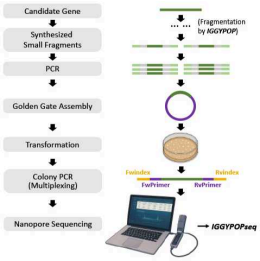
Jun 23, 2025

IGGYPOP: Rapid and Large-Scale DNA Assembly Method



DOI
https://dx.doi.org/10.17504/protocols.io.eq2lyqyzqvx9/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.eq2lyqyzqvx9/v1

Protocol Citation: Gony Dvir, Zenan Xing, Irina Beldman, Andrés ivera, Ian Wheeldon, Sean Cutler 2025. IGGYPOP: Rapid and Large-Scale DNA Assembly Method. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2lyqyzqvx9/v1

Manuscript citation:
Dvir G, Xing Z, Beldman I, Rivera A, Wheeldon I, Cutler SR. Synthesis of large single-transcript pathways from oligonucleotide pools: design of STARBURST, an autobioluminescent reporter (manuscript submitted)

Protocol status: Working
We use this protocol and it's working

Created: May 25, 2025

Last Modified: June 23, 2025

Protocol Integer ID: 218895

Keywords: DNA Assembly, Nanopore Sequencing, IGGYPOP, indexed golden gate gene assembly, golden gate gene assembly from pcr, scale dna assembly method iggypop, genes from oligonucleotide pool, using gene, synthesizing gene, golden gate cloning, gene, oligonucleotide pool, iggypop, dna, barcoded amplicon

Funders Acknowledgements:
Defense Advanced Research Projects Agency CERES
Grant ID: D24AC00011-00

Abstract

IGGYPOP (indexed golden gate gene assembly from PCR amplified oligonucleotide pools) is a pipeline for designing and synthesizing genes from oligonucleotide pools. Input sequences are fragmented into segments that can be amplified using gene-specific primers and reassembled by Golden Gate cloning. Sequence-verified constructs are then identified by nanopore sequencing of barcoded amplicons.

Protocol materials

- GoTaq® Green Master Mix **Promega Corparation** **Catalog #**M7123
- Invitrogen™ UltraPure™ BSA 50 mg/ml **Invitrogen** **Catalog #**AM2616
- Nuclease-free Water **Invitrogen - Thermo Fisher** **Catalog #**AM9937
- Flow Cell Wash Kit **Oxford Nanopore Technologies** **Catalog #**EXP-WSH004-XL
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies** **Catalog #**SQK-LSK114
- NEBNext® Companion Module v2 **New England Biolabs** **Catalog #**E7672
- BbsI HF **New England Biolabs** **Catalog #**R3539M
- T4 DNA Ligase **New England Biolabs** **Catalog #**M0202L
- Phusion™ High-Fidelity DNA Polymerase **New England Biolabs** **Catalog #**M0530L
- NEBridge Golden Gate Assembly Kit (BsmBI-v2) **New England Biolabs** **Catalog #**E1602L

Oligonucleotide Pool Design

1 **Install iggypop**
Detailed installation instructions, along with an overview of the available functions are provided on the official repository: https://github.com/cutlersr/iggypop/tree/main.

2 **Generate oligo pool**
Run iggypop, providing a file containing the sequences of interest as input and specifying a destination directory where the results will be saved. The default parameters will introduce synonymous mutations to remove internal BsaI and BsmBI sites, add external overhangs that allow cloning into the pPop vectors and BsaI sites to the 5' and 3' ends for subsequent Golden Gate cloning. You can adjust the parameters to suit the specifics of your experimental design via the command line or a .yml file. The yaml directory (https://github.com/cutlersr/iggypop/tree/main/yaml/) includes several preconfigured .yml files that provide the specific parameters for a variety of experiments. In addition to fasta formatted sequences, you can use Genbank formatted files as inputs; see the iggypop Readme file for details.

3 **Retrieve results**
Upon completion, several output files will be generated. Among these, the most important are: **_oligo_pool_to_order.fasta*, which contains the designed oligo pool for synthesis and **__pcr_primers_required.fasta*, which lists the gene-specific primers required for PCR amplification. Additionally, **_designed_seqs.fasta* contains the assembled sequences. Verify these outputs before proceeding with the experimental workflows.

Oligo Amplification

4 **Phusion PCR (96-Well Plate Format)**
The fragments required for gene assembly are amplified from oligonucleotide pools using a set of reusable indexing (i.e., gene-specific) PCR primers; the primers required are located in the file iggypop/out/[project]/[project]_index_primers_required.fasta.

4.1 **Template preparation**:
Resuspend the oligo library in **nuclease-free water** to a final concentration of **1 ng/µL**. Prepare a working dilution by diluting 1:10 in nuclease-free water to achieve **100 pg/µL (0.1 ng/µL)**.

4.2 **PCR Specifications** (based on the Phusion protocol):
Phusion™ High-Fidelity DNA Polymerase **New England Biolabs Catalog #**M0530L

	Component	Volume (µL)
	Phusion Enzyme	0.25
	HF Buffer	5
	dNTPs (10 µM)	0.5
	Primer F+R mix (10 µM)	5
	Template (0.1 ng/µL)	1
	Nuclease-free water	to 25

Thermocycling Conditions:

	Step	Temperature	Time
	Initial Denaturation	98°C	30 seconds
	30 Cycles		
	– Denaturation	98°C	10 seconds
	– Annealing	60°C	10 seconds
	– Extension	72°C	30 seconds
	Final Extension	72°C	5 minutes
	Hold	12°C	Indefinite

4.3 **Quality Check**

Run 3 µl on an agarose gel to assess amplification quality.

5 **PCR Purification with Beads**

To prepare your own beads, follow this protocol:

Protocol

NAME

A method to prepare Sera-Mag SpeedBeads for purification and size selection of nucleic acids

CREATED BY

John B. Ridenour

Preview

Use **2× the PCR volume** for purification (e.g., add 50 µL of beads to 25 µL PCR reaction).

5.1 **Post-Purification QC**

1. Use Nanodrop to quantify a few samples and estimate the average DNA yield.
2. Run 3 µL from each well of the 96-well plate on an agarose gel to check for missing or failed PCR reactions.

Golden Gate Assembly

6 **One-step assembly -- Golden Gate Assembly (BsmBI)**

Note

The default *iggypop* oligo design parameters are set up to work with pPlantPOP or pPOP plasmids, which use either BsmBI or BbsI with 5'-AATG/GCTT-3' overhangs on target genes for assemblies. If you need to use different overhangs and/or enzymes, update your *iggypop* parameters appropriately.

Using NEBridge-BsmBI

NEBridge Golden Gate Assembly Kit (BsmBI-v2) **New England Biolabs Catalog #**E1602L

1. **Reaction Setup (10 µL total):**

	Component	Amount
	pPlantPOP or pPOP-BsmBI	60 ng or 35 ng respectively
	Inserts (purified PCR product)	~5.5 ng × average number of fragments
	10X T4 DNA Ligase Buffer	1 µL
	NEB Golden Gate Assembly Mix	0.5 µL
	Nuclease-free water	to 10 µL

2. **Cycling Protocol**:
(42°C, 5 min → 16°C, 5 min) × 90 cycles → 60°C, 5 min

7 **Modification for 2-step assembly**

Although assembly of long (>2.5 kb) sequences is possible, the assembly efficiency can be low, and identifying error-free clones often requires substantially more amplicon sequencing than for smaller targets. For longer sequences **(>2 kb)**, we recommend using the two-step assembly mode, which breaks sequences into "step one" blocks

assembled using **BbsI** and **pPOP-BbsI**. Sequence validated step one clones are identified, and the final genes are constructed in a second step using **pPlantPOP** (w/ BsmBI). You need to use the 2-step YAML files for oligo pool design.

7.1 **Golden Gate Assembly -- first step is into pPlantPOP w/ BbsI**

BbsI HF **New England Biolabs Catalog #**R3539M

T4 DNA Ligase **New England Biolabs Catalog #**M0202L

1. **Reaction Setup (10 µL total):**

	Component	Amount
	pPOP-BbsI	35 ng
	Inserts (purified PCR product)	~5.5 ng × average number of fragments
	10X T4 DNA Ligase Buffer	1 µL
	BbsI	0.5 µL
	T4 DNA ligase	0.5 µL
	Nuclease-free water	to 10 µL

2. **Cycling Protocol**:
(37°C, 5 min → 16°C, 5 min) × 90 cycles → 60°C, 5 min

3. See step 22 for assembly of the final clone after obtaining sequence-verified step 1 clones.

Transformation

8
1. Prepare chemically competent E. coli using the CCMB80 method; freeze in ~ 5 mL aliquots.
2. Thaw 5 mL competent cells on ice, dispense 50 µL per well of a 96-well plate on ice
3. Add **2 µL** of the assembly reaction per well.
4. Incubate on ice for **30 minutes**.
5. Perform heat shock at **42°C for 1 min** in a thermal cycler.
6. Transfer to a 2 mL 96-well plate containing **250 µL SOC medium** per well.
7. Incubate at **37°C with shaking** for 1 hour.
8. Centrifuge at **1000 RPM for 5 minutes**, remove **200 µL SOC**, and shake again for **at least 30 minutes**.
9. **Plate the entire suspension** on 9 cm LB agar plates supplemented with **25 mg/L chloramphenicol (pPOP-BsmBI and pPOP-BbsI) or 100 mg/L spectinomycin (pPlantPOP)**

Barcoded Amplicons Generation

9 **Colony PCR with Barcoded Primers**

- The primers we use for generating barcoded amplicons with pPOP or pPlantPOP are provided **here**. After synthesizing them, they are arrayed combinatorially (https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq/tree/main/Input/PrimerAssignment) in 96-well PCR plates (10 µM, see 10.2) and used for colony PCR.

9.1 **Colony Preparation**

- Aim to screen 6 to 8 colonies per construct.
- Prepare 96-well plates with 20 µL ddH_2O per well.
- Pick colonies and resuspend in water to use in PCR reactions
- Spot colony suspensions onto single-well rectangular LB + antibiotic plates to store the bacterial stocks for recovery after sequence verification.

9.2 **Primer Preparation**:

Barcoding primers are arrayed into 96-well plates that can be used for multiple experiments

- Dilute barcoded primers to **1 µM** in a new plate.
- Mix the forward and reverse primers in separate plates following the plate layout for the specific plasmid provided **here**.

9.3 **PCR Setup (10 µL total):**

GoTaq® Green Master Mix **Promega Corparation Catalog #**M7123

	Component	Volume (µL)
	GoTaq Green Master Mix	5
	Primer F+R mix (1 µM)	2
	Colony suspension	1
	Nuclease-free water	to 10

9.4 **Stocking Colonies**:

- Use a multi-channel set at 3 µl pipette, pipette ~2 µL drops of the suspension onto the LB plate in an organized pattern.
- Add the remaining ~1 µL to the PCR reaction.

9.5

1. Seal PCR plates tightly and mix by tapping.
2. Spin the plates down.
3. **Cycling Conditions**:

	Step	Temperature	Time
	Initial Denaturation	94°C	1.5 mins
	30 Cycles		
	– Denaturation	94°C	10 seconds
	– Annealing	55°C	10 seconds
	– Extension	72°C	60–120 sec (depending on amplicon length)
	Final Extension	72°C	5 minutes
	Hold	12°C	Indefinite

9.6 **Quality Check**
Run 3 µl on an agarose gel to assess amplification quality.

PCR Product Purification

10
1. Pool 2 µL from each well into a single tube for each plate separately.
2. Purify 25 µL from each pooled tube using a 1× volume of magnetic beads using standard bead purification protocol.
3. Elute in **15 µL**.
4. Pool eluates to achieve even representation across the samples, targeting **~1 µg total DNA**.

Nanopore Sequencing

11 **Required equipment and reagents.**

- **Reagents:**

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

NEBNext® Companion Module v2 **New England Biolabs Catalog #**E7672

Invitrogen™ UltraPure™ BSA 50 mg/ml **Invitrogen Catalog #**AM2616

Nuclease-free Water **Invitrogen - Thermo Fisher Catalog #**AM9937

Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #**EXP-WSH004-XL

- **Equipment:**

1. Sequencing Device: MinION Mk1B (MIN-101B) / MinION Mk1D (**MIN-101D**)
2. Flow Cell: MinION Flow Cell (R10.4.1, **FLO-MIN114**)

12 **Check the flow cell.**
- Follow the **flow cell check protocol** provided by Oxford Nanopore Technologies.

13 **Library preparation.**
- Follow the **library preparation protocol** provided by Oxford Nanopore Technologies.

14 **Priming and loading the MinION flow cell.**
- Follow the **flow cell priming and loading protocol** provided by Oxford Nanopore Technologies.

15 **Data acquisition and basecalling.**
- Follow the **MinKNOW operation protocol** provided by Oxford Nanopore Technologies.

Note

Important Sequencing Settings:
- Basecalling: High-accuracy basecalling by Dorado
- Output file format: pod5 & fastq

16 **Wash and store the flow cell.**
- Follow the **flow cell wash protocol** provided by Oxford Nanopore Technologies.

Data Analysis

17 **Create a project workspace.**
- Make a new directory for this run (e.g., *IGGYPOP_YYYYMMDD*).
- Inside it, create one subfolder named *Input*, for the files you will prepare below.

18 **Generate SampleInfo.tsv.**
- Use **Tidy Buddy** to generate the required SampleInfo.tsv file.
- Alternatively, you may open a spreadsheet editor and enter one row per sample with the required columns, saving it as *SampleInfo.tsv* (tab-separated).

	Column	Type
	primer_index	string
	SampleID	string
	n_frags	int
	CDS_length (Optional)	int
	ReferenceName	string

	Column	Type
	ReferenceSequence	string
	FwBarcode	string
	FwPrimer	string
	RvBarcode	string
	RvPrimer	string

The detailed description of each column can be found in our GitHub repo (https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq.git).

- Move the file into the *Input/* folder.

19 **Generate passed_all.fastq.**

- Locate the base-called reads folder (usually *fastq_pass/*).
- Unzip any .gz files (skip if already in plain .fastq format), then concatenate all passed read files into one file named *passed_all.fastq*. After that, move the concatenated file into the *Input/* folder. You can use the following command to accomplish this.

```
zcat fastq_pass/*.fastq.gz > Input/passed_all.fastq
```

20 **Run the IGGYPOPseq pipeline.**

- Choose the execution environment and follow the corresponding instructions on our GitHub repo (https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq.git).

	Environment	Link to Instructions
	Linux	https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq?tab=readme-ov-file#--linux
	Docker	https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq?tab=readme-ov-file#--docker
	Compute cluster	https://github.com/cutlerlab/Construct-Validation-for-IGGYPOPseq?tab=readme-ov-file#--parallelized-analyses-with-slurm

21 **Check and store the analysis results.**

- All the results are available in the following directory: *IGGYPOP_YYYYMMDD/Analysis_Results/*.
- Store the folder in your institutional or long-term repository.

2nd assembly step for sequence-verified step 1 clones

22 **Golden Gate Assembly Using NEBridge-BsmBI into pPlantPOP**

NEBridge Golden Gate Assembly Kit (BsmBI-v2) **New England Biolabs Catalog #**E1602L

Reaction Setup (10 µL total):

	Component	Amount
	pPlantPOP	60
	Inserts (sequence validated pPOP plasmids)	~30 ng each
	10X T4 DNA Ligase Buffer	1.5 µL
	NEB Golden Gate Assembly Mix	0.5 µL
	Nuclease-free water	to 15 µL

Cycling Protocol:
(42°C, 5 min → 16°C, 5 min) × 90 cycles → 60°C, 5 min

- go to step #8 for the transformation procedure.
- Plate cells on spectinomycin (100 mg/L).
- Prep 1 - 2 colonies per clone, digest for initial confirmation; whole-plasmid sequence for final validation.

Protocol references

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