Primer Design
See Gola et.al, Yeast 2003;20:1339-1347 for diagrams

S1 and S2 (knockout) primers:
Choose 100 bp near the ATG and stop codons. Add the S1 specific sequence to the 3' end of the forward primer and the S2 specific sequence to the 3' end of the reverse primer.
S1: gaa gct tcg tac gct gca ggt c
S2: tct gat atc atc gat gaa ttc gag

Diagnostic Primers:
A2: aat gga tca gtg gca ccg gtg
512 bp from S1 primer
A3: ggg ccc att ggt taa gtt cat atg c
788 bp from S2 primer
H2: caa cga aat ggc ctc ccc tac cac ag
204 bp from S1 primer
H3: gga cga att gaa gaa agc tgg tgc aac cg
199 bp from S2 primer
U2: gtg tta cga atc aat ggc act aca gc
147 bp from S1 primer
U3: gga gtt gga tta gat gat aaa ggt gat gg
322 bp from S2 primer

G1 and G4:
G1 is usually around 200-300 bp upstream from the S1 primer, and G4 200-300 bp downstream from the S2 primer. These can be anywhere, as long as they are oustide the S1 and S2 primers, not too repetitive, and produce reasonable PCR products when paired with the A2, A3….primers.

Reconstitution primers:
Forward primer should be at least 2 kb upstream of the start codon.
Reverse primer should be about 1 kb downstream of the stop codon.
Add a NotI site and 4 additional bases to the 5' end of both primers for cloning into pDDB78 or pRYS2.
5' – 4 bp+NotI+spec.sequence-3'

Making a knockout
All solutions used should be sterile.

Day1

1. Start 3 ml overnight culture in YPD of strain to be transformed.

2. Start making 50% PEG; 5g PEG (mw 3350), in little less than 10 ml H2O. Mix on stirplate in beaker covered with plastic wrap overnight.

3. Set up 5 tubes of PCR using pFA-Arg, pFA-ura or pFA-His digested with ScaI(How to make and digest pFA):

5X Buffer	10ul
25 mM MgCl2	5 ul
10 mM dNTPs	2 ul
pFA	1 ul
S1 primer (20 uM-?mM)	1 ul
S2 primer (20 mM)	1 ul
Taq	0.5 ul
H2O	29.5 ul

1) 94C 5 min
2) 94C 1 min
3) 55C 1 min
4) 72C 4 min
to #2, 40 times (Is there a program name already?)

Day 2
1. Start 50 ml culture from overnight at an OD600 of 0.15.

2. Pool and precipitate (Which precipitation protocol?) the 5 PCR reactions. Resuspend in 20 ul H2O.

3. Make PEG, PLATE and TELiAc.

50% PEG: Bring solution started previous night to a final volume of 10 ml, and filter sterilize. Should we have actual volumes listed for 1 transformation?
TELiAc: 8 vol. H2O, 1 vol. 10X TE, 1 vol. 1 M LiAc
PLATE: 8 vol. 50% PEG, 1 vol, 10X TE, 1 vol. 1M LiAc

4. When 50 ml culture reaches and OD600 between 0.8 and 1.0 (about 4 hours), collect cells by centrifugation at 3,000 xg for 3 minutes. Discard supernatant, wash cell pellet with 10 ml sterile H2O, repellet cells, and resuspend in 500 ul TELiAc.

5. To one empty tube (neg. control) and tube containing PCR product, add 5 ul boiled herring sperm DNA. Add to each tube 100 ul of cells in TELiAc. Incubate at room temperature for 30 minutes.

6. Add 700 ul of PLATE to each tube, and incubate at room temperature overnight.

Day 3

1. Heat shock cells at 42C for one hour.

2. Pellet cells by centrufugation for 30 sec. at full speed, discard supernatant and resuspend in 200 ul H2O.

3. Plate 100 ul per plate on appropriate selective media. Only need to do one plate for negative control. Place in incubator.

Colonies should appear in 2-3 days.

Screening:
Screen by PCR using G1 paired with (A, H, or U)2 and G4 paired with (A, H, or U)3. If second round of transformation, screen each colony for both insertions (Ura and Arg). For knockouts, also screen using a set of internal primers in the coding sequence.