# Lexogen library prep 1/4 version

### *Materials needed*

- Hot plate (37°C and 42°C)
- Ice
- Charge pipettes

## First strand cDNA synthesis

1. Take out **FS1**, **FS2** from -20°C to RT. Take out **E1** from -20°C to ice.
2. Turn on hot plate at 42°C.
3. Add 1.25 µl RNA sample[1] in a new PCR plate. Add 1.25 µl **FS1** (125x8). Seal, mix, spin plate.
4. Put in Lex One program (3 min).
5. Make Master Mix 1, mix, spin and keep at 42°C for 2 min.

| | 1 sample (µl) | x8.8 | x98 |
|---|---|---|---|
| **E1** | 0.125 | 1.1 | 12.25 |
| **FS2** | 2.375 | 20.9 | 116.5 x 2 |

6. Spin plate, put back to 42°C on PCR machine, remove seal. Add 2.5 µl MM1 (2.4x32), seal. Mix, spin and incubate at 42°C for 15 min.
7. Input sample names to Excel. Mark tubes as used. Take photo.
8. Take **RS** to RT. Switch hot plate to 37°C.

## RNA removal

1. Spin plate.
2. Add 1.25 µl **RS** (125x8) to each sample, mix, seal.
3. Put in Lex Two program (10 min).
4. Prepare
   - Take out purification module (**PB**, **PS**, **EB**) from 4°C to RT.
   - Switch on BioRad PCR machine.
   - Take **SS1** to 37°C.
5. Return RNA to -70°C.
6. Spin plate, remove seal.
7. Add 2.5 µl **SS1** (viscous 2.5x32). Mix, seal, spin.
8. Put at program in BioRad machine (30 min - 15 ml).
9. Take out **SS2**.
10. Make Master Mix 2

| | 1 sample (µl) | x8.8 | x98 |
|---|---|---|---|
| **SS2** | 1 | 8.8 | 98 |
| **E2** | 0.25 | 2.2 | 24.5 |

11. Spin plate, Open. Add 1.25 µl MM2 (125x8), mix, spin. Put in Lex Three program (15 min).

[1] Assumes 60 µl elution from Qiagen plant RNA kit, should be 100 pg - 500 ng.

## Purification 1[2]

1. Vortex **PB**. Add 4 µl **PB** per reaction. Mix and incubate at RT for 5 min (4 x 48).
2. Put on magnet 2-5 min until clear supernatant.
3. Remove supernatant (6 columns with same tip).
4. Add 10 µl **EB**, remove from magnet, resuspend. Incubate 2 min at RT (10x16).
5. Add 14 µl **PS**. Mix and incubate for 5 min at RT (12x14).
6. Put on magnet 2-5 min until clear supernatant.
7. Take out PCR + i7 + i5 primers to RT.
8. Make **80% Ethanol**

| | 1 sample (µl) | x8 | plate |
|---|---|---|---|
| EtOH | 48 | 384 | 5,000 |
| $H_2O$ | 12 | 96 | 1,200 |

9. Remove supernatant (4 columns with same tip).
10. Wash with 30 µl **80% Ethanol**. Leave for 30 sec without disrupting beads, remove Ethanol (4 columns with same tip).
11. Do one more Ethanol wash.
12. Cover plate, spin, put back on magnet and individually remove leftover solution.
13. Add 8 µl **EB**. Remove from magnet, resuspend. Incubate 2 min at RT (8 x 24).
14. Put on magnet 2-5 min until clear supernatant.
15. Take 5 µl supernatant in endpoint PCR plate.

## Endpoint PCR - dual indexing

1. Prepare mastermix

| | 1 sample (µl) | x8 | x100 |
|---|---|---|---|
| cDNA | 5 | | |
| **Dual PCR** | 1.75 | 14 | 180 |
| i5 | 1.25 | 10 | 125 |
| **E3/E** | 0.25 | 2 | 24 |

2. Add 3.25 µl master mix (3.2x48) to 5 µl sample.
3. Add 1.25 µl **i7 primer** from plate to each sample.
4. Put in Lex PCR program (30 min).

## Purification 2

1. Vortex **PB**. Add 7.5 µl **PB** per reaction. Mix and incubate at RT for 5 min (7.4 x 24).
2. Put on magnet 2-5 min until clear supernatant.
3. Remove supernatant (6 columns with same tip).
4. Add 7.5 µl **EB** (7.4 x 24), remove from magnet, resuspend. Incubate 2 min at RT.
5. Add 7.5 µl **PS** (7.4 x 24). Mix and incubate for 5 min at RT.

[2] If **EB** runs out it can be replaced with dd$H_2O$ or 10mM Tris pH 8.0 (100 µl 1M TRIS pH 8.0 in unopened 10 ml $H_2O$ from Qiagen RNA extraction kit).

6. Put on magnet 2-5 min until clear supernatant.
7. Make **80% Ethanol**

| | 1 sample (µl) | x9 | plate |
|---|---|---|---|
| EtOH | 48 | 432 | 5,000 |
| $H_2O$ | 12 | 108 | 1,200 |

8. Remove supernatant.
9. Wash with 30 µl **80% Ethanol**. Leave for 30 sec without disrupting beads, remove Ethanol.
10. Wash with 30 µl **80% Ethanol**. Leave for 30 sec without disrupting beads, remove Ethanol.
11. Dry for 5-10 min.
12. Add 12 µl **EB**. Remove from magnet, resuspend. Incbate 2 min at RT (12 x 16 ).
13. Put on magnet 2-5 min until clear supernatant.
14. Take 5 µl supernatant in a PCR strip, pooling rows. Then pool all strips into new 1.5 ml tube. Store plate with remaining unpooled samples for potential future use at -20°C.

## PCR programmes

Lex One: 3 min 85°C, keep to 42°C. Machine 4 JK.

Lex Two: 10 min 95°C, keep to 25°C. Machine 4 JK.

Lex Three: 15 min at 25°C. Machine 4 JK.

Lex Four: 98°C 30 s, 14[(3)] x (98°C 10 s, 65°C 20 s, 72°C 30 s), 72°C 1 min, 12°C forever. Machine 4 JK.

BioRAD: 1 min 98°C, slow (0.5°C/sec) cool to 25°C, 25°C for 30 min. JK lab.

qPCR: 98°C 30 s, 35 x (98°C 10 s, 65°C 20 s, 72°C 30 s), 72°C 1 min. No background ROX. LH lab.

## qPCR

1. Make 2.5x SYBR working solution from original 10,000x with serial dilutions in DMSO.
2. Make qpcr master mix, distribute 29 µl per sample.

| | 1 sample (µl) | x8.8 |
|---|---|---|
| cDNA | 1 | |
| PCR mix | 7 | 61.6 |
| E | 1 | 8.8 |
| 7000 | 1.25 | 11 |
| 2.5x SYBR | 1.2 | 10.56 |
| $H_2O$ | 14.8 | 130.24 |

3. Put in qPCR program
4. Calculate: used 20% of library so in library amplification use 2 less cycles from $C_t$ calculated from machine.

[(3)] Determined by qPCR, range is 11-25.