2-stage site-directed mutagenesis

Date:

Purpose: To introduce mutations into an existing plasmid.

Methods: Two-stage site -directed mutagenesis
In this approach a single reaction is first set up as two half-reactions each containing only one of the primers.
Following an initial set of cycling parameters (Stage 1) the two half reactions are combined and another set of cycling (Stage II) is completed.

	stock conc	75 ng/ul (100 ul stock)		
Primer 1		818.6	9.16198387	90.83801613
Primer 2		954	7.86163522	92.13836478

	stock conc	25 ng/ul (10 ul stock)		
Template		330	0.75757576	9.242424242

Modified QC Protocol		**1a-1**	**1a-2**
MM2	**Final Conc.**	**1**	**1**
H20		18	18
10X PFU Buffer	1x	2.5	2.5
dNTPs	1x (~ 400uM of each)	1	1
100% DMSO	2%	0.5	0.5
primer 1 (75ng/ul)*	75-125ng/rxn	1	0
primer 2 (75ng/ul)*	75-125ng/rxn	0	1
PFU Ultra II (or TURBO)		1	1
template (25ng/ul)	25ng/rxn	1	1
		25	25

*Forward/Reverse primers added separately for 2 stage reaction
a and b from each reaction combined for stage 2. CONCENTRATIONS SHOUID BE THE SAME

STAGE1 Reaction Conditions:

		Time	Temp.
	Initial Denaturation	5 min.	95 C
	Denature	45 sec	95 C
8 Cycles	**Anneal**	45 sec	60 C
	Extend	8 min*	68 C
	Hold		4 C

STAGE 2 Cycling Conditions:

		Time	Temp.
	Initial Denaturation	5 min.	95 C
	Denature	45 sec	95 C
16 Cycles	**Anneal**	45 sec	60 C
	Extend	8 min*	68 C
	Final Extension	10 min.	68 C
	Hold		4 C

Protocol details

1 Thaw reagents and keep on ice
2 Make master mix (MM) if desired
3 combine MM (or components) with Template and appropriate primer
4 STAGE 1 parameters
5 Combine 25ul of each primer pair extension rxn.
6 Add 1-**2ul** PFU UltraII or PFU Turbo to each combined Rxn
7 Stage 2 parameters
8 Add 2ul Dpn1 to each rxn.
9 Incubate 1 hr at 37C
10 transform 2ul QC Rxn.
11 transform into DH5A**
12 Propagate and sequence 2 colonies to verify mutation

*~ 1 min/Kb of total plasmid size but base this on the polymerase you are using
** or Top10, Terri's home-made cells work well