# MOLECULAR CLONING

## Preparation of Competent E. Coli Cells Using $CaCl_2$

1. Take one colony and start a 5 ml overnight culture at 37°C, with shaking.
2. Dilute the overnight culture 1:100 into 50 ml SOB medium.
3. Grow culture at 37°C with shaking to an OD600=0.4.
4. Let the culture sit on ice for ~15 min, swirling occasionally.
5. Pour the culture into a 50 ml Falcon™ tube.
6. Centrifuge at 3500 rpm for 5 min at 4°C.
7. Remove as much as possible of the supernatant without disturbing the pellet.
8. Resuspend the pellet in 100 µL ice-cold 0.1 M $CaCl_2$ with the help of a sterilized loop.
9. Add 15 ml ice-cold 0.1 M $CaCl_2$. Mix gently by pipetting up and down a few times.
10. Incubate the cells on ice for 30 min.
11. Pellet the cells again at 3500 rpm for 5 min at 4°C.
12. Resuspend the cells in 2 ml ice-cold 0.1 M $CaCl_2$/20% glycerol.
13. Incubate for 45 min on ice.
14. Aliquot carefully in 50 µL amounts to chilled 1.5 ml tubes.
15. Snap freeze in liquid nitrogen any tubes that will not be used for transformation within a few hours.
16. Store at -80C°.

## Plasmid Preparations

OMEGA Plasmid Mini Kit I (Cat. D6943-02). and E.Z.N.A. Endo-free Plasmid Mini Kit I (Cat. D6948-02). Protocol refers to the instruction of the kit.

## Polymerase Chain Reaction (PCR)

1. Prepare a mixture of: •DNA template, 100 µg
   - •2 oligodeoxyribonucleotide primers, 0.4 µl *2
   - •10X dNTPs, 2 µl
   - •10X PCR Buffer, 2 µl
   - •rTaq polymerase, 0.1 µl
   - •dd$H_2O$, up to 20 µl
2. Set PCR program based on melting temperature, fragment length and polymerase type.

Note: We also used Takara PrimeSTAR Max/GXL Mixture. The protocol refers to the instruction of the mixture.

## 2-step PCR (for adding prefix and suffix)

1. Primers

   VEGF-A121 prefix: 5' CGGAATTCGCGGCCGCTTCTAGATGAACTTTCTGCTGTCTTGGGT 3'
   VEGF-A121 suffix: 5' AACTGCAGCGGCCGCTACTAGTACCGCCTCGGCTTGTCAC 3'

   PDGFA prefix: 5' CGGAATTCGCGGCCGCTTCTAGATGAGGACCTTGGCTTGCCT 3'
   PDGFA suffix: 5' AACTGCAGCGGCCGCTACTAGTACCTCACATCCGTGTCCTCTTCC 3'

bFGF prefix: 5’ CGGAATTCGCGGCCGCTTCTAGACCATGGCAGCCGGGA 3’
bFGF suffix: 5’ AACTGCAGCGGCCGCTACTAGTAGATCCCGTTGCAACCGC 3’

2. For the reaction mixture:
   DNA template: 200-300 ng/50ul (1 ul or 0.5 ul according to template concentration)
   Forward primer: 20 pmol (2 ul)
   Reverse primer: 20 pmol (2 ul)
   PrimeSTAR Max Premix（2×）: 25 ul
   DdH2O: up to 50 ul

3. For the reaction condition set-up:

| | |
|---|---|
| Pre-denaturalization | 98C° for 3minutes |
| Amplification cycles | 95 C° for 30 seconds |
| | 68 C° for 60 seconds |
| | Repeat for 30 cycles |
| Final elongation | 72C° for 3 minutes |

3-step PCR (for gel analysis)

1. Primer
   Forward primer for sequencing/amplifying BioBrick parts (VF2):
   5’ TGCCACCTGACGTCTAAGAA 3’
   Reverse primer for sequencing/amplifying BioBrick parts (VR):
   5’ ATTACCGCCTTTGAGTGAGC 3’

2. For the reaction mixture:
   DNA template: 2-3ng/20ul (dilute 1 ul of the plasmid in 49 ul of ddH2O, use 1 ul as template)
   Forward primer: 10pmol (1 ul)
   Reverse primer: 10pmol (1 ul)
   PrimeSTAR Max Premix（2×）: 10 ul
   DdH2O: up to 20 uL

3. For the reaction condition set-up:

| | |
|---|---|
| Pre-denaturalization | 98C° for 3minutes |
| Amplification cycles | 95 C° for 30 seconds |
| | 55 C° for 30 seconds |
| | 72 C° for 10 seconds |
| | Repeat for 30 cycles |
| Final elongation | 72 C° for 3 minutes |

Digestion

1. Make three mixes: each contains 500 ng of one of the three plasmids and ddH2O to 43 µL.
2. To each mix, add 5 µL of 10x reaction buffer for restriction enzymes.

3. Add 1 µL each of the appropriate endonucleases (two per tube) according to Fig. 25 to give a final volume of 50 µL.
4. Tap on the tubes to mix. If necessary, centrifuge for a few seconds to spin down the liquid.
5. Incubate at 37°C for 30 min.
6. Heat-inactivate the enzymes by incubating at 80°C for 20 min

### Gel Analysis

1. Close the ends of the gel tray.
2. Insert the comb into the gel tray at one end ~1 cm from the edge.
3. For a 1% 50 ml agarose gel, weigh 0.5 g of agarose in a 100 ml conical flask. Add 50 ml 1x TBE buffer.
4. To dissolve the agarose in the buffer, swirl to mix and microwave for a few minutes taking care not to boil the solution out of the flask. Remove the flask occasionally and check whether the agarose has dissolved completely.
5. Let the agarose solution cool down. Once the solution is touchable, add the DNA stain. Check the stock concentration and add the appropriate amount to give the desired final concentration.
6. Pour the gel solution into the gel tray. Remove any air bubbles with a pipette tip. Put in comb.
7. Wait for the gel to solidify while cooling down to room temperature.
8. Release the gel tray from the tape or casting stand. Place the gel tray into the buffer chamber and remove the comb carefully.
9. Add 1x TBE buffer until the gel is completely covered.
10. Take part of DNA samples and mix with loading dye.
11. Load the size marker mixed in 1x loading dye into a well.
12. Load samples into the other wells while writing down which lanes have which samples.
13. Put the lid onto the buffer chamber and connect it to the power supply.
14. Run the gel at 120 V for 30 min.
15. Stop the run and bring the gel to a UV table to visualize the gel bands.
16. Take a picture of the gel.

### Gel Extracion

OMEGA Gel Extract Kit (Cat. D2500-02) is used for gel extraction, protocol refers to instruction of the kit.

### DNA Clean-up

MicroElute® DNA Clean-Up Kit (Cat. D 6296-02) is used for DNA clean-up, protocol refers to instruction of the kit.

### Ligation

1. Add 2 µL (20 ng) of digestion mixtures to 11 µL of water.
2. Add 2 µL 10x reaction buffer for T4 DNA ligase.
3. Add 1 µL of T4 DNA ligase to give a final volume of 20 µL.
4. Incubate at room temperature (~22°C) for 30 min.

5. Heat-inactivate the enzymes by heating at 80°C for 20 min.

### Cell Glycerol Stock

1. Mix 600 µL of an overnight culture with 400 µL of 50% glycerol (to give 20% glycerol final).
2. place in the -80°C freezer.

### Transformation

1. Turn on a water bath or heating block to 42°C.
2. Thaw competent cells on ice for 15 min.
3. Add 5 µL of ligation reaction mixture or controls above to 50 µL of competent cells.
4. Incubate for 30 min on ice.
5. Heat shock for 45 s at 42°C.
6. Incubate for 5 min on ice.
7. Add 950 µL of SOB media (pre-heated to 37°C).
8. Incubate for 1–1.5 hr at 37°C, with occasional gentle mixing by inversion of the tubes.
9. For positive controls, mix gently and plate 100 µL only (=1/10th) on an agar plate containing the appropriate antibiotic as in Step 12.
10. Spin cells down from remaining 900 µL at 4000 rpm for 5 min.
11. Discard all but 100 µL of the supernatant and resuspend the pellet in the remaining 100 µL.
12. Spread the remaining suspension on an agar plate containing the appropriate antibiotic as follows:
    - •Dip the spreader into 95% ethanol.
    - •Put it into the flame for a second.
    - •Let the ethanol burn off outside the flame.
    - •Spread the bacterial suspension evenly out on an agar plate. Continue until all the inoculum has gone into the agar.
    - •Put the plates at 37°C overnight.

## REVERSE TRANSCRIPTION PCR (RT-PCR)

### RNA Extraction From Cultured Cells

1. Wash cells with PBS. Prepare cell suspension using Trypsin-EDTA.
2. Centrifuge at 1,000 rpm for 2 min, remove supernatant.
3. Add 1ml Trizol, mix by pipetting up and down a few times.
4. Add 0.2ml of chloroform, shake vigorously then allow to stand for a few minutes until phase start to separate.
5. Centrifuge at 13,000 g for 15min at 4°C.
6. Transfer the colorless upper phase to a new clean tube, avoiding the white interphase.
7. Add 0.6ml of isopropanol, mix by inverting the tube several times gently.
8. Centrifuge at 13,000 g for 10min at 4°C.
9. Remove supernatant and wash RNA by adding 0.7ml of 75% ethanol.
10. Repeat step 8 and 9.
11. Centrifuge at 13,000 g for 10min at 4°C, remove supernatant and invert the tube on a clean

kimwipe.

12. Wait for pellet to dry.
13. Resuspend pellet with DEPC water.

### Reverse Transcription

PrimeScript™ 1st Strand cDNA Synthesis Kit (Cat. 6110A) is use d for reverse transcription. Protocol refers to instruction of the kit.

## WESTERN BLOT

### Sample Lysis

1. Place the cell culture dish on ice and wash the cells with ice-cold PBS.
2. Aspirate the PBS, then add ice-cold lysis buffer (1 mL per $10^7$ cells/100 mm dish/150 $cm^2$ flask; 0.5 mL per $5x10^6$ cells/60 mm dish/75 $cm^2$ flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Alternatively, cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer in a microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. Centrifuge in a microcentrifuge at 4°C. You may have to vary the centrifugation force and time depending on the cell type; a guideline is 20 min at 12,000 rpm but this must be determined for your experiment (leukocytes need very light centrifugation).
6. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

### Sample preparation

1. Remove a small volume of lysate to perform a protein quantification assay. Determine the protein concentration for each cell lysate.
2. Determine how much protein to load and add an equal volume 2X Laemmli sample buffer.
3. Boil each cell lysate in sample buffer at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use.

### Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. Load 20–30 µg of total protein from cell lysate or tissue homogenate, or 10–100 ng of purified protein.
2. Run the gel for 1–2 h at 100 V. (The time and voltage may require optimization.)
3. The gel percentage required is dependent on the size of your protein of interest:

| Protein size | Gel percentage |
|---|---|
| 4-40kDa | 20% |
| 12-45 kDa | 15% |
| 10-70 kDa | 12.5% |
| 15-100 kDa | 10% |

| | |
|---|---|
| 25-100 kDa | 8% |

### Transferring the protein from the gel to the membrane

The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization.

### Antibody staining

1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 min each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.
5. Wash the membrane in three washes of TBST, 5 min each.
6. For signal development, follow the kit manufacturer’ s recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

## MICROPLATE READER (INTERLAB)

### OD600 Reference Point

1. Turn off the path-length correction.
2. Self-testing of instrument.
3. Prepare your 96 well plate.
4. Add 100 µl LUDOX 100 % into wells A1, B1, C1, D1.
5. Add 100 µl of H2O into A2, B2, C2, D2 .
6. Measure absorbance 600 nm of all samples in all standard measurement modes in instrument.
7. Import data into "Abs600" blue cells in provided Excel calibration sheet.(Flashes of per well: 10, Orbit averaging: not applied to our instrument, temperature: 37°C)

### FITC calibration

1. Turn off the path-length correction.
2. Self-testing of instrument.
3. Set the gain to 60%.
4. Set the excitation wavelength to 480nm.
5. Set the emission wavelength to 509nm.
6. Set the model to top optic fluorescence reading.
7. Prepare your 96 well plate.
8. Spin down FITC stock tube to make sure pellet is at the bottom of tube.
9. Prepare 2x FITC stock solution (500 µM) by resuspending FITC in 1ml of 1x Phosphate Buffer Saline (PBS).

10. Incubate the solution at 42°C for 4 hours. Properly dissolved FITC.
1. (To check this after the incubation period pipetted up and down – if any particulates are visible in the pipette tip continue to incubate overnight.)
11. Dilute the 2x FITC stock solution in half to make a 1x FITC solution (final concentration is 250 µM).
12. Add 100 µl of PBS into wells A2, B2, C2, D2....A12, B12, C12, D12.
13. Add 200 µl of FITC stock solution into A1, B1, C1, D1.
14. Transfer 100 µl of FITC stock solution from A1 into A2.
15. Mix A2 by pipetting up and down 3x and transfer 100 µl into A3.
    Mix A3 by pipetting up and down 3x and transfer 100 µl into A4.

Mix A4 by pipetting up and down 3x and transfer 100 µl into A5.
Mix A5 by pipetting up and down 3x and transfer 100 µl into A6.
Mix A6 by pipetting up and down 3x and transfer 100 µl into A7.
Mix A7 by pipetting up and down 3x and transfer 100 µl into A8.
Mix A8 by pipetting up and down 3x and transfer 100 µl into A9.
Mix A9 by pipetting up and down 3x and transfer 100 µl into A10.
Mix A10 by pipetting up and down 3x and transfer 100 µl into A11.
Mix A11 by pipetting up and down 3x and transfer 100 µl into liquid waste.
(TAKE CARE NOT TO CONTINUE SERIAL DILUTION INTO COLUMN 12.)

16. Repeat dilution series for rows B, C, D.
17. Measure fluorescence of all samples in all standard measurement modes in instrument.
18. Measure fluorescence of all of your samples.
19. Import data into "Fluorescence" blue cells in provided Excel calibration sheet.

(Flashes of per well: 10, Orbit averaging: not applied to our instrument, temperature: 37 C°)

## CYTOMETRY FLOW ANALYSIS

### Sample preparation

1. Harvest the cells in the appropriate manner and wash in PBS.
2. Fix in cold 70% ethanol. Add drop wise to the pellet while vortexing. This should ensure fixation of all cells and minimize clumping.
3. Fix overnight at 4°C.
4. Wash 2 X in PBS. Spin at 850 g in a centrifuge and be careful to avoid cell loss when discarding the supernatant especially after spinning out of ethanol.
5. Treat the cells with ribonuclease. Add 50 µl of a 100 µg/ml sock of RNase. This will ensure only DNA, not RNA, is stained.
6. Add 200 µl PI (from 50 µg/ml stock solution).

### Results analysis

1. Measure the forward scatter (FS) and side scatter (SS) to identify single cells.
2. Pulse processing is used to exclude cell doublets from the analysis. This can be achieved either by using pulse area vs. pulse width or pulse area vs. pulse height depending on the type of cytometer.
3. PI has a maximum emission of 605 nm so can be measured with a suitable bandpass filter.

# DAILY CELL TREATMENTS

## Subculture and Passage

1. Remove all medium from culture with a sterile Pasteur pipet. Wash adhering cell monolayer once or twice with a small volume of 37°C PBS to remove any residual FBS that may inhibit the action of trypsin.
2. Add enough 37°C trypsin/EDTA solution to culture to cover adhering cell layer.
3. Place plate on a 37°C warming tray 1 to 2 min. Tap bottom of plate on the countertop to dislodge cells. Check culture with an inverted microscope to be sure that cells are rounded up and detached from the surface.
4. Add isometric 37°C complete medium. Draw cell suspension into a Pasteur pipet and rinse cell layer two or three times to dissociate cells and to dislodge any remaining adherent cells. As soon as cells are detached, add serum or medium containing serum to inhibit further trypsin activity that might damage cells.
5. Add one quarter volume of cell suspension to fresh plates or flasks that have been appropriately labeled.
6. Add 4 ml fresh medium to each new culture. Incubate in a humidified 37°C, 5% CO2 incubator.
7. If necessary, feed subconfluent cultures after 1 or 2 days by removing old medium and adding fresh 37°C medium.
8. Passage secondary culture when it becomes confluent by repeating steps 1 to 7, and continue to passage as necessary.

## Freezing cells

1. Trypsinize cells from plate.
2. Transfer cell suspension to a sterile centrifuge tube and add 1 ml complete medium with serum. Centrifuge 5 min at 200 × g, room temperature.
3. Remove supernatant and add 1 ml of 4°C freezing medium. Resuspend pellet.
4. Pipet 1 ml aliquots of cell suspension into labeled 2-ml cryovials. Tighten caps on vials.
5. Place vials 1 hour to overnight in a -70C° freezer, then transfer to liquid nitrogen storage freezer.

# TRANSIENT TRANSFECTION

## Cell preparation

One day before transfection, plate 0.5-2 x 105 cells in 500 µl of growth medium without antibiotics so that cells will be 70-90% confluent at the time of transfection.

## Transfection mix

For each transfection sample, prepare complexes as follows:

1. Dilute DNA in 50 µl of Opti-MEM® I Reduced Serum Medium without serum (or other medium without serum). Mix gently.
2. Mix Lipofectamine® 2000 gently before use, then dilute the appropriate amount in 50 µl of

Opti-MEM® I Medium. Incubate for 5 minutes at room temperature. Note: Proceed to Step c within 25 minutes.

3. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine® 2000 (total volume = 100 µl). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy). Note: Complexes are stable for 6 hours at room temperature.

### Transfection

1. Add the 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.
2. Incubate cells at 37°C in a CO2 incubator for 18-48 hours prior to testing for transgene expression. Medium may be changed after 6-8 hours.

## LENTIVIRUS PRODUCTION AND INFECTION

### Cell preparation

Plate 0.5-2 x 105 cells in 500 µl of growth medium without antibiotics so that cells will be 40-70% confluent at the time of transfection.

### Transfection mix

1. In each sterile 1.5 ml tube, dilute 500 ng pMD2.G, 1 µg pSPAX2, and 2µg of pCDH expression plasmid DNA in 200 µ l of opti-MEM. Mix gently.
2. In other sterile 1.5 ml tube, dilute 2 µl Lipofectamine™ 2000 (mix gently before use) in 200µ l of opti-MEM. Mix gently and incubate for 5 minutes at room temperature.
3. After incubation, combine the diluted DNA (Step a) with the diluted Lipofectamine™ 2000 (Step b). Mix gently.
4. Incubate for 15 minutes at room temperature to allow the DNALipo2000 complexes to form.
5. Add the complex to each HEK293T cell plate well.
6. Remove and discard the medium and replace with 2ml DMEM. Incubate cells 48hours at37°C in a humidified 5% CO2 incubator.

### Cell cultivation

Set up the target cell line in target cell medium to 6 well plate so that they will be 30% confluent on the next day.

### Post-transfection

1. Harvest virus-containing supernatants and filter the viral supernatants through a 0.45 µm filter in 15 ml sterile tube.
2. Infect target cells: 1 volume of DMEM (2ml) and 1 volume of filtered virus-containing supernatants (2ml), with 4 µl polybrene (8mg/ml).
3. Select cells using drug.

## WOUND HEALING ASSAY

### Experimental procedures

1. HEK293T cells were plated on a 48-well plate and were incubated in DMEM + 10% FBS at 37 C° with 5% of $CO_2$.
2. 24 h later, artificial wounds were created by scraping on the cell monolayer with a 10 μL pipette tip. Debris and floating cells were washed with 1x PBS. Culture supernatants from our eBMSCs were added. Images of wounds were captured under light microscopy.
3. 24 h later, the wounds were imaged again.

### Data analysis

1. Images were processed with imageJ software. For measuring wound healing, the detailed procedure is: enhance contrast → find edges → convert to 8-bit → adjust threshold → measure.
2. For evaluation of cell migration velocity, 20 different in each experimental group were ranked marked manually. The migration distance was measured and the velocity was calculated as distance/time.

## EXPERIMENTS ON BMSCs

### BMSCs isolation from female rats

1. Eight-week old female Sprague-Dawley rats were sacrificed by intraperitoneal (i.p.) injection of 50% chloral hydrate solution.
2. Sterilized them in 75% ethanol.
3. Collect femurs and tibiae and then use DMEM/F12 medium to flush out bone marrow stromal cells.
4. Centrifuge suspended cells at 200 g for 5 minutes, and then seeded in DMEM/F12 media with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin at 37C° incubator with 5% CO2.
5. Remove non-adherent cells after 48 hours, and the culture medium was changed every 3 days.

### PF-127 gel formation analysis

1. Dissolve PF-127 in DMEM/F12 at 4C° overnight to prepare 40% (w/v) solution, then filter the solution through a 0.45 μM filter and finally kept at 4C° for use.
2. Dissolve 40% (w/v) PF-127 into 5% (w/v), 10% (w/v), 15% (w/v), 20% (w/v), 25% (w/v), 30% (w/v), 35% (w/v), 40% (w/v). Separate each 100μl into 1.5ml EP tube.
3. Keep the tubes at room temperature for 2min, 5min, 10min. Test whether PF-127 form gel by put the tube upside down.
4. Keep the tubes at 37C° for 30s, 2min, 5min, 10min. Test whether PF-127 form gel by put the tube upside down.

### PF-127 preparation and cell encapsulation (*in vitro*)

5. Dissolve PF-127 in DMEM/F12 at 4C° overnight to prepare 20% (w/v) solution, then filter the solution through a 0.45 μM filter and finally kept at 4C° for use.
6. BMSCs were centrifuged and re-suspended in sterile PF-127 solution with 0 / 50 / 100 μM

Vc on ice.

7. Add 200 μL of cell-hydrogel mixture to each well, and the plate then was kept in 37C° incubator (5% CO2) for 5 minutes to boost gel formation.
8. Add 0.5 mL culture medium over the gel and the plate was transferred back into the incubator

### PF-127 preparation and cell encapsulation (*in vivo*)

1. Dissolve PF-127 in DMEM/F12 at 4C° overnight to prepare 20% (w/v) solution, then filter the solution through a 0.45 μM filter and finally kept at 4C° for use.
2. eight-week female rats were randomly divided into five groups, namely sham (no surgery), control (IUA without injection of BMSCs or F-127/Vc), BMSC encapsulation (IUA and injection of BMSCs capsulated with PF-127/Vc ), BMSC plus Vc (IUA and injection of BMSCs only with Vc) and PF-127 plus Vc ( IUA and injection of only F-127/Vc).
3. Cells (8×105) were injected in a total volume of 200 μL

### FACS analysis

1. Digest the third passage of rat BMSCs with 0.05% trypsin-EDTA and 105 cells were re-suspended in 100 μL DMEM/F12.
2. Incubate re-suspended cell with 5 μL of phycoerythrin (PE)–conjugated anti-rat CD34 and anti-rat CD45 in the dark on ice for 30 minutes
3. Use a flow cytometer to perform cytometric analysis.

### CCK cell proliferation assay

Assess BMSCs proliferation by *TransDetect*™ cell counting kit (TransGen Biotech, Beijing, China), following the manufactory' s instruction.

1. Seed BMSCs at a density of 5×105/mL with different encapsulation conditions.
2. Cell growth of the 3rd day and 7th day after seeding were tested by adding 10% CCK to each well and incubated for 2 hours.
3. Measure the absorbance at 450 nM using a microplate reader

### Cell apoptosis assay

Use TansDetect Annexin V-FITC/PI Apoptosis Detection Kit (TransGen Biotech, Beijing, China) to evaluate cellular apoptosis, according to manufacturer' s instructions.

1. Seed BMSCs at a density of 5×105/mL with different encapsulation conditions for 3 days and 7 days.
2. Trypsinize and suspend the cells in binding buffer, labeled with 5 μL Annexin V-FITC and 5 μL PI for 15 minutes in the dark on ice.
3. Add 300 μL binding buffer to each sample and the cells were evaluated by flow cytometer.
4. Positive control was carried out using 100 μM H2O2 treated for 15 minutes.

## ANIMAL EXPERIMENTS

### Rat IUA model and BMSC transplantation

Use mechanical damage method to establish rat uterine cavity adhesion model.

1. Anesthetize rats with 10% chloral hydrate (300 mg/kg), and open the abdominal cavity to expose the uterus.
2. A 2 mm transverse incision in the left uterus was prepared at the upper end, and a 1.5-2.0 cm rough-feeling endometrial damage was generated by a scraping spoon.

3. Inject 200 μL PF-127 encapsulated BMSCs with Vc or other controls into the uterine horn while establishing the IUA model
4. After the surgery, rat abdomen was sutured and the rat was recovered for eight weeks followed by further examination.
5. The right side uterine without damage was considered as sham control.

### Histological analysis

Eight weeks after surgery, the rats were sacrificed and the tissues underwent standard paraffin embedding, section cutting and HE staining.

1. The morphological changes were observed under the light microscope. Five fields in each image were selected for counting. Image Pro-Plus 6.0 (IPP 6.0) was applied to analyze the thickness of endometrium, the total number of the endometrial glands and the area of interstitial fibrosis of the endometrium.
2. Endometrial fibrosis was revealed by Masson' s trichrome staining.

(1) Eight weeks after surgery, the rats were sacrificed and the tissues underwent standard paraffin embedding, and 4 μM serial sections were prepared.

(2) Sections were immune-labeled with anti-interleukin-1β antibody (IL-1β, 1:150, rabbit, Bioss, Beijing, China), anti-Pan Cytokeratin antibody (Pan Cytokeratin, 1:50, mouse, Boster, Wuhan, China) and anti-von Willebrand factor antibody (vWF, 1:70, mouse, Boster, Wuhan, China).

(3) Percentages of positive staining area were quantified using the Image-Pro Plus software (Media Cybernetics, Rockville, MD).

## InterLab Study Protocols

We did the InterLab experiment according to the protocol here:
http://2017.igem.org/Competition/InterLab_Study/Plate_Reader

### OD600 Reference Point

Turn off the path-length correction.

Self-testing of instrument.

Prepare your 96 well plate.

Add 100 μl LUDOX 100 % into wells A1, B1, C1, D1.

Add 100 μl of H2O into A2, B2, C2, D2 .

Measure absorbance 600 nm of all samples in all standard measurement modes in instrument.

Import data into "Abs600" blue cells in provided Excel calibration sheet.(Flashes of per well:

10, Orbit averaging: not applied to our instrument, temperature: 37°C)

**FITC calibration**

Turn off the path-length correction.
Self-testing of instrument.
Set the gain to 50%.
Set the excitation wavelength to 480nm.
Set the emission wavelength to 509nm.
Set the model to top optic fluorescence reading.
Prepare your 96 well plate.
Spin down FITC stock tube to make sure pellet is at the bottom of tube.
Prepare 2x FITC stock solution (500 µM) by resuspending FITC in 1ml of 1x Phosphate Buffer Saline (PBS).
Incubate the solution at 42°C for 4 hours. Properly dissolved FITC.
(To check this after the incubation period pipetted up and down – if any particulates are visible in the pipette tip continue to incubate overnight.)
Dilute the 2x FITC stock solution in half to make a 1x FITC solution (final concentration is 250 µM).
Add 100 µl of PBS into wells A2, B2, C2, D2....A12, B12, C12, D12.
Add 200 µl of FITC stock solution into A1, B1, C1, D1.
Transfer 100 µl of FITC stock solution from A1 into A2.
Mix A2 by pipetting up and down 3x and transfer 100 µl into A3.
Mix A3 by pipetting up and down 3x and transfer 100 µl into A4.
Mix A4 by pipetting up and down 3x and transfer 100 µl into A5.
Mix A5 by pipetting up and down 3x and transfer 100 µl into A6.
Mix A6 by pipetting up and down 3x and transfer 100 µl into A7.
Mix A7 by pipetting up and down 3x and transfer 100 µl into A8.
Mix A8 by pipetting up and down 3x and transfer 100 µl into A9.
Mix A9 by pipetting up and down 3x and transfer 100 µl into A10.
Mix A10 by pipetting up and down 3x and transfer 100 µl into A11.
Mix A11 by pipetting up and down 3x and transfer 100 µl into liquid waste.
(TAKE CARE NOT TO CONTINUE SERIAL DILUTION INTO COLUMN 12.)
Repeat dilution series for rows B, C, D.
Measure fluorescence of all samples in all standard measurement modes in instrument.
Measure fluorescence of all of your samples.
Import data into "Fluorescence" blue cells in provided Excel calibration sheet.
(Flashes of per well: 10, Orbit averaging: not applied to our instrument, temperature: 37 C°)

**Cell measurements**

**Day 1**

transform Escherichia coli DH5αwith these following plasmids:
Positive control
Negative control
Test Device 1: J23101+I13504

Test Device 2: J23106+I13504

Test Device 3: J23117+I13504

Test Device 4: J23101.BCD2.E0040.B0015

Test Device 5: J23106.BCD2.E0040.B0015

Test Device 6: J23117.BCD2.E0040.B0015

**Day 2**

Pick 2 colonies from each of plate and inoculate it on 5mL LB medium + Chloramphenicol.

Grow the cells overnight (16-18 hours) at 37°C and 220 rpm.

**Day 3**

Cell growth, sampling, and assay

Set your instrument to read OD600 (as OD calibration setting)

Measure OD600 of the overnight cultures

Record data in your notebook

Import data into Excel (Dilution Calculation) Sheet_1 provided

Dilute the cultures to a target OD600 of 0.02 in 12 ml LB medium + Chloramphenicol in 50 mL falcon tube covered with foil.

Incubate the cultures at 37°C and 220 rpm.

Samples each culture at 0, 2, 4, and 6 hours of incubation and measure the samples' OD600 and fluorescence.

Import data into Excel (cell measurement tab) Sheet_1 provided

## Bricks construction

**2-step PCR(for adding prefix and suffix)**

Primers

VEGF-A121 prefix: 5' CGGAATTCGCGGCCGCTTCTAGATGAACTTTCTGCTGTCTTGGGT 3'

VEGF-A121 suffix: 5' AACTGCAGCGGCCGCTACTAGTACCGCCTCGGCTTGTCAC 3'

PDGFA prefix: 5' CGGAATTCGCGGCCGCTTCTAGATGAGGACCTTGGCTTGCCT 3'

PDGFA suffix: 5' AACTGCAGCGGCCGCTACTAGTACCTCACATCCGTGTCCTCTTCC 3'

bFGF prefix: 5' CGGAATTCGCGGCCGCTTCTAGACCATGGCAGCCGGGA 3'

bFGF suffix: 5' AACTGCAGCGGCCGCTACTAGTAGATCCCGTTGCAACCGC 3'

For the reaction mixture:

DNA template: 200-300ng/50ul (1ul or 0.5ul according to template concentration)

Forward primer: 20pmol (2ul)

Reverse primer: 20pmol (2ul)

PrimeSTAR Max Premix（2×): 25ul

Dd$H_2O$: up to 50ul

For the reaction condition set-up:

Pre-denaturalization

98 C° for 3minutes

Amplification cycles

95 C° for 30 seconds

68 C° for 60 seconds

Repeat for 30 cycles

Final elongation

72 C° for 3 minutes

**3-step PCR(for gel analysis)**

Primer

Forward primer for sequencing/amplifying BioBrick parts (VF2): 5' TGCCACCTGACGTCTAAGAA 3'

Reverse primer for sequencing/amplifying BioBrick parts (VR): 5' ATTACCGCCTTTGAGTGAGC 3'

For the reaction mixture:

DNA template: 2-3ng/20ul (dilute 1ul of the plasmid in 49ul of ddH2O, use 1ul as template)

Forward primer: 10pmol (1ul)

Reverse primer: 10pmol (1ul)

PrimeSTAR Max Premix（2×): 10ul

$DdH_2O$: up to 20ul

For the reaction condition set-up:

Pre-denaturalization

98C° for 3minutes

Amplification cycles

95 C° for 30 seconds

55 C° for 30 seconds

72 C° for 10 seconds

Repeat for 30 cycles

Final elongation

72 C° for 3 minutes

**Digestion**

Reaction mixture

2ug DNA

2ul EcoRI (Fast digest enzyme, from Thermo Fisher Scientific LTD.)

2ul PstI (Fast digest enzyme, from Thermo Fisher Scientific LTD.)

4ul fast digestion buffer (from Thermo Fisher Scientific LTD.)

DdH2O to a total volume of 40ul

or

3ug DNA

3ul EcoRI (Fast digest enzyme, from Thermo Fisher Scientific LTD.)

3ul PstI (Fast digest enzyme, from Thermo Fisher Scientific LTD.)

6ul fast digestion buffer (from Thermo Fisher Scientific LTD.)
DdH2O to a total volume of 60ul

Reaction condition
37C° for 1 hour.

Perform gel extraction or clean-up using E.Z.N.A.® gel extraction Kit from Omega Bio-tek LTD.

**Ligation**

Reaction mixture
50ng(~0.03pmol) plasmid backbone(cut from InterLab test device 1 plasmid with EcoRI and PstI)
~0.21pmol insert (PCR product cut with EcoRI and PstI)
0.5ul T4 ligase (from Thermo Fisher Scientific LTD.)
2ul T4 ligase buffer (from Thermo Fisher Scientific LTD.)
DdH2O to a total volume of 20ul

Reaction condition
Ligate at 22C° for 1 hour.

The mixture is used to transformed 100ul of DH5-alpha competent cell.