



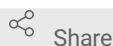
Jul 26, 2022

DIMPLE library generation and assembly protocol

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ABSTRACT

This is a protocol for generating and QCing mutagenic libraries using the DIMPLE protocol.

DOI

dx.doi.org/10.17504/protocols.io.rm7vzy7k8lx1/v1

PROTOCOL CITATION

Christian Macdonald, David Nedrud, Patrick Rockefeller Grimes, Donovan Trinidad, James Fraser, Willow Coyote-Maestas 2022. DIMPLE library generation and assembly protocol. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.rm7vzy7k8lx1/v1>

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CREATED

May 02, 2022

LAST MODIFIED

Jul 26, 2022

PROTOCOL INTEGER ID

61826

MATERIALS TEXT

Enzymes and cells

[PrimeSTAR GXL DNA Polymerase](#) Contributed by
users Catalog #R050A

[MegaX DH10B T1R Electrocompetent Cells](#) Life
Technologies Catalog #C6400-03

[NEB Golden Gate Assembly Mix](#) **New England**
Biolabs Catalog # E1601S

(Bsal)

Kits (NEB kits given only as examples - any comparable kit should work)

[Monarch PCR and DNA Cleanup Kit - 50 preps](#) **New England**
Biolabs Catalog #T1030S

[Monarch® DNA Gel Extraction Kit](#) **New England**
Biolabs Catalog #T1020

[Monarch Plasmid Miniprep Kit - 250 preps](#) **New England**
Biolabs Catalog #T1010L

Media and chemicals

[SOC Outgrowth Medium - 100 ml](#) **New England**
Biolabs Catalog #B9020S

[PCR Water \(nuclease free\)](#) Contributed by
users Catalog #PCPW

Equipment and consumables

- Thermocycler
- Electroporator
- Shaker
- Horizontal electrophoresis system
- Benchtop centrifuge
- OD meter

- Electroporation cuvettes (0.1 cm)
- Cuvettes for OD measurement
- Selection agar plates
- Agarose
- Optional: large BioAssay plates for plasmid purification

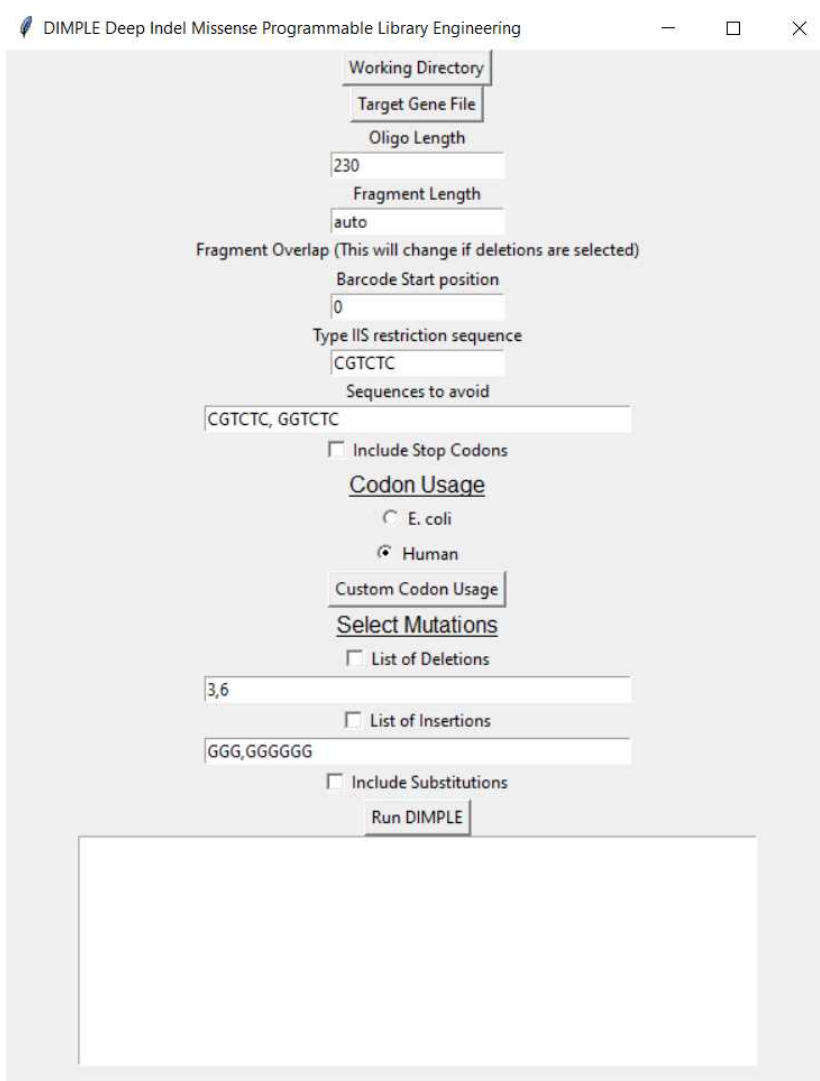
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Preparation

1 Use DIMPLE to generate mutagenic oligos and primers.



The screenshot shows the DIMPLE Deep Indel Missense Programmable Library Engineering GUI. The interface includes the following fields and options:

- Working Directory
- Target Gene File
- Oligo Length: 230
- Fragment Length: auto
- Fragment Overlap (This will change if deletions are selected)
- Barcode Start position: 0
- Type IIS restriction sequence: CGTCTC
- Sequences to avoid: CGTCTC, GGTCTC
- ☐ Include Stop Codons
- Codon Usage
 - ☐ E. coli
 - ☒ Human
- Custom Codon Usage
- Select Mutations
 - ☐ List of Deletions: 3,6
 - ☐ List of Insertions: GGG,GGGGGG
 - ☐ Include Substitutions
- Run DIMPLE

Snapshot of the default DIMPLE GUI

Important note: DIMPLE breaks a gene up into sub-library fragments and generates mutagenic

- 2 insert oligo pools, where each oligo contains barcodes, Type IIS restriction cutsites, and a sub-region of the gene. Be sure to review your library generation vector and gene sequences and look for pre-existing Type IIS restriction sites. Use site-directed mutagenesis to remove unwanted off-target sites.
- 3 **Input wild type gene sequence:** Input your gene sequence, including backbone, in fasta format. Place all genes to be mutagenized in the same fasta file. You can define which positions your genes start and end in the fasta header. Note that this uses 0 indexing: for example, if your first nucleotide is 884 on snappene, use 883 in the fasta header).
 - **For example:** >geneA start:35 end:250

```

test_smaller - Notepad
File Edit Format View Help
bTSHR start:657 end:2885
gtcattagttcatagcccatataggagttccggttacataactacggttaaatggccgctggctgaccgccaacgaccccgccattgacgtcaataatgacgtatgttcccatagtaacgccaataggaggt
GACCAAGGTGTATCTCACCGACATATCTTTATTTGGAGATCACCGATAACCCCTATATGACAAGTATTCCTGTCAATGCTTTTCAGGGGTGTGCAACGAAACCCGTACGCTCAAGTTGTACAATAACGGGTTCCAC
ATCGACTGGCAGACCGGACGAGGTGCAACACAGCCGGATTTTACCCTGTTTCGCTTCTGAGCTGTCCGTGTACACTCTGACTGTCAATTACATTGGAACGGTGGTATGCAATCACATTCCGCATCGCACTCGACCGGA
tcagcgaacatgatcatcgctcatccaggccagcccaagcccggaatttctgaatctcggaagcgtactcggcttccgctcgcgagatcgacggcgatctgttgatcaagatctcgcacatctcgatccac
accaataggccgaatcgcaaaatcccttataaatcaaaagaatagaccgagatagggttgagtggtttccagtttgaacaagagtcactattaaagaacgtggactccaacgtcaaaagggcgaaaaacgtcta
cggctgtcagcgcagggcgccgggtctttttgtaagaccgactgtccgggtgacctgaatgaactgcaagacgagcgagcggtatcgtggctggccacgacggcggttcttgcgcagctgtgtcgactgt
ggggccaatagcccggttcttcttcttcccccaccccccccaagttcgggtgaaggccagggctcgagccaacgtcggggcgagggccctgcatagcctcaggttactcatatatactttagattgattt
>ABCG2 start:15 end:1979
AGACAGCTCTCACCATGTCTCCAGTAATGTGCAAGTTTTATCCAGTGTACAAAGGAACCAATGGCTTCCCGCGACAGCTTCCAATGACCTGAAGGCATTTACTGAAGGAGCTGTGTTAAGTTTTCATAACAT
CAACTCCTCTCTTACAAAGAAACAAAAGCTGAATTACATCAACTTTCGGGGGTGAGAAGAAAGAAAGATCACAGTCTTCAAGGAGATCAGCTACACCACTCCTTCTGTCACTCAACTCAGATGGGTTTCAAGCGT
ATGGTCATAGCTGTTTCTGTGTGAATTTGTTATCCGCTCAGAGGGCACAATCCTATTCCGCGCTATCCGACAAATCTCAAGACATTAGGTGGAGTTCAGTTCCGGCTATGGCATATGTCTGGAAGAACATGTGAG
AGGCAGTTCATAGGATGGCAAGATCTCGTATCGGTCTGCGATTCCGACTCGTCAACATCAATACAACCTTAATTTCCCTCGTCAAAAATAAGGTATCAAGTGAGAAATCACCATGAGTGACGATGAATCCG
ACGACGGCCAGTCATGCAATAATCCGACGATCTGGAATAAGGAAGTGCCATTCCGCTGACCT
  
```

*If you do not define your start & end position, the software will analyze different ORFs and ask you to define these positions.

```

Analyzing Gene:pmV306_mOP_EccD3
ORF#1 MSENTVMPIVRVAVLAAAGDDGGRLT...VGLFSLVLDR - length 475, strand 1, frame 2
ORF#2 MGTAEDSAQREDPSLIRQPAGRCEV...RGAPHLPGAR - length 102, strand 1, frame 2
ORF#3 MPRSEESHKSCRPLERKMARGRKHP...PESCTGVATR - length 89, strand 1, frame 3
ORF#4 MDAEAWLAGEKRLIEMWTPPDRAK...EAMSKLAKTS - length 330, strand 1, frame 3
ORF#5 MSHIQRETSCSRPRLNSNMADLYG...QFHLMDEFF - length 271, strand 1, frame 3
ORF#6 MLLAAGLVPTHRAAVLALAARRGS...WRHLCDRHFT - length 311, strand -1, frame 1
ORF#7 MVGRGINSVSQFSLTISSVTSLATL...SGAWGFYKPR - length 43, strand -1, frame 2
ORF#8 MVVMFKSPVEHAEQADQQRHHRD...LLNSAAGSEL - length 497, strand -1, frame 2
ORF#9 MPRGPCSSSYGSPSCALGSSPRRAE...THAPELASSL - length 166, strand -1, frame 3
ORF#10 MQYAHGEAFALTRATEPATNPST...VFSIDILKLDL - length 367, strand -1, frame 3
Which ORF are you targeting? (number):1
MSENTVMPIV
Is this the beginning of your gene?(position 883) (y/n):y
VGLFSLVLDR
Is the size of your gene 1425bp? (y/n):n
Enter nucleotide length of your gene:1428
GLFSLVLDR*
Is this end correct? (y/n):y
  
```

It is important to include backbone so that the software can avoid making primers that nonspecifically recognize a region outside of your gene

```

Non specific Fragment:8
[162, 159, 159, 159, 156, 156, 159, 159, 159]
Creating Gene:pmV306_mOP_EccD3 --- Fragment:2-54
no thermodynamic data for neighbors 'AT/GT' available. Please check position manually:352 reverse
Primer:ATAGGTCTCgcccgatccggccgc
Match: ggccgcgacgcccgcacccggccgc
Found non-specific match at 790bp:
match:cgaccacgcccgcacccggccgc
primer:ATAGGTCTCgcccgatccggccgc Tm:35.03620585066153
----- Fragment size swapped due to non-specific primers -----
  
```

4 Using the DIMPLE GUI

- 4.1 *Working Directory:* When you open the DIMPLE GUI, first designate your working directory. This is the folder you wish your mutagenic oligo & primer outputs to be saved in.
- 4.2 *Target Gene File:* Upload your gene fasta text file with the Target Gene File button.
- 4.3 *Oligo length:* Designate the length of your oligo. This will include the barcodes, Type IIS restriction cutsite, and a region of your target gene that will be mutagenized. So, target gene +50bp.
- 4.4 *Fragment length:* You can adjust the length of your fragments, we recommend leaving the fragment length set to auto.
 - DIMPLE will automatically break up your gene into roughly the same fragment sizes, and will determine which lengths work best to avoid matching overhangs.
- 4.5 *Fragment overlap:* The number of base pairs shared between sub-regions. We have seen that setting overlap to 0 base pairs leads to errors. The overlap is set to 4 base pairs by default.
- 4.6 *Barcode start position:* The software selects from a set pool of barcodes when designing oligos. You can define which number barcode in the list this starts from.
- 4.7 *Type IIS restriction sequence:* You can select which Type IIS restriction cutsite you'd like to append to your inserts and backbone. You can choose between the BsmBI (CGTCTC) or BsaI (GGTCTC) sequences.
- 4.8 *Sequences to avoid:* This will allow the code to design fragments without the cutsite you selected in the *Type IIS restriction sequence* section. If there is an error when running, this implies you have a cutsite in your gene or vector that needs to be removed.

```
Exception in Tkinter callback
Traceback (most recent call last):
  File "C:\Program Files\WindowsApps\PythonSoftwareFoundation.Python.3.10.3.10.1520.0_x64_qbz5n2kfra8p0\lib\tkinter\__init__.py", line 1921, in __call__
    return self.func(*args)
  File "C:\Users\ASUS\Desktop\Fraser_CoyoteMaestas_Lab\DMS\DIMPLE-master\run_dimple_gui.py", line 45, in run
    OLS = addgene(app.geneFile)
  File "C:\Users\ASUS\Desktop\Fraser_CoyoteMaestas_Lab\DMS\DIMPLE-master\DIMPLE\DIMPLE.py", line 41, in addgene
    tmpOLS.append(DIMPLE(gene, start, end))
  File "C:\Users\ASUS\Desktop\Fraser_CoyoteMaestas_Lab\DMS\DIMPLE-master\DIMPLE\DIMPLE.py", line 142, in __init__
    raise ValueError('Unwanted Restriction cut sites found. Please input plasmids with these removed.' + str(DIMPLE.avoid_sequence)) # change codon
ValueError: Unwanted Restriction cut sites found. Please input plasmids with these removed.[Seq('GGCTCT')]
```

4.9 *Codon usage:* Your oligos can be codon optimized to your organism of choice. The default options are *E. coli* or *Human*, but you can also upload a codon usage table for any organism by pressing "Custom codon usage."

4.10 *Select mutations:*

Deletions: If you'd like to generate a library with deletions across your gene, select "List of deletions". Enter how many base pairs you'd like to be deleted across your library.

- **For example,** if you'd like deletions that are one codon long, enter "3" for 3 nucleotides.
- **For example,** If you'd like to include both deletions that are one codon long AND two codons long in the same pool, enter "3,6" for both 3, and 6 nucleotide long deletions.

Insertions: If you'd like to generate a library with different amino acids inserted across your gene, select "List of Insertions".

- **For example,** If you'd like to insert glycines across your gene, include "GGG" in the box.
- **For example,** If you'd like to insert glycine-serine across your gene, include "GGGTGC" in the box.
- **For example,** if you'd like to insert glycine AND glycine-serine across your gene, include "GGG,GGGTGC" in the box.

Substitutions: If you'd like to generate a library where an amino acid at each position is swapped with every other possible amino acid, select "Include Substitutions."

4.11 *Run DIMPLE:* Hit "Run DIMPLE" when you are ready to generate your pool.

5 Examples of what the running code and outputs look like:

The code will first iterate the ideal fragment length sizes across each gene of interest.


```

----- Analyzing Gene:pMV306_mOP_EccD3 -----
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:1
[162, 156, 159, 159, 159, 159, 159, 159, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:2
[162, 159, 156, 159, 159, 159, 159, 159, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:3
[162, 159, 159, 156, 159, 159, 159, 159, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:4
[162, 159, 159, 159, 156, 159, 159, 159, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:6
[162, 159, 159, 159, 156, 156, 162, 159, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:7
[162, 159, 159, 159, 156, 156, 159, 162, 156]
----- Fragment size swapped due to matching overhangs -----
Non specific Fragment:8
[162, 159, 159, 159, 156, 156, 159, 159, 159]

```

- 5.1 Next, barcodes are assigned to each fragment. The code will update how many barcodes it attempted to use to generate each fragment pool, and will remove these from the remaining barcode pool.

This will continue for each gene included in your fasta file.

```

Creating Gene:Hikeshi --- Fragment:43-81
Barcodes used:9
Barcodes Remaining:1066
Creating Gene:Hikeshi --- Fragment:83-119
Barcodes used:3
Barcodes Remaining:1063
Creating Gene:Hikeshi --- Fragment:121-158
Barcodes used:6
Barcodes Remaining:1057
Creating Gene:Hikeshi --- Fragment:160-198
no thermodynamic data for neighbors 'GT/AG' available. Please check position manually:258 forward
Primer:ATAGGTCTCAGACAGCAAATGATGAAGCAAATTGTAG
Match: CACGAATGGGAAGCCAAGTGCATCTTCAAATTTTCAG
Barcodes used:9
Barcodes Remaining:1048

```

- 5.2 There is a final QC check for each primer set generated.


```
Running QC for barcode primer specificity
Checking primer set:TSHR_oligoP_DMS-1
Checking primer set:TSHR_oligoP_DMS-2
Checking primer set:TSHR_oligoP_DMS-3
Checking primer set:TSHR_oligoP_DMS-4
Checking primer set:TSHR_oligoP_DMS-5
Checking primer set:TSHR_oligoP_DMS-6
Checking primer set:TSHR_oligoP_DMS-7
Checking primer set:TSHR_oligoP_DMS-8
Checking primer set:TSHR_oligoP_DMS-9
Checking primer set:TSHR_oligoP_DMS-10
Checking primer set:TSHR_oligoP_DMS-11
Checking primer set:TSHR_oligoP_DMS-12
Checking primer set:TSHR_oligoP_DMS-13
Checking primer set:TSHR_oligoP_DMS-14
Checking primer set:TSHR_oligoP_DMS-15
Checking primer set:TSHR_oligoP_DMS-16
Checking primer set:ABCG2_oligoP_DMS-1
Checking primer set:ABCG2_oligoP_DMS-2
Checking primer set:ABCG2_oligoP_DMS-3
Checking primer set:ABCG2_oligoP_DMS-4
Checking primer set:ABCG2_oligoP_DMS-5
Checking primer set:ABCG2_oligoP_DMS-6
Checking primer set:ABCG2_oligoP_DMS-7
Checking primer set:ABCG2_oligoP_DMS-8
Checking primer set:ABCG2_oligoP_DMS-9
Checking primer set:ABCG2_oligoP_DMS-10
Checking primer set:ABCG2_oligoP_DMS-11
```

5.3 All outputs are saved in your working directory.

If you are using DIMPLE to mutagenize several genes, there will be separate files for the primers and mutagenic inserts for each gene, as well as a master list of **all** oligos and primers.

All_Oligos	FASTA File	16,745 KB
All_Primers	FASTA File	19 KB
GPR161_Gene_Primers	FASTA File	2 KB
GPR161_DMS_Oligo_Primers	FASTA File	2 KB
GPR161_DMS_Oligos	FASTA File	2,655 KB
GPR161_mutations	Microsoft Excel Co...	20 KB
Hikeshi_DMS_Gene_Primers	FASTA File	1 KB
Hikeshi_DMS_Oligo_Primers	FASTA File	1 KB
Hikeshi_DMS_Oligos	FASTA File	996 KB
Hikeshi_mutations	Microsoft Excel Co...	21 KB

For each gene, DIMPLE generates a list of:

- **EXAMPLE_DMS_Oligos.** This is the list of sub-library oligo pools, where each oligo contains barcodes, Type IIS restriction cutsites, and a sub-region of the gene.

GPR161_DMS_Oligos - Notepad

File Edit Format View Help

```
>GPR161_DMS-1_Ser2Cys
GAATAGGTGGCAAAACGCTGAGATAGATGCGGTCTCCgatatgTGCctgaactcaagtc
tgtcctgccgaaaaaactcttaacctcacagaggaggaagggggagaaggcggcgtga
taattacgcagttcattgccatcatcgtgataaccatattcgtatgtttgggaaacctcg
tcatttggtgaGGAGACCACTAAACGTGACGGGTGCGCGTAAAGTGAGC
>GPR161_DMS-1_Ser2Asp
GAATAGGTGGCAAAACGCTGAGATAGATGCGGTCTCCgatatgGACctgaactcaagtc
tgtcctgccgaaaaaactcttaacctcacagaggaggaagggggagaaggcggcgtga
taattacgcagttcattgccatcatcgtgataaccatattcgtatgtttgggaaacctcg
tcatttggtgaGGAGACCACTAAACGTGACGGGTGCGCGTAAAGTGAGC
>GPR161_DMS-1_Ser2Ser
GAATAGGTGGCAAAACGCTGAGATAGATGCGGTCTCCgatatgTCCctgaactcaagtc
tgtcctgccgaaaaaactcttaacctcacagaggaggaagggggagaaggcggcgtga
taattacgcagttcattgccatcatcgtgataaccatattcgtatgtttgggaaacctcg
tcatttggtgaGGAGACCACTAAACGTGACGGGTGCGCGTAAAGTGAGC
```

Screenshot of mutagenic inserts generated by DIMPLE

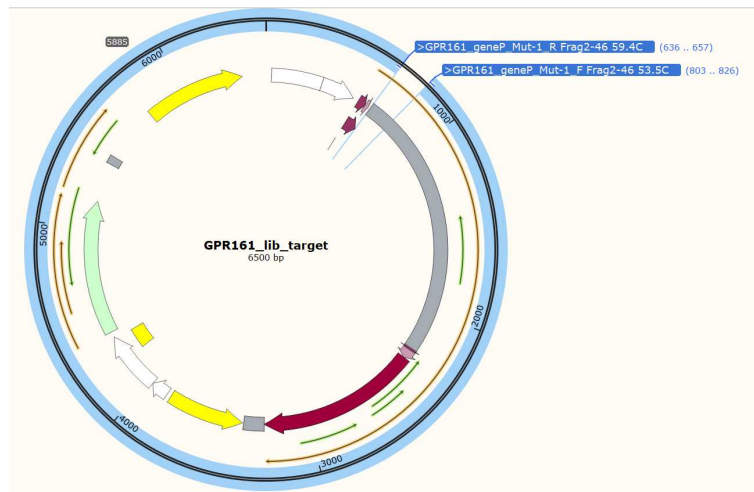
- **EXAMPLE_DMS_Gene_Primer**s. These are primers used to add cutsites to and amplify the backbone each mutagenic insert will be ligated into.

GPR161_DMS_Gene_Primer - Notepad

File Edit Format View Help

```
>GPR161_geneP_Mut-1_R Frag2-46 59.4C
ATAGGTCTCtatcgatgctggcgtcatcatc
>GPR161_geneP_Mut-1_F Frag2-46 53.5C
ATAGGTCTCggtgaccctgtataaaaaatcata
>GPR161_geneP_Mut-2_R Frag48-90 57.6C
ATAGGTCTCcgaggtttccaaacatacgaal
>GPR161_geneP_Mut-2_F Frag48-90 60.3C
ATAGGTCTCgatctttggagtcgtgtggtgc
>GPR161_geneP_Mut-3_R Frag92-134 61.2C
ATAGGTCTCttctgattgaactagtcacgacaaaagg
>GPR161_geneP_Mut-3_F Frag92-134 61.1C
ATAGGTCTCgaaaataacaggcaatcgcgcc
>GPR161_geneP_Mut-4_R Frag136-178 57.2C
ATAGGTCTCacaccatagggtacagaacgg
>GPR161_geneP_Mut-4_F Frag136-178 56.3C
ATAGGTCTCctggcaccgggaaccc
```

Screenshot of backbone primers generated by DIMPLE. Each primer name also lists its melting temperature.



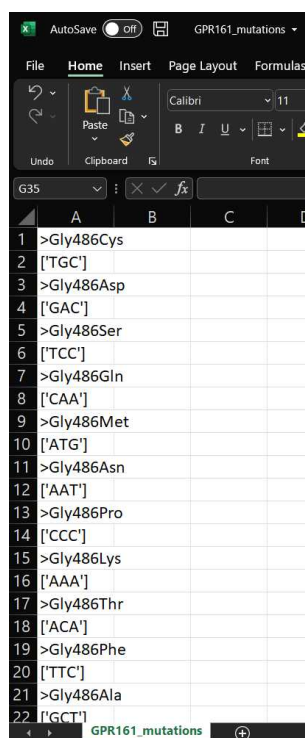
Each primer pair linearizes the backbone

- **EXAMPLE_DMS_Oligo_Primer**s. These are primers used to amplify the mutagenic inserts from your oligo pool.

GPR161_DMS_Oligo_Primer - Notepad

```
File Edit Format View Help
>GPR161_oligoP_DMS-1_F Frag2-46_57.9C
GAATAGGTGGCAAACGCTGA
>GPR161_oligoP_DMS-1_R Frag2-46_58.3C
GCTCACTTTACGCGCACC
>GPR161_oligoP_DMS-2_F Frag48-90_58.6C
AGTTTGGGGATCTCGTACACG
>GPR161_oligoP_DMS-2_R Frag48-90_57.8C
TGAGGACTTTTGCCTTCGAC
>GPR161_oligoP_DMS-3_F Frag92-134_55.9C
ATCCATAGCGTCCCTATTGC
>GPR161_oligoP_DMS-3_R Frag92-134_58.7C
TTCCCCGTCATGCTTAGGGA
>GPR161_oligoP_DMS-4_F Frag136-178_58.6C
CTCTAACGCGTGACACTTAG
>GPR161_oligoP_DMS-4_R Frag136-178_58.5C
TTGGCTAATAGTGGGAGGCG
```

- **EXAMPLE_mutations**. This is the list of mutations that will be present in your final library.



Library assembly

3d

6 PCR amplification of oligos, backbone

Thaw all components beforehand and follow general directions provided by manufacturer.

Amplification of mutagenic inserts and backbones should ideally be performed in parallel, assuming one has two thermocyclers. Alternatively, the backbone amplification can be performed first, then the the insert amplification can be done during the gel purification of the backbone.


7 PCR amplification of backbone.

Prepare a master mix with PrimeSTAR GXL polymerase:

A	B	C	D	E
Component	Total amount in master mix (μL)	Amount/reaction (μL)	Comment	Number of regions
dNTP	20	4		5
5X buffer	50	10		
Template (vector)	5	1	~10 ng	
Enzyme	5	1		
Nuclease-free H ₂ O	160	32		
Primers		2	Fwd & reverse combined at 20 μM (each)	
Total	240	50		

Mix by vortexing and spin down.

7.1 Transfer  **48 μL** of master mix into separate tubes for each reaction.

7.2 Add reaction-specific primers to each tube:  **2 μL (paired at 20 μM, each)** . Mix and spin down.

7.3 Place on thermocycler and amplify:

A	B	C
Step	T (° C)	Time (sec)
1	98	10
2	55	15
3	68	60 per kb
4	Repeat 1-3 24 times	
5	10	Hold

Note: the number of cycles can be optimized. Increasing cycles introduces more PCR bias and error. An initial comparison of 20, 22, and 24 cycles can be used to find a minimum number of cycles which yields sufficient DNA and no more.

7.4 Purify amplified product by gel extraction.

Prepare an agarose gel for gel purification (0.5% - 0.75%). Ideally, it should be of sufficient size to load all samples.

7.5 Load and run backbone products.

7.6 Use a scalpel or razor blade to (carefully!) cut out each product. Using a gel extraction kit, purify the product and elute in **10 µL TE**.

8 PCR amplification of mutagenic inserts.

Prepare a master mix with PrimeSTAR GXL polymerase:

A	B	C	D	E
Component	Total amount in master mix (µL)	Amount/reaction (µL)	Comment	Regions
5X buffer	50	10		5
dNTP	20	4		
Oligo pool	5	1		
Enzyme (PrimeSTAR GXL)	5	1		
Primers		2	Fwd & rev combined, 20 µM (each)	
Nuclease-free H ₂ O	160	32		
Total	240	50		

Mix by vortexing.

8.1 Transfer **48 µL insert master mix** into separate tubes for each reaction.

8.2 Add reaction-specific primers to each tube: **2 µL (paired at 20 µM, each)**. Mix and spin down.

8.3 Place on thermocycler and amplify:

A	B	C
Step	T (° C)	Time (sec)
1	98	10
2	55	15
3	68	60
4	Repeat 1-3 29 times	
5	10	Hold

8.4 Use a PCR cleanup kit to purify each product. Elute in 10 µL TE .

- 9 **Important QC step** - run a gel with each PCR product and visualize it. Ideally, each reaction should ideally give a single strong band at the expected size. Failure to do so may require changing PCR conditions.

10 Golden gate assembly

Prepare a master mix for the assembly. Use the table below to calculate: adjust number of regions and amount of necessary backbone and insert for desired amounts per reaction. We have found 3 µL backbone and 1 µL oligo (insert) are usually good.

A	B	C	D	E
Component	Total amount (µL)	Amount/reaction (µL)	Notes	Number of regions
10X buffer	20	4		5
Enzyme	15	3		
Backbone		3	300 ng	
Insert		1	2:1 molar ratio: for example libraries here, 100-200 ng	
Nuclease-free H ₂ O	145	29		
Total	200	40		

Vortex to mix.

10.1 Transfer appropriate master mix into separate tubes for each reaction.

Example: if each reaction has 3 μL backbone and 1 μL insert, then transfer **36 μL** to each tube.

10.2 To each tube, add the appropriate vector/insert pair:

For this example:

 **3 μL backbone (purified)**

 **1 μL insert (purified)**

Mix well and spin down.

10.3 Place on thermocycler and run the following program:

A	B	C
Step	T ($^{\circ}\text{C}$)	Time (min)
1	37	5
2	16	5
3	Repeat 1-2 29 times	
4	60	5
5	10	Hold

10.4 Use a PCR cleanup kit to purify each product. **Elute in**



 **10 μL nuclease-free H_2O .**

Important: using elution buffer or TE may cause electroporation to fail!

10.5 **Important QC step:** before electroporating libraries, transform 1 μL of each library into chemically competent cells and plate. If no or very few colonies are observed, this could suggest a failure during assembly. Further check the assembly by running a small portion on a gel.

Transformation and recovery of sublibraries

2d

- Using a high-efficiency electrocompetent strain of *E. coli*, such as MegaX DH10B, prepare a transformation for each assembly:  **3 μL assembly product** with  **50 μL electrocompetent cells** in a 0.1 cm cuvette. Follow the specific instructions for voltage and outgrowth media corresponding to your cells and electroporator. Outgrow cells for 1

hour before proceeding.

12 Essential QC step: Count transformants for ensuring adequate coverage.

Prepare 6 10-fold serial dilutions: for each reaction, add **90 µL** H₂O to six 500 µL microcentrifuge tubes. Add **10 µL** of the outgrowth to the first, then mix well and then **10 µL** of this to the second tube. Repeat for the rest of the tubes.

- 12.1 Plate the remainder (**90 µL**) of the last three dilutions (1:10000, 1:100000, 1:1000000) on LB-agar with appropriate antibiotic. Grow overnight and count colonies the next day. Calculate the total number of transformants and determine the variant coverage per reaction. At least 50-fold coverage is important.

Example plates and calculations

- 12.2 **Optional QC step:** pick single colonies from the QC plates and use Sanger sequencing to check for mutations.

- 13 Add the remaining outgrowth culture to **30 mL** LB supplemented with appropriate antibiotic for further outgrowth in either a Falcon tube or glass culture tube. Shake until the culture reaches OD 0.6-0.7.

Note: it is important to harvest the cells in the exponential phase. This takes 4-6 hours in our experience, but may vary (including between samples in the same day).

- 14 Harvest DNA from cultures using a miniprep kit, splitting each reaction across 3 columns. Elute each column in **40 µL** elution buffer and combine elutions corresponding to a single sublibrary.

Sublibrary pooling and subcloning

2d

- 15 Measure each sublibrary concentration with a fluorometric method, such as Qubit. With the lowest concentration as the target concentration, prepare a dilution of each sublibrary in H₂O. Mix **2 µL** of this dilution from each sublibrary to create an equimolar library pool.
- 16 Subclone library to transfer from library vector to selection vector. Depending on construct design, this may require digestion/ligation or a Golden Gate reaction.

- 17 Use PCR cleanup kit to purify reaction, and elute in **10 µL** H₂O.
- 18 Using a high-efficiency electrocompetent strain of *E. coli*, such as MegaX DH10B, transform **3 µL subcloned product** with **50 µL electrocompetent cells** cells in a 0.1 cm cuvette. Follow the specific instructions for voltage and outgrowth media corresponding to your cells and electroporator. Outgrow cells for 1 hour before proceeding.
- 19 Repeat transformation and isolation following steps 9-11.