PROTOCOL FOR: The At-Home Collection of Urine, Extraction of RNA by HiVE and Detection of Gene Transcripts by RT-PCR.

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LEGEND

⇨ *ATTENTION*

* *HINT*

REST

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)

PROCEDURE

Include a step-by-step guide to the procedure, using the legend above to add hints, attention points, and rest stages.

The At-Home Collection Procedure

The manufacturer's safety information for the preservative states that it is Class 2a, the same as for ethanol and the 23andme home genomic testing kit. Any potential skin contact is minimal as the urine preservative is dried down to the bottom of the tube and takes 20min to fully dissolve in urine.

A letter of introduction is sent to the patient informing them that the kit will be sent to them a week later. This letter contains a brief description of the kit and a description of the study. The information on provision of a urine sample requests that the patient provides a sample after a minimum of 3 hours sleep.

The Home collection kit is posted to the patient. If the recipient agrees to be part of the study he will sign the two consent forms, keep one for himself and place the other

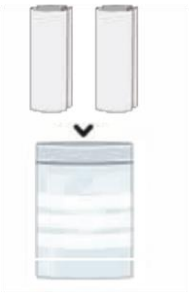
in the documents section of the Safebox to be returned with the urine samples.
The participant provides two 30ml urine samples, one from the first micturition of the day and the second 1-hour later - using the 1-hour timer provided. Both samples are placed inside a plastic sealable bag containing wadding. This bag is then placed inside the leakproof sealable compartment, the lid is attached and the whole kit can be returned in the post (Figure 1).

Figure 1. Instructions for posting Urine samples

>1

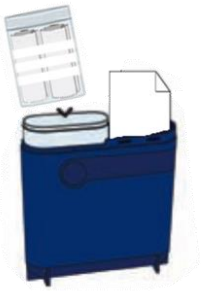
Step one
Place Samples in padding

>2



Step two
Place in plastic self-seal bag and seal.

>3

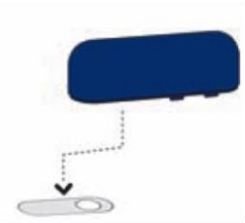


Step three
Put the whole package in the clear plastic compartment. Place any documentation in the other compartment.

>4

Step four
Important: Check that all the contents you want to send are inside the package before closing as once it has been closes, it can't be reopened without destroying it

>5

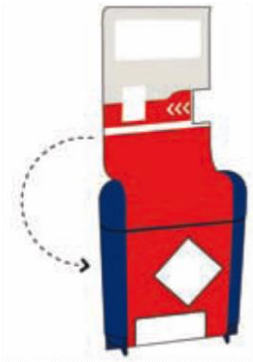


Step five
Remove the cardboard separator from the lid and dispose of it.

>6

Step six
Place the lid over the top of the containter and firmly press shut.

>7



Step seven
Peel the otuer backing from the self-adhesive label. Then wrap around the Safebox™.

>8

Step eight
Make sure the package is addressed correctly and the return address has been completed. **It's ready to go.**

>9

Step nine
You can put First Class Safebox™ in any post-box. Safebox™ posted using Special Delivery ™ needs to be taken to any Post Office

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

Summary:
Cell sediment is removed from the urine sample by centrifugation followed by a 0.8μm filter. 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.
The 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).

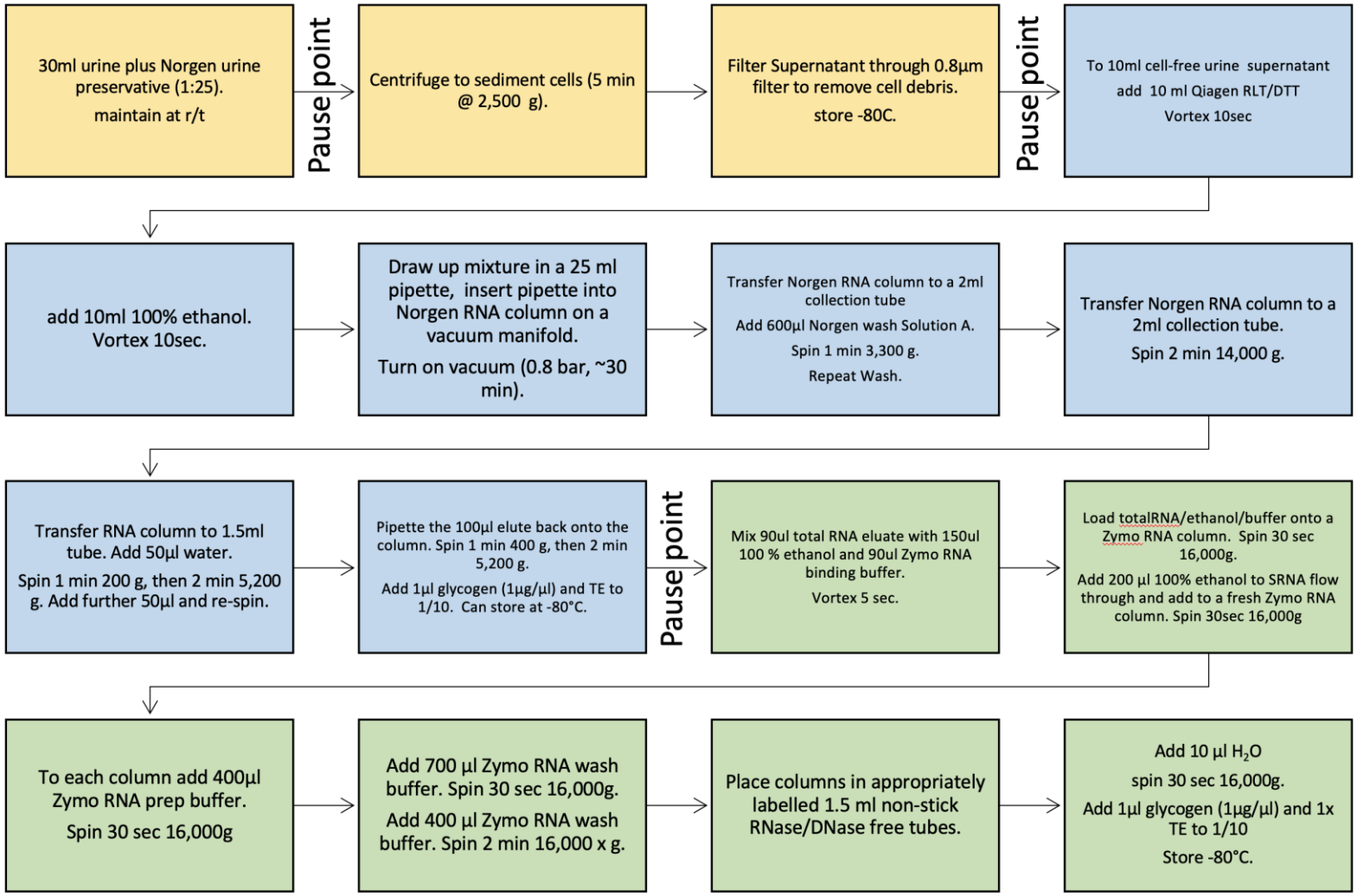


Figure 2) Overview of HiVE RNA extraction. The procedures can be split into three sections; yellow – Urine collection and preparation, blue Total-RNA extraction, and green – Large and Small-RNA separation. Pause points are indicated.

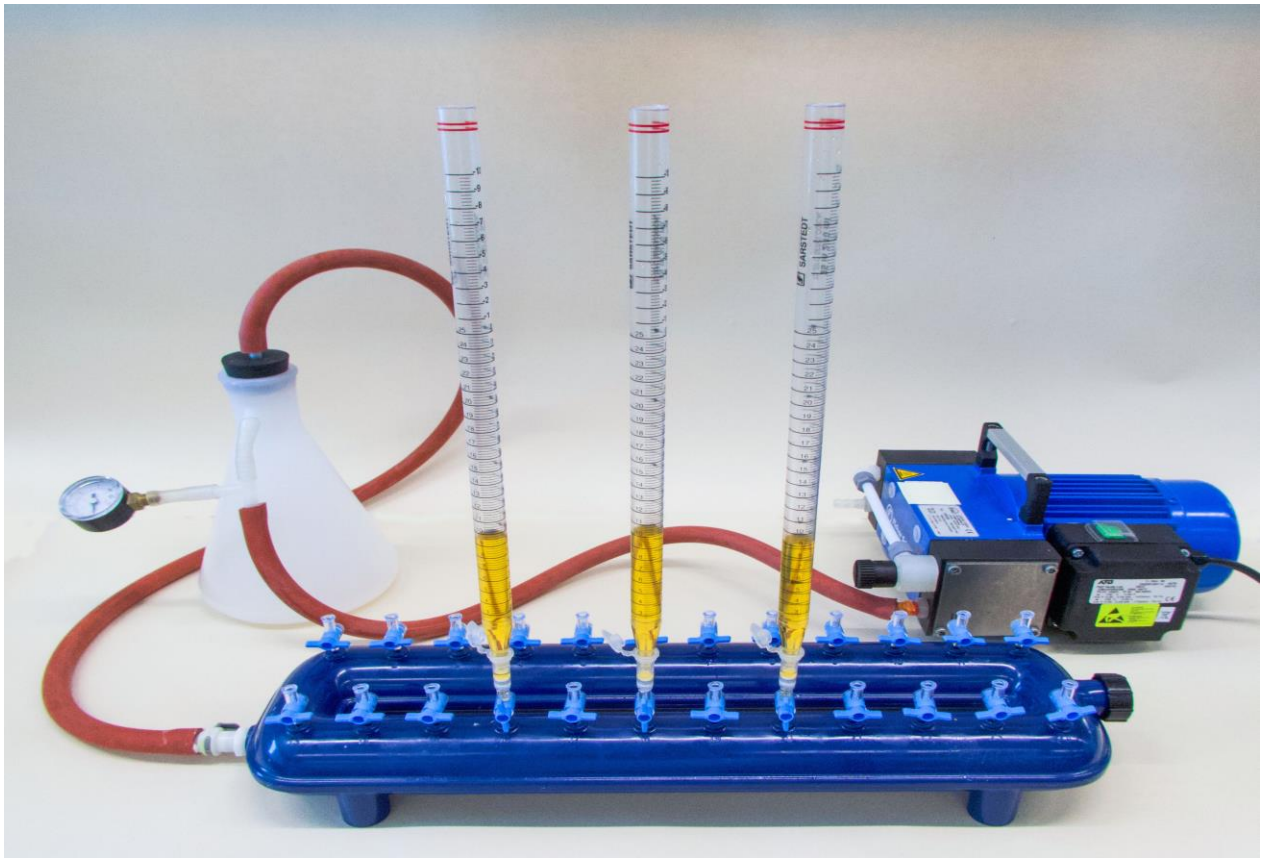


Figure 3a. Set up of the HiVE equipment .

Figure 3b. Close up of the pipette/reservoir, RNA binding column, VacValve and QIAVac vacuum manifold for HiVE.

HiVE RNA Extraction

1. Remove Urine cell sediment by centrifugation 5 min @ 2,500 g

2. The urine supernatant is loaded into a disposable 50ml syringe and passed through 0.8µm filter into 50ml tube.
3. Transfer 10ml supernatant to a fresh 50ml tube. (Store remainder -80C).
4. Add 10ml Qiagen RLT/DTT mixture, vortex 10 sec.
5. Add 10ml 96-100% Ethanol, vortex 10 sec.
6. Place Norgen RNA binding column (or Qiagen RNeasy column) into a VacValve on the vacuum manifold.
7. Draw up 30 ml urine/RLT/DTT into a plastic disposable pipette. Insert pipette tip into the Norgen RNA column.
8. Open VacValve and switch on vacuum (0.8Mbar) to draw the sample over the RNA binding column.
9. When the sample has passed through the column (~40 min), close the VacValve. More urine/RLT/DTT can be draw up into the pipette and the above procedure repeated as required.
10. Remove all pipettes and transfer the RNA binding columns into 2ml collection tubes.
11. Add 600µl Norgen wash solution A. Centrifuge 1 min 3,300 g, discard flow through.
12. Repeat wash step.
13. Transfer the column to a fresh collection tube, centrifuge 2 min 14,000 g.
14. Transfer column to a 1.5 ml non-stick RNase/DNase-free collection tube (Ambion).
15. Add 100µl RNase/DNase free H_2O to the column, centrifuge 1 min 200 g, followed by 2 min 5,200 g.
16. Reload water from 1.5ml tube onto column, replace column into the same tube.
17. Centrifuge 1 min 400 g, then 2 min 5,800 g.
18. Samples (~90µl) can now be stored at -80C for up to one week before separating into small-RNA and large-RNA fractions as below.

Separation of Total RNA into Small (<200nt) and Large-RNA (>200nt) fractions

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

1. To each 100µl Total-RNA sample add 267µl of a mixture of Zymo RNA binding Buffer (100µl) plus Ethanol (167µl).
2. Pipette onto a Zymo RNA binding column placed into a 2ml collection tube, centrifuge 30 sec 10,00 g.

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

3. To the Small-RNA eluate add 200µl Ethanol. 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

4. To each column add 400µl Zymo RNA Prep buffer, centrifuge 30 sec 10,000 g. Discard flow through.
5. Add 700µl Zymo RNA wash buffer, centrifuge 30 sec 10,000 g. Discard flow through.
6. Add 400µl Zymo RNA wash buffer, centrifuge 2 min 16,000 g. Discard flow through.
7. Transfer column into a 1.5 ml non-stick RNase/DNase free collection tube to which has been added 1µl of 1µg/µl glycogen (Sigma)
8. Add 10µl RNase/DNase free H_2O, centrifuge 30 sec 10,000 g.
9. Quantify RNA with a Bioanalyzer or Qubit as manufacturer's instructions, store at -80°C
10. *REST*

3) Nugen Whole Transcriptome Amplification method

Overview (Total 4hr 40min for whole protocol)

1) Generation of First Strand cDNA (1 hr 15 min)

First strand cDNA is prepared from total RNA using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase (RT). The primer mix contains a unique mixture of random and oligo dT primers such that priming occurs across the whole transcript. RT extends the 3´ DNA end of each primer generating first strand cDNA. The resulting cDNA/mRNA hybrid molecule contains a unique RNA tag sequence (SPIA tag) at the 5´ end of the cDNA strand which will be used as a priming site for the SPIA process.

2) Generation of a DNA/RNA Heteroduplex Double Strand cDNA (2 hours)

Fragmentation of the mRNA within the cDNA/mRNA complex creates priming sites for DNA polymerase to synthesize a second cDNA strand, which includes DNA complementary to the 5´ SPIA tag sequence from the first strand chimeric primers. The result is a double-stranded cDNA with a DNA/RNA heteroduplex corresponding to the SPIA tag at one end.

3) SPIA® Amplification (1 hr 35 min)

SPIA is a rapid, simple and sensitive strand-displacement amplification process de- veloped by NuGEN. It uses a DNA/RNA chimeric primer (SPIA primer), DNA poly- merase and RNase H in an isothermal assay. RNase H removes the RNA portion of the heteroduplex SPIA tag sequence, revealing a site for binding the SPIA primer. DNA polymerase synthesizes cDNA starting at the 3' end of the primer, displacing the existing forward strand. Priming with the chimeric SPIA primer recapitulates the heteroduplex SPIA tag, creating a new substrate for RNase H and the initiation of the next round of cDNA synthesis. The process of SPIA DNA/RNA primer binding, DNA replication, strand displacement and RNA cleavage is repeated in a highly processive manner, resulting in rapid accumulation of micrograms of amplified cDNA from picograms of total RNA.

Table 2 Thermal Cycler Programming

First strand cDNA Synthesis
1) Primer annealing: 85°C 30 sec, 80°C 3 min, 65°C 2 min, hold at 4°C (minimum 5 min)
2) First Strand synthesis: 4°C 2 min, 25°C 30 min, 42°C 15 min, 50°C 5min, 55°C 5 min, 65°C 5 min, 70°C 15 min, hold at 4°C
Second Strand Synthesis:
3) Second strand Synthesis: 4°C – 1 min, 25°C – 10 min, 50°C – 30 min, 55°C 10 min, 65°C 10 min, 80°C – 20 min, hold at 4°C.
4) SPIA Amplification: 4°C 1min, 47°C 75 min, 95°C 5min, hold at 4°C.

A) First Strand Synthesis:

1. Take the First Strand Primer Mix (blue: A1 VER 8), First Strand Buffer Mix (blue: A2 VER 3), First Strand Enzyme Mix (blue: A3 VER 1) and the Nuclease-free Water (green: D1) from the –20°C storage.
2. Spin down the contents of A3 and place on ice.
3. Thaw the other reagents at room temperature, mix by vortexing, spin and place on ice. Leave the Nuclease-free Water at room temperature.
4. Add 2 μl of A1 to a 0.2 ml PCR tube.
5. Add 5 μl of total RNA sample (500 pg to 50 ng) to the primer.
6. Mix by pipetting 5 times, spin and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Primer Annealing; see Table 1):
8. Remove the tubes from the thermal cycler and place on ice.
9. Once Primer Annealing (Step 7) is complete, prepare a master mix by combining of Blue top A2 First Strand buffer Mix (2.5μl) and of Blue top A3 First Strand Enzyme Mix (0.5μl) in a 0·5 ml capped tube.
10. Add 3μl (actually 2.9μl) of the First Strand Master Mix to each tube.
11. Mix by pipetting 5 times, spin and place on ice.
12. Place the tubes in a pre-cooled thermal cycler programmed to run Program 2 (First Strand cDNA Synthesis; see Table 1):
13. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
14. Continue immediately with the Second Strand cDNA Synthesis protocol.

B) Second Strand cDNA Synthesis

1. Take the Agencourt RNAClean XP purification beads (supplied with the Ovation PicoSL WTA System V2) from 4°C storage and place at r/t.
2. Thaw Second Strand Buffer Mix (yellow: B1 VER 3) at r/t, vortex, spin and place on ice.
3. Take the Second Strand Enzyme Mix (yellow: B2 VER 2) from -20°C, spin and place on ice.
5. Make a master mix by combining Yellow B1 (9.7μl) and Yellow B2 (0.3μl) in a 0·5 ml capped tube.
6. Add 10 μl of the Second Strand Master Mix to each First Strand reaction tube.
7. Mix by pipetting 5 times, spin and place on ice.
Place the tubes in a pre-cooled thermal cycler programmed to run (Second Strand cDNA

Synthesis; see Table 1)

Remove the tubes from the thermal cycler and spin to collect condensation.

Continue immediately with the Purification of cDNA protocol.

C) Purification of cDNA

1. Ensure the Agencourt RNAClean XP beads have completely reached room temperature before proceeding.
2. Prepare a 70% ethanol wash solution. It is critical that this solution be prepared fresh on the same day of the experiment from a recently opened stock container. Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce amplification yield.
3. Resuspend the beads by inverting and tapping the tube. Ensure that the beads are fully resuspended before adding to the sample. After resuspending do not spin the beads. A large excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.
4. At room temperature, add 32 µl (1·6 volumes) of the bead suspension to each reaction and mix by pipetting 10 times.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Keeping the tubes on the magnet, carefully remove only 45 µl of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.
8. With the tubes still on the magnet, add 200 µl of freshly prepared 70% ethanol and allow to stand 30 sec.
Note: The beads should not disperse; instead they will stay on the walls of the tubes. Significant loss of beads at this stage will impact cDNA yields, so ensure beads are not removed with the binding buffer or the washes.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the wash 2 more times.
Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least 2 pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
11. Air-dry the beads on the magnet for 15 to 20 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
It is critical that all residual ethanol be removed prior to continuing with SPIA Amplification.
12. Continue immediately with the SPIA Amplification protocol with the cDNA still bound to the dry beads.

D) SPIA Amplification

1. Take the Red C1 SPIA Primer Mix, Red C2 SPIA Buffer Mix and Red C3 SPIA Enzyme Mix from –20°C storage.
2. Thaw C3 on ice and mix the contents by inverting gently 5 times, spin and place on ice. Ensure the enzyme is well mixed without introducing bubbles.
3. Thaw reagents C1 and C2 at room temperature, mix by vortexing, spin and place on ice.
4. Make a master mix by sequentially combining C2 (20µl), C1 (10µl) and C3 (10µl) in an appropriately sized capped tube.

Note: Make sure the addition of C3 is at the last moment.

5) Add 40 µL of the SPIA Master Mix to each tube containing the double-stranded cDNA bound to the dried beads. Mix thoroughly by pipetting at least 8 to 10 times. Attempt to get the majority of the beads in suspension and remove most of the beads from the tube walls. Note: Beads may not form a perfectly uniform suspension, but this will not affect the reaction. The addition of SPIA master mix will elute the cDNA from the beads.
6) Place the tubes in a pre-cooled thermal cycler programmed to run Program 4 (SPIA Amplification, see Table 1)
7) Remove the tubes from the thermal cycler, spin and place on ice. Do not re-open the tubes in the pre-amplification workspace.
Note: If using the Agencourt RNAClean XP Kit for final SPIA cDNA cleanup, skip steps 8–10 below and go directly to the Agencourt RNAClean XP Kit protocol on 22. It is not necessary to recombine the half-reactions or to remove the beads at this point.
8) Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
9) Carefully remove all of the cleared supernatant containing the eluted SPIA cDNA and transfer to a fresh tube. The beads may now be discarded.
10) Continue immediately with the Purification of SPIA cDNA protocol or store the reaction products at –20°C prior to continuing.

REST

E) Purification of SPIA cDNA

The SPIA cDNA product can be purified using various methods listed in Appendix A. Purification is required if the SPIA cDNA is intended for use in an Encore labeling module or other supported labeling protocol. We recommend that the SPIA cDNA be purified prior to qPCR analysis.

F) Measuring SPIA cDNA Yield and Purity

1) Mix the purified SPIA cDNA sample by brief vortexing and spinning prior to checking the concentration.
2) Measure the absorbance of the SPIA cDNA at 260, 280 and 320 nm. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3) Purity: Subtract the A320 value from both A260 and A280 values. The adjusted (A260 – A320) / (A280 – A320) ratio should be >1.8.
4) Yield: Assume 1 A260 unit = 33 µg/mL for single-stranded cDNA. To calculate: (A260 – A320 of diluted sample) X (dilution factor) X 33 (concentration in µg/mL of a 1 A260 unit solution) X 0.03 (final volume in mL) = total yield in micrograms
5) Alternatively, you may measure the concentration and purity of the SPIA cDNA with a Nanodrop, using the ssDNA setting or using 1 A260 unit = 33 µg/ mL as the constant.
6) The purified SPIA cDNA may be stored at –20°C.

4) PCR primers and conditions

1) TaqMan primers

PCA3: cat.no. 04685130001

PCA3 Forward: GGAAGCACAAAAGGAAGCAC

PCA3Reverse: CATCGATGACCCAAGATGG

KLK3: cat. no. 04688988001
KLK3 Forward: TCCGTGACGTGGATTGGT
KLK3 Reverse: CAGGGTTGGGAATGCTTCT

OR51E2: cat. no. 04687639001
Or51E2 Forward: CCTCCCCTGGAATCTAAAGC
OR51E2 Reverse: TGACTGGAGAGGGTGAGGTC
TaqMan was set up as manufacturers' instructions in triplicate.

2) Semi quantitative PCR primers and conditions

TMPRSS2:ERG **primer sequences:**
Forward: CAGGAGGCGGAGGCGGA
Reverse: GGCGTTGTAGCTGGGGGTGAG
PCR conditions:
1 x 94ºC 30 sec, 35 x(94ºC 20 sec, 68ºC 60 sec), 1 x 68ºC 7 min, hold at 4ºC
As Clark *et al.,* ref

PCA3 primer sequences:
Forward: CCATCTGAGGCCACACATCTGC
Reverse: GTCATCGATGAGCCTCGC
PCR conditions:
1 x 94ºC 30 sec, 35 x (94ºC 20 sec, 60ºC 10 sec, 68ºC 30 sec), 1 x 68ºC 7 min, hold at 4ºC

KLK2 primer sequences:
Forward: GCAGGAAGTCAAACCTATTAGGCTG
Reverse: GCAGTGCAGGGCTGCTG
PCR conditions:
1 x 94ºC 30 sec, 35 x (94ºC 20 sec, 60ºC 10 sec, 68ºC 30 sec), 1 x 68ºC 7 min, hold at 4ºC.

PCR master mix components:

Component	Volume (µl)
10 x PCR Buffer (-Mg)	2.5
50 mM $MgCl_2$	0.75
10 mM dNTP mix	0.5
10 µM Forward Primer	0.5
10 µM Reverse Primer	0.5
Template DNA (30 ng/µl)	0.5
Platinum *Taq* (10 U/µl)	0.1

RECIPES

EQUIPMENT

Ambion non-stick tubes 1.5ml (Fisher – AM12450), 2ml (Fisher – AM12475)
100kDa cut-off microfiltration device (Merck – UFC910024)
Royal Mail SafeBox (Royal Mail – SBFC)
0.8μm filter (Sartorius – 16592K),
20ml disposable syringe (BD - 300296)
50ml tube, Falcon
RNeasy kit (Qiagen – 74004)
Norgen RNA extraction column (Urine Cell-Free Circulating RNA Purification Mini Kit, (Norgen – 56900)
Hologic Urine preservative Hologic – 105575)
Norgen Urine Preservative (Norgen Biotek - 18124)
Norgen Urine Collection and Preservative 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).
Ovation Pico WTA System V2 kit (TECAN – 3312-48)

The At-Home Collection Kit components
Invitation Letter

Information Sheet
Two Urine Collection Tubes (30ml) containing Norgen dried preservative
2x Consent Forms
disposable non-allergenic glove
Pen
Timer
Sealable plastic bag with wadding
Preaddressed postage paid Safebox for returning samples

TROUBLESHOOTING

Include any hints & tips to other users