference can also be edited.

29. _____ More (tab at bottom of window) – click to introduce restriction enzyme sites.

30. _____ Add NdeI at 5' terminus. Press three dot box […] to go to cutting sites. Click on NdeI. CATATG appears in site window.

31. _____ IMPORTANT – Remove ATG from the site in window. Otherwise, the sequence with primer added will have 2 ATGs (reading CATATGATG).

32. _____ Add 3' terminus enzyme site. Click on desired site.

33. _____ Click OK. In PCR Analysis, left pane, primer pairs are displayed (one or more).

34. _____ Verify primers for full lengh product have been created, in steps 35 - 38.

35. _____ Remove highlighting from text pane.

36. _____ Right click on #1 Product of length…

37. _____ Pull down to Find PCR Product.

38. _____ Verify in graphic and/or text pane that entire sequence is selected.

39. _____ Create gene sequence file with primer and restriction enzyme sites, steps 40 – 46.

40. _____ Right click on Product of length nnn, for desired pair of primers (usually highest rating pair).

41. _____ Pull down Save as Molecule in Database. Info window opens.

42. _____ General tab – name file, eg. Rv.nnnn.PCR.

43. _____ Comments – Rv.nnnn with PCR primers including eg, NdeI and HindIII.

44. _____ Click OK.

45. _____ At request for Save to location, choose the project subset.

46. _____ Verify that Vector NTI text and graphic windows text display sequence with primers (blue arrows).

47. _____ Create plasmid map in Vector NTI window (steps 48 – 68).

48. _____ File → Pull down Create New Sequence → Using Construct /Design Procedure (DNA/RNA)

49. _____ In Construct Molecule window, type Name – eg Rv.nnnn.pET23b-

50. _____ Select **Circular**.

51. _____ At bottom of Construct Molecule window, press Mol Frag button. Fragment of Molecule window appears.

52. _____ Select the vector in Fragment of Molecule window, steps 53 – 59.

53. _____ Parent Molecule – click […] button.

54. _____ Go to project subset for this plasmid.

55. _____ Select vector, eg pET23b(-), – click OK.

56. _____ Press Left Terminus […].

57. _____ Press Restriction site, add the 3' site, eg HindIII, press OK.

58. _____ Back in Fragment of Molecule window press Right Terminus […].

59. _____ Press Restriction site, add the 5' site, eg. NdeI, press OK.

60. _____ In Fragment Molecule window, verify left terminus is 3' site and right terminus is 5' site. Press OK.

61. _____ Again press Mol Frag button at bottom of Construct Molecule window.

62. _____ Select Parent Molecule, this time to select gene insert.

63. _____ In project subset, highlight the PCR product file created in step 23. press OK.

64. _____ Left terminus – restriction site: select site at 5' terminus of this file – typically, NdeI. Press OK.

65. _____ Right terminus – restriction site: select site at 3' terminus of this file – eg HindIII, XhoI, BamHI. Press OK.

66. _____ Construct Molecule window now has two fragments. Verify that the enzyme sites are in reverse orientation in the two fragment and that the gene sequence has the NdeI at 5' end.

67. _____ Press Construct in upper right corner.

68. _____ Insert Molecule into project subset. Press OK. Plasmid appears.

69. _____ Change start position of new plasmid (note 7) to match parent vector.

70. _____ In Vector NTI window, pull down File → Open → parent vector, eg pET23 b(-).

71. _____ In text pane of parent vector sequence, highlight and copy the first 20 nucleotides.

72. _____ Minimize the parent vector window.

73. _____ In the new plasmid window, position cursor in text pane.

74. _____ In toolbar, pull down Edit → Find Sequence.

75. _____ Paste sequence from start of parent vector into Find what: Click Find Next.

76. _____ Close Find window.

77. _____ Place cursor in at start of the text pane in the recombinant plasmid.

78. _____ Pull down File → Molecule Operations → Advanced DNA/RNA →Change starting coordinate → OK.

79. _____ Verify starting coordinate change.

80. _____ Save As. Vector NTI will query Rename or Overwrite – choose Overwrite.

81. _____ Add gene sequence (CDS) as a marked feature, steps 82 – 88.

82. _____ Highlight the coding sequence in the text pane from the graphic map pane (note 8). Tricky – cursor moves quickly.

83. _____ Start at the final gene sequence base (at first base just upstream of the 3' enzyme site).

84. _____ Continue to the ATG of the NdeI site (highlight only the ATG of the NdeI site – omit the CAT).

85. _____ In graphic pane, click Add Feature (the left arrow in the Active Pane toolbar).

86. _____ In Feature Type list, click on CDS.

87. _____ In description window, state Rv.nnnn coding sequence. Click OK.

88. _____ Verify that map now displays broad arrow denoting CDS.

89. _____ Click on arrow – CDS will be selected in text pane.

90. _____ Confirm sequence is properly positioned (steps 91 – 98).

91. _____ Translate selected sequence to verify protein (steps 92 – 96).

92. _____ Click on CDS (broad arrow) in graphic pane.

93. _____ Extend selection to include fusion sequence (eg – 6 his and stop codon for pET23b).
_M_
94. _____ Click on ATG in Active Pane toolbar. Translation appears.

95. _____ Scan for inappropriate stop codons.

96. _____ Confirm first and last five amino acid residues against protein sequence in Tuberculist.

97. _____ Confirm that signal sequence has been correctly removed and sequence begins at desired residue.

98. _____ Confirm that N or C-terminal fusion is in-frame.

99. _____ Place cursor in text pane.

100. _____ Select Print Preview.

101. _____ Print pages showing translation for records.

102. _____ Move cursor to graphic pane.

103. _____ Print map for records.

Notes:

1. Search Tuberculist for coding sequence of gene of interest.
   a. Enter Rv and number in Free Text box, left pane. Press Search. Data appears.
   b. Blue screen may list more than one gene. Select gene of interest.
   c. Yellow screen holds data for individual gene. Verify correct gene is displayed.
   d. Scroll to bottom of yellow screen.
   e. Select DNA + 200 bp. Click Get Data.
   f. Data appears in separate window. Maintain this window for VNTI file creation.
2. Vector NTI operates in two windows – data management (VNTI window) and file management (Exploring).
   Date window:
   a. Left upper pane is file description information.
   b. Right pane is graphic pane.
   c. Bottom pane is sequence as text pane.
   Exploring window (the Local Database): This window accesses files (sequence, plasmid, etc.).
   a. Upper left control window: Click DNA/RNA molecules.
   b. Left pane: The DNA/RNA table lists Subsets containing files for experimenters and other grouped sets.
   Click on a subset to view files in the right pane.
   c. Right pane: files in the selected subset.
3. Alternative process to transfer gene sequence to Vector NTI:
   a. Download Tuberculist DNA (with or without upstream/downstream sequence).
   b. Open as Windows default.
   c. Save window content as seq file to personal domain.
   d. In Vector NTI Exploring window, pull down DNA/RNA in top toolbar.
   e. Press Import → Molecule from Text File.
   f. Press FastA – OK
   g. Navigate to file. Select file. Open
   h. In VectorNTI Exploring window, pull down DNA/RNA tab → Import file and direct file to desired subset.
   i. Remove stop codon, signal sequence, etc.
4. Vectors typically used in TB contract are pET15b(-), pET23b(-), and PVV16 rc. rc = reverse complement.
5. This process uses the gene sequence file (step 12) to create a new file with primer sequences and restriction enzyme sites included.
6. Generally, cloning primers must be selected at start and end of sequence file. Best practice is to search for forward and reverse primers with Tm (temperature of melting) within 5 degrees of each other. It may be necessary to change parameters to 10 or 15 degrees before VNTI will assign primers. In such cases, the amplification may be challenging.
7. The start position of the parent vector is not preserved in map creation, creating a break in sequence number in regions that will be sequenced. In that event, sequence alignment will be incomplete. To move all sequence relevant to the insert and fusion tags into a region of unbroken ascending number, we reapply the parent sequence start coordinate.
8. Clicking on a section of the map in graphic pane highlights the sequence in text pane.