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iGEM09: Assembling devices made of several parts - Aar1 shuffle:

Goal: Something we need to do often is connecting several pieces of DNA. Here we want to connect 3 pieces in one reaction. We want to connect each A-B piece via the B-C piece to each C-D piece and ligate those into an A-D vector backbone.

Overview: you will digest the **A-B** and **C-D** DNA elements with Aar1 to generate the required elements, purify the DNA and ligate into an Aar1 cut vector. The backbone pHO189 and the B-C piece is ready for ligation as is. DO NOT DIGEST THEM FURTHER. **SOMETIMES YOU MIGHT USE A DIFFERENT B-C PIECE OR BACKBONE- DOUBLE CHECK WITH BUDDY!**

Materials: see appendix 1

GENERAL RULE FOR ALL YOUR REACTIONS: ADD ENZYME LAST!

1) Digest parts with Apa1 (check with buddy first!)

Before we generate the respective ends with Aar1 we digest the vectors containing the elements with Apa1. This enzyme cuts just outside the Aar1 site and will reduce the background of religated original vector in your experiment.

Let's set up the reactions!
Calculate the needed amount of water and DNA first and start with that.

DNA:	7-10 ug
Buffer 4:	15 ul
BSA:	1.5 ul
Apa1:	7ul
Sterile H2O:	x ul
	150ul

Leave on the bench (25C) for 3-4 hours. O/n is also possible.

2) PCR purification:

Next we need to get rid of buffer 4 to digest with Aar1.
Follow the Quiagen PCR purification protocol (can be used for DNA fragments – not only PCR). Elute with 50ul sterile H2O.
Possible to store now.

3) Digest with Aar1 (o/n):

Now here comes the real thing. We digest with Aar1 to generate the A-B and C-D fragments. (Why Aar1? We use the restriction enzyme Aar1 because it cuts 4 bases away from where it sits and generates non palindromic overhangs. We have standardized the sequence of the overhangs generated and call them A,B,C and D).

For each reaction use the 50ul DNA from the previous digest.

DNA:	50 ul
10x Aar1 Buffer:	13.5ul
50x oligonucleotide	2.7 ul (a weird thing to add to a digest, but Aar1 is unusual ☺)
Aar1	3.5 ul
Sterile H2O	65.3ul
	135ul

digest for at least 3-4 hours (o/n works well in my hands). Aar1 should then be inactivated by incubation for 20 min at 65C (otherwise Aar1 can remain bound to digested DNA).

4) Gel purification:

After cutting we want to separate the vector backbone from our insert of interest.

Pour a 0.8% agarose gel containing SYBR Safe (1:20000); add 6ul DNA loading dye to each sample.

CAUTION! The point of the following loading scheme is to be able to cut DNA out of the gel, yet avoiding to use the DNA you have exposed to UV in your ligation later on. Dicty DNA is very sensitive to UV and usually can not be used for cloning after exposure (UV induces crosslinks, double strand breaks,…)
Make sure you understand the following before you begin any practical work:

Load 6ul DNA ladder in lane 1. Load 6ul of DNA sample 1 in lane 2 (marker lane). Load the rest (50ul) of that particular sample in well 3 and 4. (You can also tape two adjacent teeth together to make one large well/product to be purified instead of well 3+4). Leave one well (5) empty. Load 6ul of DNA sample 2 in lane 6 (marker lane sample 2) and 50 ul in well 7 and 8 (or taped together well).

Draw your scheme here:

Run gel (I use 100 volts). Once the product has been resolved, cut out the marker lane(s) of the gel and examine on a UV trans-illuminator. **Do not expose the DNA that you wish to purify to UV.** (You will see 2 bands: Why? What will the sizes be? Which band do you want?) Use a clean razor blade to mark the location of the top and bottom of the fragment of interest in the marker lane (be careful not to cut all the way across the lane as this will cause it to fall apart when moved). Take the lane back to the lab and align with appropriate lane formed by the large well in the gel. Using the cut marks as guide, take a clean scalpel and remove the agarose containing the PCR product from the lane formed by the large well. Place the agarose slice in a clean 1.5ml microfuge tube.

Purify DNA from gel slice using Qiagen Gel Extraction Kit. Follow instructions (I always add around 20µl of 3M Sodium Acetate pH5.0 to make sure QG buffer is the correct pH for binding i.e. yellow in colour) except elute with 30µl of sterile H2O.

Measure OD (260 nm) of each of your samples. (Usual yield is 1.5-15% of amount you started out with in the very beginning.) If you do not have the possibility of measuring OD, let me know we can do it for you at UCSF.

5) Ligations:

Now it's time to fuse various pieces together.
Remember you want to fuse each A-B via the B-C piece to each C-D piece. (How many reactions are there going to be?)

CONSIDERATIONS:

You want to use 50 ng of pHO189 (the backbone) and a 2 fold molar excess of each of your pieces to insert (this works out as 1ul of the B-C piece I prepared for you). How much do you need of each A-B and C-D insert?

Let's assume pHO189 is approximately 3000 bp. A quick way to calculate the molar ratio is to find the ratio of sizes between the BACKBONE and the INSERT. For example, if your INSERT is 500bp, it is $1/6^{th}$ the size of the vector. For a 1:1 ratio that would be $1/6^{th}$ of 50 ng, or ~8.3 ng INSERT. But you want a 2 fold molar excess, or 16.6 ng.
(Should you find that you do not have enough DNA, try equimolar amounts).

Example: You want to fuse pHO43(A-B, 400bp), B-C and pHO50 (C-D, 1000bp) into pHO189. Apply the strategy I just described to calculate the amounts needed:

pHO189: 50 ng = 1ul
pHO143:13.3 ng = x ul
B-C = 1 ul
pHO50: 33.3 ng = x ul
(Of course x depends on what you measure as the DNA concentration to be.)
Easy, isn't it ?

Now the actual ligation reaction will look like this: calculate the amounts needed and fill to 20 ul with water.

pHO189:	1ul
B-C:	1ul
A-B:	x ul
C-D:	x ul
10 buffer:	2 ul
Ligase:	1 ul
	20 ul

Once you have assembled the reaction, flick the tube to mix, and spin down. Then incubate at room temperature for at least 30 minutes.
You can start thawing your cells for the transformation while this is occurring.

Transformations into TG1 cells:

Thaw the cells on ice (never let them get warm, until you do the heat shock).
Each tube has enough cells for 3-4 transformations.

Once the cells are thawed flick tube to resuspend cells and add 50 ul of cells to each ligation. Pipette up/down once gently (the less handling the better) and put tubes back on ice for 20 minutes.

Heat shock: take tubes directly from ice to a 37 degree water bath for 20 seconds. Place cells directly back on ice for 2 minutes. Add 500 ul LB media to each tube and shake for 1hour at 37 degree (~210rpm). Spread each 500 ul transformation on one plate, put (without lid) into a 37 degree incubator until agar surface is dry (~30 minutes). Put lid on and leave o/n.

Minipreps:

Pick 4 colonies of each transformation and grow them in LB (add kanamycin 1:1000) o/n. Perform miniprep as described in Quiagen protocol.

Diagnostic PvuI digest:

You want to do a diagnostic digest with PvuI on each of your DNA preparations to see whether your cloning worked. PvuI cuts inside pHO189 and linearizes your construct. A clone showing only a 3 kb band indicates pHO189 without insert, a larger band indicates a fused construct.

1 reaction:		10x mastermix:
DNA:	3 ul	
Buffer 3:	2 ul	20 ul
BSA:	0.2 ul	2 ul
PvuI:	0.3 ul	3 ul
H2O:	14.5 ul	145 ul
	20ul	170 ul

incubate at 37 degrees for 30-60 minutes. Run a 0.8% agarose gel.

Take a picture of this gel. Get any clones that might be positive to me and I will sequence to check integrity of the borders.

GOOD LUCK!

Ant questions?

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Appendix 1:

Materials

backbone A-D:

	rough size:
pHO189	~ 3 kb

All the following pieces reside inside a 2.8 kb vector. The size indicated is the size of the actual piece which adds on top of the 2.8 kb.

A-B pieces:

pHO43	0.4kb
pHO44	0.5kb
pHO45	0.3kb
pHO46	0.3kb
pHO48	1.6kb
ALW148	1kb?
ALW150	1kb?

B-C piece:

oligos HO232/HO233 (60nt)
or oligos HO362/HO363

C-D pieces:

pHO50	1kb
pHO55	1kb
pHO66	1.6kb
pHO92	4.7kb
pHO93	3.6kb
pHO97	2.3kb
pHO99	1.5kb
pHO132	0.6kb
pHO143	0.5kb
pHO144	0.5kb