Experiment: **RNA-seq** DATE:

Purpose: Make mRNA-seq libraries for sequencing.

Methods and Materials:

1.TrisHCl 1M, pH 7.0(Purchased from TEKNOVA/UCSD Core)

2. TrisHCl 1M, pH 7.5(Purchased from TEKNOVA/UCSD Core)

3. LiCl 10M

4. EDTA 0.5M, pH 8.0: 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 (make fresh)

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 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 DEPC-treated H20, aliquoted in 500μL and store them in -20^{o}C. (**one tube from Don's Lab**)

9. dUNTPs 10mM 100μL: 10μL dATP 100mM, 10μL dCTP 100mM, 10μL dGTP 100mM, 10μL dUTP 100mM,60ul DEPC H2O.
(**tubes in mRNA-seq box in Zhen's -20 C**)

Prepare RNA 2μg – 10μg of total RNA per sample per library
We used the following samples:

Sample	Concentration	Vol. for 10μg or more	Add DEPC H_20

I then added the respective volume of DEPC H20 in each sample. With this dilution, you have **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^{o}C

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^{o}C, 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^{o}C.

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! Put the rack on vertexer,Votex).

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

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

FS mix 2.1x	**µL for 1x for 20µL reaction**	**µL for 11x**
Reverse transcription buffer 10x	2.0	22.0
dNTPs 10mM	1.0	11.0
MgCl2 100mM	1.0	11.0
DTT 100mM	2.0	22.0
Random primers	1.6	17.6
Oligo dT primer	1.0	11.0
Actinomycin D Add to enzyme mix	0	0
	8.6	86.0

2. Mix in individual 0.2ml PCR tubes:

FS reaction	amount of oligo dT RNA	FS mix 2.1X	Total µl
Sample	**10.4***	**9.6**	**20**

*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**: 12µl RNAseOUT, 12µl Superscript III and 12µl Actinomycin D.

4. In the PCR machine add 3µL of FS enzyme mix, with the 10µl pipette and use a different tip (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 in Zhen's 4°C refrigerator).
- 1.7ml tubes(EPPENDORF DNA Lowbinding tube,it is easy to be cut) with cross-cutted caps
- Yellow tips with inserted 4x4mm2 piece of GF/C(filter paper in zhen's bench drawer)

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**	**µL for 11x**
Reverse transcription buffer 10x	1	11.0
Second strand synthesis buffer	15	165.0
MgCl2 100mM	0.5	5.5
DTT 100mM	1	11.0
dUNTPs 10mM	2	22.0
E. coli DNA ligase	0.5	5.5
DNA polymerase I	2	22.0
Rnase H	0.5	5.5
	22.5	247.5

SS reaction						
eluate						
SS mix 22.5ul/ea						
H20 to 75uL						

Incubate for 2hrs at 16°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;
- 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 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 50µl 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 **2x26** µl).
Left in -20°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). New sonicator
Before starting, I kept 2µL of sample R-ZY#13 and R-ZY#22 to check for the efficiency of the sonication.

1. Go to glass lab to use the 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).

3. Set Green/Red indicators to 0.5min/1min (meaning on sonication 30sec, then off for 1min). 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 15 min x 4 times, when each 15 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 @150V for 3-5 min.
(2ul ladder/2ul samples+2ul H2O+1ul loading dye)

7. Looked like it is working. They were all sonicated enough.

8. Keep samples at -20^{o}C overnight.

Step2. End repair

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

ER reaction mix	**µL for 1x**	**µL for 11x**
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	106.7

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^{o}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).

Step3.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**	**µl for 11X**
Klenow buffer 10x (=NEB buffer 2)	5	55
dATP 1mM	10	110
H20	0.5	5.5
Klenow 3'-5' exo minus (5U/µl)	0.5	5.5
total	16	176

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;
- 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 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 **10**µl EB to the centre of membrane, wait for ~3 min, centrifuge ~13krpm (~18kg), 2 min (did not do it with two aliquots of EB this time because the volume was too small).

Step4.<u>Adaptor ligation</u>

1. Set temperature of the Thermomixer at 20°C.
2. First prepare the Adaptor mix (13pmol/uL) as follows:

Adapter 1 (100pmol/ µL)	3.25 µL
Adapter 2 (100pmol/ µL)	3.25 µL
DEPC water	18.52 µL
total	25µL

3. Then add 2µL of the adaptor mix to the cDNA, mix carefully and let stand for 5min (on ice):

4. Add 15µL of DNA ligase buffer 2x (quick ligase) per sample.

5. Add 3µL of DNA ligase (quick ligase) per sample. Finally in each tube you have the following:

Adaptor Ligation reaction	**µL for 1x**	**Conc. in reaction**
cDNA (20ng/uL)	10	3.3ng/uL
Adapter mix (13pmol/uL)	2	0.9pmol/uL
DNA ligase buffer 2x	13	1x
DNA ligase	1	
total	26	

6. Incubate 30min at 20°C (fast ligation) in Thermomixer.
7. Perform QIAquick-QG purification as follows:
1. Add **90µL** (3 volumes) QG buffer
2. Mix & wait 1-3 min
3. Add **30µL (**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 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 **30**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 16** µl).

Step5.Size selection

1. Prepare a 2% high-resolution agarose gel in the medium size gel (from the big BioRad tank) with 26-well combs; two rows. Prepared 330mL agarose in TBE 1x (diluted in sterile ddH20) but did not use all of it.

2. Use the tank with medium length tray and.

3. Add **12µl** of 6x OrangeG loading buffer to your samples.

4. Prepare markers as follows:

	1x	10x
marker 50bp	0.4	4
H20	7.6	76
6x Orange Dye	2	20
	10	100

	1x	2x
marker 1kb	1	2
H20	7	14
6x Orange Dye	2	4
	10	20

5. Load the samples on the gel (everything, using P20 in two loads per well 21ul/ea well).

Well	1	2	3	4	5	6	7	8	9	10	11	12	13
Sample	marker	empty	**ZY13**	**ZY13**	empty	marker	empty	**ZY14**	**ZY14**	empty	marker	empty	**ZY15**
	50bp					50bp					50bp		

14	15	16	17	18	19	20	21	22	23	24	25	26
ZY15	empty	marker	empty	**ZY16**	**ZY16**	empty	marker	empty	**ZY17**	**ZY17**	empty	marker
		50bp					50bp					**1kb**

Well	1	2	3	4	5	6	7	8	9	10	11	12	13
Sample	marker	empty	**ZY18**	**ZY18**	empty	marker	empty	**ZY19**	**ZY19**	empty	marker	empty	**ZY20**
	50bp					50bp					50bp		

14	15	16	17	18	19	20	21	22	23	24	25	26
ZY20	empty	marker	empty	**ZY21**	**ZY21**	empty	marker	empty	**ZY22**	**ZY22**	empty	marker
		50bp					50bp					**1kb**

4. Run the gel: 120V, ~1.5 hours.

5. Stain gel in 500mL 1xTBE + 50µL SYBR Gold for 15min at room temperature. In the meantime, prepare two 2 ml tubes for the main and reserve gel fragments.

6. Cut out **150-300bp main** fragment
7. QIAquick-QG purification of main fragment:

Weigh the gel piece _____mg

	150-300bp		
Sample	Tube	Tube+Gel	Gel

R-ZY13	1.07	1.42	0.35
R-ZY14	1.06	1.35	0.29
R-ZY15	1.07	1.32	0.25

We continued with the 150-200bp fragments right away.
Add **4x** volume of QG buffer: _____µl
`

	150-300bp		
	Gel weight (g)	QG vol (mL)	Isopropanol (mL)
R-ZY13	0.35	1.4	0.455
R-ZY14	0.29	1.16	0.377
R-ZY15	0.25	1	0.325

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
Remove ~2µl of QG from the column by pipetting
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 droplet of PE from the column with the pipette
- 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).

If the start material is 2ug-5ug, elute in 30ulEB.
Samples were stored at -20^{o}C overnight .

Step6.UNGase 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:

UNGase reaction	**µL for 1x**	**Conc. in reaction**
Non-amplified library	50	
(UNGase buffer 10x) used TE 1x	5.7	Tris 1x, EDTA 0.1x…
(UNGase 1U/µL) used AmpEraseung (UNG) from AB SybrGreen kit	1	minimum 1U per 1µg DNA
	56.7	

3. Incubate 30min at 37°C.

Cool samples down and quick spin.

Step 7: Real-Time PCR check of the non-amplified sequencing library

1. Prepare the real-time PCR reaction mix for 4 controls (-):

Real-Time PCR reaction	**uL for 1x**	**µL for 35x**
SYBR-Green buffer Master Mix(Roche)	10	350
primer PCR 2.1 10µM	0.8	28.0
primer Ad_1 10µM	0.8	28.0
H20	7.4	259
total	19	665

2. Distribute 19µl of the master mix into optical PCR tubes;
3. Add **1µl of non-amplified library** to each well (triplicates per sample), or 1µl of H2O to the (-) control tube

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

4. Start the PCR program on LC480 RT- PCR System:

Temperature	Time	Cycle No.
95°C	10:00	
95°C	0:15	39 cycles
65°C	0:30	
72°C	0:30	
melt curve analysis		

5. Determine number of cycles necessary to reach the middle of the logarithmic phase:
For s3 150-200bp A=**9.23** (Ct from RT-PCR)
6. Estimate the library complexity:
~0.1µg / (2A * 0.175kb * 103 * 2 * 330g/mol) * NAvogadro[1/mol] = 1011/(2A * 0.175kb)

Go to: http://seq.molbiol.ru/lib_ampl.html and add the cycle number at the algorithm – calculates complexity and PCR cycle number automatically.
7. Determine the number of cycles for large-scale PCR (N):

Sample	Ct - mean	Cycles	Complexity/µL of unamplified library

Step8.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 95°C, put the tubes in and resume the program.

Used the 20µM primer solution prepared before from the 100µM stock: 20µL stock + 80µL H20.

1. Set up the large-scale PCR reaction:

		Old	**Make a new Master mix**
Large-scale PCR reaction	**uL for 1x**	**uL for 12x**	**uL for 12x**
Non-amplified library	(10)		
primer PCR 1.1 20µM	0.6	7.2	7.2
primer PCR 2.1 20µM	0.6	7.2	7.2
PCR Master Mix 2x	15	180	180
H20	3.8	45.6	45.6
Mix total	20	240	240

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	8 cycles
65°C	0:30	
72°C	0:30	
4°C	hold	

Take out samples after the number of cycles shown at the table above.
Transfer to new 0.65mL tubes.
(You could Keep samples at -20°C overnight.)

3. Prepare 2% high-resolution agarose gel in TBE 1x (350mL TBE + 7g agarose).
4. For the samples: add 12µl of 6x OrangeG loading buffer to the 30µl PCR reaction.

5. Prepare markers as follows:

	1x	7x
marker 50bp	0.4	2.8
H20	7.6	53.2
6x Orange Dye	2	14
	10	70

	1x	2x
marker 1kb	1	2
H20	7	14
6x Orange Dye	2	4
	10	20

6. Load samples on the gel, using P20 (2x20µL).(Drop the samples on the ground.)
7. Run the gel: 120V, ~1.5-2 hours;
8. Stain gel in 500mL 1xTBE + 50µL SYBR Gold for 15min at room temperature.
9. All samples had a consistent smear of about **120-300bp**, which we have cut out. There was no visible smear above 200bp for all our samples, so we did not take that gel slice.

	120-300bp		
Sample	Tube	Tube+Gel	Gel

10. QIAquick-QG purification of main fragment:
I had to transfer the gel pieces to FACS tubes before adding QG buffer.
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

	150-200bp		
	Gel weight	QG vol (mL)4x	Isopropanol (mL)1.3X

Mix and wait 1-3 min
Load on column (in <0.75ml aliquots):
Centrifuge ~13krpm (~18kg) 0.5 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):
- 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

Elution:
- Insert column into elution tube
- Add 15-**30**µl EB(depends the bands' brightness) 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 16 µl).

Keep samples at -20^{o}C overnight.

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
Stock Concentration: your samples'conc(ng/ul)
Stock Volume: 10ul
Final Concentration: 5.2nM
Final Volume: 10ul
Molecular Weight: average size which you 170x 660(g/mol)=112200
Calculate 5.2nMx3=15.6nM=1.75ng--1.8ng(150bps-200bps)
5.2nMx3=15.6nM==2.5ng(200bps-300bps)

Sample	Qubite-Concentration	Vol. for 1.75ng-1.8ng