PROTOCOL FOR the Extraction of RNA by HiVE and Detection of Gene Transcripts by RT-PCR.

- Dr Jeremy Clark, Martyn Webb and Mark Winterbone, Norwich Medical School, University of East Anglia, Norwich. UK.

LEGEND

⇨ *ATTENTION*

* *HINT*

REST

NB: Using the Norgen column binds all sizes of RNA but vacuum flow of urine through the column is slow. The vacuum flow through the Qiagen RNeasy column is a lot faster but does not bind small RNA. If you use the Qiagen RNeasy columns then wash the columns as per the standard Qiagen protocol except for the DNase step – instead used the improved DNase step that is described in the main method below.

REAGENTS AND MATERIALS

- RLT (Qiagen – 79216)
- DTT (Sigma-Aldrich – 43816)
- Ethanol (96-100%) (VWR – 20821.330)
- RNase/DNase free water (Fisher AM9932)
- glycogen (1µg/µl) (Manuf Catno)

- TE (Sigma-Aldrich – 93283)
- PBS (Sigma-Aldrich – 79378)
- Chemgene HLD4H, Chemgene.
- Platinum Taq (Fisher – 100021273)

1) HiVE (High volume Vacuum Extraction) method for cfRNA.

Summary:
Cell sediment is removed from the urine sample by centrifugation followed by passing through a 0.8μm filter attached to a syringe. Cell-free urine is mixed with a lysis buffer and ethanol and drawn over an RNA extraction column (Norgen Biotek) by vacuum. The column is then washed with an ethanol-based wash solution before Total RNA is eluted in 100μl water.
Total RNA is then separated into small (<200 nt) and large (>200 nt) RNA species which are each eluted in 10μl using Zymo RNA columns.
See Figures 2 and 3 for a visual overview of the extraction steps and the vacuum equipment set up.

HiVE Equipment/Reagents Required:

- Norgen RNA extraction column (Urine Cell-Free Circulating RNA Purification Mini Kit, (Norgen - 56900)
- Norgen Urine Collector (Norgen – 18111)
- Zymo RNA clean and concentrator-5 (Cambridge Bioscience - R1013)
- Buffer RLT (Qiagen – 79216) (10ml per sample)
- DTT 1M (400μl per sample)
- 96 – 100% Ethanol (10ml per sample)
- 25ml disposable pipettes
- 0.8μm filter (Sartorius)
- 50ml disposable syringe (BD 300296)
- ‘QIAvac 24-Plus’ vacuum manifold (*Qiagen[19408, 19413]*)
- VacValve (Qiagen)
- Vacuum Pump – capable of 0.8mBar

- Chemgene 10ml in the vacuum trap (minimum 2% final volume).

Figure 1) Overview of HiVE RNA extraction. The procedures can be split into three sections;

yellow – Urine collection and preparation, blue Total-RNA extraction, and green – Large and Small-RNA separation. Pause points are indicated.

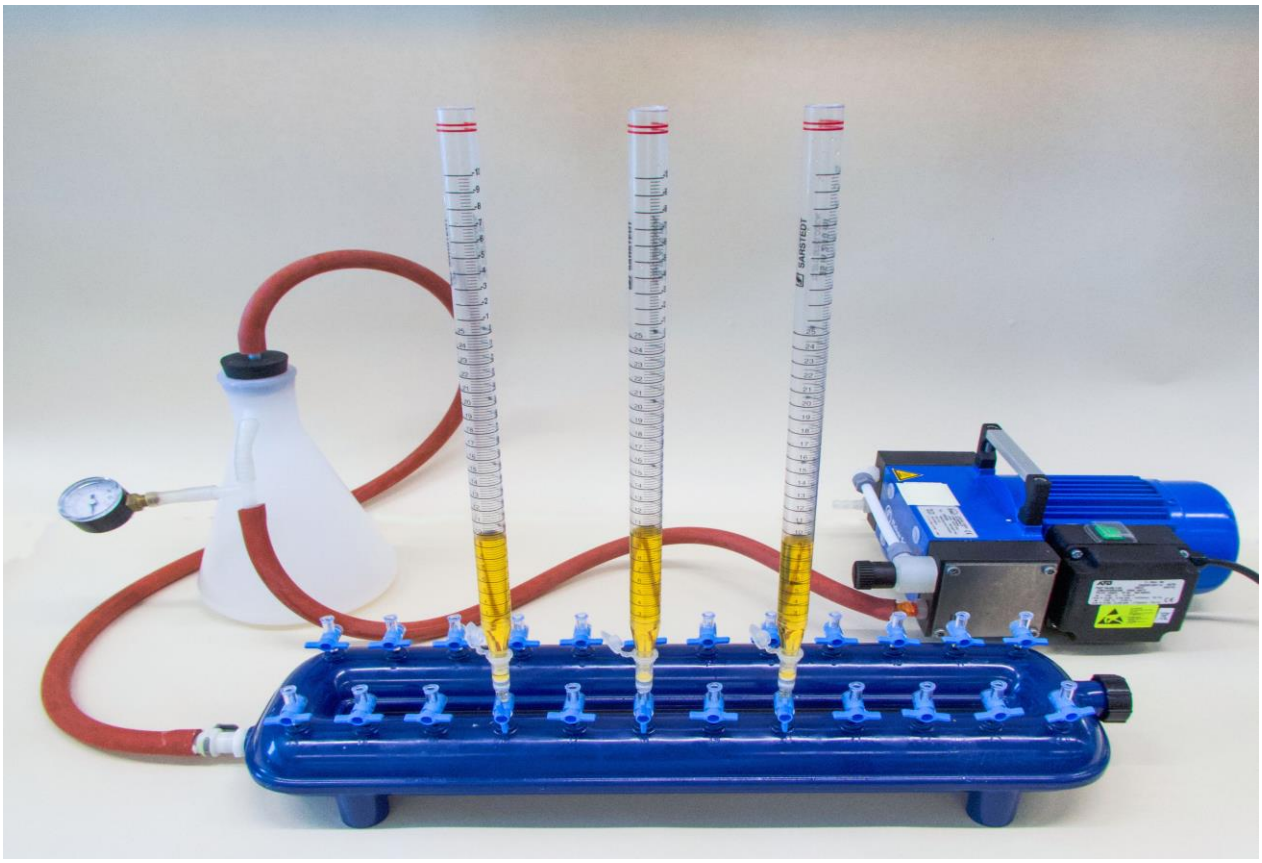

Figure 2a. Set up of the HiVE equipment .

Figure 2b. Close up of the pipette/reservoir, RNA binding column, VacValve and QIAVac vacuum manifold for HiVE.

HiVE RNA Extraction.

A) Set Up

1. Thaw 1xPBS (for cell pellets, 500µl per sample).
2. Record the sample IDs, approximate urine volume, date and time of sample provision and date of sample processing.
3. Print labels for Urine Collection and Urine RNA extraction.
4. Add 10ml Chemgene to the 1 litre plastic conical vacuum trap (minimum 2% final v/v).
5. Set up, close lids and label the following tubes with Sample IDs:

   Collection:

   WU (Whole (Urine) 2x 2ml flip top tube, 1x 30ml centrifuge tube.
   CP (Cell Pellet)1x 2ml flip top tube
   SN (Supernatant) 1x 30ml tube for -80°C

   Extraction:

   1x 100ml tube
   1x Norgen RNA binding column
   2x 2ml Qiagen collection tube (no label required)
   1x 1·5ml non-stick collection tube (Ambion).

- 6. Prepare the DNase mix: Qiagen RNeasy micro kit (before first use): Prepare 'DNase I' stock solution. Dissolve the DNase 1 stock in 550µl of Ambion (Nuclease-free) water (inject into the vial using a needle and syringe). Divide into aliquots for long-term storage, store at -20°C (up to 9 months). Thawed solutions can be stored at 4°C (must be used within 6 weeks).

B) Sample Processing and cfRNA Extraction

1. Set up the tubes (capped) and label them on side and lid.
2. Invert urine sample 10 times, aliquot 2x1·5ml Whole Urine in 2ml flip top tubes, freeze aliquots at -80°C.

3. Sediment the cells: Spin the whole urine in a 30ml polypropylene centrifuge tube, centrifuge 5 min @ 2,500 g, Acceleration ‘9’, Brake ‘2’, Eppendorf 5810R centrifuge.
4. Transfer the supernatant into a 30ml polypropylene tube and freeze at -80°C or proceed to step 6 below for RNA extraction procedure.
5. Resuspend the cell pellet in 500µl filter sterilised PBS (PBS is stored in aliquots at -20°C).
   If the pellet gets stuck on or inside the pipette tip then place the pipette tip into the 2ml tube, place this inside the Universal collection tube and spin at 2500g 5 min. Freeze cell pellet/PBS -80°C.

   ***Pause point**, can freeze Whole Urine, Cell Pellet and Supernatant at -80°C now.

   Set up: Thaw supernatant in a waterbath at r/t ~10-15min. Set up the tubes for RNA extraction whilst thawing.

6. Draw up the urine supernatant into a disposable 50ml syringe with a sterile 13cm Kwill attached. Pass urine through a 0·8µm filter into a 1x100ml tube.
7. Add 1 vol Qiagen RLT/DTT mixture, vortex 10 sec.
8. Add 1 vol 96-100% Ethanol, (it is important to swirl the mixture while adding ethanol) vortex 10 sec.
9. Place Norgen RNA binding column into a VacValve on the vacuum manifold.
10. Draw up 30 ml Urine/RLT/DTT into a plastic disposable pipette (25ml and 50ml pipettes work well). Insert pipette tip into the Norgen RNA-binding column.
11. Open VacValve and switch on vacuum (80mBar) to draw the sample over the RNA-binding column.
12. When the sample has passed through the column (~20 min for 3ml urine), close the VacValve. More Urine/RLT/DTT can be draw up into the pipette and the above procedure repeated as required.
13. Remove all pipettes and transfer the RNA binding columns into 2ml collection tubes.
14. Add 600µl Norgen wash solution A or Qiagen RW1 (both work equally well). Centrifuge 1 min 3,300 g, discard flow through.
15. Repeat wash step.
16. Perform DNase step: Prepare 10µl of ‘DNase I’ stock solution in 70µl of ‘Buffer RDD’ for each column. Mix by inversion.

17. Add 80µl of the DNase/RDD mix directly to the membrane of each RNA-binding column. Leave at room temperature for 15 min.
18. Add 600ul RWT to column, spin 15sec, **RELOAD** the flowthrough back onto column, spin 15sec. This readdition step minimises the massive loss of RNA that you get with the standard Qiagen protocol at this point.
19. Transfer the column to a fresh collection tube, centrifuge 2 min 14,000 g.
20. Transfer column to a 1.5 ml non-stick RNase/DNase-free collection tube (Ambion).
21. Add 100µl H_2O to the column, spin 1 min 200g, followed by 2 min 5,200g.
22. Reload RNA/water flowthrough onto the column, replace column into the same tube.
23. Centrifuge 1 min 400 g, then 2 min 5,800 g.
24. ***Pause Point!** Total RNA samples (~90µl) can now be stored at -80°C for up to one week before separating into small-RNA and large-RNA fractions as below.
25. **NB:** if using Qiagen RNeasy column then the small RNA will not be harvested. Eluted RNA volume can be reduced in a SpeedyVac, or using a Zymo column as below.

C) Separation of Total RNA into Small- and Large-RNA fractions

Set up:

2x Zymo tubes per sample

1x non-stick 1.5ml tube containing 1µl glycogen (1ug/µl)

The total-RNA (>17nt) eluted from the Norgen column can be divided into small (<200nt) and large (>200nt) RNA fractions using a Zymo RNA-binding column and Zymo 'Clean & Concentrator 5' reagents. This also reduces the RNA volume from 100µl to 10µl.

1. Make the volume of each Total-RNA sample up to 100µl.
2. Add 265µl of a mixture of Zymo RNA-binding Buffer (100µl) plus Ethanol (165µl).
3. Pipette onto a Zymo RNA binding column placed into a 2ml collection tube, centrifuge 30 sec 10,000 g.

Retain the flow-through, it contains small-RNA (<200nt), the large RNA (>200nt) remains bound to the column

4. To the Small-RNA eluate add an equal volume of Ethanol (~200µl). Pipette onto a fresh Zymo RNA binding column placed into a 2ml collection tube, centrifuge 30 sec 10,000 g. Discard flow through.

The following steps are for both the small- and large-RNA fractions

5. To each column add 400µl Zymo RNA Prep buffer, centrifuge 30 sec 10,000 g. Discard flow through.
6. Add 700µl Zymo RNA wash buffer, centrifuge 30 sec 10,000 g. Discard flow through.
7. Add 400µl Zymo RNA wash buffer, centrifuge 2 min 16,000 g. Discard flow through.
8. Transfer column into a 1.5 ml non-stick collection tube to which has been added 1µl of 1µg/µl glycogen (Sigma)
9. Add 10µl RNase/DNase free H_2O, centrifuge 30 sec 10,000 g.
10. Quantify RNA with a Bioanalyzer or Qubit as manufacturer's instructions, store at -80°C
11. *REST*
12. Equipment clean-up: the urine/Chemgene in the vacuum trap is left for 1 hour and then is poured down the sink in the fume hood with the tap running.

EQUIPMENT

Ambion non-stick tubes 1.5ml (Fisher – AM12450), 2ml (Fisher – AM12475)
0.8µm filter (Sartorius – 16592K),
20ml disposable syringe (BD - 300296)
50ml tube, Falcon
RNeasy micro kit (Qiagen – 74004)
Norgen RNA extraction column (Urine Cell-Free Circulating RNA Purification Mini Kit, (Norgen – 56900)
Norgen Urine Preservative (Norgen Biotek - 18124)
Norgen Urine Collection and Preservation Tube (Norgen – 18111)
'QIAvac 24-Plus' vacuum manifold (Qiagen – 19413)
VacValve (Qiagen - 19408)
'25ml' disposable plastic pipette (Sarstedt – 86.1685.001)
Zymo RNA column (RNA Clean & Concentrator-5, Zymo Research – R1013)
Qubit 2.0 Fluorometer (Fisher Scientific – Q32866)
Qubit RNA HS Assay (Fisher Scientific – Q32852)
Bioanalyzer 2100 (Agilent – G2939BA)
Bioanalyzer RNA 6000 Pico kit (Agilent – 5067-1513).
Bioanalyzer kit for sRNA (Agilent – 5067-1548).
Kwills Sterile Filling Tubes (13cm/5") (GP Supplies SKU18271)

Gosselin Straight 125mL Container, Polypropylene, Clear with Blue Screw Cap, (Scientific Laboratory Supplies – CON1018).