Directed evolution.

California Institute of Technology
Dr. F.H. Arnold's lab

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Mutagenic PCR

-[Mn]

The amount of Mn used in the reaction should be titrated to produce the desired mutagenic rate. Libraries that have close to 30% inactive clones are typically desired (best balance between amount of parent and amount of dead clones) although this may vary depending upon the gene and the property being evolved. For shorter genes (1 kb or less, we typically use 0.15 mM $MnCl_2$ in the reaction. For longer genes, use less. In the case of pNB esterase (2kb) no Mn was used in the mutagenic PCR reaction. Do not add the $MnCl_2$ stock to the PCR buffer - keep them separate until setting up the reactions.

-pH

The protocol as described by Caldwell & Joyce uses pH 8.3. The Taq Promega buffer we typically use is pH 9.0. We have found that using pH 9.0 is more mutagenic than 8.3 (and pH 8.5 is between the two). So once you have optimized your reaction conditions, don't switch buffers.

-[Mg]

The final concentration of $MgCl_2$ in the reaction should be 7 mM. Several Taq buffers come with some Mg added, while some (such as the Promega buffer) do not so supplement accordingly.

-dNTPs

Higher concentrations of CTP and TTP (1 mM final concentration) are required to promote their misincorporation, while ATP and GTP are present at 0.2 mM.

Example reactions:

Example 1 - pNB esterase.
For one 100 µl reaction (number in parentheses is the concentration of the stock solution):

10 µl 10X buffer at pH 8.5 - (10 X buffer has 7.5 mM $MgCl_2$)
25 µl $MgCl_2$ (25 mM)
2 µl ATP (10 mM)
2 µl GTP (10 mM)
6 µl CTP (10 mM)
6 µl TTP (10 mM)
1 µl Taq polymerase (Promega)
2 µl primer 1(20 µM)
2 µl primer 2(20 µM)
1 µl template DNA
43 µl dd H_20

Combine PCR buffer, $MgCl_2$, dNTP mix, DNA and dd H_20. Vortex slightly to mix, there is detergent in the PCR buffer so vortexing too much will lead to foaming.
Add 1 µl (5U) *Taq* and mix gently.

Cycling protocol:
25 cycles of:
94 °C 1 min.
48 °C 1 min.
72 °C 2 min.

After PCR is done, run a small amount on a gel to be sure that you have product. Standard PCR should be run as a control.

Example 2 - subtilisin E.
For one 100 µl reaction
10 mM Tris-HCl (pH 8.3 at 25°C)
50 mM KCl
7mM $MgCl_2$
0.01% (wt/vol) gelatin
0.2mM dGTP
0.2mM dATP
1mM dCTP
1mM dTTP
3µM of each primer
5 ng template
5 U *Taq* DNA polymerase (Promega).

$MnCl_2$:
Unlike pNB esterase, subtilisin mutagenic PCR *does* require $MnCl_2$. $MnCl_2$ concentration depends on the error rate you require. As a guide 0.15 mM gives ~35% inactive clones, 0.5 mM gives ~60% inactive clones.

Cycling protocol:
13 cycles of:
94°C 1 min
50°C 1 min
72°C 1 min

Notes - If the subtilisin gene is contained in a large plasmid (>5 kb) an initial step of 2 min at 94°C should be added prior to the first cycle. This allows time for the strands to dissociate.

The error rate depends heavily on the number of cycles. For example, 20 cycles of the schedule given above results in ~70% inactive clones. If you want to adjust the error rate it is generally better to play with the $MnCl_2$ concentration than cycle number, because $MnCl_2$ concentration can be more finely adjusted.

In general there is no way to predict the optimal conditions for a new gene. A series of different $MnCl_2$ concentrations must be tried. To avoid having to sequence sample clones for each test condition, it is useful to note that a relationship appears to exist between error rate and the number of active clones. This relationship may differ from gene to gene. For subtilisin E, an error rate of two base changes per gene results in ~65% active clones. In any case, screening the resulting library for activity is easier than sequencing.

Reference: Caldwell & Joyce in *PCR Methods & Applications* Cold Spring Harbor Laboratory Press (1994)

DNA shuffling

This method of gene recombination is based on gene fragmentation and reassembly by cross hybridization and extension. There are 3 major steps in this process: fragmentation, assembly, and amplification. This assembly reaction is a PCR like reaction which lacks flanking primers. This reaction generates a mixture of products (which runs as a smear on an agarose gel), from which the desired full length genes are amplified in the amplification reaction (a PCR reaction which includes flanking primers).

To begin this method, you need plenty of DNA so start with a standard PCR protocol, or purify your insert from a restriction digest of plasmid.

Template amplification:

Do 2 X 100 µl standard PCR reaction for each mutant you plan to shuffle.

Mix for 6 reactions (concentration of stock solution is in parentheses):

60 µl 10X Promega Buffer (no Mg)	
36 µl $MgCl_2$	(25 mM)
30 µl dNTPs	(4 mM)
12 µl primer 1	(20 µM)
12 µl primer 2	(20 µM)
2 µl Taq	
444 µl dd H_2O	

Aliquot 100 µl/tube and add 0.5 µl of plasmid template to each tube

Run:

94 °C 2 min. (initial DNA denaturation, run only once)
Repeat the following for 12 cycles:
94 °C 30 sec (denature DNA)
50 °C 30 sec (anneal)
72 °C 1 min (extend)

Using a small amount of each reaction, check the PCR results on a gel.

Purify the DNA (using a Promega PCR Wizard Prep or other purification method).

Fragmentation Protocol:

Mix the DNA (PCR product or insert purified from restriction digestion) for the genes you want to shuffle. Then mix with Tris buffer and Mn stocks:

40 µl DNA
2.5 µl 1.0 M Tris HCl, pH 7.5
2.5 µl 200 mM $MnCl_2$

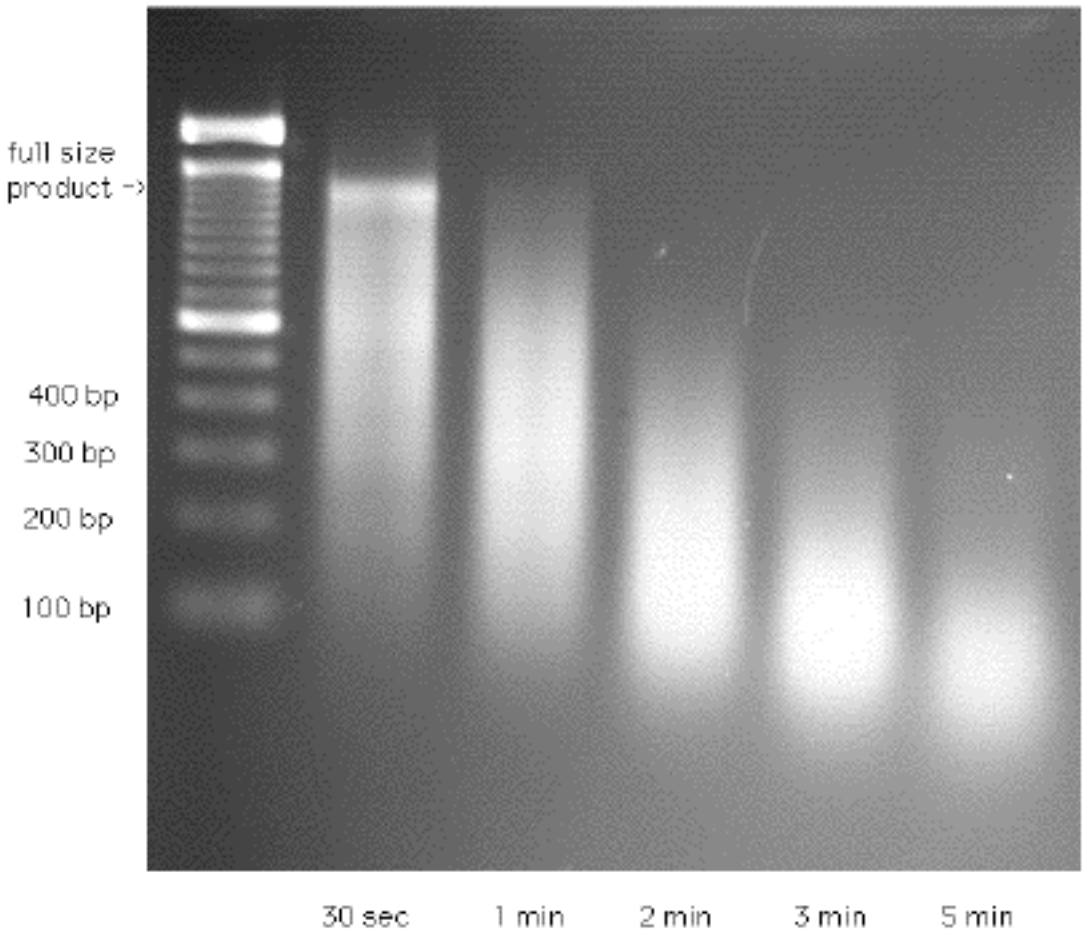
Cool this mixture to 15 °C then add:

1.5 µl 1:200 DNase I (10 U/µl enzyme diluted in water)

Continue incubation at 15 °C. Take 10 µl aliquotes after 30 seconds, 1, 2, 3 and 5 minutes and immediately mix them with 10 µl 0.1 M EDTA to stop digestion.

Run digestion products on 2% agarose gel. Elute DNA fragments of desired size (the Qiaex II (Qaigen) kit or DEAE paper).

Sample 2% agarose gel of DNase I digestion products.



Adjust DNase I concentration and/or incubation time if necessary.

Assembly reaction

Mix:
- 5 µl 10X PCR buffer
- 5 µl $MgCl_2$ (25 mM)
- 2.5 µl dNTP (4 mM)
- 0.3 µl Taq
- 10-30 µl DNA fragments
- dd H_2O up to 50 µl

Run: 96 °C 3 min (perform only once for initial DNA denaturation)
Do 40 cycles of:
94 °C 30 sec (denature DNA)
55 °C 30 sec (anneal)
72 °C 1 min. + 5 sec. per cycle

Check a small amount of the reaction on a gel. You should see a smear. More cycles can be done, or the annealing temperature lowered, if a smear does not appear. The smear should extend through the size of your full-length product.

Amplification

Now you want to run a PCR reaction with primers in order to amplify the full length product.

10 µl 10X PCR buffer (Promega)
6 µl $MgCl_2$ (25 mM)
5 µl dNTPs (4 mM)
1 µl from previous reaction
4 µl primer 1 (10 µM)
4 µl primer 2 (10 µM)
0.5 µl Taq
60 µl dd H_2O

Run:
20 cycles
94 °C 30 sec
50 °C 30 sec
72 °C 30 sec

Check for full size product on a gel.

If amplification reaction is not successful and you get smear instead of discrete band repeat amplification with smaller amount of template. Run reactions with several dilution of the template: 1:10, 1:20 and 1:50 and select the most successful one for cloning.
Another solution to the smear problem can be use of nested primers, separated by 50-100 bp from the original primers.

StEP recombination

This method for gene recombination is based on cross hybridization of slowly growing gene fragments. Template genes are mixed and flaking primers are added. Primers anneal and extend in PCR-like cycles that have been optimized for very short extensions. Through multiple cycles, the growing fragments anneal to and extend from different parents, creating novel recombinants.

StEP

Mix together 1 µl of each of the templates you wish to shuffle. Use 0.5 µl of this mix as template in the StEP reaction. These templates should be plasmids which have been purified from a *dam*-positive strain (DH5α or XL1-Blue), as this allows removal of template sequences with a *Dpn* I digest. While the number of primers can be varied from 1 to many (we most commonly use 2), the total amount should be kept at 100 ng.

For one reaction:

5 µl 10X PCR buffer (Promega, no Mg)
3 µl $MgCl_2$ (25 mM)
2.5 µl dNTPs (4 mM)
0.5 µl plasmid template
5 µl primer 1 (10 ng/µl)
5 µl primer 2 (10 ng/µl)
0.5 µl Taq
28 µl dd H_2O

Set up a control reaction containing no primers.

Run:

90 cycles:

94 °C 30 sec (denature DNA)
55 °C 15 sec (anneal and extend DNA, keep this step short)

Short templates like GFP (about 700 bp size) may require shorter annealing ti

Check a small amount on an agarose gel. The StEP reactions may or may not show a smear of DNA fragments which are smaller in length than the original template. The presence of the smear does not guarantee success.

Dpn I digest:

At this point you want to get rid of the plasmid DNA template which is still in your reaction mixture. If you do not get rid of the plasmid DNA you will end up with a lot of parent DNA in your transformations.

Mix:

2 µl StEP reaction
6 µl dd H_2O
1 µl 10X PCR buffer
1 µl DPN

Run at 37 °C for 1 hour - also do a DPN digest of the no primer control reaction.

Standard PCR with primers:

Now you want to PCR with primers in order to amplify the full length product.

For 1 reaction:

10 µl 10X PCR buffer (Promega)
6 µl $MgCl_2$ (25 mM)
5 µl dNTPs (4 mM)
5 µl from DPN digest
2 µl primer 1 (20 µM)
2 µl primer 2 (20 µM)
0.5 µl Taq
64 µl dd H_2O

Run 20 cycles of : 94 °C 30 sec
50 °C 30 sec
72 °C 30 min

Check for full sized product on a gel. A band should be visible in the reaction in which the first step (the StEP PCR) was performed with primer. There should be no band visible for no primer control reaction. If there is a band in this negative control, your product is simply amplified parent template.

If amplification reaction is not successful and you get smear instead of discrete band repeat amplification with smaller amount of template. Run reaction with several dilution of the template: 1:10, 1:20 and 1:50 and select the most successful one for cloning.
Another solution to the smear problem can be use of nested primers, separated by 50-100 bp from the original primers.