SEQUENCING DOUBLE STRANDED TEMPLATE

3-5 MICROGRAMS OF PLASMID DNA/ ANNEALING RXN

ALKALINE-DENATURATION METHOD

1) DENATURE DNA BY ADDING 0.1 VOLUMES OF 2M NaOH, 2 mM EDTA
   ------> INCUBATE AT 37 C FOR 30 MIN

2) NEUTRALIZE THE REACTION BY ADDING 0.1 VOLUMES OF 3M SODIUM ACETATE (pH 4.5 -5.5)  
   ------> VORTEX  
   ------> ADD 2X VOLUME OF ETHANOL  
   ------> -70 C FOR 15 MIN  
   ------> SPIN  
   ------> WASH W/ 70% ETHANOL  
   ------> REDISSOLVE IN 7 MICROLITERS OF WATER

QUANTITY AND PURITY OF DNA IS THE MOST IMPORTANT FACTOR

ANNEALING TEMPLATE AND PRIMER

1) FOR EACH SET OF FOUR SEQUENCING LANES, A SINGLE ANNEALING REACTION IS USED

   PRIMER (1 PICOMOLE) 1 µL
   REACTION BUFFER 2 µL
   DNA PELLET + WATER 7 µL

2) CONTROL REACTION

   M13 CONTROL DNA 5 µL
   WATER 4 µL
   PRIMER 1 µL

3) WARM THE TUBES TO 65 C FOR 2 MIN ON A HOT BLOCK/ WATER BATH  
   ------> COOL SLOWLY TO RT OVER 30 MIN.  
   WHEN TEMPERATURE IS BELOW 35 C ANNEALING IS COMPLETE.  
   PLACE TUBE ON ICE. USE W/IN 4 HOURS

LABELING REACTION

1) DILUTE LABELING MIX 5-FOLD W/ WATER 4µL MIX TO 16µL OF WATER.  
   FOR SEQUENCING CLOSE TO THE LABELING MIX SHOULD BE DILUTED 15 FOLD OR THE Mn BUFFER SHOULD BE USED.
2) DILUTE SEQUENASE VERSION 2.0 ENZYME 1:8 IN ICE COLD ENZYME DILUTION BUFFER.

3) TO THE ANNEALED TEMPLATE-PRIMER ADD THE FOLLOWING (ON ICE):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>TEMPLATE/PRIMER</td>
<td>10.0 µL</td>
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<tr>
<td>DTT 0.1M</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>DILUTED LABELING MIX</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>[ a-35S] dATP</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>DILUTED SEQUENASE 2.0</td>
<td>2.0 µL</td>
</tr>
</tbody>
</table>

MIX THOROUGHLY AND INCUBATE AT RT FOR 2 - 5 MIN. NO HIGHER THAT RT OTHERWISE ARTIFACTS ARISE.

ISOTOPE CONC = 10 µCi /µL AND 10 µM

TERMINATION REACTIONS

1) FOR EACH LABELING RXN HAVE FOUR TUBES LABELED G, A, T, AND C

2) PLACE 2.5 µL OF THE ddGTP TERMINATION MIXES IN THEIR RESPECTIVE TUBES.

3) PREWARM THE TUBES AT 37 C FOR AT LEAST 1 MIN

4) WHEN THE LABELING RXN IS COMPLETE, PLACE 3.5 µL INTO EACH OF THE 4 TERMINATION MIXES. MIX THOROUGHLY, SPIN, AND INCUBATE AT 37 C FOR 3 - 5 MIN. INCUBATION CAN EXTEND TO 30 MIN W/O PROBLEMS.

5) ADD 4µL OF STOP SOL’N TO THE TERMINATION REACTIONS. MIX AND STORE AT -20 C UNTIL READY TO RUN.

6) WHEN GEL IS READY FOR LOADING, HEAT THE SAMPLES TO 75-80 C FOR 2 MIN AND LOAD IMMEDIATELY. USE 2 - 3 µL IN EACH LANE.