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

- RZ1032: dut<sup>s</sup>, ung, tet<sup>r</sup>
- Bw313:

**dut**: deoxy uracil transphosphatase (enzyme required for formation of thymidine precursor)
**ung**: uracil-DNA-glycosylase (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<sub>260</sub> = 20 µg/ml.

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

2a. Add the following:

- 13λ dH<sub>2</sub>O
- 2λ oligo DNA (approx. 10 ng)
- 2λ 10X T4 ligase buffer (Boeh. Mann.)
- 2λ 10mM ATP
- 1λ 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<sup>2+</sup> competent RZ1032 cells (these cells can't be made electrocompetent).
3d. Transfect template (ds DNA) into Ca<sup>2+</sup> competent RZ1032 cells:

- add 0.1 µg of template ds DNA to a cold eppendorf tube.
- add 50λ of thawed CA<sup>2+</sup> 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 100\(\lambda\) of the shaker culture and add 2 ml of 2XYT media containing 20\(\lambda\) of helper phage (either M13K07 or VCSM13 strain), 10 \(\mu\)g/ml Tet and 0.25 \(\mu\)g/ml fresh Uridine (no Amp or Tet is added to media at this time).

**NOTE**: assume 1 \(\mu\)g of ss DNA from 1 ml of phage culture, e.g., to recover 10\(\mu\)g of ss DNA start with 10 ml of phage culture (5x 2 ml cultures). Remember, one site-directed mutagenesis reaction requires a minimum of 5\(\mu\)g 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\(^\circ\)c shaker incubator.

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

3i. Incubate phage-antibiotic culture for 8 hrs in 37\(^\circ\)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\(^\circ\)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 \(dH_2O\) (200\(\lambda\)), wash the rotor wall covered by the PEG film until the PEG dissolves completely into \(dH_2O\) 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 500\(\lambda\) 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\(^\circ\)c freezer for a minimum of 2 hrs.

3q. Pellet the precipitate ss DNA at 12000 rpm, 4\(^\circ\)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 50\(\lambda\) 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 ddH₂O
- 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 dH₂O
- 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 100\(\mu\)l of elongation/ligation reaction, add 12.5\(\mu\)l of 7.5 M Ammonium Acetate and 375\(\mu\)l 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 20\(\mu\)l TE.

6e. Electroporate 1\(\mu\)l of ss DNA with 50\(\mu\)l of electrocompetent TGI cells.

6f. Streak electroporation reaction Hi/Lo on LB + Amp plates.
STEP 2. OLIGO KINASE REACTION (20μl total volume)
* SEE ALTERNATIVE PROTOCOL

2a. Add the following:

- 13μl dH₂O
- 1μl oligo DNA (final conc. = 250ng/μl)
- 2μl 10X Salt Stock (500mM Tris pH 7.8, 100mM MgCl₂)
- 2μl 10X ATP/DTT (10mM ATP, 100mM DTT)
- 2μl T4 kinase (NEB: New England Biolab)
- 20μl

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 (20μl total volume)
* SEE ALTERNATIVE PROTOCOL

4a. Add the following:

- 6μl dH₂O
- 10μl template ss DNA
- 2μl kinased oligo (500ng)
- 2μl 10X Salts (500mM Tris, 100mM MgCl₂)
- 20μl

4b. Prepare ss DNA, no primer, but elongated negative control reaction by replacing kinased oligo volume with equal volume of dH₂O.
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 dH2O
- 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 annealing reaction

\[ 105λ \text{ total volume, elongation/ligation reaction} \]

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