# Tagging GluR5 with GFP; a random insertion strategy that uses the Tn5 transposon.

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## *Day 1.*

**Reagents, Supplies and Solutions:**

pBNJ24.6 plasmid (map at: http://momotion.cns.montana.edu)
Hind III enzyme
Hind III buffer (10X)

*Pfu* buffer (10X)
*Pfu* enzyme
Nucleotides
ME primer #63288 (5' CTGTCTCTTATACACATCT 3')
Eppendorf tubes (0.5 ml)

pcDNA6.2DESTGluR5 DNA (100 ng/μl)

Tn5 transposase (available through www.epicentre.com)
10X EZ::TN buffer
Stop solution

Qiagen MinElute PCR Purification kit

Electrocompetent E. coli
SOC
0.1 gap width cuvette for electroporation
Falcon 5029 tube or equivalent
1 LB Amp plate
8 LB Amp + Kan plates

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**The goal of the day is:**

The first step is to create enough of the transposon. This can be done by amplifying it from the plasmid pBNJ24.6 with PCR. This reaction requires only a single primer that is complimentary to the outer ends (Mosaic Ends or MEs) of the transposon. We will be cutting the plasmid we are using for template before the PCR however. This is simply to minimize the number of possible plasmids that we might recover in subsequent transformation steps.

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## I) Linearize the plasmid carrying the transposon.

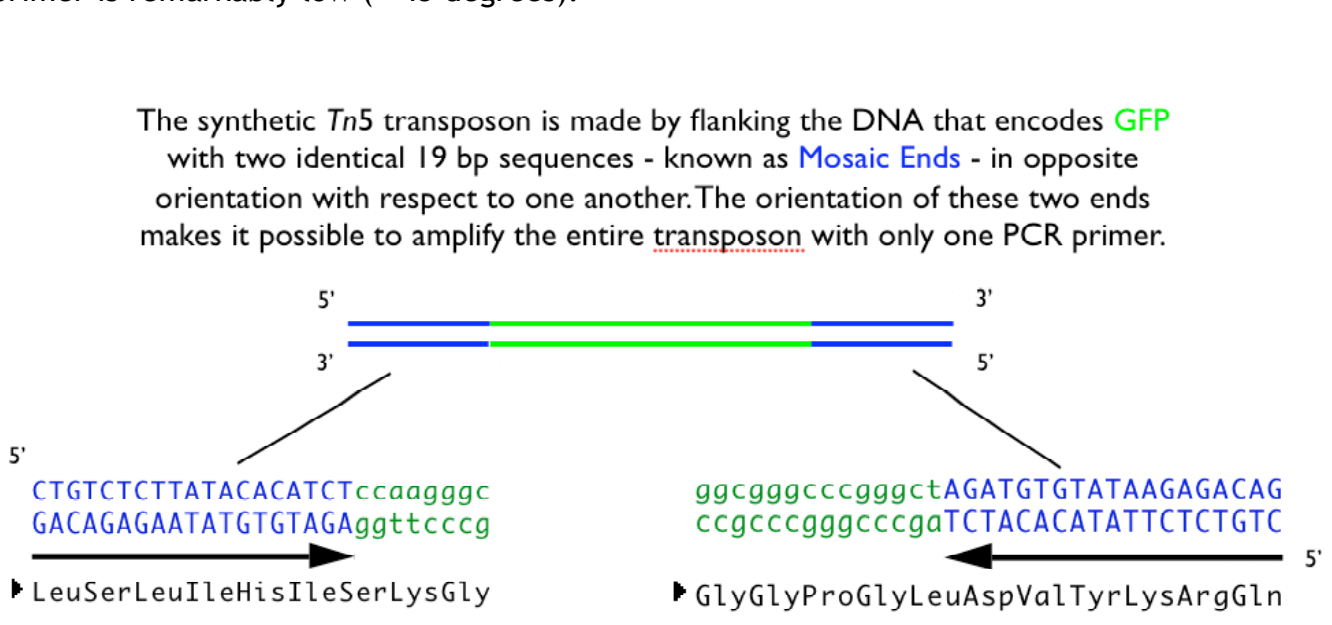
- Digest ~100 ng of pBNJ24.6 with *Hind* III. Mix the following, in order, in a 0.5 ml tube on ice. Remember to mix the final reaction mix by pipetting up and down a few times.

| | |
|---|---|
| DH20 | 7.5 µl |
| Transposon template | 1 µl |
| Hind III | 0.5 µl |
| 10X NEB buffer #2 | 1 µl |
| Total Volume | 10 µl |

- Incubate at 37 $^0$C for 1 hr.
- Heat inactivate the reaction at 65 $^0$C for 20 minutes.
- Store the excess reaction at -20 $^0$C.

## II) Amplify the Transposon from the Linearized Vector with PCR.

Use a single primer complementary to the EZ::TN transposon Mosaic end (ME), to amplify the transposon with the high fidelity polymerase Pfu. This works because the MEs of the transposon are an inverted pair of identical 19 bp sequences on either end of the transposon. The only strange part of this is that the annealing temperature of the ME primer is remarkably low ( ~45 degrees).



□ Mix the following, in this order, in a thin walled tube suitable for PCR cycling.

| | |
|---|---|
| $ddH_20$ | 106 μl |
| 10X Pfu buffer | 12.5 μl |
| dNTPs (25 mM) | 1 μl |
| Digestion Reaction from step 1 | 2.0 μl |
| ME primer | 2.5 μl |
| Pfu Polymerase (2U/μl) | 2.5 μl |
| total volume | 125 μl |

□ When you have mixed the reaction well, by pipetting up and down, amplify the transposon with the following temperature protocol:

| 94 $^0$C | 47 $^0$C | 72 $^0$C | Cycles |
|---|---|---|---|
| 4 min.. | 0 min.. | 0 min.. | 1 |
| 1 min.. | 30 sec. | 5.30 min. | 24 |
| 0 | 0 | 10 min. | 1 |

□ Use the MinElute PCR Purification Kit to remove excess nucleotides and primers. Transfer one half of your PCR reaction to a new, larger tube and add 5 times the volume of Buffer PB to the tube. Put the spin column in the collection tube, and then transfer the PCR reaction the spin column. Centrifuge for 1 minute, remove the spin column and discard the flow-through. Replace the cartridge, and add 750 μl of the PE buffer. Centrifuge for a minute, remove the flow through, and then centrifuge again to remove any traces of the PE buffer. Now transfer the spin column to a fresh tube, add 20 μL of EB to the center of the membrane, wait one minute, and then centrifuge. Label the tube.

□ Resolve 4 μl of the PCR reaction on an agarose gel (1%) along with a lane of markers and 4 lanes of 1, 2, and 4μl of the quantified standard that we will give you. *Remember to wear appropriate eye protection when looking at the gel on the transilluminator*. Photograph the gel for documentation in your notebook and use a comparison of your sample with the standards to estimate the concentration of your PCR product.

*A 1 to 1 mole ratio of target/transposon during transposition is optimal for the transposition reaction. Deviation from this ratio can result in either low yield of transposed clones or increased frequency of multiple transposon insertions in a given plasmid.*

## III) Transpose the Target Plasmid.

- Using the estimate from your gel, set up a transposon reaction with your amplified transposon and your pGluR5DEST target plasmid. Your are aiming at getting 200 ng of the target plasmid (~7,000 bp) and 50 ng of transposon (1,900 bp) in the same tube with the transposace and buffer.

*It is critical that the DNA used in this reaction, both transposon PCR product and target plasmid, has NOT been previously exposed to Ethidium Bromide and a transilluminator. This will kill the magic and waste a lot of your time....*

| | |
|---|---|
| dd$H_2$0 | --- |
| 10X EZ::TN buffer | 1 μl |
| target plasmid | 200 ng |
| Transposon (your PCR product) | ? μl |
| EZ::TN transposase | 1 μl |
| total volume | 10 μl |

- Incubate at 37$^0$C for 2 hr.
- Add 1 μl of 10x Stop Solution.
- Heat inactivate at 70 $^0$C for 10 min. (Reaction can be stored at -20 $^0$C for >6 months)

*Never skip the step where you add the Stop Solution (1% SDS) and heat inactivate the transposition reaction. The transposon/transposase complex is very stable and will readily transpose the E.coli genome after transformation. This will give you lots of Kan resistant E.coli and desperately few transposed plasmids.*

## IV) Transform E. coli with Transposition Reaction.

It is critical to use electroporation to transform with the transposition reaction. Chemical transformations don't work. After transformation and an hour of growth, you plate the reaction on Amp + Kan as well as on plates that only contain Amp. A comparison of the two tomorrow will provide you with a way of calculating the efficiency of your transposition reaction.

- Pre chill a 0.1cm gap cuvette on wet ice, pre warm the SOC media to 37 $^0$C, put one Amp and eight Amp + Kan plates in the incubator to warm and dry.
- Carefully pipette 0.5 μl of the transposition reaction onto the back wall of a pre

chilled 0.1 cm gapped cuvette. Now add 75 μL (or the manufacturer's recommended amount) of electrocompetent *E. coli* and pipette gently to mix the DNA and cells. Shock the cuvette contents with a 1.5 kV pulse and then quickly add 1 ml of pre warmed SOC. Pipette up and down to mix, transfer to a Falcon 5029 tube, and incubate at 37 0C, shaking, for 40 minutes.

*This part of the process is all about competence. The transposition reaction only inserts a transposon into ~1% of the target plasmids in the tube: you need very competent cells to get a decent yield.*

- ☐ Plate 10 μl (dilute 10 μl of the transformation with 90 μl of fresh broth) on Ampicillin only plates as a control.

- ☐ Plate 100 μl each on 8 LB Agar plates with 100 mg/mL Ampicillin and 50 mg/mL Kanamycin. Remember to label the bottom of your plates with your name, and the page number of your lab notebook.

  *This process generate a lot of tube and a ton of plates. A week from now you'll be looking at the plate wondering what on earth it is. The page number of your notebook will save you when nothing else can.*

- ☐ Incubate plates at 37 $^0$C overnight (~ 14 hr.).

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## *Day 2.*

**Reagents, Supplies and Solutions:**

2.5 ml of LB with Amp (100 mg/ml)
125 ml LB with Amp + Kan (100 mg/ml, 50 mg/ml)
Autoclaved wooden toothpicks
96 deep well growth plates from the EppendorfPerfectPREP 96-well DirectBind VAC kit.

Multichannel pipette and tips (100 μl - 1200 μl range)
Flask of HEK 293 cells
(MEM-E + 10% FBS)
Trypsin-EDTA solution
hemocytometer
96 well, glass-bottom tissue culture plate (Nalge-NUNC cat. # 164588)
LB Amp plate with colonies of pWay19 (map at http://momotion.cns.montana.edu)
LB Amp plate with colonies of pFIg262 (map at http://momotion.cns.montana.edu)

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**The goal of the day is:**

Twofold. First you'll want to figure out if your transposition worked, and how well it worked. Then you want to pick clones from the reaction for growth. At the same time, you'll want to prepare your tissue culture ahead of time. This involves plating cells now so that you can transfect them tomorrow.

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## I) Analyze the efficiency of the transposition reaction.

- ☐ Count the number of colonies on your Amp + Kan plate as well as your control Amp only plate. How efficient was your transposition reaction? How efficient was your transformation?

## II) Inoculate Cultures.

- ☐ Aliquot 1.25 ml of LB with Amp into wells A1 and A2 of the 96-well deep-well culture plates. Aliquot 1.25 ml of LB with Amp + Kan into the remaining 94 wells.

- ☐ Use the wooden toothpicks to inoculate the first well (A1) of your plate with the clone pWay19. This is a plasmid that produces robust GFP signals that cannot be missed. Inoculate the second well of your plate (A2) with clone pFlg262. This is a clone encodes a GFP tagged glutamate receptor. It is dim, and represents a worst case scenario in terms of signal. This will be very useful tomorrow.

- ☐ Using toothpicks, inoculate the rest of the 94 wells of the clones on your LB amp + kan plates. It helps to leave the toothpicks in the wells as you're doing this to avoid the space out factor that inevitably leads to inoculating a well twice.

- ☐ Incubate at 37 $^0$C, shaking, for 20-22 hours.

## III) Plate HEK 293 Cells for Transfection

- ☐ Plate ~6 x 104 HEK293 cells in 100 μl of culture media (MEM-E + 10% FBS) per well in a 96-well optical glass-bottom tissue culture plate. The advantage of using this glass bottom plate is that you can use more efficient lenses and will be able to see dim constructs that you would not see if you were using plastic bottom dishes.

*Day 3.*

**Reagents, Supplies and Solutions:**

Centrifuge adapted for 96 well plates
EppendorfPerfectPREP 96-well DirectBind VAC kit
Eppendorf manifold system and Vacuum source.
Eppendorf electronic, programmable, 8 channel pipette (100μl - 1200μl range)
2 racks of pipette tips for the 8 channel pipette
20% filter sterilized glycerol in water (10 ml)
standard 350 μl 96 well plate

Lipofectamine 2000
OptiMem I solution (10 ml in a sterile test tube)
96 well PCR plate
Multichannel Pipettes (0-10μl range and 10 - 100μl range)
Sterile tips for multichannel pipettes

**The goal of the day is:**

Today is pretty involved. First you need to miniprep the 96 clones you picked yesterday. In turn, you'll use a small part of this miniprep DNA to transiently transfect the HEK 293 cells that you plated yesterday.

## I) Minipreps in a 96-well Format

This protocol is miniprep intensive. One could do it with something like the Qiagen spin kits for minipreps, but it would be very time consuming and staggeringly boring. We use a 96 well format that is sold by Eppendorf. With practice, a single plate of 96 clones can be processed in ~ 45 minutes, and should the spirit move you 4 plates take only an hour and a half. The DNA it produces is sufficient, in terms of quality and quantity, for sequencing reactions and more importantly transient expression in HEK 293 cells.

- Using the multichannel pipette (5 to 100 μl), aliquot 100 μl of 20% filter sterilized glycerol into each well of a standard 96 well plate. Then use the pipettor to transfer 100 μl of each of your cultures to the plate. Put the plate in the -70 freezer for 20 minutes to freeze, and then put it in ziplock bag in the freezer for safe keeping.

  *You can skip this step if you don't think you'll ever want this clone again. There are times that we just miniprep the whole thing, and if we need a particular clone again we just transform with the miniprep DNA. However, doing the glycerol stocks at this*

*point can save you a whole lot of transformations and time in the future and it doesn't take that long.*

- ☐ Prepare the manifold you will be using. Place the short adaptor plate in the manifold chamber, then position the Filter plate DB over the adaptor. Close the lid of the manifold. Make sure that the plates are in the same orientation. Place the Filter plate A on the top.

- ☐ Make sure that your Purification Solution has been diluted with ethanol.

- ☐ Pellet the over night cultures. Put the deep well plate of overnight cultures - with the lid on - in the centrifuge and spin at 1900 x g for 5 minutes. Check that there are good pellets in the wells by holding the plate over your head. Quickly invert the plate to drain media, place the plate upside down on a paper towel to drain off excess media.

  *This is a great place to stop for the day. You can freeze these pellets and process the minipreps later. Some in our lab swear that the frozen pellets miniprep better than the fresh ones.*

- ☐ Using program 1 on the pipettor (50 - 1200 μl range), add 150 μl of solution 1 into each well.

- ☐ Put the sealing tape onto the top of the plate, and let it sit for 5 minutes. Then vortex the entire plate for ~ 2 minutes to re suspend the culture. Check that it is completely re suspended by looking at the bottom of the dish while you hold it over your head. Good re-suspension is critical for a good yield.

- ☐ Remove the sticky tape and using program 1 on the pipettor add 150 μl of Solution 2 to each well. Tap the plate onto the palm of your hand 10 times, rotate the plate 180 degrees and tap 10 times more. This is sufficient to mix the reagents. (Do not leave in solution 2 for more than five minutes)

- ☐ Using program 1 on the pipettor, add 150 μl of solution 3 to each well. This neutralizes the bacterial lysate. Put a new piece of sticky tape on the top again, and then use the inverted lid of the deep well growth plate to maintain pressure across the entire top of the plate to hold the sticky tape in place. Invert this entire sandwich very gently 10 times to mix.

- ☐ Prepare the Vaccum manifold. You want to open up the manifold and put a small spacer plate. Then you put in the DB plate. Now close the manifold and put the Filter plate A on top. The plan here is to add the lysate to the filter plate, pull a vaccum, and have only the liquid and DNA end up in the DB plate below.

- ☐ Carefully peel off the sticky tape - to avoid well to well contamination - and then using program 2 of the pipettor, transfer the lysate (450 μl) to the corresponding wells of Filter plate A on the vacuum manifold.

- Gradually apply vacuum to the manifold and maintain for five minutes. Then allow the vacuum to gradually bleed off by first backing off the bleed screw and then turning off the vacuum. DO NOT use the black switch to close the vacuum line. A fast change in the vacuum will cause the plates to bounce, rattle and rumble, ruining your samples and your day.

- Remove the Filter plate A from the manifold and throw away. Open the manifold and carefully lift out the filter plate DB. Take out the short adaptor plate, and place the used, deep well culture plate in the manifold to catch the waste in the manifold. Close the manifold and place the filter plate DB on top. Using program 3 on the pipettor, add 300 μl of binding buffer to each well in the filter plate DB. Be careful at this step to hold the pipette tips just above the well, not in it, because the binding buffer will fill the well right to the top. Gradually apply vacuum to the manifold by turning on the vacuum and slowly closing the bleed screw.

- When all the wells of the plate are empty, slowly bleed of the vacuum by opening the bleed screw and then turning off the vacuum. Using program 4, add 400 μl of purification solution (that you have previously diluted with ethanol). Gradually apply vacuum for a minimum of five minutes.

- Gradually release the vacuum, remove the filter plate DB, and gently blot the bottom of the filter plate on paper towels until all traces of ethanol are gone from the bottom of the plate. You can tap the plate on the paper towels, but if you slam it down you'll mess up the pointy tips on the bottom of the plate (not good).

- Place a new 350 μl collection plate on top of a tall adapter inside the vacuum manifold. Close the manifold chamber and place the filter plate DB on top of the manifold lid. Make sure both plates are in the same orientation.

- Using program 5 on the pipettor, add 70 μl of water to each well. Take care to add the water straight down the well so it lands on squarely on the bottom rather than on the side of the well. Wait for one minute, then gradually apply vacuum for five minutes. Gradually release the vacuum and recover the collection plate. Seal the plate with a new piece of sticky tape. Label this plate (not just the lid) with your name and the page number of your lab notebook. This is your master plate.

## II) Transfection of HEK293 Cells in a 96-well Format

*The goal here is to create a mix of 350-600 ng of DNA and 0.3 μL of Lipofectamine 2000 in 50 μL of OptiMEM I per well in a 96 well plate and then transfer this mix to the plate with your cells. All of this work is done in a sterile tissue culture hood.*

- Using the multichannel pipette, aliquot 19 μl of OptiMEM I (Gibco) per well in a 96-well PCR plate.

- Using the multichannel pipettor, add 6 μl of miniprep DNA (~350-600 ng DNA).
- Put 2500 μL of OptiMEM I in a separate, sterile tube, and then add 30 μL of Lipofectamine 2000 (Invitrogen). Mix by pipetting up and down.
- Incubate for 5 minutes at room temp.
- Using the multichannel pipettor, add 25 μl of Lipofectamine/OptiMEM I mix to each well of DNA mix.
- Incubate at room temp for 20 min.
- Add transfection mixture to the cells in 100 μl of media plated yesterday.

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*Day 4.*

## Reagents, Supplies and Solutions:

Epifluorescence microscope fitted with FICT optics (480 excitation, 530 long pass emission)

*Srf* I enzyme & universal buffer (Stratagene)

Qiagen MinElute PCR purification kit

Fast link ligation kit (Epicenter)

Electrocompetent E. Coli
0.1 cm gapped cuvette for electroporation
SOC
LB Amp plate
LB Amp + Kan plate

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**The goal of the day is:**

Today you will use the fluorescence microscope to search for clones that encode fluorescent fusion proteins. When you find them, you will return to your mini prep DNA to digest and ligate the clones to create the full length, tribrid, fusion protein.

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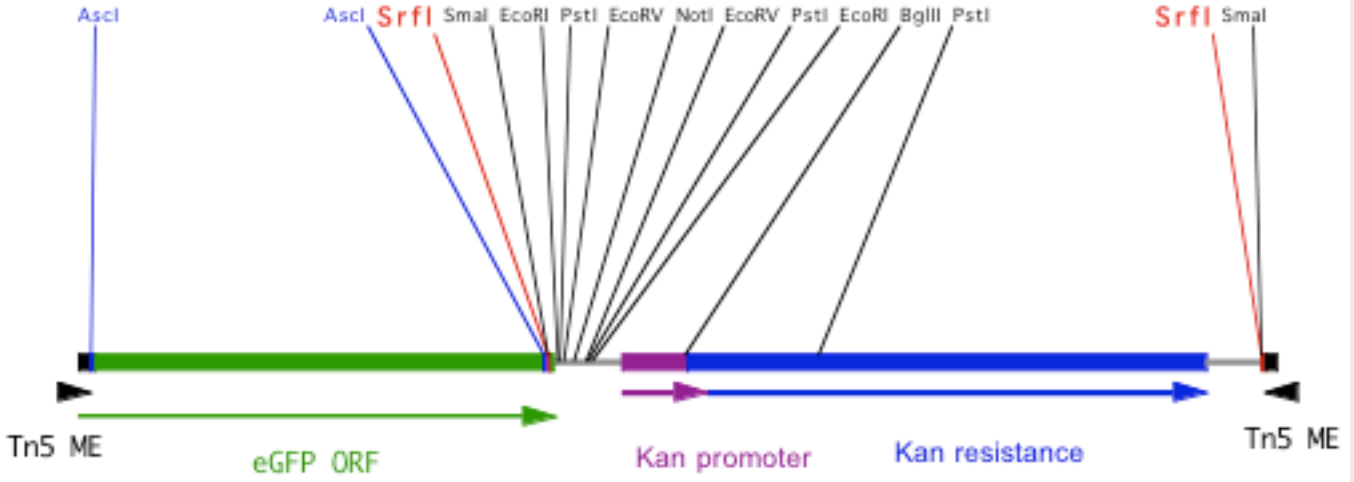
## I) Screen live HEK 293 cells for GFP fluorescence.

The screen for fluorescent colonies involves systematically going through the plate from one well to the next to look for fluorescence. Use DIC optics or Phase contrast to make sure that you are actually in the right focal plane, and then switch to fluorescence illumination (any FITC compatible filter set will work for this; to order your own set, see http://chroma.com/ or http://www.omegafilters.com). If the clone you are using does not express that well, you will need to use objectives with a high numerical aperture (1.2 to 1.4) or you will miss some the positive clones. The first control well (A1) that you inoculated with the GFP expression plasmid pWay19 will show you the best possible signal you can hope for, the second well (A2) is a worst case scenario. Make sure that you can see both clearly before pressing on. Then systematically work through the plate looking for fluorescent cells. If you have doubts about the signal, and auto fluorescence can at times generate a dim yellow that is confusing, switch to the RITC optics. Auto fluorescence will still be visible, the GFP signal will not. Keep careful records of which clones produced fluorescent cells at this point or all hope of success is lost.

## II) Digest to remove the Kanamycin cassette from the clones that produce fluorescent signals.

- The Kan cassette in the transposon is flanked by *Srf* I sites. *Srf* I digestion will remove this cassette so that you can create the full length fluorescent protein.




- Now that you have identified the clones that produce a fluorescent fusion protein, go back to your master plate and use the miniprep DNA to set up the necessary digests to remove the Kanamycin cassette within the transposon. If you have more than one clone to process, make up a reaction mix and then add aliquots of the reaction mix to tubes with each of your clones.

| | Single Reaction | 10 Reaction Master mix |
|---|---|---|
| dd$H_20$ | 4.5 μl | 45 μl |
| 10X Stratagene Universal buffer | 1 μl | 10 μl |
| Srf I | 0.5 μl | 5 μl |
| Plasmid DNA | 4 μl | -- |
| total volume | 10 μl | aliquot @ 6 μl |

- Digest for 1 hour at 37 $^0$C.
- Clean up with the MinElute PCR clean up kit. Elute with 10 μl of dd$H_20$.
- Re-ligate the digested plasmid

Mix, in order, on ice, the following ligation reaction.

| | |
|---|---|
| ddH20 | 7.75 μl |
| 10X fast link buffer | 1.5 μl |
| 10 mM ATP | 0.75 μl |
| Srf I digestion reaction | 4 μl |
| fast link ligase | 1 μl |
| Total volume | 15 μl |

*The efficiency of ligating two different pieces of DNA together is a directly related to the insert/vector ratio AND the molar concentration of free ends. If the concentration of free ends is high enough, you will get recombinants in which two different pieces are joined to one another. The converse of this rule is true too. If you dilute the sample enough, you will favor the plasmid closing on itself without an insert. Here we simply dilute the restriction digestion we just did and set up a ligation reaction that favors the plasmid closing on itself without the KAN cassette.*

- Incubate at room temperature for 15 minutes.
- Heat inactivate at 70 $^0$C for 15 minutes (failure to inactivate can cause decreased transformation efficiencies).
- Transform electrocompetent E. coli with 0.5 μl of the ligation reaction using the transformation protocol from day 1. Plate 100 μl of the transformation on an LB Amp

plate and 100 μl on an LB Amp + Kan plate.

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## *Day 5*

**Reagents, Supplies and Solutions:**

LB with Kan + Amp
LB with Amp
culture tubes
Inoculation loop
Qiagen miniprep spin kit
Eppendorf tubes
Pipette tips

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**The goal of the day is:**

Pretty simple. You are looking for the clone that no longer contains KAN resistance and then you'll grow it up and miniprep it. The simplest way to check each clone for the loss of the Kan cassette is to grow each clone in duplicate tubes of broth with Amp OR with Amp + Kan.

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- ☐ Using your plates from yesterday, calculate the proportion of clones that still have the Kanamycin cassette. Using this calculation, estimate how many clones you will have to grow up to ensure that one of them does not have the Kanamycin cassette.
- ☐ Set up pairs of culture tubes with 1 ml of LB Amp and 1 ml of LB Amp + Kan.
- ☐ Using a Sterile inoculation loop, pick individual colonies from your plate and inoculate first the tube with Amp and then the other tube with Amp + Kan. Grow up the clones in the shaking incubator for at least 12 hours.
- ☐ Find the pair of tubes in which the clone has Ampicillin resistance but no Kanamycin resistance.
- ☐ Using the Quigen miniprep kit, miniprep the clone.
- ☐ Use a *Sma* I digest to check your clone and verify the loss of the Kan cassette. This works because Sma I recognizes the inner six base pairs of the Srf I site set up digestions with both your clones as well as the parent plasmid.

**Notes & References for further reading**

The hyperactive transposace is available through Epicentre (http://www.epicentre.com/main.asp). Epicentre has generously provided this and many other reagents to our course over the years. They offer both this enzyme as well as a number of prepared transposons that are remarkably useful tools for rapidly sequencing plasmids, for creating sublibraries of BACs, and for creating deletions. There are a variety of hyperactive transposons available at this point, but Tn5 is one of the few that has fortuitous reading frames that cross the outer, or mosaic, ends.

To learn more about the Tn5 tranposon, there are several excellent reviews.

Reznikoff, W. S. (2003). Tn5 as a model for understanding DNA transposition. Mol Microbiol *47*, 1199-1206.

Reznikoff, W. S., Bhasin, A., Davies, D. R., Goryshin, I. Y., Mahnke, L. A., Naumann, T., Rayment, I., Steiniger-White, M., and Twining, S. S. (1999). Tn5: A molecular window on transposition. Biochem Biophys Res Commun *266*, 729-734.

If you're intriguied by transposons ingeneral, CSHL press has recently published a really superb book on the subject:

Bushman, Frederic (2001) Lateral DNA Transfer: Mechanisms and Consequences. CSHL Press.

To read the original paper in which we describe this entire procedure, see:

Sheridan, D. L., Berlot, C. H., Robert, A., Inglis, F. M., Jakobsdottir, K. B., Howe, J. R., and Hughes, T. E. (2002). A new way to rapidly create functional, fluorescent fusion proteins: random insertion of GFP with an in vitro transposition reaction. BMC Neurosci *3*, 7.

For more background on tribrid fusion proteins, here are several interesting applications of the approach:

Manoil, C., and Traxler, B. (2000). Insertion of in-frame sequence tags into proteins using transposons. Methods *20*, 55-61.

Nelson, B. D., Manoil, C., and Traxler, B. (1997). Insertion mutagenesis of the lac repressor and its implications for structure-function analysis. J Bacteriol *179*, 3721-3728.

Ross-Macdonald, P., Sheehan, A., Roeder, G. S., and Snyder, M. (1997). A multipurpose transposon system for analyzing protein production, localization, and function in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A *94*, 190-195.

Ross-Macdonald, P., Coelho, P. S., Roemer, T., Agarwal, S., Kumar, A., Jansen, R., Cheung, K. H., Sheehan, A., Symoniatis, D., Umansky, L., *et al.* (1999). Large-scale analysis of the

yeast genome by transposon tagging and gene disruption. Nature *402*, 413-418.