March, 05

RNeasy Micro Kit (Qiagen, cat#: 74004)

Reagents to be used:

RLT (sample lysis buffer)
Ethanol (70% and 80%)
RW1 (washing buffer)
RDD buffer (store in fridge)
DNase in RDD buffer
RPE
RNase free water

- Add 10µl Mercaptoethanol/1ml RLT (RLT is stable for a moth at RT after addition of mercaptoethanol).
- Add 4Vol of absolute ethanol to RPE buffer.
- Dissolve DNase in 550 µl of RNase free water. Do not vortex (as DNase is physically sensitive). Aliquote and store at -20C.
- Use carrier RNA when the starting material is less than 10 µg. Use carrier DNA and the sample +RLT before homogenization.
- Dissolve carrier RNA (310 µg) in 1ml RNase free water (store at -20C).

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(1) Add **350 µl** of **RLT** (mercaptoethanol added) to your sample prior to homogenization. If the sample is <10 µg tissue, add 20ng (i.e. 5µl of 4ng/µl) of carrier RNA

(2) Spin at >10,000 rpm for 3min. Transfer the supernatant to a new tube (i.e. the lysate).

(3) Add **350 µl** (i.e. 1 vol) of 70% **ethanol**. Pipet up and down but **do not centrifuge**. If ppt appears after ethanol adding, don't worry about it.

(4) Apply the sample (with any ppt) to **RNeasy spin column** in 2ml collection tube. Spin for 15second at >8000 xg (i.e. >10,000 rpm) and discard the flow through.

(5) Add 1 vol **RW1** buffer, spin (15sec at >8000g).

(6) Prepare **DNase** solution (as **10 µl DNase in 70 µl RDD buffer**). **Do not vortex** but gently pipette up and down. **DNase is physical sensitive**. Pour the 80µl of DNase + buffer to RNase column and **incubate for 15min** at RT. Mix well both DNase/RDD + silica gel membrane so the reaction takes place.

(7) Add **350 µl RW1** (after genomic DNA digestion). Centrifuge for 15s/8000xg

(8) Transfer the silica column in a new 2ml collection tube. Pipet **500 µl**of **RPE buffer** (ethanol added) and spin 15s/8000xg.

(9) Add **500 µl** 80% ethanol and spin for 2min (2min/ > 8000 xg). Remove RNeasy column carefully from the collection tube, so it does not touch the ethanol.

(10)Transfer the column to a new 2ml collection tube. Open the cap and spin at the highest speed for 5min. Discard the flow-through (to insure that all ethanol is gone).

(11)**Elution step:** Transfer the silica column to a new 1.5ml tube and add **14 µl of RNase free water**. Close the tube and spin for 1min at maximum speed (you can use less volume to have more conc RNA).

This protocol will extract the total RNA but enrich the mRNA, since any RNA < 200 bases (i.e. rRNA and tRNA) will be in the flow-through. Any RNA >200 (i.e. mainly mRNA) will be trapped in the silica gel.

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Real time RT-PCR

Superscript III Platinum 2step qRT-PCR with SYBR Green (invitrogen)

Cat#: 11735-032 (100 rxn)
11735-040 (500 rxn)

RT enzyme mix: contains RT superscript III which reduces RNaseH activity and synthesizes cDNA at 45-60 °C. This enzyme is not inhibited by tRNA or rRNA and thus, it can be used to synthesize cDNA from total RNA.

2XRT reaction mix: contains 2.5µM oligo $(dT)_{20}$, 2.5ng/µl random hexamers, 10mM $MgCl_2$ and dNTPs.

Platinum SYBR Green qPCR superMix-UDG: contains:
(1) SYBR green (binds with dsDNA and its fluorescent signal is related to the amplified DNA).
(2) 60U/ml Platinum Taq DNA polymerase: has hot start technologyas it is inactivated by antibodies at ambient temperature.
(3) 40U/ml UDG, 6mM $MgCl_2$ and 400µM of each dNTPs (including dUTP). dUTP is included to ensure that the amplified DNA contains uracil. UDG removes uracils from ssDNA or dsDNA and preventing dU containing DNA to serve as a template in future PCR.

ROX: is used to normalize the fluorescent signal between reactions.

RNaseH: this *E. coli* recombinant enzyme will eliminate single stranded RNA from RNA:cDNA hybrid after cDNA formation to increase the PCR sensitivity.

RT protocol:

Respect the order of adding the components:

2X RT rxn mix	10µl
* RT enzyme mix	2µl
RNA	?
DEPC treated water	to 20µl

* RT enzyme mix contains RNaseOUT. If this is finished, you can order RNaseOUT and RT enzymes separately.

Program the PCR machine as follows:

1	25°C	10min
2	42°C	50min
3	85°C	5min
4	Suspend to Chill on ice for 1min and add 1µl (2U) of RNaseH	
5	37°C	20min
6	End → store at -20C until processing (i.e. PCR)	

PCR (after RT rxn):

Use primers at 10µM working concentration. It is so critical to use master mix (i.e. all reagents mixed together except the cDNA template ≈ RT product). Master mix will help reducing error of pipetting.

(1) Prepare the mater mix:

Platinum SYBR Green Superscript UDG	12.5µl x (# of samples)
F-primer (10µM)	0.5µl x (# of samples)
B-primer (10µM)	0.5µl x (# of samples)
DEPC treated water	9µl x (# of samples)
	22.5µl x (# of samples)

(2) Add 2.5µl of RT product (i.e. cDNA template) to each tube to get total volume of 25µl. Thus, the RT product consists of 1/10 of the total volume of PCR rxn tube.

(3) Gently mix

(4) Spin briefly

(5) Do Real time PCR.

Real time protocol [using qPCR SmGPCR primers (see next page)]:

1st Hold	50C	2min
2nd Hold	94C	2min
40-45cycles		
Denature	95C	15sec
Annealing	51.1C	30sec
Extension	70.2C	30sec

Real time protocol [using qPCR *S. mansoni* α-tubulin primers (see next page)]:

1st Hold	50C	2min
2nd Hold	94C	2min
40-45cycles		
Denature	95C	15sec
Annealing	56C	30sec
Extension	75.4C	30sec

Primers for qRT-PCR (to evaluate RNAi of SmGPCR):

qPCR SmGPCR **sense**(22nt): 5'-**TCACTCAAAAAACCTAAATCAC**-3'

qPCR SmGPCR **antisense** (25nt): 5'-**GAGATGTCAAAGAAAATTCTCTATC**-3'
These primers are designed to target and amplify **349**nt of the SmGPCR gene in the il3 region out of the RNAi target region.

The positions of these primers are: 958-1306 of the SmGPCR.

PCR product
Position : 958 - 1306
Length : 349 bp
GC% : 29.8 %
Tm(product) : 70.2°C
Tm(annealing) : 51.1°C

Primers for qRT-PCR of *S. mansoni* α-Tubulin (M80214) 1422bp

(169 sense): **CTTATCGTCAACTTTTCCATCC** (22nt)
(170 antisense): **GGAAGTGGATACGAGGATAAGG** (22nt)

This will amplify the region between 245-802 (i.e. **558bp**)

According to oligo Analyzer software, both primers have no self-annealing, can form sense-antisense dimers that can be broken easily (i.e. less than -6Kcal).

The **annealing temperature (Tm) is 56°C**.

PCR product
Position : 245 - 802
Length : 558 bp
GC% : 40.9 %
Tm(product) : 75.4°C
Tm(annealing) : 56.0°C

Standard curve of known plasmid, using rotor gene instrument

The plasmid that I will draw a standard curve from is pCIneo-SmGPCR (0.03µg/µl).

(1) Make 4 serial dilutions of the known plasmid concentration.
(2) Prepare the master mix for 4samples. According to the table in the previous page, it will be:

Platinum SYBR Green Superscript UDG	50µl (i.e. 12.5µl x4samples)
F-primer (10µM)	2µl (0.5µl x 4)
B-primer (10µM)	2µl (0.5µl x 4)
DEPC treated water	36µl (9µl x 4)
	90 µl (22.5µl x 4)

(3) Split the master mix into 4tubes (the Rotor-gene thin walled tubes). Each tube should receive 22.5µl

(4) Add 2.5µl of different dilutions per tube. This makes the total volume of 25µl/tube.

To prepare serial dilutions of plasmid stock:
Let's make 1:10, 1:20, 1:40 and 1:80 (2X dilution for 4samples)

Take 2µl of the plasmid stock and mix it with 18µl of DEPC treated water. This is your first 1:10 dilution.

Take 10µl of 1:10 dilution and mix it with a new 10µl water→ you got 1:20

Take 10µl of 1:20 dilution and mix it with a new 10µl water→ you got 1:40

Take 10µl of 1:40 dilution and mix it with a new 10µl water→ you got 1:80
