Experiment: **RNA-seq** DATE: 07-01-2012

Purpose: true-seq primer testing:

Making multiplex mRNA-seq lib by using ilmn 3-pimers multiplex system

Methods and Materials:

Last time we had made stock solutions and aliquoted reagents:

Stock solutions made with ster. ddH_20 and filter sterilized.

1. TrisHCl 1M, pH 7.0
2. TrisHCl 1M, pH 7.5
3. LiCl 10M(from MGR lab)
4. EDTA 0.5M, pH 8.0: Purchased from Invitrogen (Ultra Pure)

Recipe: 23.86g EDTA (was all there was left) in 128mL ster. ddH_20, filtered. Add EDTA powder with about 80mL, put on a stirrer and add NaOH pellets until it is dissolved. Check pH and adjust to 8.0 (I had to put it down with HCl).
Purchased from Invitrogen(Ultra Pure)

5. **Binding buffer, 10mL**: 200μL 1M Tris pH 7.5, 1mL LiCl 10M, 40μL 0.5M EDTA, 8.76mL DEPC H_20 (mixed fresh from stock solutions, on 02/11/2010, right before the experiment).

6. **Washing buffer B, 10mL**: 100μL 1M Tris pH 7.5, 15μL 10M LiCl, 20μL 0.5M EDTA, 9.865mL DEPC H_20 (mixed fresh from stock solutions, on 9.09.09, right before the experiment).

7. **10mM Tris HCl pH 7.5, 10mL**: 100μL 1M Tris pH 7.5, 9.9mL DEPC H_20. (**make fresh and filtered**)

8. Actinomycin D 120ng/μL: 2mg in 16mL, aliquoted in 500μL and store them in -20^oC. (**used stock from last time**)

9. dUNTPs 10mM 100μL: 10μL dATP 100mM, 10μL dCTP 100mM, 10μL dGTP 100mM, 10μL dUTP 100mM,60ul DEPC H2O.
(**used stock from last time**)

Prepare RNA – 10µg of total RNA per sample per library

We used the following samples:

Samples' ID	Sample description	Concentration	Vol. for 10µg or more	Add DEPC H_20	index
RZY#162		2.9ug/ul	5	95	Ilmn #7
RZY#163		2.4ug/ul	5	95	Ilmn#8
RZY#164		160ng/ul	35	65	Ilmn #9
RZY#165		147ng/ul	35	65	Ilmn#10
RZY#166		2.5ug/ul	5	95	Ilmn #11
RZY#167		2.8ug/ul	5	95	Ilmn#12

I then added the respective volume of DEPC H20 in each sample. With this dilution, you have 3-**5-10µg of RNA in a total of 100µL of DEPC H20**.

Add **100µl** of Binding Buffer to the RNA samples.

Sample 1. polyA$^+$ RNA isolation from total RNA with oligo dT dynabeads

Step 1: Wash the beads

Set the Thermomixer to 65^oC

1. Resuspend the Dynabeads Oligo (dT)25 thoroughly in the vial to obtain a uniform brown suspension. Transfer **200µl (1 mg)** of beads to a tube for each sample.

2. Place tubes on a magnet (Dynal MPC™) for 1-2min. The Dynabeads Oligo (dT)25 will migrate to the side of the tube nearest the magnet.

3. Remove the supernatant with a pipette while the tube remains on the magnet.

4. Remove the tube from the magnet and add **100µl Binding Buffer** (20 mM Tris-HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA) to resuspend the beads. Wash beads by using the magnet to pull the beads to the side of the tube.

5. Again place the tube on the magnet for 1-2 min.

6. Remove the supernatant while the tube remains on the magnet.

7. Resuspend the beads in **100µl Binding Buffer**.

Step 2: Add the total RNA

Is the Thermomixer to 65^oC, ready?

1. Take the RNA samples containing 7ug total RNA and diluted as shown on the table above (total volume is 200uL).
2. Heat to 65°C for 2 min to disrupt secondary structures. Immediately place on ice for 5 min.
Once you are done with this step, set the Thermomixer to 78^oC.
3. Add the **200µl** of total RNA to the **100µl** washed beads.
4. Mix thoroughly and anneal by rotating continuously on a mixer for 5 min at room temperature (I did this by hand!).

5. Place the tube on the magnet for 1-2 min and carefully remove all the supernatant (I kept this sup to check concentration later).

6. Remove the tube from the magnet and add **200µl** Washing Buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). Mix by using the magnet to pull the beads to the side of the tube. Carefully remove all the supernatant.

7. Repeat the washing step as described in step 7. (I did NOT keep the washes to check concentration).

8. After the last wash, place all samples on ice and proceed with elution **one-by-one**.

9. To elute mRNA from the beads, remove the washing solution, quick spin, place on magnet and remove the remaining of the washing buffer.

10. Take the magnet out and add the desired amount (12 µl) of cold 10 mM Tris-HCl directly on the beads.

11. Heat to 78°C for 2 min and place the tube immediately on the magnet. Quickly transfer the eluted mRNA to a new RNase-free tube. (I did it one sample after each other). Keep on ice until you are done with all samples.

12. Once all samples are eluted prepare the mastermix for the first-strand cDNA synthesis.

2. cDNA synthesis from polyA$^+$ RNA

Made two programs at the PCR machine #4 (user magda, fs1 and fs2) for the first synthesis steps below).

1. Prepare fresh 2.1x First Strand synthesis mix with primers:

FS mix 2.1x	**µL for 1x for 20µL reaction**	**8**
Reverse transcription buffer 10x	2.0	16
dNTPs 10mM	1.0	8
MgCl2 100mM	1.0	8
DTT 100mM	2.0	16
Random primers	1.6	12.8
Oligo dT primer	1.0	8
Actinomycin D	0.0	0
	8.6	

2. Mix in individual 0.2ml PCR tubes:

FS reaction	amount of oligo dT RNA	FS mix 2.1X	Total µl
Sample	**10.4***	**8.6**	**19**

*That was all the eluted volume from the oligo dT beads.

3. Put tubes in a PCR machine with the following program (**Zhen's folder/program: fs1 – lasts for approx. 20min**):

Step	**Temperature**	**Time**
RNA denaturation	98°C	1:00
	70°C	5:00
Primer annealing	step down to 15°C by 0.1°C/sec	
Enzyme loading	15°C	hold

In the meantime prepare **FS enzyme mix**: 1µl RNAseOUT, 1µl Superscript III and 1ul Actinomycin D .

4. In the PCR machine add 3µL of FS enzyme mix, with the 10µl pipette and use a different3.5ti(20ul) for mixing. Mix samples and proceed with the program (**zhen folder/ program: fs2 – lasts for 1hr and 30 min):**

Step	**Temperature**	**Time**
cDNA synthesis at gradually increasing temperature	0.1°C/sec to 25°C	
	25°C	10:00
	0.1°C/sec to 42°C	
	42°C	45:00
	0.1°C/sec to 50°C	
	50°C	15:00
Enzyme inactivation	75°C	15:00
	15°C	Hold

1. Prepare in advance:
- Autoclaved 5ml aliquots of G-50 suspension in 1mM TrisHCl, pH 7.0 (used the suspension that was prepared on 8.13.09 and were kept at 4°C).
- 1.7ml tubes with cross-cutted caps
- Yellow tips with inserted 4x4mm2 piece of GF/C

2. Prepare before use:
- Mix well the G50 suspension
- Put 100µl of suspension into the yellow tip with filter
- Shake solution down
- Add another 150 µl of G50 suspension
- Insert yellow tip into the precutted cap of 1.7 ml tube.

When ready, take samples out.

Purify the reaction on the freshly prepared gel-filtration columns:

1. Spin down the empty column: 0.5 min at 2 krpm
2. Insert the column into a new collection tube (with opening on the cap)
3. Load the sample and spin down 1 min at 2 krpm
4. Measure volume of the eluate (was 28µL in the course) and transfer it to a 1.5 ml tube – kept in -20°C until ready to continue with the protocol.
5. Put tubes on ice while you prepare SS-mix
6. Prepare fresh 3.3x SS-mix (Second Strand synthesis mix):

SS mix 3.3x	**µL for 1x**	**X8**
Reverse transcription buffer 10x	1	8
Second strand synthesis buffer	15	120
MgCl2 100mM	0.5	4
DTT 100mM	1	8
dUNTPs 10mM	2	16
E. coli DNA ligase	0.5	4
DNA polymerase I	2	16
Rnase H	0.5	4
	22.5	

Incubate for 2hrs at 16^{o}C in Thermomixer.
Purify cDNA using the QIA-quick-QG purification as follows:
(for Qiagen columns the minimal DNA size is limited by column-loading buffer: ~60bp for QG-buffer and ~90bp for PB-buffer)
1. Add 3 volumes of QG buffer: **225µL**
2. Mix & wait 1-3 min
3. Add 1 volume isopropanol: **75µL**
4. Mix & briefly spin down on minicentrifuge.
5. Load on column (in <0.75ml aliquots)
- Centrifuge ~13krpm (~18kg) 1 min
- Change collection tube;
6. Washing (take a new collection tube for each wash):
- Add **0.75ml** PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Clean rotor's cover, rotor, tube rack with a wet paper towel
- Add **0.75ml** PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Centrifuge ~13krpm (~18kg), 0.5 min to remove excess of PE from the column
- Change collection tube
- Remove all traces of PE: centrifuge 1 min
7. Elution:
- Insert column into elution tube
- Add 54µl EB to the centre of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min (better to do it with two aliquots of EB: i.e. I did **2x27** µl).
Left in -20^{o}C overnight.

Did not measure DNA concentration. We sonicated all 50µL for all nine samples.

3. RNA-seq library preparation

Step 1: ultrasonic digestion with Diagenode Bioruptor (Ren lab bath sonicator in cold room).
Before starting, I kept 1.5µL of all samples to check for the efficiency of the sonication.

1. Use the Ren lab Biorupters in cold room.

2. Insert the 1.5 ml tubes with cDNA tightly into floating holder; fill empty holes with water filled tubes (total of 6 positions in the holder).
Insert the 0.5 ml tubes with cDNA tightly into floating holder; fill empty holes with water filled tubes (total of 10+2tubes blank positions in the holder).

3. Set Green/Red indicators to 0.5min/0.5min (meaning on sonication 30sec, then off for 30sec). Set power knob at H (high, for 100% amplitude).

4. Put the holder with your tubes inside the water bath sonicator, add ice to water bath. The water+ice level is appropriate (covers the tube, but does not touch the metal part of the holder).

5. Start sonication for 10 min x 5 times, when each 10 min done, I will add new ice to water bath and spun down samples and sonicated for next 15min.

6. Run the samples in flash gel @200V for 3-5 min.
7. Looked like it is working. They were all sonicated enough.
8. Keep samples at -20^{o}C overnight.

Step 2: End repair

1. Set temperature of the Thermomixer at 20°C.
2. Prepare fresh ER mix:

ER reaction mix	**µL for 1x**	**X11**
T4 DNA ligase buffer with 10mM ATP	6	66
dNTP's mix (10mM)	1.5	16.5
T4 DNA polymerase 3U/**u**L	1	11
Klenow DNA polymerase 5U/**u**L	0.2	2.2
T4 PNK	1	11
	9.7	

Add **9.7µ**L of the ER reaction mix to the tube with the 50**µ**L sheared cDNA and mix carefully.

Incubate 30min at 20°C in Thermomixer.

Purify cDNA using the QIA-quick-QG purification as follows:
1. Add **180µL** (3 volumes) QG buffer
2. Mix & wait 1-3 min
3. Add **60µL (**1 volume) isopropanol
4. Mix & briefly spin down on minicentrifuge
5. Load on column (in <0.75ml aliquots)
- Centrifuge ~13krpm (~18kg) 1 min
- Change collection tube;
- Remove ~2µl of QG from the column by vacuum pump with yellow tip;
6. Washing (take a new collection tube for each wash):
- Add **0.75ml** PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Clean rotor's cover, rotor, tube and rack with a wet paper towel
- Add **0.75ml** PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Centrifuge ~13krpm (~18kg), 0.5 min to remove excess of PE from the column
- Change collection tube
- Remove ~2µl of PE from the column by vacuum pump with a NEW yellow tip
- Remove all traces of PE: centrifuge 1 min
7. Elution:
- Insert column into elution tube
- Add 34µl EB to the centre of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min (better to do it with two aliquots of EB: i.e. **2x 18** µl).

Step 3: A-tailing

1. Set temperature of the Thermomixer at 37°C.
2. Prepare A-tailing reaction mix as follows:

A-tailing reaction mix	**µL for 1x**	**X11**
Klenow buffer 10x (=NEB buffer 2)	5	55
dATP 1mM	10	110
Klenow 3'-5' exo minus (5U/µl)	1	11
total	16	160

Add 16µL of the A-tailing reaction mix in each tube containing 34µL of DNA.

3. Incubate 30 min at 37°C in Thermomixer.
4. Purify cDNA using the **MinElute**-QG purification as follows:

1. Add **150µL** (3 volumes) QG buffer
2. Mix & wait 1-3 min
3. Add **50µL (**1 volume) isopropanol
4. Mix & briefly spin down on minicentrifuge
5. Load on column (in <0.75ml aliquots)
- Centrifuge ~13krpm (~18kg) 1 min
- Change collection tube;
6. Washing (take a new collection tube for each wash):
- Add 0.75ml PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Clean rotor's cover, rotor, tube rack with a wet paper towel
- Add 0.75ml PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Centrifuge ~13krpm (~18kg), 0.5 min to remove excess of PE from the column
- Change collection tube
- Remove ~2µl of PE from the column by vacuum pump with a NEW yellow tip
- Remove all traces of PE: centrifuge 1 min
7. Elution:
- Insert column into elution tube
- Add **11**µl EB to the centre of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min

Step 4: Adaptor ligation

1. Set temperature of the Thermomixer at 20°C.
2.

Adaptor Ligation reaction	**µL for 1x**	**Conc. in reaction**
cDNA (20ng/uL)	11	3.3ng/uL
Illumina PE Adapter mix or IndexPE adapter	1	
DNA ligase buffer 2x	13	1x
DNA ligase	1	
total	26	

5. Incubate 15 min at 20C.

Step 5: Size selection

1. Prepare a 2% high-resolution agarose gel in the medium size gel (from the big BioRad tank) with 20-well combs; two rows. Prepared 160mL agarose in TBE 1x (diluted in sterile ddH20)+16ul Sybr safe.
2. Add **8µ**l of 6x OrangeG loading buffer to your samples. Load the samples on the gel .
3. Run the gel: 120, 5min,and 100V for 50min.
4. Cut out **150-400bp main** fragment
5. QIAquick-QG purification of main fragment:

Weigh the gel piece _____mg
Add **4x** volume of QG buffer: _____µl
Dissolve the gel slice completely by rotating at RT for ~10min
Check, that color is yellow
Add **1.3x** volume of Isopropanol: _____µl
Mix and wait 1-3 min
Load on column (in <0.75ml aliquots):
Centrifuge ~13krpm (~18kg) 1 min
Change collection tube and add a fresh collection tube
Add another 0.75ml on the column and repeat as many times as needed in order to load everything on the column
Centrifuge 1 min
Change collection tube
Wash with **0.5 ml QG**
Centrifuge ~13krpm (~18kg) 0.5 min
Change collection tube
Washing (take a new collection tube for each wash):
- Add **0.75ml** PE to the column
- Centrifuge ~13krpm (~18kg), 1 min
- Change collection tube
- Clean rotor's cover, rotor, tube rack with a wet paper towel
- Centrifuge ~13krpm (~18kg), 0.5 min to remove excess of PE from the column
- Change collection tube
- Remove all traces of PE: centrifuge 1 min

Elution:
- Insert column into elution tube
- Add **50**µl EB to the centre of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min (better to do it with two aliquots of EB: i.e. **2x 26** µl).

Samples were stored at -20^{o}C overnight.

Step 6: UDGase treatment of the non-amplified sequencing library

1. Set temperature of the Thermomixer at 37°C.
2. Add following reagents to the non-amplified library and mix carefully:

UDGase reaction	**µL for 1x**
Non-amplified library	50
(UDGase buffer 10x) used TE 1x	5.7
(UDGase 1U/µL) used AmpEraseung (UNG) from AB SybrGreen kit	1
	56.7

3. Incubate 30min at 37°C.
Cool samples down and quick spin.

Step 8: Library amplification

Amplification tips:

Prepare the reaction mixture on ice.

Do not use the final extension step (5-10 min 70-72°C). It does not help, but may induce problems because of the exonuclease activity of the proofreading polymerase.

Do manual hot-start: start the PCR program, pause at 98°C, put the tubes in and resume the program.

1. Set up the large-scale PCR reaction:
2.

Large-scale PCR reaction	**uL for 1x**
Non-amplified library	(10)
PCR primer InPE 1.0	1
PCR primer InPE 2.0	1
KAPA PCR Master Mix 2x	13
Mix total	25
PCR primer Index # (add individually)	1

Take 20μL of the master mix per tube and add 10μL of the non-amplified library, or H20 for negative control (H20 was amplified for 10 cycles).

2. Start the PCR program on PCR machine:

Temperature	**Time**	**Cycle No.**
98°C	0:30	
98°C	0:15	**9-13** cycles (depends the start total RNA)
65°C	0:30	
72°C	0:30	
4°C	hold	

If you start 10ug total RNA, you may only need run 9 cycles. Less start martials, more PCR cycles.

Load the PCR product directly to the gel

Second-time Size selection

1. Prepare a 2% high-resolution agarose gel in the medium size gel (from the big BioRad tank) with 20-well combs; two rows. Prepared 160mL agarose in TBE 1x (diluted in sterile ddH20)+16ul Sybr safe.

Add **4μ**l of 10x OrangeG loading buffer to your samples. Load the samples on the gel.

3. Run the gel: 120V, 45-60 min.
4. Cut out **150-400bp main** fragment and
5. QIAquick-QG purification of main fragment:

Weigh the gel piece _____mg

We continued with the 130-300bp fragments right away.

Add **3x** volume of QG buffer: _____μl

Dissolve the gel slice completely by rotating at RT for ~10min
Check, that color is yellow
Add **1x** volume of Isopropanol: _____µl
Mix and wait 1-3 min
Load on column (in <0.75ml aliquots):
Centrifuge ~13krpm (~18kg) 1 min
Change collection tube and add a fresh collection tube
Add another 0.75ml on the column and repeat as many times as needed in order to load everything on the column
Centrifuge 1 min
Change collection tube
Wash with **0.5 ml QG**
Centrifuge ~13krpm (~18kg) 0.5 min
Change collection tube
Remove ~2µl of QG from the column by pipetting
Washing (take a new collection tube for each wash):
Elution:
- Insert column into elution tube
- Add 25ul EB to the center of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min (better to do it with two aliquots of EB: 14ul and 10ul).

OD the samples by using Qubit.(2ul orig. sample +198ul dye-mix)
The website for calculation is listed below:
http://www.idtdna.com/analyzer/Applications/DilutionCalc/Default.asp
Molecular Weight: average size which you 250x660=165000

Sample	Qubite-Concentration	Dilute 10nM (org. + EB)
RZY#162		
RZY#163		
RZY#164		
RZY#165		
RZY#166		
RZY#167		

The concentration is **10nM**

The following samples were given to Samantha to sequence on the Hi-seq sequencer on 07/09/2012

Multi-RZY#29 **(10nM) PE**	RZY#162	Index#7
	RZY#163	Index#8
	RZY#164	Index#9

Multi-RZY#30 **(10nM) PE**	RZY#165	Index#10
	RZY#166	Index#11
	RZY#167	Index#12

PS: you could group you samples as you need. You could do up to 6 samples in one lane .