DNASCRIPT

DIY Gene Assembly by PCA
Quick Guide

A do-it-yourself guide for gene assembly using oligos synthesized by enzymatic DNA synthesis

This quick guide provides an easy-to-follow, step-by-step protocol to assemble genes and gene fragments by polymerase cycling assembly (PCA), starting from oligo design to the final assembled gene construct.

Note: The guide is an example protocol that users can use as a starting point to guide their experimental design and planning. It is not a definitive guide to gene assembly and the protocol should be optimized depending on the users' gene length and complexity.

GOAL

- **Assemble a full gene** using polymerase cycling assembly (PCA) into a cloning vector, starting from oligos printed by enzymatic DNA synthesis (EDS) on the SYNTAX STX-200 platform.
- Compare two approaches: start from **oligos of either 60 nucleotides (nt) or 120nt** in length.
- Incorporate an **error correction step on blocks** within the PCA assembly.

The guide describes the workflow by demonstrating the **successful assembly of the 1,698bp hemagglutinin (HA) gene** from Influenza A H1N1 virus (A/Puerto Rico/8/1934) into the PJET1.2 blunt vector.

MATERIALS & TOOLS USED

Prior to starting the step-by-step protocol for the HA gene assembly, below is the list of sequences, materials and tools that were used during the gene assembly process.

DNA Sequences

Sequence name	Accession page
Hemagglutinin (HA) gene [1,698 bp]	https://www.ebi.ac.uk/ena/browser/view/ABD77675
pJET1.2 blunt vector [2,974 bp]	https://assets.thermofisher.com/TFS-Assets/LSG/brochures/pJET1.2-plasmid-sequence.txt

Kits and Reagents

Item	Catalog number
pJET1.2 blunt vector (part of the CloneJET PCR Cloning Kit)	ThermoFisher Scientific, K1231
Q5 High-Fidelity DNA Polymerase	New England Biolabs, M0491S
Mastercycler nexus gradient thermal cycler	Eppendorf, 6331000025
CorrectASE enzyme and reaction buffer	ThermoFisher Scientific, A14972
Monarch PCR and DNA Cleanup Kit	New England Biolabs, T1030S
NEBuilder HiFi DNA Assembly Master Mix	New England Biolabs, E2621X

Web Tools

Purpose	Webpage	Link/access
Build blocks from gene sequence Design homology primers	NEBuilder Assembly Tool (v2)	https://nebuilder.neb.com/#!/
Design PCA oligonucleotides	DNAWorks (v3.2.4)	https://helixweb.nih.gov/dnaworks/

STEP-BY-STEP PROTOCOL

Figure 1 presents the experimental workflow that was used for HA gene assembly. The overall process can be split into four steps: **1) block design**, **2) oligo design and synthesis**, **3) block assembly by PCA and error correction**, and **4) gene assembly into vector and sequence verification**.

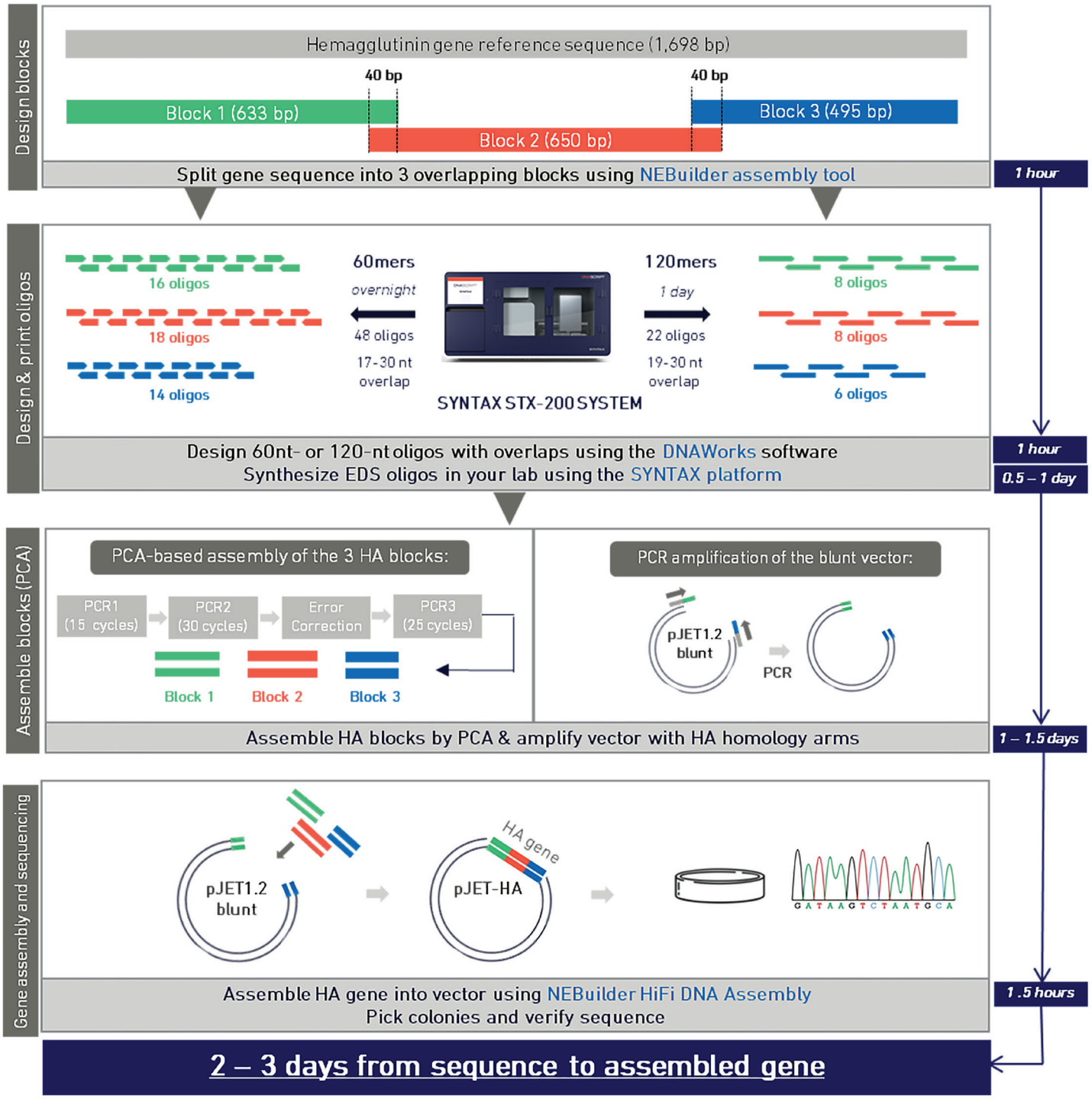

Figure 1. Experimental workflow for assembly of the hemagglutinin (HA) gene. (1) The 1.7kb HA gene sequence was split into 3 blocks (green, orange, and blue) with a 40-nt overlap using NEBuilder Assembly tool. (2) DNAWorks v3.2.4 was used to design 48 or 22 oligos (60nt and 120nt assembly approaches respectively) as starting material for PCA assembly into blocks. DNA oligos were printed on the SYNTAX STX-200 platform. (3) Oligos were assembled into 3 dsDNA blocks by PCA assembly in 3 PCR steps, including an error correction step using the CorrectASE enzyme (Thermo Fisher Scientific). The pJET1.2 vector was PCR-amplified with gene-overlapping primers designed to contain 25nt-homology with blocks 1 and 3 using the NEBuilder Assembly tool. (4) Blocks were then purified and assembled into the HA gene and cloned into the pJET1.2 blunt vector using the NEB HiFi DNA Assembly kit. Resulting vectors containing the assembled HA gene were transformed into TOP10 bacteria cells. The resulting sequences from picked colonies were analyzed by rolling circle amplification (RCA)- Sanger sequencing.

Note: The entire process from sequence to assembled gene should typically take from 2 to 3 days, depending on the length of oligos to be synthesized. Additional time may be required for cloning, optional QC steps during the PCA process, and depending on sequencing facilities for sequence verification.

STEP 1. Design blocks

1. Load the HA sequence into the NEBuilder assembly tool 2.0 (https://nebuilder.neb.com/#!/) and specify that the fragment DNA will be produced by synthesis.
2. Split the single sequence into multiple overlapping synthetic sequences (blocks), specifying the maximum block length and minimum overlap between blocks (here: 650bp and 40bp respectively).*
3. Add the pJET1.2 blunt vector sequence as a new fragment and specify that fragment DNA will be produced by PCR.
4. Edit settings to specify the minimum overlap length of the assembly primers used to assemble the gene into the vector (here: 25nt overlap) and the polymerase used (here: Q5 High-Fidelity DNA polymerase).
5. Download resulting sequences (here: 3 HA overlapping blocks of 633, 650, and 495bp and assembly primers with 25nt overlap with blocks 1 and 3).

***Note:** NEBuilder assembly tool suggests a 40bp overlap between synthetic fragments but this can be modified. Block length typically ranges from 200-700bp and can be adapted depending on sequence structure/complexity and sequencing needs. Typically, fewer fragments result in higher assembly efficiencies.

STEP 2. Design and print oligos

1. Load the HA blocks sequences into the DNAWorks oligo design software (https://helixweb.nih.gov/dnaworks/) and specify parameters such as the desired oligo length and annealing temperature range (here: 62-65°C annealing temperature; 58-62nt oligo length for 60-mer assembly approach, 117-123nt for 120-mer assembly approach).
2. Repeat the process to obtain a list of overlapping oligos for each block to be built by PCA (here: 48 oligos for 60-mers, 22 oligos for 120-mers).
3. Design PCR primers at the extremities of each block for amplification
4. Synthesize assembly oligos and PCR primers on the SYNTAX STX-200 platform using the SYNTAX 96 Hi-Fidelity Kits.

STEP 3. Assemble blocks by PCA and perform error correction

1. For each block to assemble, pool corresponding oligos at a final concentration of 150nm each in H20.
2. **PCR1: Oligo primary assembly**

Set up the following reaction in a 50µL final volume:

Reagents	Final Concentration	50µL reaction
5X Q5 reaction Buffer	1X	10µL
Oligonucleotide pool (0.15µM)	30 nM each	10µL
10mM dNTPs	200µM each	1µL
Q5 High Fidelity DNA polymerase	0.02U/µL	0.5µL
Water	-	to 50µL

Perform PCR1 in a thermocycler using the following cycling parameters:

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	15
Annealing	64°C*	15sec	
Exension	72°C	20sec**	
Final extension	72°C	2min	1

*adapt annealing temperature to Tm from DNAWorks output
**adapt extension time to block length (20-30sec/kb)

3. PCR2: Amplification PCR

Using the product from PCR1, set up the following reaction in a 50µL final volume:

Reagents	Final Concentration	50µL reaction
5X Q5 reaction Buffer	1X	10µL
Forward primer	0.5µM	variable
Reverse primer	0.5µM	variable
10mM dNTPs	200µM each	1µL
Q5 High Fidelity DNA polymerase	0.02U/µL	0.5µL
Water	-	to 50µL
Product from PCR1	-	5µL

Perform PCR2 in a thermocycler using the following cycling parameters:

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	30
Annealing	60-63°C*	10sec	
Exension	72°C	20sec**	
Final extension	72°C	2min	1
*adapt annealing temperature to PC primer Tm (for each block to amplify) **adapt extension time to block length (20-30sec/kb)			

4. Confirm successful block assembly by running 5µL of PCR2 product on a 1% agarose gel.
5. Quantify the PCR product using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

6. Error correction

6.1 In a PCR tube, dilute the PCR2 product to 25ng/µL in 1X CorrectASE reaction buffer (Thermo Fisher Scientific) in a 50µL reaction volume.

6.2 To create mismatches, denature and re-anneal the diluted DNA using the following cycling parameters: 98°C for 2 minutes; 4°C for 5 minutes; 37°C for 5 minutes; 4°C hold. Place on ice.

6.3 Transfer 10µL of product to a separate PCR tube and add 1µL of CorrectASE enzyme.

6.4 Incubate the reaction mix at 25°C for 30 minutes* and immediately place on ice to stop the reaction. Add 1µL of 5mM EDTA.

6.5 Proceed to PCR 3 immediately.

*CorrectASE incubation time may be optimized depending on block/gene length. Refer to Thermo Fisher protocol for additional information.

7. PCR3: final PCR amplification

Using the error-corrected product, set up the following reaction in a 50µL final volume:

Reagents	Final Concentration	50µL reaction
5X Q5 reaction Buffer	1X	10µL
Forward primer	0.5µM	variable
Reverse primer	0.5µM	variable
10mM dNTPs	200µM each	1µL
Q5 High Fidelity DNA polymerase	0.02U/µL	0.5µL
Water	-	to 50µL
Error-corrected DNA	-	5µL

Perform PCR3 in a thermocycler using the following cycling parameters:

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	25
Annealing	60-63°C*	10sec	
Exension	72°C	20sec**	
Final extension	72°C	2min	1
*adapt annealing temperature to PC primer Tm (for each block to amplify) **adapt extension time to block length (20-30sec/kb)			

8. Purify resulting error-corrected blocks prior to assembly (using the Monarch PCR & DNA Cleanup kit according to manufacturer's instructions: New England Biolabs, Cat No T1030S).
9. Quantify assembled blocks using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

STEP 4. Assemble the gene into the vector and transform bacteria cells for sequence verification

1. Using assembly primers designed with DNAWorks in STEP 1 and printed with the STX-200 platform in STEP 2, perform a PCR reaction to amplify the 3kb pJET blunt cloning vector with the appropriate homology arms at its ends (here: 25nt complementarity with ends of blocks 1 and 3 of the HA gene).

The following reaction and cycling conditions were used:

Reagents	Final Concentration	50µL reaction
5X Q5 reaction Buffer	1X	10µL
Foward pJET assembly primer	0.5µM	variable
Reverse JET assembly primer	0.5µM	variable
10mM dNTPs	200µM each	1µL
Q5 High Fidelity DNA polymerase	0.02U/µL	0.5µL
Water	-	to 50µL
PJET blunt vector (5ng/µL)	5ng	1µL

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	25
Annealing	58°C*	10sec	
Exension	72°C	1min30**	
Final extension	72°C	2min	1
*adapt annealing temperature to assembly primer Tm **adapt extension time to vector length (20-30sec/kb)			

2. Purify amplified vector using the Monarch PCR & DNA Cleanup kit and quantify it using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) following manufacturer's instructions.

3. Assembly of blocks into the HA gene and into vector

Follow the instruction manual for NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Cat No E2621S) to assemble the HA gene into the pJET blunt vector.
Useful recommendations for reaction protocol for assembly of 4 fragments (3 blocks + 1 linearized vector):

- Use 0.2 pmol total DNA amount, at a 1:1:1:1 vector: inserts ratio (0.05 pmol each)
- Incubate samples for 60 minutes at 50°C to perform the assembly reaction.

4. Transform chemically competent cells using 2µL of the chilled assembled product, following the manufacturer's recommendations. One Shot TOP10 Chemically Competent *E. Coli* cells were used here (Thermo Fisher Scientific Cat No C404010).
5. Verify DNA sequences of the resulting colonies to confirm the successful assembly of the gene into the vector. In this case, approximately 70 individual colonies were picked for each condition (with or without error correction, using the 60nt or 120nt oligo approach). They were inoculated in 300µL LB containing 100µg/mL ampicillin in a 2mL 96-deep well plate and grown for 8 hours. 10µL aliquots of each saturated culture were transferred to agar plates, and individual plasmid DNA was sequenced by automated rolling circle amplification (RCA) – Sanger sequencing.

Following this protocol, we successfully assembled the 1.7kb HA gene in less than 3 days starting from the DNA sequence, oligo design, and synthesis all the way through to the assembly of the gene into the vector, using both the 60nt- or 120-nt oligo strategies to build the complete HA gene. Detailed results are provided in the Application Note.

Note: This quick guide is to be used as a general workflow to help users develop their specific assembly protocol, which should be adapted to the gene/sequence to assemble. The total time from sequence to assembled product may vary depending on sequence characteristics and the number of fragments required for the assembly. Furthermore, optional checkpoints may be included throughout the process, such as oligo quality control, sequencing, and gel visualization of assembled products at each step of the PCA process.

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