iGEM Protocols

Double Digest Protocol (NEB)

Materials

A "Typical" Restriction Digestion

Reagent	Volume
Nuclease free water	Add up to 50 µl
10X NEBuffer	5 µl (1:10 dilution)
DNA	1 µg

Restriction Enzyme	generally 1µl (10 units) per enzyme
Total Reaction Volume	50 µl

Procedure

1. Get an ice bucket and fill it with ice
2. Place enzymes, cutsmart buffer, DNA, nuclease free water on ice
3. Calculate amount needed for each component (water would be 50µl - (all other components)
4. Add nuclease free water
5. Add 5ul cutsmart buffer
6. Add DNA
7. Add 1ul enzyme(s)
8. Flick or pipette up and down or briefly centrifuge to mix (Make sure everything is well combined!)
9. Incubate at 37C for 1 hour
10. Stop the reaction by heat inactivating at specific temperature and time

Other notes:

Enzyme

- Keep on ice when not in the freezer
- Should be the last component added to reaction
- Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.
- In general, we recommend 5–10 units of enzyme per µg DNA, and 10–20 units for genomic DNA in a 1 hour digest.

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol
- Smaller reaction volumes can be used

Alternative Volumes for Restriction Digests

	Restriction Enzyme*	DNA	10X NEBuffer
10 µl rxn	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

Incubation Time

- Incubation time is typically 1 hour (for our purposes, usually at 37C)
- Can often be decreased by using an excess of enzyme
- It is possible, with many enzymes, to use fewer units and digest for up to 16 hours.

Ligation (NEB)

Material & Methods:

NEB ligation calculator here

Reagent	20 µl REACTION
Nuclease-free water	to 20 µl
T4 DNA Ligase Buffer (10X)*	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
T4 DNA Ligase	1 µl

Procedure (~30-40 min)

1. Get an ice bucket and fill it with ice
2. Put T4 DNA Ligase, buffer, DNA, and nuclease free water on ice
3. Take a microcentrifuge tube and combine x uL nuclease free water, 2uL buffer, insert and vector DNA, and 1uL T4 ligase in this order.
4. Gently mix the reaction by pipetting up and down and microfuge briefly.
5. For cohesive (sticky) ends, incubate at 16°C or room temperature for ~~10 minutes~~ overnight.
6. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours *(alternatively, high concentration T4 DNA Ligase can be used in a 10 minute ligation)*.
7. Heat inactivate at 65°C for 10 minutes.
8. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

Chemical Transformation (iGEM)

Materials

Reagent	Amount (per transformation)
DNA to be transformed (sample DNA or 10pg/uL positive control)	1 uL
Competent cells	50 uL
LB/SOC media w/o antibiotics	950 uL
LB agar plates with antibiotics	1

Method

1. Resuspend DNA in selected wells in the Distribution Kit with 10µl dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
2. Label 1.5ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.
3. Thaw competent cells on ice: This may take 10-15min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
4. Pipette 50µl of competent cells into 1.5ml tube: 50µl in a 1.5ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.
5. Turn on the water bath at 42 C
6. Thaw competent cells (50 uL aliquot) on ice and label them accordingly
7. Pipette 1µl of resuspended DNA into the competent cells: Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
8. Pipette 1µl of control DNA into another tube of competent cells: Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
9. Don't need to pipette anything into the negative control.
10. Incubate on ice for 30min: Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
11. Heat shock tubes at 42°C for 45 sec: 1.5ml tubes should be in a floating foam tube rack. Place in a water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
12. Incubate on ice for 5min: Return transformation tubes to ice bucket.
13. Pipette 950µl LB/SOC media to each transformation: LB/SOC can be warmed to room temperature before use. Check for contamination.
14. Incubate at 37°C for 1 hours, shaking at 200-300rpm
15. Spin down cells at 6800 rcf for 3mins and discard 850µL of the supernatant. Resuspend the cells in the remaining 150µL, and pipette each transformation onto petri plates Spread with sterilized spreader or glass beads immediately. This increases the chance of getting colonies from lower concentration DNA samples.

16. Incubate transformations overnight (14-18hr) at 37°C: Incubate the plates upside down (agar side up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
17. Pick single colonies: Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.
18. Count colonies for control transformation: Count colonies on the 100µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of 1.5x10^8 to 6x10^8 cfu/µg DNA.

Electroporation (NEB)

Materials

Reagent	Amount (per transformation)
DNA to be transformed (sample DNA or 10pg/uL positive control)	1 uL
Electro-competent cells	50 uL
LB/SOC media w/o antibiotics	950 uL
LB agar plates with antibiotics	1

Equipment

- Electroporator
- Cuvette

Procedure

1. Take an ice busket and fill it with ice
2. Chill the cuvettes on ice
3. Aliquot 950 uL SOC media for each transformation sample
4. Thaw Electrocompetent cells on ice (about 10 min) and mix cells by flicking gently. Transfer 25 µl of the cells (or the amount specified for the cuvettes) to a chilled microcentrifuge tube. Add 1 µl of the DNA solution.

5. Carefully transfer the cell/DNA mix into a chilled cuvette without introducing bubbles and make sure that the cells deposit across the bottom of the cuvette.
6. Electroporate using the following conditions for BTX ECM 630 and Bio-Rad GenePulser electroporators: 2.0 kV, 200 Omega, and 25 µF. The typical time constant is 4.8 to 5.1 milliseconds.
7. Immediately add 975 µl of 37°C NEB 10-beta/Stable Outgrowth Medium to the cuvette, gently mix up and down twice, then transfer to the 17 mm x 100 mm round-bottom culture tube.
8. Shake vigorously (250 rpm) or rotate at 37°C for 1 hour.
9. Dilute the cells as appropriate then spread 100-200 µl cells onto a pre-warmed selective plate.
10. Incubate plates overnight at 37°C.

Agarose Gel Electrophoresis

Material

Reagent	Amount
DNA	50 uL
Agarose	1%
1X TAE buffer	(for making the agarose gel) 50 mL (for covering the gel) 1200 mL
DNA ladder	2 - 5 uL
6X Loading dye	10 uL (1:6 dilution)
SYBR Safe	2.5 uL per 50 mL gel

Equipment

- Gel casting platform
- Electrophoresis box
- Comb
- Power source

Procedure

1. Weigh 0.5g Ultra pure agar and put it in a 250 mL flask
2. Add 50mL 1X TAE to the flask
3. Melt the agarose in a microwave for 90s
4. While the agarose is cooling, prepare the gel casting platform by taping the two opening sites
5. When the agarose is comfortable to touch, add 2.5 uL SYBR Safe stain and swirl gently to mix
6. Pour the agarose into the casting platform which has the comb inserted
7. Remove air bubbles
8. Let the gel solidify ~10 minute
9. Remove the tape and orient the casting platform such that the comb is close to the anode
10. Remove the comb
11. Pour 1X TAE to the gel box to just covering the gel
12. Load all of each sample into a separate lane and keep track of what sample in what lane
13. Load 2-5 uL DNA ladder in one of the lanes
14. Perform the electrophoresis at 100 V for 1 hour
15. Visualize the gel using a transilluminator

PCR

Material

Reagent	Amount
dH2O	9.5uL
Forward primer (10uM)	1.25uL
Reverse primer (10uM)	1.25uL
Template plasmid (~10ng)	0.5uL
Q5 mastermix (2x)	12.5uL

Equipment

- Thermal cycler
- PCR tubes
- Pipettes and tips

Procedure

1. First add the designated volume of water, template DNA, and primers
2. With all reaction tubes *on ice,* add the designated volume of Q5 mastermix
3. Run PCR program on thermocycler for 30-35 cycles

Golden Gate Assembly

Material

For a 10uL reaction;

Reagent	Amount

DNA fragments (to insert)	40fmol *each**
DNA backbone	20 fmol*
T4 ligase	0.5uL
10x T4 ligase buffer	1uL
BsaI-HFv2 enzyme	0.2uL
10x BSA buffer	1uL
dH2O	Add until final reaction volume is 10uL

*

Equipment

Procedure

Media Preparation

1. LB media

Reagent / Protocol	Amount / Time
LB powder	12.5 g (21.25g for 850ml)
Water	500 ml
Autoclave	Liquid 20

2. LB - Agar media

- Makes ~22 plates per 500 ml

Reagent / Protocol	Amount / Time
LB powder	12.5 g (21.25g for 850ml)
Agar powder	7.5 g (12.75g for 850ml)
Water	500 ml
Autoclave	Liquid 20
Antibiotics	500 uL

Equipment

- Autoclave machine
- A 500ml bottle

Procedure

- Weigh the desired amount of LB and agar
- Add 500ml of milliQ
- Put the bottle in a plastic basket and add some amount of water to the basket (like covering ~1/10 of the bottle)
- Autoclave at liquid 20
- Cool down the media to touchable temperature
- Add antibiotics
- Swirl and make sure the antibiotics is dissolved
- Pour media to petri dish under the flame to ~½ full

3. Bacterial Glycerol Stock

Reagent / Protocol	Amount
Cell overnight culture	1000 uL (1mL)
50% glycerol (equal parts of glycerol and water, can be any volume)	1000 uL (1mL)

Equipment

- -80C freezer
- 1.5mL microcentrifuge tube (epi tube)

Procedure

- Add 1000uL of liquid bacterial culture to a 1.5mL epi tube.
- Add 1000ul of 50% glycerol to the same tube.
- Mix thoroughly by pipetting up and down, and store in -80C.

*1000uL volume can be adjusted as long as the tube to freezer contains equal parts of culture and 50% glycerol.

4. Antibiotics media

- Note:
  - Stock media should have a concentration of 1000X of the working concentration
  - Expected concentration list: https://www.addgene.org/protocols/pouring-lb-agar-plates/

Antibiotic	Recommended Stock Concentration	Recommended Working Concentration
Ampicillin	100 mg/mL	100 µg/mL
Bleocin	5 mg/mL	5 µg/mL
Carbenicillin*	100 mg/mL	100 µg/mL
Chloramphenicol	25 mg/mL (dissolve in EtOH)	25 µg/mL
Coumermycin	25 mg/mL (dissolve in DMSO)	25 µg/mL
Gentamycin	10 mg/mL	10 µg/mL
Kanamycin	50 mg/mL	50 µg/mL
Spectinomycin	50 mg/mL	50 µg/mL
Tetracycline	10 mg/mL	10 µg/mL

Equipment

- 2 falcon tubes
- 1 syringe
- 1 syringe filter
- Microcentrifuge tubes

Procedure

1. Weight desired amount of antibiotics to a falcon tube
2. Add desired amount of miniQ to the falcon tube
3. Attach a syringe to a syringe filter (make sure not to touch the bottom of the filter as we want to keep it sterile)
4. Withdraw the media into the syringe and dispense the media to another falcon tube
5. Aliquot ~1ml to a microcentrifuge tube
6. Label antibiotic_name + concentration and date and store at -20 deg

Column-pure Miniprep protocol (ABM)

Column-pure Gel purification (ABM)

Column-pure PCR purification (ABM)

Making DNA into usable stocks

1) Resuspending primers from IDT

Equipment

- Freeze dried primer from IDT
- Pipettes and tips
- dH2O

Procedure

- Record the nmol of each primer (printed on the side of the tube).
- Multiply each nmol value by 10. This is the volume (uL) of H2O to add to the dried primer.
- Pull up the volume of H2O needed from step 2 and place the tip at the bottom of the tube, which should be conical and end in a tip. Resuspend the primer by pipetting up and down 5-6 times.

*This will result in a 100uM solution of primer. For PCR, 10uM is required. To dilute to 10uM, mix 90uL of dH2O with 10uL of primer stock.

2) Utilizing DNA from an iGEM distribution kit

Equipment

- iGEM Distribution Kit plate
- Pipettes and tips
- dH2O

Procedure

- Locating the well of interest:
    a. When looking at the kit plate with the iGEM logo and title facing you, A1 will be on the top right corner. However, you can only access the wells when the aluminum foil side is facing you.
    b. Consequently, when the aluminum foil side is facing you, the A1 well is on the top left corner. Moving horizontally to the right increases the number, moving vertically towards the bottom increases the letter.
    c. You can mark the sides of the foil with a sharpie as you go along, and circle / mark the well you need with a Sharpe as well.
- Resuspending the DNA:
    a. Puncture the aluminum foil covering the well of interest with a pipette tip.
    b. Add 10uL dH2O to the bottom of the well. When the tip is touching the bottom, resuspend by pipetting up and down 5-6 times.

GeneJET Plasmid Miniprep Kit (Thermo)

https://tools.thermofisher.com/content/sfs/manuals/MAN0012655_GeneJET_Plasmid_Miniprep_UG.pdf

Making competent cells (Salmonella)

https://drive.google.com/file/d/1PLdirC-dptxXFguN_wHtW551FuK0arg_/view?usp=sharing

Making competent cells (E. coli)

https://jemi.microbiology.ubc.ca/sites/default/files/Chang%20et%20al%20JEMI-methods%20Vol%201%20pg%2022-25.pdf