Lexogen library prep 1/4 version

Materials needed

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

Sample preparation

0.5 µl RNA samples in pcr plate and Excel doc showing sample organisation on it (100 pg - 500 ng in kit volume)

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. Mix 75 µl molecular grade H_2O and 125 µl **FS1**. Dispense 2 µl (2x18) to 96-well plate. Add 0.5 µl RNA sample. 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.

		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. Check next step!

RNA removal

1. Take **RS** to RT. Switch hot plate to 37°C.
2. Spin plate.
3. Add 1.25 µl **RS** (125x8) to each sample, mix, seal.
4. Put in Lex Two program (10 min).
5. Prepare
   - Take out purification module (**PB**, **PS**, **EB**) from 4°C to RT.
   - Switch on BioRad PCR machine.
   - Take **SS1** to 37°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

		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).

Purification 1[1]

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.
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 (14x14).
6. Put on magnet 2-5 min until clear supernatant.
7. Take out PCR + i7 primers at room temperature.
8. Make **80% Ethanol**

		x8	plate
EtOH	48	384	5
H_2O	12	96	1.2

9. Remove supernatant.
10. Wash with 30 µl **80% Ethanol**. Leave for 30 sec without disrupting beads, remove Ethanol.
11. Wash with 30 µl **80% Ethanol**. Leave for 30 sec without disrupting beads, remove Ethanol, individually.
12. Dry for 5-10 min.
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

		x8	x100
cDNA	5		
Dual PCR	1.75	14	175
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.
4. Add 7.5 µl **EB**, remove from magnet, resuspend. Incubate 2 min at RT.
5. Add 7.5 µl **PS**. Mix and incubate for 5 min at RT.
6. Put on magnet 2-5 min until clear supernatant.

[1] If EB runs out it can be replaced with ddH_2O or 10mM Tris pH 8.0

7. Make **80% Ethanol**

		x9	plate
EtOH	48	432	5
H_2O	12	108	1.2

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 10 µl supernatant in a fresh PCR plate. Pool 1 µl per sample in tube, and store plate at -20°C with new seal.
15. Optional Qbit with HS kit.

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[2] 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.

		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.

[2] Determined by qPCR, range is 11-25.