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Site-Directed Mutagenesis of the Nitrogenase Fe-Protein from Azotobacter vinelandii

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SITE-DIRECTED MUTAGENESIS OF THE NITROGENASE FE-PROTEIN FROM *Azotobacter vinelandii*

By

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Site-Directed Mutagenesis

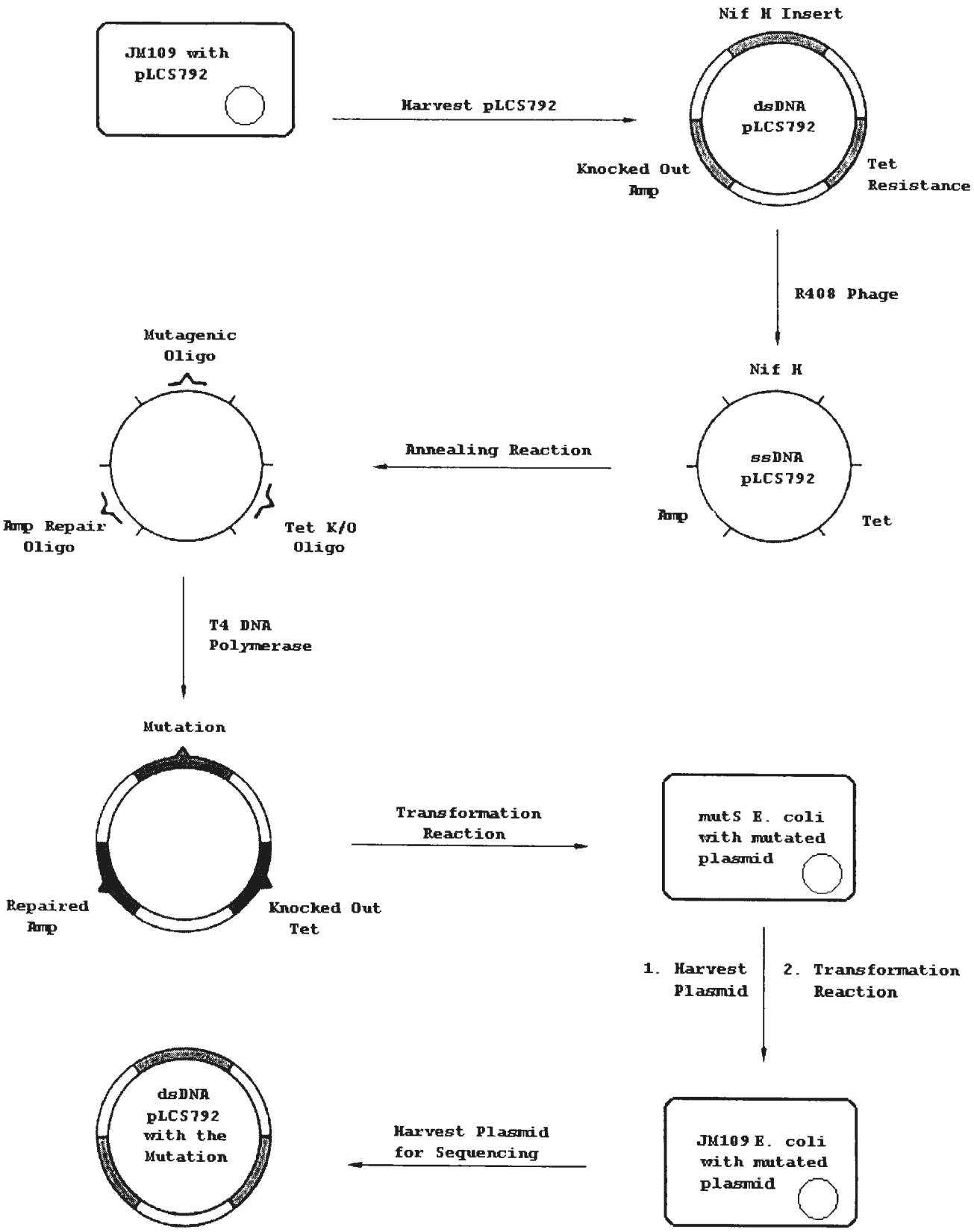

Figure #1

Nitrogen fixation is the chemical reaction of dinitrogen (N_2) being reduced to ammonia (NH_3). This reaction occurs naturally by nitrogen fixing microorganisms which contain the enzyme nitrogenase. The overall reaction is written below.

$$8e^- + N_2 + 16MgATP + 8H^+ \rightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

Nitrogenase is composed of two proteins: molybdenum-iron protein (MoFe-protein) and iron-protein (Fe-protein). N_2 binding and fixation occurs within the MoFe-protein, but the reaction is dependent on the electrons brought to MoFe-protein by the [4Fe-4S] cluster of the Fe-protein. The interaction between the two components is dependant upon conformational changes induced by nucleotide binding. Evidence suggests that the Fe-protein component of nitrogenase exists in three conformations: a non-nucleotide conformation, MgATP-bound conformation, and a MgADP-bound conformation. In a non-nucleotide conformation, the redox potential of the [4Fe-4S] cluster is approximately -300mV and is able to accept an electron from a precursor protein. Two MgATP molecules can then bind to the Fe-protein causing a conformational change which both lowers the redox potential and increases affinity to the MoFe-protein. Once the Fe-protein docks with the MoFe-protein it can then transfer an electron and undergo ATP hydrolysis. At this point the Fe-protein is bound to two molecules of MgADP which create the third conformation. This conformation decreases affinity for the MoFe-protein and facilitates the release of the Fe-protein from the MoFe-protein. Once free, Fe-protein can then release the nucleotides and return to a non-nucleotide conformation. One electron is shuttled to the MoFe-protein with every dock and release of Fe-protein. Therefore, in order to complete the fixation the Fe-proteins must dock and release with Mo-Fe-protein eight times.

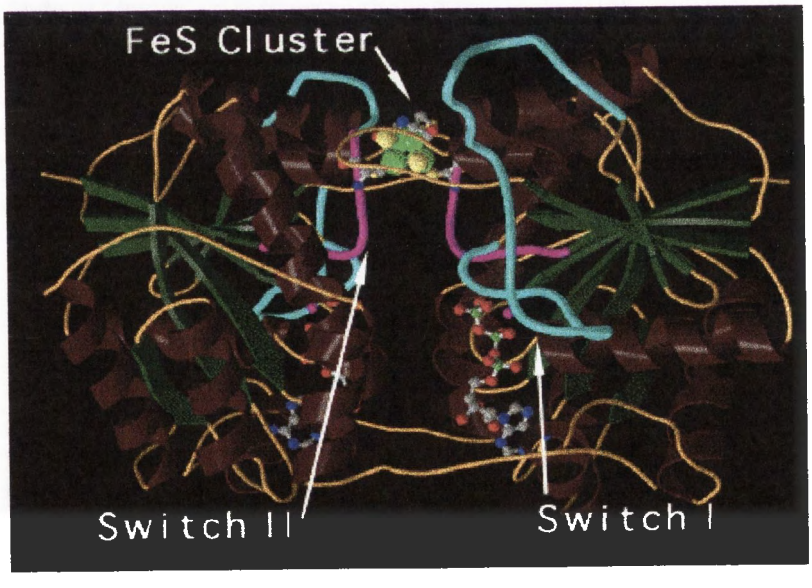

Figure 2

The nucleotide induced conformational changes are facilitated by the leverage-like movements of two protein regions surrounding the [4Fe-4S] cluster. As seen in figure 2 above

the region between Asp39 to Val67 is referred to as switch I. The region between Asp125 to Cys132 is referred to as switch II. The characterizations of site-directed mutations within switch II suggests that nucleotide binding causes switch II to reposition the [4Fe-4S] cluster. This new conformation facilitates electron transfer to MoFe-protein. Switch I is less characterized, but it is believed that the movement of switch I, due to nucleotide binding and hydrolysis, facilitates docking and release of Fe-protein to MoFe-protein. Further characterizing of Fe-protein altered with site-directed mutations in switch I should help elucidate the interaction between nucleotides and conformational changes involved in docking. The purpose of this report is to explain the process of site-directed mutagenesis, and provide a detailed protocol for performing site-directed mutagenesis on Fe-protein of *Azotobacter vinelandii*.

Site-directed-mutagenesis is the tool used to change the amino acid sequence of a protein. In Dr. Lance Seefeldt's lab, *Azotobacter vinelandii* is used to study nitrogenase, but *A. vinelandii* does not lend itself to metagenesis. Mutations in Fe-protein therefore require placing the gene for Fe-protein (nif H) into a plasmid. The plasmid can then be stored, and mutated using *E. coli*.

A plasmid is a relatively small circular strand of DNA which can be transferred from one bacteria to another. The plasmid carrying the nif H gene was constructed by Dr. Lance Seefeldt and is named pLCS792. The nomenclature used to name this plasmid can be understood as: p refers to plasmid, LCS refers to Lance Seefeldt, and 792 refers to the page in Dr. Seefeldt notebook recording how he made the plasmid. pLCS792 contains three genes of interest. Two of the genes code for the antibiotic resistance of ampicillin and tetracycline. The third gene codes for nif H (see figure 1). The ampicillin region of pLCS792 has been knocked out (a single codon mutation) to prevent ampicillin resistance. This will play a major role in the metagenesis process. The tetracycline region of pLCS792 provides tetracycline resistance for *E. coli*. This resistance prevents non-plasmid carrying *E. coli* from forming colonies on LB_{tet} plates, and allows for the selection of colonies containing pLCS792 plasmid.

```
5' GATGTCGGGCCATTGTTCGGTTGTAGCAATTACAACAGTCGGAGTAGGGGG 3'
   ----------------------------------------------------
3' CTACAGCCCGGTAACAAGCCAACATCGTTAATGTTGTCAGCCTCATCCCCC 5'

   D  V  G  P  L  F  G  C  S  N  Y  N  S  R  S  R  G
    M  S  G  H  C  S  V  V  A  I  T  T  V  G  V  G  G
     C  R  A  I  V  R  L  *  Q  L  Q  Q  S  E  *  G  D
```

Figure 3

It will be helpful to discuss some of the conventions and nomenclature of genetics, before going on to mutagenesis. Shown above in figure 3 is a small portion of a double stranded plasmid. The top strand is referred to the coding strand and by convention reads 5' to 3'. The bottom strand is referred to the non-coding or the template strand and reads 3' to 5'. Transcription of RNA occurs on the bottom strand (thus making it the template), but the RNA is analogous with the top strand (thus making it the coding strand). Mutagenesis does not involve

transcription, but the nomenclature will help describe mutagenic replication. Underneath the double-stranded DNA (dsDNA) are the three possible reading frames of that sequence. The appendix has a copy of the entire nif H sequence and identifies the proper reading frame.

Site-directed-mutagenesis of the nif H gene begins with pLCS792 in *E. coli*. The first step as seen in figure 1 involves harvesting a single stranded DNA (ssDNA) from *E. coli*. This is done by growing the plasmid carrying bacteria in LB_{tet} media with R408 helper phage. R408 is a bacterial virus which encapsulates the coding strand of pLCS792. These capsules can then be separated from the bacterial cells, lyced, and the ssDNA collected using a Qiagen mini-prep (see page 10 of protocol). Mutations are made as we build the non-coding strand piece by piece.

Oligonucleotides (oligos) are short pieces of ssDNA (17-25 bases) complementary to regions of the ssDNA plasmid. Oligos can be annealed to the single stranded plasmids by hydrogen bonding through a heat step of 75°C followed by a slow cooling step. The annealed oligo serves as a primer for polymerase to finish building the non-coding strand. However, oligos used for mutagenesis are not exactly complementary. Oligos are synthesized one base at a time by a machine in the Biotech Center. At the center of the oligo is a non-complementary change in sequence. The change may include: addition of a codon, deletion of a codon, or a change in a codon. The final result is the addition, deletion, or change in the amino acid sequence. Annealing of the oligo remains possible by the 8-12 complementary bases on either side of the mutation. Below is an example of an mutagenic oligo annealed to the pLCS792 single stranded plasmid.

```
   D  V  G  P  L  F  G  C  S  N  Y  N  S  R  S  R  G
5' GATGTCGGGCCATTGTTCGGTTGTAGCAATTACAACAGTCGGAGTAGGGGG 3'
                  |||||||||  |||||||||
               3'AACAAGCCAAACTCGTTAAT 5'
                 L  F  G  L  S  N  Y
```

The result of this mutagenic oligo would change a cystine into a leucine.

Figure 4

For mutagenesis of pLCS792 three oligos are used: an oligo knocking out the tetracycline resistance, an oligo repairing the ampicillin resistance, and an oligo changing the amino acid sequence of Fe-protein. After completion of the annealing step T4 DNA polymerase, T4 DNA Ligase, and a mixture of nucleotide bases are added to the reaction. T4 DNA polymerase attaches the 5' ends of the nucleotide bases to the 3' ends of the mutagenic oligos until the non-coding strand has been completed. The result is a double-stranded pLCS792 plasmid with mutations in the non-coding strand.

The new mutated strand also contains nicks at the 5' end of the oligos. In order for these nicks to be repaired, the 5' end requires a phosphate group. Synthesized Oligos lack a 5' phosphate group. Before oligos are annealed they should be phosphorylated with T4 Poly-

Nucleotide Kinase (see step 3 of protocol). With the phosphate in position, T4 DNA Ligase can then repair the nicks left over by T4 DNA Polymerase. The final result of the reaction is double-stranded plasmid ready to be transformed into *E. coli*.

If the new plasmid is transferred into wild type *E. coli* the repair mechanisms of *E. coli* will repair the new mutations. To prevent this, Promega provides mutS *E. coli*. These bacteria lack repair enzymes; therefore, they are used for the initial replications. The mutated plasmid is transformed into mutS only to replicate a relatively small number of complementary double-stranded plasmids. After one round of replication, the non-coding mutagenic strand replicates into a completely complementary dsDNA plasmid. By growing transformed mutS in LB_{amp}, media, only *E. coli* with a repaired ampicillin gene can divide and replicate. The hope is that the single-stranded plasmids, which accepted the amp-repair oligo, are also likely to have accepted the mutation oligo. The mutation will not be confirmed until the plasmid has been sequenced. The risk of further mutations, makes mutS a poor choice for storage and colony growth of pLCS792. Therefore, the plasmid is harvested with a Promega mini-prep and transformed into JM109 *E. coli* (see step 7 of protocol). JM109 is a hardy bacteria which carries DNA repair mechanisms. Within JM109 the plasmid is safer from unwanted mutations. Plasmids can be indefinitely stored and/or amplified while in JM109 *E. coli*. Note that in order for mutS and JM109 to take up the plasmid they must first become competent (see making competent *E. coli* section of protocol)

Transformed JM109 are grown up on LB_{amp} plates. Colonies are chosen at random and harvested for their plasmids. The plasmids can then be sequenced at the biotech center using dideoxy phosphorescence sequencing. This method can sequence only 300-400 bases accurately and is dependant on placement of primers (oligos). In order to detect the desired mutation, a primer must be chosen up-stream of that mutation. They can be complementary to the coding and non-coding strand. Primers complementary to the non-coding strand will sequence the coding strand in the 3' direction. Primers complementary to the coding strand will provide the sequence to the non-coding strand also in the 3' direction. A list of primers and their sequencing region is provided in the appendix.

Once sequencing has been completed and the desired mutation found, the plasmid must be transformed into *Azotobacter vinelandii* in order for the nifH gene to express itself. At this point, a little background of *A. vinelandii* would be helpful.

A. vinelandii can obtain its nitrogen from two sources: organic nitrogen found in the media or elemental dimolecular nitrogen found in the air. It prefers organic nitrogen, but in the lack of organic nitrogen, *A. vinelandii* begins to express its nitrogen fixating genes. These genes allow for *A. vinelandii* to fixate nitrogen. Bacteria which can fix dinitrogen are referred to as nif-plus (nif+). Bacteria are refer to as nif-minus (nif-) if they can not fix N_2. Some mutations in the nif H gene only slow fixation, referred to as nif-slow, while other mutations render *A. vinelandii* nif-. Nif+ and nif-slow *A. vinelandii* can survive without nitrogen in their media (B plates). Nif- *A. vinelandii* requires organic nitrogen (BN plates).

The existence of the desired mutation can be determined by sequencing potential colonies, but the consequence of the mutational effect on nitrogenase activity has yet to be determined. For this reason two methods of transformation exist: Marker Rescue and Congression. Marker rescue identifies those mutations which are nif+ or nif-slow. The process is dependant on two types of mutated *A. vinelandii*: DJ54 and DJ525. These two mutants have a large region of the nif-H gene excised. The region where the mutagenesis reaction took place determines which Marker Rescue strain to use. If the mutation resides between amino acids 1-68, DJ525 is used. All other mutation require DJ54. The idea of Marker Rescue is based on the fact that DJ54 and DJ525 are both nif-. If the mutagenesis reaction created a nif+ or nif-slow plasmid then transformation of that plasmid into the proper marker rescue strain would result in nif+ or nif-slow *A. vinelandii* growing on B-plates. In this case, transformation of the mutagenic plasmid occurred and site-directed mutagenesis has been completed. If no growth occurred on B-plates then the mutation must be nif-minus.

When the mutation has been identified as nif-, Congression is required to transform the plasmid. Congression transforms the nif- plasmid into wild-type *A. vinelandii* (AV-trans). The result is nif+ AV-trans cells becoming nif-. Very few plasmids get transcribed into AV-trans. An additional a rifampacin resistant plasmid (pDB303) is added to the transformation reaction to assist identification of transformed cells. Only cells which have taken up pDB303 will be able to grow on BN_{rif} plates. The idea is that those cells that took up the pDB303 are likely to have taken up the mutagenic plasmid. Colonies forming on BN_{rif} plates are then struck on both B-plates and BN-plates. Those colonies which grow on BN-plates but not B-plates have the transformed plasmid, completing site-directed mutagenesis.

Protocol for

Site-Directed Mutagenesis

on the

Fe-Protein from

Azotobacter vinelandii

A list of needed materials is listed in the appendix.

It is crucial to use sterile technique and to label and document every thing throughout the entire protocol.

Step 1 Designing the mutagenic oligo

Oligos should be about 21-24 nucleotide long with the mutation in the middle.
Fill out the Biotech. order form. Choose the 50_nmol synthesis scale unless your oligo is more than 50% G and has a string of G's. In that case, choose the 200_nmol synthesis scale, and have the oligo purified.

Step 2 Resuspending the Oligo.

The oligo from the Biotech. comes as a dry pellet of DNA and must be resuspended in SddH_2O Avoid disturbing the pellet while handling.

Very carefully open the eppendorf tube with the oligo and add 200_ul SddH_2O. Using your pipet stir gently. Allow the mixture to sit for one hour at room temp.

Determine the concentration of the oligo. In a separate eppendorf tube add 5_ul of the Oligo to 495_ul of ddH_2O. Mix the solution by sucking and reinjecting the mixture several times. Using the 200_ul quartz cuvette obtain a spec reading at 260_nm. (blank the spec to ddH_2O) Use the following formula to determine the concentration.

$$(\text{Concentration in pmol/ul}) = \text{Spec}_{260} \div \sum_{260} \times (100) \times (1000)$$

$$\sum_{260} - (\#\text{ of A} \times 15) + (\#\text{ of G} \times 11.8) + (\#\text{ of C} \times 7.4) + (\#\text{ of T} \times 8.8)$$

Store the oligo at -20 °C

Step 3 Phosphorylating the oligo.

Get two water baths ready, one at 37°C the other at 70 °C.
Make up fresh 10_mM ATP. Chill 1.5_ml SddH_2O on ice. Add 5.5 mg of ATP to a Eppendorf tube. Take 1_ml of the chilled water and add to the ATP. Syringe filter the solution into a sterile eppendorf tube - keep it on ice.

Add the following to an eppendorf tube...

100_pmol	Oligo
2.5_ul	Kinase 10x buffer
1.5_ul	T4 polynucleotide Kinase
3 ul	10_mM ATP
SddH_2O	to a total volume of 25_ul.

Incubate the reaction in the 37°C bath for 30_min.
Incubate the reaction in the 70°C bath for 10_min.

If you are going on to the anealing reaction keep the solution on ice otherwise store the phosphorolated oligo at -20°C.

Step 4 Annealing the Oligo to the ssDNA (It is helpful if this is started in the morning.)

Ready a 75 °C water bath with a half full 500_ml beaker
In a eppendorf tube add..

0.1- 0.05 pmol	ssDNA
1_ul	repair oligo (usually amp)
1_ul	knockout oligo (usually tet)
2-ul	mutogenic oligo (amount may very, you want plenty)
2_ul	Annealing 10x Buffer
SddH_2O	to a total volume of 20_ul.

Pulse for a minute, then place the eppendorf tube in a foam holder to float the tube in a half full 500_ml beaker at 75 °C for 5_min. Then remove the beaker with the reaction from the water bath and allow for it to cool to 45 °C on the counter (should be about 30-40 min.). At 45 °C place the beaker on ice and allow it to cool to room temp. Place the reaction mixture on ice and add in order...

3_ul	Synthesis 10x Buffer
1_ul	T4 DNA Polymerase
1_ul	T4 DNA Lygase
5_ul	SddH_2O

Incubate the reaction at 37 °C for 90 min. The resulting product is a dsDNA plasmid contaning the mutation ready to be transformed into mutS *E. coli*.

Step5 Transforming the newly annealed plasmid into mutS *E. coli.*

Prepair a water bath at exactly 42 °C.

Pre-chill on ice a 15_ml falcon tube for each transformation. Retreave from the -80 °C enough aliquoted mutS competent cells for each annealing reaction and a controle and place them on ice to thaw. (about 5_min.)

Transfer 100_ul of the cells into the pre-chilled falcon tubes.

Add 2_ul of the annealing reaction to the mutS. Gently ster the mixture with the pipet while dispencing the plasmid. (For a controle add 2_ul of SddH_2O)

Place the Falcon tubes quickly back on ice for 10_min.

Heat shock the cells by holding them in the 42 °C water bath for 45-50 sec.
Quickly place the tubes back on ice for 2_min. (DO NOT SHAKE)

Add 900_ul of room temp. LB broth to each falcon tube and incubate the cells for 30_min at 37 °C 225_rpm

In sterile 50_ml flasks add 4.5_ml of LB broth and either 5_ul of amp_{125} stock or 3.5_ul of Tet_{15} stock solution. (resultant LB+antibiotic broth should be either 125_ug/ml Amp or 10_ug/ml Tet.). After the 30_min. incubation add 500_ul of the transformation reaction into the 4.5_ml LB+antibiotic media. Incubate the culture over night at 37 °C 225_rpm about 15 hours for Amp. and 20 hours for Tet. Growth should only accure where the plasmid was added. (for extra ashurance inoculate a LB+antibiodic plate with 75_ul of the tranformation reaction) The mature cultures are ready for the mini-prep.

Step 6 Harvest the Plasmid from mutS using the Promega Mini-prep.

Pour the 5_ml cultures in to 15_ml falcon tubes. Centrifuge the media in a table top centrifuge (it's in the cold room) for 15_min. Decant the supernatant.

With a inoculation loop resuspend the pellet in 250_ul of resuspention solution.

Add 250_ul of Lysis solution. Cap tightly and invert 10-12 times without getting the mixture in the lid. Incubate the mixture for 30_min at 37 °C - no shaking

After the 30_min incubation transfer the mixture to a sterile eppendorf tube. Add 250_ul of the Neutralization solution. A white slime should form. Mix the solution well yet gently and centrifuge at full speed for 5_min. Decant the supernatant into an other sterile eppendorf tube and centrifuge again at full speed for 5_min.

Pour the supernatant into 15_ml falcon tubes and add 1_ml of the resin solution (mix well before use). Invert the samples as before and let sit 5_min while setting up the syringe-colomns on the pig.

To setup the syringe-columns on the pig, pull the plunger on the syringes. Then attach the white columns provided in the kit to 3_cc syringes and jam the tips of the columns in to the pig.

Gently invert the samples as before and pour them into the syringes. Use the aspirator vacuum to pull the solution through. Keep the vacuum on until the columns begin to dry.

Add 1.5_ml of wash solution and let the vacuum pull it through. (this should go slowly)

Remove the columns and place them on eppendorf tubes. Spin the tubes with the columns on top to fully dry them. Remove the column from the eppendorf tubes and place on fresh sterile labeled eppendorf tubes.

Add 35_ul of the 65 °C SddH_2O to each column. Wait 1_min. then add 40_ul more of the 65 °C SddH_2O. Wait another minute and centrifuge the eppendorf tubes with the columns at full speed for 3_min. to pull the water with the plasmid in to the tube. After obtaining The plasmid is now ready to be transferred into competent JM109 *E. coli*.

Determine the concentration of the plasmid by making a 20 fold dilution of the plasmid DNA and ddH_2O (10_ul DNA into 190_ul ddH_2O). Take a spec. reading at 260_nm. and enter that reading into the following equation.

(concentration in ug/ul)=($Spec_{260}$) x (50) x (20) X (.001)

It works out that the spec. reading equals the concentration in ug/ul.

$spec_{260} \div spec_{280}$ = relative purity (should be about 1.8)

Note: This DNA can be stored at -20 °C if you can not go on to the next step.

Step 7 Transforming the plasmid into JM109 *E. coli*

Prepair a water bath at exactly 42 °C.

Pre-chill on ice a 15_ml falcon tube for each transformation. Retrieve from the -80 °C freezer enough aliquoted JM109 competent cells for each annealing reaction and a control and place them on ice to thaw. (about 5_min.)

Transfer 100_ul of the cells into the pre-chilled falcon tubes.

Add 3_ul from the mini-prep to the JM109. Gently ster the mixture with the pipet while dispensing the plasmid. (For a control add 2_ul of SddH_2O)

Place the Falcon tubes quickly back on ice for 30_min.

Heat shock the cells by holding them in the 42 °C water bath for 45-50 sec.
Quickly place the tubes back on ice for 2_min. (DO NOT SHAKE)

Add 900_ul of room temp. SOC media to each falcon tube and incubate the cells for 90_min at 37 °C 225_rpm

After the incubation spread 75_ul of the SOC culture onto LB+antibiotic plates. Growth should only occur on the plates with the plasmid. (JM109 grow much faster than mutS. in 12 hours you should have a lot of solid single colonies.

Step 8 Isolating single colonies

Use a single colony from the over night LB+antibiotic plate to streak out a fresh LB+antibiotic plate in such a way as to get single colonies. streak out five plates from five colonies for each reaction. Seal with paraffin the overnight plate and store it in the cold room (4 °C) Watch carefully the growth of the JM109. If they grow too much it becomes difficult to obtain single colonies and non-mutated satellites begin to grow when antibiotics are used up. (About 14 hours)

From the five plates taken from single colonies find another single colony and streak it onto another fresh LB+antibiotic plate (your going for a lawn on this plate.) After these plates have grown they are ready to be harvested and archived. (Be sure every thing is well Labeled and documented)

Use the **mini-prep protocol** to harvest and determine the concentration of the new plasmid. Archive the cells using the *E. coli* archival protocol. The plasmid is now ready to be sequenced to check for the mutation.

Step 9 Sequencing

Aliquot 50_ul of each plasmid into a sterile labeled eppendorf tube. Use the sequencing chart to choose the proper primer. It should have a sweet spot where the mutation occurred. If the mutation is not found in any of the plasmids repeat step 7. If the plasmid contains the mutation proceed with the tramsformation or congression into Azotobacter vinilandi.

Harvisting dsDNA with a Promega Mini-prep

In a sterile 50_ml flask inoculate, with a loop of cells, from an overnight plate, 15_ml of LB+antibiotic media. Allow them to incubate over night (16_hr max) at 37 °C/ 300_rpm

Pour the cultures into 15_ml falcon tubes. Centrifuge the media in a table top centrifuge (it's in the cold room) for 15_min. Decant the supernatant.

With a inoculation loop resuspend the pellet in 500_ul of resuspention solution.

Add 500_ul of Lysis solution. Cap tightly and invert 10-12 times without getting the mixture in the lid. Incubate the mixture for 30_min at 37 °C - no shaking

After the 30_min incubation transfer the mixture to a sterile eppendorf tube. Add 500_ul of the Neutralization solution. A white slime should form. Mix the solution well yet gently and centrifuge at full speed for 8_min. Decant the supernatant into an other sterile eppendorf tube and centrifuge again at full speed for 5_min.

Pour the supernatant into 15_ml falcon tubes and add 2_ml of the resin solution (mix well before use). Invert the samples as before and let sit 5_min while setting up the syrenge-colomns on the pig.

To setup the syringe-columns on the pig, pull the plunger on the syringes. Then attach the white columns provided in the kit to a 3_cc syringes and jam the tips of the columns into the pig.

Gently invert the samples as before and pour them into the syringes. Use the aspirator vacuum to pull the solution through. Keep the vacuum on until the columns begin to dry.

Add 3_ml of wash solution and let the vacuum pull it through. (this should go slowly)

Remove the columns and place them on eppendorf tubes. Spin the tubes with the columns on top to fully dry them. Remove the column from the eppendorf tubes and place on fresh sterile labeled eppendorf tubes.

Add 55_ul of the 65 °C SddH_2O to each column. Wait 1_min. then add 100_ul more of the 65 °C SddH_2O. Wait another minute and centrifuge the eppendorf tubes with the columns at full speed for 2_min. to pull the water with the plasmid in to the tube. The plasmid is now ready to be transferred into competent JM109 *E. coli*.

Determine the concentration of the plasmid by making a 20 fold dilution of the plasmid DNA and ddH_2O (10_ul DNA into 190_ul ddH_2O). Take a spec. reading at 260_nm. and enter that reading into the following equation.

(concentration in ug/ul)=($Spec_{260}$) x (50) x (20) X (.001)

It works out that the spec. reading equals the concentration in ug/ul.

$spec_{260} \div spec_{280}$ = relative purity (should be about 1.8)

Transforming plasmid back into *A. vinelandii.*

To save time **Maker Rescue** and **Congression** are performed at the same time

Streak out on a BN_{Mo-} plate AV-trans(for Congression) and DJ54 or DJ525 depending on the region of the mutation(for marker rescue). Use DJ525 for mutations between amino acids 1-68, all other mutation require DJ54). Grow these cells on BN_{Mo-} plates for several generations (4-5).

4:00pm
From the 4th or 5th generation streak out a fresh BN_{Mo-} plate for each cell type. After 24 hours the growth should be fluorescent green.

Acid wash and sterilize four 125_ml flasks.

4:00pm next day
From these 24_hr plates obtain a small pellet and innoculate 50_ml of BN_{Mo-Fe-} media in the acid washed 125_ml flasks. Innoculate 2 flasks for each cell type. Incubate the cells for 20-25 hours (preferably 20_hr.) at 30°C/170_rpm.

11:00am
The media should now be a bright flourescent green. These cells are now competent and ready for Marker Rescue and Congression.

Marker Rescue Transformation Reaction.

In a sterile eppendorf tube add the following:

200_ul	1X MOPS
200_ul	competent DJ54/DJ525
20_ul	Mutant DNA

Also make a positive control with 20_ul of pLCS792 and a negative control without any DNA.

Allow the mixture to stand for 20_min.
Make a dilution series for the mutation transformation reaction:
0 (no DNA), 1:5 (60ul/240ul $SddH_2O$),1:25 (12ul/288ul $SddH_2O$)

Spread over B-plates 75_ul from each dilution.

Incubate the plate at 30°C. Healthy greenish growth should occur after 2-3 days on the positive control. No growth should occur on the Negative control. If growth appears after 2-3 days on the B-plates then the mutation is nif+. If growth appears after 4-6 days then the mutation is nif-slow. In either case you can disregard any result from the Congression. If no growth occurred the mutation is nif- and you must continue with the Congression

If growth occurred, take 6 colonies and streak them on fresh B-plates. Allow them to grow up for 2-3 days. Then streak them out again on fresh B-plates and allow them to grow for another 2-3 days. If they still look good streak out BN-plates from the b-plates in preparation for archiving the colonies. When a carpet of green cells appears, archive the cells mutated AV-cells.

Congression Transformation Reaction.
(do this with Marker rescue)
In a sterile eppendorf tube mix 40_ul of mutant DNA with 100_ng of pDB303. Now add the Following:

200_ul	1X MOPS
200_ul	Competent AV trans

Also make a positive control by adding only pDB303 to the AV trans/MOPS mixture, make a negative control by adding nothing to the AV trans/MOPS mixture

Allow the mixture to stand for 20_min.

Make a dilution series for the mutation transformation reaction:
0 (no DNA), 1:5 (60ul/240ul $SddH_2O$),1:25 (12ul/288ul $SddH_2O$)

Spread 75_ul of each dilution onto BN_{rif10} plates. Steak two plates for the 0 and 1:5 dilution (You may need the extra colonies later).

Incubate the plates at 30°C for 2-4 days. Growth should only occur on plates with the added pDB303.

To find nif- mutations, colonies must be grown on both B and BN plates. Those colonies that grow on BN-plates but not B-plates have the nif- mutation.

For each mutation reaction label five B and BN plates. The label should identify one B-plate to one BN-plate, and identify the top of each. Find a BN_{rif10} that has well defined isolated colonies. Line that plate next to a B-plate and a BN-plate. With the fat end of a sterile toothpick take a single colony from the BN_{rif10} plate and make a small streak (about 1cm) onto **first** the B-plate then onto the BN-plate. Repeat that step taking different colonies until you have filled all five B and corresponding BN plates with about 50 streaks each. (bring some music this take a long time)

After 2-3 days growth will occur on both plates. Every thing will grow on the BN-plates, but hopefully on the B-plates two or three streaks will not grow per plate. These streaks contain the nif- mutation. If the Congression was successful, out of five plate you should have about 5-15 nif- colonies.

Select six nif- colonies. Use the fat end of a sterile toothpick to remove the corresponding colonies growing on the BN-plates and streak it onto fresh B and BN plates. If growth only occurs on the BN-plate, streak out another BN-plate in preparation for archiving the cells.

QIAGEN protocol for ssDNA

Day 1 7:00am

From a frozen stock culture of JM109 pLCS792 (-80) inoculate a $LB_{+antibiotic}$ Incubate for 12-16 hours at 37° C
Make up some sterile 1_mM Tris pH 8.5 for tomorro

9:00pm

In two 50 ml vials innoculate 10_ml of $TYP_{+antibiotic}$ media with a loop of cells from the LB+Antibiotic plate. Incubate at 37° C, 300rpm, for 12-16 hours

Day 2 11:00am

Aliquot 5_ml of $TYP_{+antibiotic}$c media into three 50_ml flasks. Inoculate the media with 100_ul of the over-night culture. Incubate for 30 min. at $37°C/_{300rpm}$

Add 40_ul of R408 phage (-20). (Do not shake the R408) Return the flasks to the shaker and incubate for 5 hours.

5:30pm

Transfer each 5_ml cultures into three eppendorf tubes. Centrifuge the tubes at 5000_rpm for 15 min.

Pour the supernatant into a 15_ml Falcon tube. Be sure not to get any of the pellet. Centrifuge twice if needed. Add 50_ul of M13 Precipitation Buffer (1/100 volume). Vortex and incubate at room temp. for at least 2 min.
(This helps the phage stick to the column)

Setup two QIAprep spin columns by placing the QIA 2_ml centrifuge tubes in the centrifuge and inserting the QIA column into the 2_ml tubes. In 700_ul aliquotments spin the supernatant through the spin columns for 15_sec. at 8000 rpm. (set the timer to 8000_rpm and hold the timer at Zero for 15_sec.) After each spin drain the 2_ml tube so that the you can run the next aliquotment.

Wash the column by adding 700_ul of M13 Lysis and Binding Buffer. Spin at 8000_rpm for 15_sec. Add another 700_ul of the M13 Lysis and Binding Buffer and allow it to incubate at room temp. for at least 1 min. Spin and drain as before. (This lyses the protein capside and exposes the SSDNA.)

Add 700_ul of PE Wash Buffer. Spin and wash as before. Spin again for 15 sec. to be sure all of the PE Wash Buffer is out. (You don't want any residual ethanol)

Place the QIAprep spin column in a clean 1.5_ml eppendorf tube. Add 100_ul of 1_mM tris pH 8.5, incubate for 10_min, and centrifuge for 30_sec. (This pulls the DNA off of the column and into the solution.

Determin the concentration by making a 20 fold dilution (10_ul in 190_ul), taking a spec reading at 260_nm and using the following formula

Consentration in ug/ul $=(20)x(33)x(.001)x(spec._{260})$

Run an agarose gel to besure of the product.

Lable and store at -20°C

Running Agarose Gels.

Making the gel.

0.4_g agarose in 50_ml TAE buffer.

Mix in a 125_ml flask. Place a 50_ml flask upside down on top of the 125_ml flask for a lid. Heat the mixture in the microwave for 50_sec watching carefully for boiling. As soon as it begins to boil stop the microwave, thoroughly mix the agarose by swirling the flask, and continue heating the gel in the microwave until you are sure that the agarose has been completely dissolved. Allow the solution to cool slightly before pouring the gel in the gel setup.

Once the gel has hardened fill the gel holder with TAE buffer until it just covers the gel. Remove the comb and load the gel.

For double stranded DNA mix..

8_ul	TAE Buffer
2_ul	6x dye
2_ul	dsDNA

load 6_ul

Add 6_ul KB marker along the side.

For single stranded DNA mix:

10_ul	ssDNA
2_ul	6x dye

Load 10_ul

Add 6_ul KB marker along the side.

Run agarose gels at 60V until the dye passes the third red bar(2.5_in.)

When the gel has completely ran pour the TAE buffer and the gel into a Tupperware container and add 10_ul ethidium bromide for staining.

View the gel with UV light and draw a diagram of the gel in your notebook. (use protective eyeware when viewing the gel)

Protocol for Competent mutS. & JM109

Day 1 6:00pm

- In a sterile 10_ml Erlenmeyer flask aliquot 2.5_ml of sterile LB broth. Inoculate the broth with **ONE** colony (one dot) of mutS *E. coli*. Incubate the cells at 37°C at 225_rpm.

Make up 250_ml of LB + 20mM Mg SO_4 and sterilize. Also sterilize one of the 250_ml bent-necked centrifuge bottles. Bring warm cloths to lab for Day 2.

Day 2 8:00am

- Put the SLA-1500 rotor in the cold room.
- Pour the 2.5_ml of culture into a 1_L flask with 250_ml of sterile LB+20mM $MgSO_4$ media. Monitor the growth by taking a spec. reading. Go on to the next step when the cells reach 0.4-0.6 at 600_nM Blank the spec. to LB broth These cells grow very fast. check them after the first hour and every 20_min from there. They double about every 15_min. JM109 grow faster than mutS. Sometime before the cells are done start the refrigeration on the centrifuge.

- Pour the culture into a sterile 250_ml centrifugation canister. Fill another with water for balance. Using the SLA-1500 rotor spin the sample at 7000_rpm for 6_min at 4°C.

- Drain the supernatant and resuspend the pellet in 100_ml of T FB1 solution. Incubate the cells on ice, in the cold room, for 5_min.

- Pellet the cells again by spinning cells at 7000_rpm for 6_min at 4°C (remember to balance the other side).

- Resuspend the cells in 10_ml of TFB2 and incubate on ice in the cold room for about 60_min. During the incubation period prepare an isopropanol dry ice bath in a large tupperware or Styrofoam box. Place an eppendorf freezer box in the bath.

- After 60_min. aliquot the 10_ml into 110_ul samples (use the rubber gasket capped freezer tubes) Quick-freeze in the isopropanol bath. Make enough to fill a whole box and a few extra to test their competency. To test the competency follow the transformation step in the metageneses protocol. (Step 4 and 6)

Store the cells at -80°c . mutS should be good for at least three months.
JM109 should be good for one year.

Appendix

Things you need before you begin.

- The entire promega mutagenesis kit, including R408 phage.
- Harvested single stranded and double stranded pLCS792.
- Harvested pDB303 .
- Several sleeves of LB_{amp125} and Lb_{tet15} .
- Sterile LB media.
- Sterile Soc media.
- A lot of sterile eppendorf tubes.
- Soluble ATP
- Mutagenic Oligo
- Mops 1X buffer

Protocols for making these reagents can be found in the black protocol book.

(Linear) MAP of: Nif.Seq check: 2201 from: 1201 to: 2200

LOCUS AVINIFC 28795 bp DNA BCT 04-APR-1995
DEFINITION A.vinelandii major nif gene cluster encoding nitrogen fixation complex, complete cds.
ACCESSION M20568
NID g758356
KEYWORDS nif gene; nif gene cluster. . . .

March 24, 1997 12:14 ..

```
      CGAACGTTCAAGTGGAAATGCAACCTGAGGAAATTAACTATGGCTATGCGTCAATGCGCC
1201  ---------+---------+---------+---------+---------+---------+ 1260
      GCTTGCAAGTTCACCTTTACGTTGGACTCCTTTAATTGATACCGATACGCAGTTACGCGG

      R  T  F  K  W  K  C  N  L  R  K  L  T  M  A  M  R  Q  C  A   -6
                                                 └#1 Start of nif H


     ATCTACGGCAAAGGTGGTATCGGTAAGTCCACCACTACTCAGAACCTGGTGGCAGCCCTG
1261  ---------+---------+---------+---------+---------+---------+ 1320
      TAGATGCCGTTTCCACCATAGCCATTCAGGTGGTGATGAGTCTTGGACCACCGTCGGGAC

      I  Y  G  K  G  G  I  G  K  S  T  T  T  Q  N  L  V  A  A  L   -26

                                                          Primer #2, ←
      GCTGAGATGGGCAAGAAGGTCATGATCGTTGGTTGTGACCCGAAAGCTGACTCCACCCGC
1321  ---------+---------+---------+---------+---------+---------+ 1380
      CGACTCTACCCGTTCTTCCAGTACTAGCAACCAACACTGGGCTTTCGACTGAGGTGGGCG

      A  E  M  G  K  K  V  M  I  V  G  C  D  P  K  A  D  S  T  R   -46

          ——→
      CTGATCCTGCACTCCAAGGCCCAGAACACCATCATGGAAATGGCTGCCGAAGCCGGTACC
1381  ---------+---------+---------+---------+---------+---------+ 1440
      GACTAGGACGTGAGGTTCCGGGTCTTGTGGTAGTACCTTTACCGACGGCTTCGGCCATGG

      L  I  L  H  S  K  A  Q  N  T  I  M  E  M  A  A  E  A  G  T   -66


      GTGGAAGATCTGGAGCTGGAAGACGTGCTGAAGGCTGGCTACGGCGGCGTCAAGTGCGTT
1441  ---------+---------+---------+---------+---------+---------+ 1500
      CACCTTCTAGACCTCGACCTTCTGCACGACTTCCGACCGATGCCGCCGCAGTTCACGCAA

     V  E  D  L  E  L  E  D  V  L  K  A  G  Y  G  G  V  K  C  V   -86


      GAGTCCGGTGGTCCGGAGCCGGGCGTTGGCTGCGCCGGCCGTGGTGTTATCACCGCCATC
1501  ---------+---------+---------+---------+---------+---------+ 1560
      CTCAGGCCACCAGGCCTCGGCCCGCAACCGACGCGGCCGGCACCACAATAGTGGCGGTAG
           ←——  Primer #1
      E  S  G  G  P  E  P  G  V  G  C  A  G  R  G  V  I  T  A  I   -106
```

```
     AACTTCCTGGAAGAGGAAGGCGCCTACGAAGACGATCTGGACTTCGTATTCTACGACGTG
1561 ---------+---------+---------+---------+---------+---------+ 1620
     TTGAAGGACCTTCTCCTTCCGCGGATGCTTCTGCTAGACCTGAAGCATAAGATGCTGCAC

     N  F  L  E  E  E  G  A  Y  E  D  D  L  D  F  V  F  Y  D  V   -126

                                  Primer #14 →
     CTGGGCGACGTGGTGTGTGGCGGCTTCGCCATGCCGATCCGCGAGAACAAGGCCCAGGAA
1621 ---------+---------+---------+---------+---------+---------+ 1680
     GACCCGCTGCACCACACACCGCCGAAGCGGTACGGCTAGGCGCTCTTGTTCCGGGTCCTT

     L  G  D  V  V  C  G  G  F  A  M  P  I  R  E  N  K  A  Q  E   -146

     ATCTACATCGTCTGCTCCGGTGAGATGATGGCCATGTACGCCGCCAACAACATCTCCAAG
1681 ---------+---------+---------+---------+---------+---------+ 1740
     TAGATGTAGCAGACGAGGCCACTCTACTACCGGTACATGCGGCGGTTGTTGTAGAGGTTC

       Y  I  V  C  S  G  E  M  M  A  M  Y  A  A  N  N  I  S  K   -166

     GGCATCGTGAAGTATGCCAACTCCGGCAGCGTGCGTCTGGGCGGCCTGATCTGCAACAGC
1741 ---------+---------+---------+---------+---------+---------+ 1800
     CCGTAGCACTTCATACGGTTGAGGCCGTCGCACGCAGACCCGCCGGACTAGACGTTGTCG

     G  I  V  K  Y  A  N  S  G  S  V  R  L  G  G  L  I  C  N  S   -186

                                  Primer #15 →
     CGTAACACCGACCGCGAAGACGAGCTGATCATCGCTCTGGCCAACAAGCTGGGCACCCAG
1801 ---------+---------+---------+---------+---------+---------+ 1860
     GCATTGTGGCTGGCGCTTCTGCTCGACTAGTAGCGAGACCGGTTGTTCGACCCGTGGGTC

                  ← Primer #25
     R  N  T  D  R  E  D  E  L  I  I  A  L  A  N  K  L  G  T  Q   -206

     ATGATCCACTTCGTGCCGCGTGACAACGTCGTGCAGCGCGCCGAAATCCGCCGCATGACC
1861 ---------+---------+---------+---------+---------+---------+ 1920
     TACTAGGTGAAGCACGGCGCACTGTTGCAGCACGTCGCGCGGCTTTAGGCGGCGTACTGG

     M  I  H  F  V  P  R  D  N  V  V  Q  R  A  E  I  R  R  M  T   -226

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1921 ---------+---------+---------+---------+---------+---------+ 1980
     CACTAGCTTATGCTAGGCTTTCGGTTCGTTCGGCTGCTTATGGCGCGAGACCGGGCGTTC

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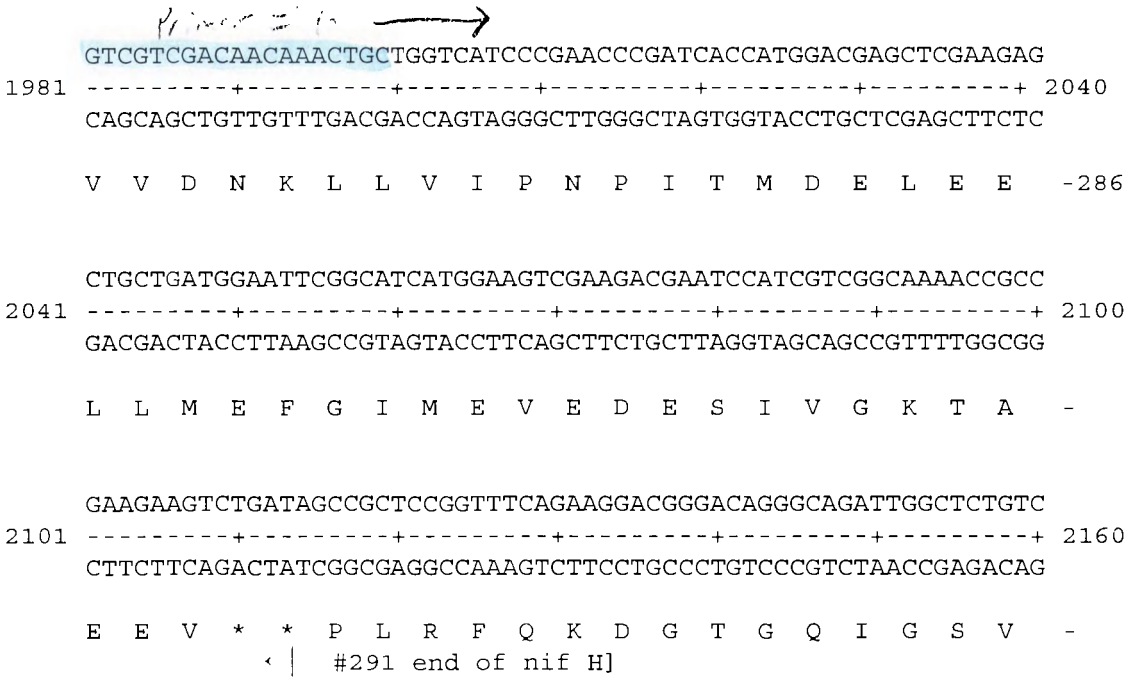
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1981 ---------+---------+---------+---------+---------+---------+ 2040
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V V D N K L L V I P N P I T M D E L E E -286
CTGCTGATGGAATTCGGCATCATGGAAGTCGAAGACGAATCCATCGTCGGCAAAACCGCC
2041 ---------+---------+---------+---------+---------+---------+ 2100
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L L M E F G I M E V E D E S I V G K T A -
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2101 ---------+---------+---------+---------+---------+---------+ 2160
CTTCTTCAGACTATCGGCGAGGCCAAAGTCTTCCTGCCCTGTCCCGTCTAACCGAGACAG
E E V * * P L R F Q K D G T G Q I G S V -
#291 end of nif H]