

# Wet Lab Tutorial: Genelet Circuits

This tutorial will introduce the *in vitro* transcriptional circuits toolkit. The tutorial will focus on the design, synthesis, and experimental testing of a synthetic oscillator built using components from this toolkit. First, we will provide a brief theoretical introduction to transcriptional circuits, with overviews of

1. The main features transcriptional circuits, including achievable dynamic behaviors
2. The general design process for genelet circuits, with focus on two and three node oscillators
3. The protocols and experimental techniques needed to implement genelet circuits in the lab.

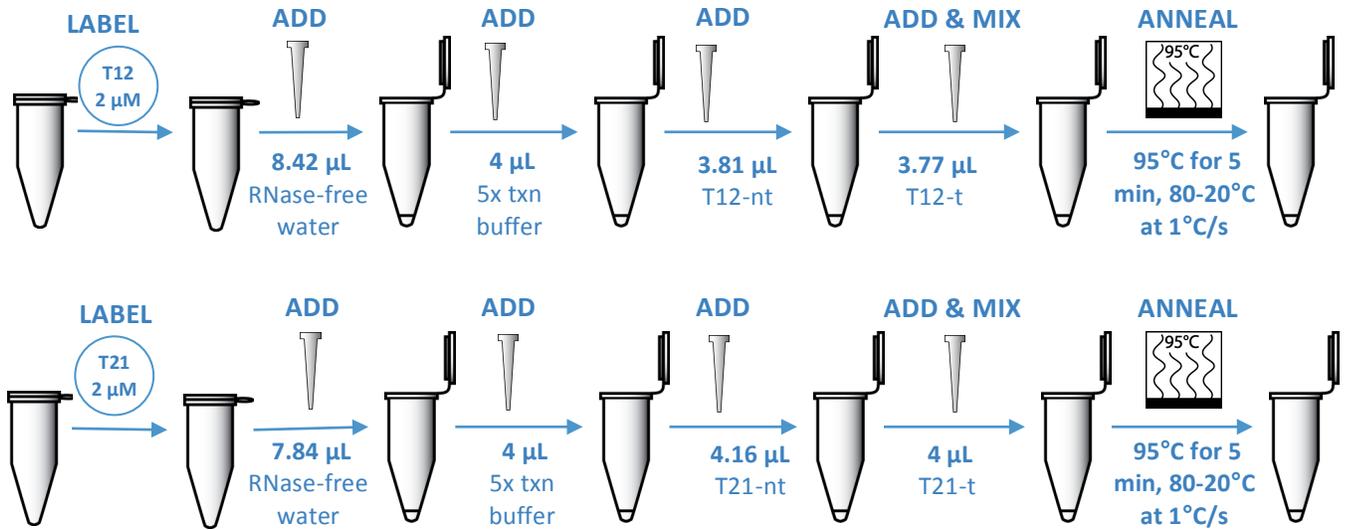
Also, we will describe some of the software tools that can be used to design and simulate transcriptional circuits. Participants will be encouraged to pursue a design project of their choice using the software (options/project outlines will be provided).

The protocol for a laboratory session is provided below. The participants will individually run the protocol and collect oscillatory trajectories data for a two-node oscillator.

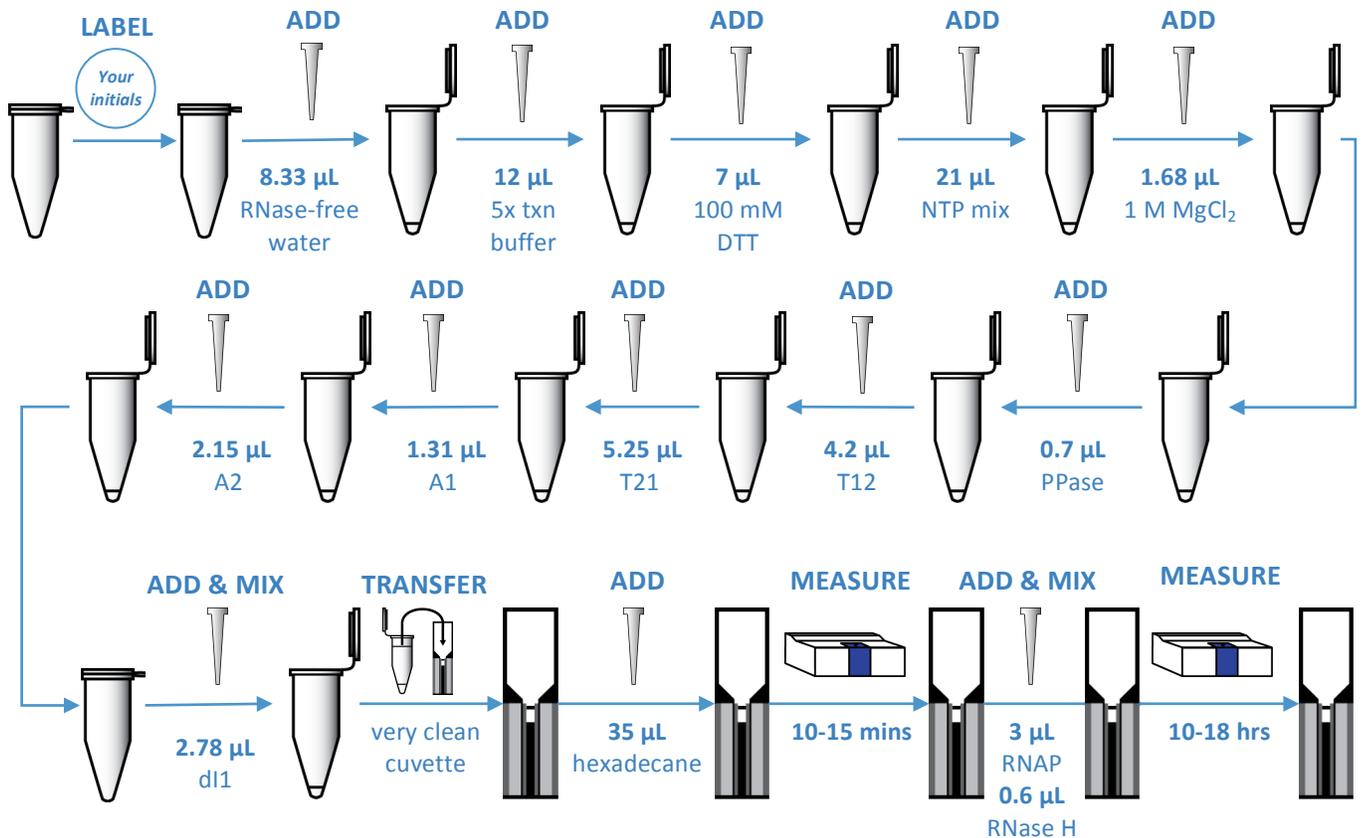
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# WET LAB TUTORIAL: TWO-NODE GENELET OSCILLATOR

## PART 1: ANNEALING



## PART 2: ASSEMBLING



# 1. WET LAB TUTORIAL PROTOCOLS

## 1.1. PRE-LAB PREPARATION

1. After lunch, set the spectrofluorometer water bath to 37°C.

## 1.2. MATERIALS

DNA, ENZYMES, REAGENTS & BUFFERS	SUPPLIES & EQUIPMENT
<i>DNA (all strands are from IDT)</i>	<i>Supplies</i>
T12-t	(2) PCR tubes
T21-nt	(1) Eppendorf tube
T21-t	(1) cuvette
A1	<i>Equipment</i>
A2	Thermocycler
dl1	Spectrofluorometer
<i>Enzymes</i>	
T7 RNA Polymerase (RNAP) (EpiBio, TM910K)	
E. coli Ribonuclease H (RNaseH) (Ambion, AM2293)	
Inorganic Pyrophosphatase (PPase) (Sigma Aldrich, I1891-100UN)	
<i>Reagents &amp; Buffers</i>	
Nucleoside triphosphates (NTPs) (EpiBio, RN02825)	
5x Transcription buffer (EpiBio, BP1001)	
10x dithiothreitol (DTT) (EpiBio, BP1001)	
RNase-free water	

*Note: Product I1891 is lyophilized powder.*

*To resuspend, use 20 mM Tris-HCl pH 7.2, 1 mM MgCl<sub>2</sub>, 50% glycerol (v/v) to 1.5 U/uL.*

*Note: Product BP1001 is discontinued.*

## 1.3. MORNING PROTOCOLS

### ANNEALING GENELET CIRCUIT COMPONENTS

This protocol describes the method for preparing the genelet components of a two-node oscillator by annealing, from DNA stocks provided to you. Due to lab space constraints, groups of four will work in two teams of two each. Each team of two will share the following reagents:

	reagent	volume	concentration
DNA strands	T12-nt	15 µL	10.5 µM
	T12-t	15 µL	10.6 µM
	T21-nt	15 µL	9.6 µM
	T21-t	15 µL	10.0 µM
	A1	20 µL	10.7 µM
	A2	20 µL	11.4 µM
	dl1	30 µL	12.6 µM
Transcription reagents	Txn buffer	60 µL	5x
	10x DTT	30 µL	100 mM
	NTP mix	60 µL	25 mM each NTP
	MgCl <sub>2</sub>	20 µL	1 M
	RNase-Free H <sub>2</sub> O	0.5 mL	-

Note: All of the DNA reagents you will receive have already been quantitated, but those interested may practice quantitation using the included protocol.

Each group of four will also share the following reagents:

	reagent	volume	concentration
Enzymes	RNAP	25 $\mu\text{L}$	-
	RNase H	6 $\mu\text{L}$	-
	PPase	6 $\mu\text{L}$	-

Note: Enzymes should be kept at freezing temperatures at all times.

Okay, start annealing!

1. Label two (2) PCR tubes T12 and T21.
2. Prepare annealing reactions to a target concentration of 2  $\mu\text{M}$  in PCR tubes. The volume of each DNA strand needed depends on the relationship:

$$\text{vol } (\mu\text{L}) = \frac{\text{Target concentration } (\mu\text{M})}{\text{Stock concentration } (\mu\text{M})} \times \text{Total volume } (\mu\text{L})$$

Use the following table as a checklist for your additions.

	RNase-free water	5x txn buffer	T12-nt	T12-t
T12	8.42 $\mu\text{L}$	4 $\mu\text{L}$	$20 \times 2/10.5 =$ 3.81 $\mu\text{L}$	$20 \times 2/10.6 =$ 3.77 $\mu\text{L}$
	RNase-free water	5x txn buffer	T21-nt	T21-t
T21	7.84 $\mu\text{L}$	4 $\mu\text{L}$	$20 \times 2/9.5 =$ 4.16 $\mu\text{L}$	$20 \times 2/10.0 =$ 4.00 $\mu\text{L}$

- a. Add (20 (1 - 2/<stock conc.>))  $\mu\text{L}$  of RNase-free water to each tube.
  - b. Add 4  $\mu\text{L}$  of 5x transcription buffer to each tube.
  - c. Add (20  $\times$  2/<stock conc.>)  $\mu\text{L}$  of non-template strand.
  - d. Add (20  $\times$  2/<stock conc.>)  $\mu\text{L}$  of template strand.
3. Vortex each tube thoroughly (approx. 1 minute).
  4. Spin down each tube briefly in microcentrifuge.
  5. Load tubes into thermocycler.
    - a. In Keck 040, start thermocycler program 'Anneal'
    - b. In Moore 210, start thermocycler program 'NPRTF'

## DNA QUANTITATION

The typical DNA quantitation assay involves measuring the absorbance of a DNA solution at 260 nm ( $A_{260}$ ). The exact protocol depends on the equipment being used to perform the assay, but the measured concentration of DNA is given by the relationship

$$\text{DNA concentration } (M) = \frac{A_{260} \times \text{path length } (cm)}{\text{extinction coefficient } (M^{-1} \cdot cm^{-1})}$$

Note: IDT's Oligo Analyzer web app is useful for obtaining extinction coefficients.

Here are protocols for using a Nanodrop (Keck 040) and a Biophotometer (Moore 210).

#### *Nanodrop (Keck 040)*

1. Start the Nanodrop software. Select **Nucleotides**.
2. Blank the Nanodrop.
  - a. Add **2  $\mu$ L** of **RNase-free water** to the Nanodrop sample pedestal.
  - b. Click **Blank**.
  - c. Clean the pedestal with a Kimwipe.
3. Measure your sample.
  - a. Add **2  $\mu$ L** of **DNA sample** to the Nanodrop sample pedestal.
  - b. Click **Measure**.
  - c. Record the  $A_{260}$  value.
  - d. Clean the pedestal with a Kimwipe.
4. Clean the Nanodrop.
  - a. Add **2  $\mu$ L** of **RNase-free water** to the Nanodrop sample pedestal.
  - b. Click **Measure**.
  - c. Check to see that the  $A_{260}$  value is close to **0**. If not, repeat steps **4a** and **b**.
  - d. Clean the pedestal with Kimwipes.

#### *Biophotometer (Moore 210)*

5. Blank the Biophotometer.
  - a. Add **100  $\mu$ L** of **RNase-free water** to a cuvette and place in Biophotometer.
  - b. Click **Blank**.
6. Measure your sample.
  - a. Prepare sample in a fresh cuvette and place in Biophotometer.
    - i. Add **98  $\mu$ L** of **RNase-free water** to cuvette.
    - ii. Add **2  $\mu$ L** of **DNA sample** to cuvette.
  - b. Click **Measure**.
  - c. Record the  $A_{260}$  value and multiply by dilution fact (i.e. **50**)

In both cases, repeat measurements twice. If measurements differ by more than 5% (allows for standard pipetting errors), then mix the DNA sample thoroughly and repeat the procedure.

## 1.4. AFTERNOON PROTOCOLS

### ASSEMBLING GENELET CIRCUITS

Assembling a genelet network for experimental study involves the careful pipetting of annealed genelets; DNA activator and inhibitor strands; buffers, transcription reagents, and water; and enzymes at the appropriate time.

Additionally, the target concentrations/quantities of all components need to be carefully chosen to ensure the desired dynamic behavior is observed in your experiment, and so that you know the necessary volumes to pipette in advance. Here are the DNA concentrations and enzyme quantities that we have seen result in oscillations:

	reagent	concentration
DNA	T12	120 nM
	A2	350 nM
	T21	150 nM
	A1	200 nM
	dl1	500 nM
enzymes	RNAP	3 $\mu$ L
	RNase H	0.6 $\mu$ L

*Note: All of the DNA reagents you will receive have already been quantitated, but those interested may practice quantitation using the included protocol.*

Okay, start assembling!

1. Combine the buffers and transcription reagents in a fresh Eppendorf tube. (Label the tube with your initials, e.g. J.B). Use the following table as a checklist for your additions:

	RNase-free water	5x txn buffer	100 mM DTT	NTP mix (25 mM each NTP)	1 M MgCl <sub>2</sub>	PPase
J.B.	8.33 $\mu$ L	12 $\mu$ L	7 $\mu$ L	21 $\mu$ L	1.68 $\mu$ L	0.7 $\mu$ L

*Note: We are aiming for 30 mM final Mg<sup>2+</sup> (6 mM final Mg<sup>2+</sup> comes from just 5x txn buffer) such that Mg<sup>2+</sup>:NTP ratio is 1:1. You can keep Mg<sup>2+</sup>:NTP ratio a little more than 1:1 without seeing inhibition of transcription.*

*Note: The total reaction volume will be 70  $\mu$ L with the enzymes, which we will add after starting the data collection.*

2. Combine the DNA components in a fresh Eppendorf tube. (Label the tube with your initials, e.g. J.B.). Use the following table as a checklist for your additions.

	T12 (2 $\mu$ M)	T21 (2 $\mu$ M)	A1 (10.7 $\mu$ M)	A2 (11.4 $\mu$ M)	dl1 (12.6 $\mu$ M)
J.B.	4.2 $\mu$ L	5.25 $\mu$ L	1.31 $\mu$ L	2.15 $\mu$ L	2.78 $\mu$ L

3. Vortex to mix thoroughly, about **1 minute**.

## MEASURING GENELET CIRCUIT BEHAVIOR

We use a *spectrofluorometer* (Horiba Jobin-Yvon Fluorolog 3) for real-time measurement of the two genelet states within the oscillator. (Two spectrofluorometers, actually, located in Moore 222 and Keck 040.)

We observe the activities of genelets T21 and T12 via the fluorophore labels TYE665 (649nm/665nm max. excitation/emission) and TYE563 (549nm/563nm max. excitation/emission), respectively, and their interaction with the quenchers labeling the activators A1 and A2 (i.e. an activated, or ON, genelet has low fluorescence, while a deactivated, or OFF, genelet has high fluorescence). We set the excitation and emission monochromators on the spectrofluorometer to these wavelengths, with a 5 nm slit width.

*Note: When using multiple fluorophores, you must determine the extent of the crosstalk between channels for each fluorophore. (Luckily, there's a negligible crosstalk contribution for the case of TYE563 and TYE665.)*

1. Make sure the water bath is set to **37°C** and the slit width is **5 nm**.
2. Transfer your (mostly) **assembled genelet circuit** from the tube labeled with your initials to **very** clean cuvette.
3. Add **35 µL** of **hexadecane** on top of your sample to prevent evaporation. Place the cuvette in one of the sample holders in the turret inside the spectrofluorometer.
4. Start data collection in the Datamax software with the help of the tutorial leaders.
  - a. Collect data every **1-2 minutes**.
  - b. Establish a baseline for the enzyme-free, completely off circuit by collecting data for **10-15 minutes**.
5. Pause the data collection to add the enzyme components. Use the following table as a checklist for your additions.

cuvette	RNase H 0.6 µL	RNAP 3 µL
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- a. Pipette each enzyme into the middle of sample in the cuvette and pipette up and down rapidly to mix thoroughly.
  - b. *Optional: mix even more thoroughly by rapidly stirring your sample with the pipette tip for several more seconds.*
6. Restart the data collection. We'll check on the fluorescence time-courses again after the software tools session.

*Note: After completing the spectrofluorometer measurements, the fluorescence data are typically normalized from arbitrary units (cps: counts per second) to normalized signal between 0 and 1, using the maximum and minimum fluorescence measured for each genelet. The maximum/minimum ratio for each fluorophore is determined off-line, i.e. without enzymes, by quenching. The minimum or maximum fluorescence from an experimental run and the quenching ratio from the off-line quenching study can be used together to calculate the normalized genelet signal.*

## 1.5. APPENDIX

### LIST OF DNA SEQUENCES

T21-nt (101mer)

5'-\ TYE665 \-CAT TAG TGT CGT TCG TTC ACA GTA ATA CGA CTC ACT ATA GGG AGA  
GTA AAA CGG ATT GAA GCA AGG GTA AGA TGG AAT GAT AAT ACT GAC AAA GTC  
AGA A(A)-3'.

T21-t (74mer)

5'-TTT CTG ACT TTG TCA GTA TTA TCA TTC CAT CTT ACC CTT GCT TCA ATC CGT TTT  
ACT CTC CCT ATA GTG AGT CG-3'.

A1 (36mer)

5'-TAT TAC TGT GAA CGA ACG ACA CTA ATG AAC TAC TAC-\ Iowablock RQ \-3'.

dl1 (38mer)

5'-GTG TGT AGT AGT AGT TCA TTA GTG TCG TTC GTT CAC AG-3'.

T12-nt (106mer)

5'-\ TYE563 \-AAG CAA GGG TAA GAT GGA ATG ATA ATA CGA CTC ACT ATA GGG AGA  
AAC AAA GAA CGA ACG ACA CTA ATG AAC TAC TAC TAC ACA CTA ATA CTG ACA AAG  
T(CA GAA A)-3'.

T12-t (79mer)

5'-TTT CTG ACT TTG TCA GTA TTA GTG TGT AGT AGT AGT TCA TTA GTG TCG TTC GTT  
CTT TGT TTC TCC CTA TAG TGA GT CG-3'.

A2 (35mer)

5'-TAT TAT CAT TCC ATC TTA CCC TTG CTT CAA TCC GT-\ IowaBlack FQ \-3'.

*Note: IDTDNA no longer synthesizes long oligos with NHS-ester modifications such as TAMRA and Texas Red. Also, oligos longer than 100-mer have special pricing as ultramers. Here, we use 100-mer nt strands (the bases within brackets are part of design but are not included in the DNA synthesis order).*