# PROTOCOLS

**PREPARATION OF DNAZYMES**

**Preparation of beads**

1. Take two 1.5 mL centrifuge tubes. Take 50 µL of magna beads in each.
2. Prewash the beads with 100 µL of solution A twice and soup out the liquid.
3. Prewash the beads with 100 µL of solution B twice and soup out the liquid clearly.
4. Take 1 µL of biotinylated BCl-2 mRNA from 100 µM stock solution and add into 100 µL of 2X binding and washing buffer.
5. Incubate the mRNA (100 µL) into beads at 37°C for 15 min at 150 rpm shaking condition.
6. Soup out unbound mRNA.
7. Wash the unbound mRNA twice with 1X wash buffer.

**Preparation of DNA library:**

1. Take 1 µL of DNA library from 100 µM stock solution and dilute into 100 µL of binding buffer.
2. Heat the library at 95ᵒC for 10 minutes and snap chill it.
3. Then incubate snap chilled library with beads at 37ᵒC for 10 minutes with 150 rpm in incubator shaker.
4. After 10 minutes soup out the unbound library.
5. Wash the beads 8 times with wash buffer.
6. Add 100 µL of cleavage buffer (150mM KCl, 2 mM MgCl2)
7. Incubate at 37°C for 3 hrs with 150 rpm shaking condition.
8. Soup out the cleaved molecules.
9. Set up the following PCR reaction in 3 tubes.

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| --- | --- |
| Contents | Quantity |
| Master Mix | 25 µL |
| Cleaved molecules | 24.5µL |
| (100μM) Forward primer library | 0.25µL |
| (100μM) Reverse primer library | 0.25µL |
| Total volume per tube | **50μL** |

So, the master reaction will be,

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| Contents | Quantity |
| Master Mix | 75 µL |
| Cleaved molecules | 73.5µL |
| (100μM) Forward primer library | 0.75µL |
| (100μM) Reverse primer library | 0.75µL |
| Total volume | **150μL** |

1. Run the 5 µL of PCR product in 1.5 % gel using urea-PAGE electrophoresis.

**Control Reaction:** Add 100μL wash buffer to beads and incubate for 3 hrs at 37°C and 150 rpm.

**UREA PAGE GEL ELECTROPHORESIS**

We ordered readymade urea page gel and loaded the sample with TBE Urea sample buffer.

***Crush and soak method.***

Single or double stranded DNA molecules once separated, were extracted from the urea PAGE gel by crush and soak method. The principle involved was separation of DNA molecules by simple diffusion method. Gel piece of interest was cut down from the gel, dried and the gel was made into fine particles. 3 to 4 volume of the 1mM EDTA +0.5M ammonium acetate solution was added to the dried and fine powdered urea gel and incubated for a period of 6 to 8 hours at 550 c.After the incubation time the sample was centrifuged at 12000 rpm for 5 minutes and collected the supernatant that contains DNA.

**TRANSFECTION OF DNAZYMES**

1. Heat DNAzyme samples to 95°C to denature and make it single stranded.
2. In eppie tubes, add 10μL each of DNAzyme to 175μL of optiMEM.
3. Also prepare a control consisting of 10μL milliQ water and 175μL optiMEM.
4. Prepare oligofectamine mix.

* In a 6 well culture plate, add 6μL of oligofectamine and 45μLof optiMEM to each well and pipette it 15 times.
* Incubate at room temperature for 8 minutes.

1. Combine DNAzyme mix with oligofectamine mix (15μL/well)
2. Incubate for 20 min at room temperature.
3. Remove growth media and wash once with serum free media.
4. Replenish with 800μL of serum free media.
5. Add 200μL of complexes drop by drop
6. Incubate at 37°C for 4hrs.
7. Prepare recovery media: 3mL FBS +7mL media
8. After 4hrs, add 500μL of recovery media to each well.

**ISOLATION OF RNA USING TRIZOL REAGENT**

1. **Harvesting of cells**
   1. Rinse cells with cold PBS once.
   2. Scrape the cells with cell scraper and PBS.
   3. Centrifuge the cell suspension at 2000 rpm for 5 minutes.
   4. Remove PBS and take cell pellet.
2. **Cell lysis**
   1. Add 1mL of Trizol for cell pellet of 5 to 6×106 cells. Vortex thoroughly and pipette several times.
   2. Incubate homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complex.
3. **Phase separation**
   1. Add 0.2mL of chloroform per mL of Trizol reagent
   2. Vortex sample vigorously for 15 sec and incubate at room temperature for 2-3 minutes.
   3. Centrifuge sample at 12,000xg for 15 minutes at 2-8°C.
   4. Three phases will form – lower red phenol-chloroform phase, an interphase and an upper aqueous phase which contains RNA.
   5. Take the aqueous phase out carefully without disturbing.
4. **RNA precipitation** 
   1. Add 0.5mL isopropyl alcohol for 1mL of Trizol.
   2. Centrifuge at 12,000xg for 10 minutes at 2-4°C.
5. **RNA Wash**
   1. Add 1mL 75% ethanol for 1mL Trizol.
   2. Centrifuge at 7,500xg for 5 minutes at 2-8°C
   3. Remove ethanol.
   4. Airdry RNA pellets for 5- 10 minutes
   5. Re-suspend RNA with DEPC treated water.

**cDNA SYNTHESISUSING VERSO cDNA SYNTHESIS**

**Pre prep**

* Prepare crushed ice.
* Label tubes and keep in ice.
* Set the temperature of dry bath to 70°C.
* Set the temperature of water bath to 42°C.

1. Prepare 8μL primer cocktail with random hexamer and oligodT in 3:1 ratio.
2. In 1steppie tube, add and mix

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| --- | --- |
| Contents | Quantity |
| RNA | 1μL |
| Primer cocktail | 1μL |
| Nuclease free water | 11μL |
| Total volume | **13μL** |

1. Incubate mixture at 70°C for 5 minutes
2. Snap chill in crushed ice.
3. In 2ndeppie tube, add and mix,

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| --- | --- |
| Contents | Quantity |
| 5X buffer | 4μL |
| Dntp | 2μL |
| Enzyme mix | 1μL |

Make upto 20μL by adding 13μL of the reaction mixture from the 1steppie tube.

1. Incubate at 42°C for 1 hr in water bath.
2. Deactivate at 72°C for 5 min in dry bath.
3. Store at -20°C.

**RESTRICTION DOUBLE DIGESTION**

1. Wipe down the working surface with 70% ethanol.
2. Place the buffers and restriction enzymes in ice or in minicooler.
3. In an eppie tube, keep the following digestion mixture

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| --- | --- |
| Contents | Quantity |
| PCR purified product | 28μL |
| Cut smart buffer (10x) | 4 μL |
| PstI | 1.5 μL |
| NF water | 6.5 μL |
| Total volume | 40 μL |

1. Incubate at 37°C for 2hrs.
2. Deactivate the enzyme in dry bath at 80°C for 10-15 minutes.
3. Load the whole reaction mixture in 1% agarose gel and run the gel in electrophoresis.
4. Perform gel extraction.
5. Confirm the presence of product by running 2μL of the gel extracted product on 1% agarose gel.
6. In a fresh eppie tube, prepare the following digestion reaction.

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| --- | --- |
| Contents | Quantity |
| PCR purified product | 28μL |
| FastDigest smart buffer (10x) | 4 μL |
| ECoRI | 1.5 μL |
| NF water | 6.5 μL |
| Total volume | 40μL |

1. Incubate at 37°C for 1 hr.
2. Deactivate the enzyme on 80°C for 10-15 minutes.
3. Load the whole reaction on 1% agarose gel and run the gel in electrophoresis.
4. Cut out 100bp band and perform gel extraction.
5. Confirm gel extracted product by performing agarose gel electrophoresis on 1%gel.
6. The insert is ready for ligation.

**LIGATION**

1. Mix the following in an eppie tube

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| --- | --- |
| Contents | Quantity |
| Insert | 10μL |
| pSB1C3 (plasmid backbone vector) | 5 μL |
| T4 ligase buffer (10x) | 2 μL |
| T4 ligase enzyme | 1 μL |
| Nuclease Free water | 2 μL |
| Total Volume | 20μL |

1. Incubate the reaction mixture at 22°C (room temperature) for 1 hr or

4°C overnight.

1. Take 10μL of ligated product and perform transformation using DH5α competent cells.