Dut/ung *in vitro* Site-directed Mutagenesis Protocol

(originally written by M. Davis and P. Bjorkman) revised: 12/20/95

Favored *E. coli* strains for site-directed mutagenesis using uracil replacement:

RZ1032: dut^{ts}, ung, tet^{r}
Bw313:

dut: deoxy uracil transphosphatase (enzyme required for formation of thymidine precursor)
ung: uracil-DNA-glycosylyase (enzyme which normally will proof and replace uracil in DNA)

STEP 1. GEL PURIFY MUTAGENESIS OLIGOS (IF REQUIRED)

1a. Purify crude oligos from 1% agarose gel.
1b. Desalt oligo prep using NAP 25 column.
1c. EtOH precipitate oligo DNA and resuspend into TE to give final concentration of 1.8 µg/λ.

NOTE: For ss DNA, 1 OD_{260} = 20 µg/ml.

STEP 2. ALTERNATIVE OLIGO KINASE REACTION (20λ total volume)

2a. Add the following:

13λ	dH_2O
2λ	oligo DNA (approx. 10 ng)
2λ	10X T4 ligase buffer (Boeh. Mann.)
1λ	10mM ATP
2λ	T4 kinase (Boeh. Mann.)
20λ	

2b. Incubate for 3 hr at 37°c.
2c. Store kinased oligos at -70°c until ready for use.

STEP 3. PREPARATION OF TEMPLATE SINGLE STRAND DNA (ss DNA)

3c. Prepare Ca^{2+} competent RZ1032 cells (these cells can't be made electrocompetent).
3d. Transfect template (ds DNA) into Ca2+ competent RZ1032 cells:

- add 0.1 µg of template ds DNA to a cold eppendorf tube.
- add 50λ of thawed CA^{2+} comp. RZ1032 cells, invert to mix reaction contents.
- incubate reaction 5 min on ice, then 5 min at 37°c.
- immediately add 1 ml of 2XYT media and incubate reaction 1 hr at 37°c.
- streak reaction Hi/Lo on LB + Amp + Tet plates, place plate in 37°c incubator until colonies appear.

3e. Pick a single colony from transfect reaction plate and grow in 2 ml of LB (or 2XYT) media containing 100 µg/ ml Amp, 10 µg/ ml Tet, and 0.25 µg/ml fresh Uridine in a 37°c shaker incubator.

3f. After approximately 8 hrs (usually overnight, O/N), removed 100λ of the shaker culture and add 2 ml of 2XYT media containing 20λ of helper phage (either M13K07 or VCSM13 strain), 10 µg/ml Tet and 0.25 µg/ml fresh Uridine (no Amp or Tet is added to media at this time).

NOTE: assume 1 µg of ss DNA from 1 ml of phage culture, e.g., to recover 10µg of ss DNA start with 10 ml of phage culture (5x 2 ml cultures). Remember, one site-directed mutagenesis reaction requires a minimum of 5µg of ss template DNA. If using phage culture larger volumes (10-20 ml), use 50 ml SS-34 rotor tubes with yellow caps for incubating (see step 3j).

3g. Incubate phage culture 1 hr in 37°c shaker incubator.

3h. After 1 hr incubation, add to phage culture Amp (final conc. = 50 µg/ml) and Kanamycin (final conc. = 50 µg/ml).

3i. Incubate phage-antibiotic culture for 8 hrs in 37°c shaker incubator.

3j. Pellet the phage-antibiotic culture at 1900xg for 10 min (4000 rpm using SS-34 rotor).

3k. Pool supernatants from multiple culture tubes into one sample. Add 1 vol. of 20% PEG, 2.5 M NaCl for each 4 vol. of supernatant. Mix by inverting tube and incubate reaction overnight at 4°c.

3l. Pellet PEG/NaCl reaction at 7650xg for 20 min (8000 rpm using a SS-34 rotor).

3m. Remove and discard supernatant. The pellet containing the precipitated phage particles should be clearly present at base of the rotor tube. In addition, there may a slight whitish film of PEG lining the rotor tube wall. Using a very small amount of dH_20 (200λ), wash the rotor wall covered by the PEG film until the PEG dissolves completely into dH_20 wash. Repeated washings may be required to remove the PEG film. Be careful to not disturb the phage pellet.

3n. Resuspend the phage pellet with 500λ TE pH 8.0 and transfer suspension to 1.5 ml eppendorf tube. Do not use a TE with a lower pH as your ss DNA will dissolve into the phenol phase in the extraction procedure following this step.

3o. Extract the phage pellet resuspension with an equal volume of buffer phenol pH 8.0. Remove the aqueous (top) phase containing the ss DNA and repeat 1:1 volume phenol extraction until the interface does not contain a whitish ppt. Follow the phenol extractions with one chloroform extraction. Add two volumes of chloroform for each vol. of ss DNA in TE.

3p. Precipitate the ss DNA by adding 1/10 vol. of 3M NaAc and 2 vol. of 100% EtOH. Place reaction in -70°c freezer for a minimum of 2 hrs.

3q. Pellet the precipitate ss DNA at 12000 rpm, 4°c for 30 min using microfuge.

3r. Wash ss DNA pellet using 80% EtOH and dry the pellet by speedvac.

NOTE: place parafilm across 1.5 ml tube opening to prevent pellet being lost when releasing speedvac vacuum.

3s. Resuspend the ss DNA pellet in 50λ TE.

3t. Measure concentration and purity of ss DNA by $OD_{260/280}$ ratio. Verify quality and quantity of ss DNA by running a sample on a 1% agarose gel containing a known quantity of ss DNA.

***STEP 4. ALTERNATIVE TEMPLATE-PRIMER ANNEALING REACTION (10λ total volume)**

4a. Add the following:

2λ	autoclaved ddH_2O
6λ	template ss DNA (5 µg)
1λ	kinased oligo (500ng)
1λ	10X T4 DNA ligase buffer (NEB)
10λ	

4b. Incubate 70°c for 2 min, chill on ice for 2 min, incubate at 22°c (RT) for 10 min.

***STEP 5. ALTERNATIVE ELONGATION/LIGATION REACTION (100xλ total volume)**

5a. Add the following to the 10λ alternative annealing reaction:

40λ	dH_2O
25λ	2mM dNTP
10λ	10mM ATP
10λ	10X T4 DNA ligase buffer (Boeh. Mann.)
3λ	T4 DNA polymerase (Boeh. Mann.)
2λ	T4 DNA ligase (Boeh. Mann.)
90λ	
+ 10λ	alternative annealing reaction
100λ	total volume, elongation/ligation reaction

5b. Incubate reaction at 22°C (RT) for 2 hrs.

5c. Incubate reaction at 37°c for 1 hr.

5e. Check result of elongation/ligation reaction by running a sample on a 1% agarose gel. Include the following controls:

i) ss DNA, no primer, no elongation

ii) ss DNA, no primer, but elongated

iii) ds DNA which is equal expected kilobase length of ds mutagenesis DNA product

STEP 6. PRECIPITATE AND RECOVER MUTAGENIC DS DNA

6a. Precipitate ds DNA from elongation/ligation reaction. For each 100λ of elongation/ligation reaction, add 12.5λ of 7.5 M Ammonium Acetate and 375λ of 100% EtOH. Incubate precipitate reaction at -80°c for a minimum of 1 hr.

6b. Pellet precipitated ds DNA at 12000 rpm for 30 min using microfuge.

6c. Wash ds DNA pellet using 80% EtOH and dry the pellet by speedvac.

NOTE: place parafilm across 1.5 ml tube opening to prevent pellet being lost when releasing speedvac vacuum.

6d. Resuspend the ds DNA pellet in 20λ TE.

6e. Electroporate 1λ of ss DNA with 50λ of electrocompetent TGI cells.

6f. Streak electroporation reaction Hi/Lo on LB + Amp plates.

STEP 2. OLIGO KINASE REACTION (20λ total volume)
** SEE ALTERNATIVE PROTOCOL*

2a. Add the following:

13λ	dH_2O
1λ	oligo DNA (final conc. = 250ng/λ)
2λ	10X Salt Stock (500mM Tris pH 7.8, 100mM $MgCl_2$)
2λ	10X ATP/DTT (10mM ATP, 100mM DTT)
2λ	T4 kinase (NEB: New England Biolab)
20λ	

2b. Incubate for 3 hr at 37°c.

2c. Store kinased oligos at -70°c until ready for use.

STEP 4. TEMPLATE-PRIMER ANNEALING REACTION (20λ total volume)
** SEE ALTERNATIVE PROTOCOL*

4a. Add the following:

6λ	dH_2O
10λ	template ss DNA
2λ	kinased oligo (500ng)
2λ	10X Salts (500mM Tris, 100mM $MgCl_2$)
20λ	

4b. Prepare ss DNA, no primer, but elongated negative control reaction by replacing kinased oligo volume with equal volume of dH_2O.

4b. Incubate 70°c for 2 min, chill on ice for 2 min, incubate at 22°c (RT) for 10 min.

STEP 5. ELONGATION/LIGATION REACTION (105λ total volume).
** SEE ALTERNATIVE PROTOCOL*

5a. Add the following to the 20λ annealing reaction:

35λ	dH_2O
25λ	2mM dNTP
10λ	ATP/DTT stock (10mM ATP, 100mM DTT)
10λ	10X Salts stock
3λ	T4 DNA polymerase (Boeh. Mann.)
2λ	T4 DNA ligase (Boeh. Mann.)
85λ	
+ 20λ	annealing reaction
105λ	total volume, elongation/ligation reaction

5b. Incubate reaction at 16°c for 10 min.

5c. Incubate reaction at 22°C (RT) for 2 hrs.

5d. Incubate reaction at 37°c for 1 hr.

5e. Check result of elongation/ligation reaction by 1% agarose gel electrophoresis. Include the following controls:

i) ss DNA, no primer, no elongation

ii) ss DNA, no primer, but elongated

iii) ds DNA which is equal expected kilobase length of ds mutagenesis DNA product