Chapter 10

Measuring the Expression of microRNAs Regulated by Androgens

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Abstract

The discovery of microRNAs (miRNAs) provided yet another mechanism of gene expression regulation. miRNAs have recently been also implicated in many diseases, including prostate cancer (PC). As PC is a highly androgen-dependent disease, extensive effort has been invested to identify the miRNAs that are androgen regulated. However, relatively few of them have been shown to be directly androgen regulated in PC. In this chapter we introduce the commonly used techniques to study the androgen regulation of miRNAs. The most cost-effective tool to profile global miRNA expression is microarray-based hybridization, whereas real-time quantitative reverse transcription PCR (qRT-PCR) is commonly used for the study of individual miRNAs.

Key words microRNA, Prostate cancer, Androgens, Androgen receptor, Gene expression, Microarray, qRT-PCR

1 Introduction

miRNAs have been found to be differentially expressed in many human malignancies [1], and it has been shown that they can exert tumor-suppressive and oncogenic functions [2, 3]. Several miRNAs play an important role in the development of prostate cancer (PC) [4]. For example miR-101 and miR-15a/16-1 [5, 6] have been shown to function as tumor suppressors, whereas miR-21, miR-221, and miR-32 function as androgen-regulated oncogenes, driving the castration-resistant PC phenotype [7–11].

Androgen receptor (AR) is known to target hundreds of genes, including several kallikreins, such as *KLK2* and *KLK3*, also known as PSA, and *TMPRSS2*. However, little is known about miRNAs that are directly targeted by AR. It seems that some of the androgen-regulated miRNAs are regulated through other mechanisms as well, thus making it difficult to assess the significance of the androgen dependence. For example, miR-21, which is known to be androgen regulated, is highly expressed in many AR-negative tissues as well.

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Most of the studies on androgen regulation are based on the LNCaP cell line model. However, at least for miR-21, 29a, 29b, and 221 and for miR-17, 18b, 19b, 20a, 20b, 93, and 148a, androgen regulation has been observed in several AR-positive cell lines and xenografts as well [7, 12–14]. In addition, in at least one study [15] neoadjuvant treatment with goserelin and bicalutamide was used to identify androgen-regulated miRNAs in vivo in patients who had undergone radical prostatectomy. The published literature on androgen regulation of miRNAs often shows some discrepancies, which may be due to the use of different ligands, induction times, and cell lines. Relatively long androgen exposure time is needed to detect the induction of miRNA expression and this may be due to either slow biogenesis from the transcribed pri-miRNAs to pre- and mature forms by drosha and dicer enzymes (reviewed by Bartel [16]), or indirect androgen regulation mechanism. Yet another source of variability in the data is introduced by the method used to detect the gene expression. Some of the tools measure the mature form of the miRNAs specifically, whereas others can detect pre-miRNAs as well. miRNAs are also known to be polymorphic, and some of the detection tools, such as microarrays by Agilent Technologies, are sensitive to 3′ end length polymorphisms. In addition, different methods of normalization in microarray analyses and the use of different reference genes in qRT-PCR assays can easily result in discrepancies in the outcome.

We have recently identified miR-32 (officially hsa-miR-32-5p) as an androgen-regulated miRNA, which is overexpressed in castration-resistant prostate cancer [11]. The miR-32 expression data were obtained by both qRT-PCR and microarray in prostate cancer cell lines as shown in Fig. 1a. The direct androgen regulation was demonstrated with various means. Expression of miR-32 was shown to be induced by androgens (Fig. 1b), and the AR-binding site (ARBS) was first identified with chromatin immunoprecipitation-sequencing (ChIP-seq) [17], followed by validation with ChIP-PCR (Fig. 1c).

The introduction of hybridization techniques to detect the expression of mature miRNAs has expanded the number of studies on miRNAs. The microarrays are still the most cost-effective method to study large-scale miRNA expression, as reviewed by Li and Ruan [18]. And the qRT-PCR technique is still most commonly used for studying the expression of individual miRNAs. Other techniques worth mentioning are in situ hybridization and Northern blot, the latter providing the only reliable tool to validate the actual expression of mature miRNAs in some applications [19]. The new next-generation sequencing-based techniques provide yet another set of tools to detect gene expression as well as transcription factor binding and chromatin structure. However, these approaches lie out with the present discussion.

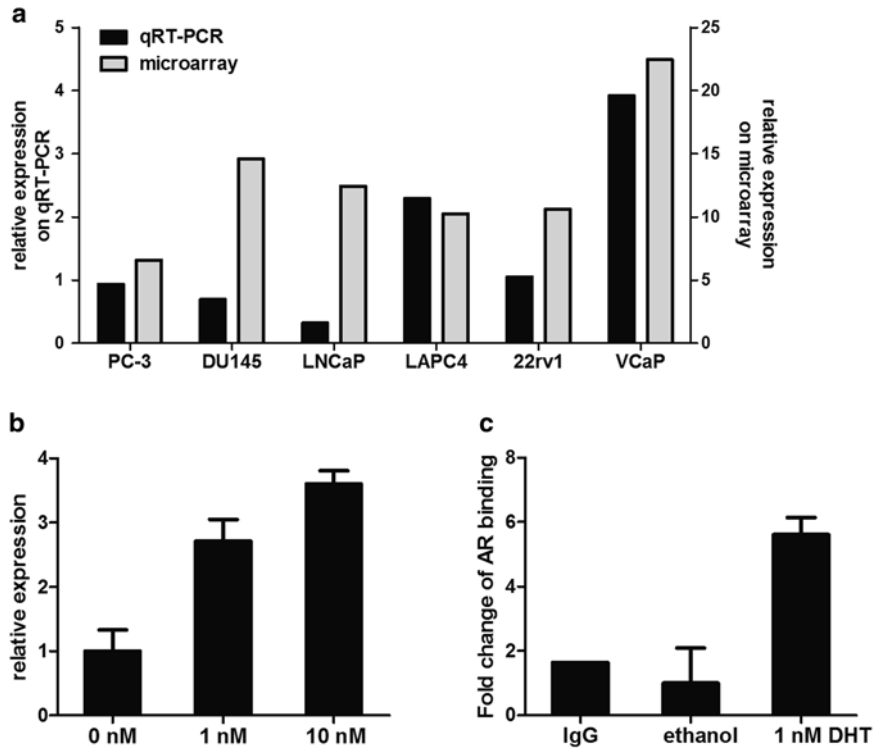

Fig. 1 miR-32 is an androgen regulated miRNA. (**a**) Comparison of qRT-PCR (TaqMan) and microarray (Agilent Technologies) analysis in measuring the expression levels of miR-32 in different prostate cancer cell lines. (**b**) Induction of miR-32 expression in LNCaP cells upon stimulation with 0, 1, and 10 nM of dihydrotestosterone (DHT) for 6 h. The expression of miR-32 was measured with qRT-PCR (TaqMan). The miR-32 values were normalized against RNU44 expression. (**c**) AR binds to an ARBS near miR-32 genomic location. LNCaP-ARhi cells stably expressing high levels of AR [21] were stimulated with 1 nM DHT for 2 h. ChIP-qPCR was performed [11]

In this chapter, we describe in detail how LNCaP prostate cancer cells are stimulated by androgens and used for measuring androgen-responsive miRNA expression by microarray (Agilent platform) and qRT-PCR (TaqMan assay).

2 Materials

Proper cell culturing techniques have the primary importance for the study of androgen regulation. For example, the use of different forms of androgens, as well as the exposure time and cell lines, affects the results. Use only sterile supplies, media, and reagents and work always under a cell culture hood when handling cells.

Prepare all solutions using ultrapure, sterile water and cell culture-grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials. In RNA work use only RNase-free water and aseptic gloves. Avoid all possible RNase contamination and work promptly.

2.1 Cell Culturing Reagents

1. Use ATCC LNCaP PC cell line (Clone FGC, ATCC® CRL-1740™) (*see* **Note 1**).
2. Cell culturing medium for LNCaP cells: ATCC-formulated RPMI-1640 Medium (Catalog No. 30-2001), supplied with fetal bovine serum (FBS, Gibco®, Life Technologies™, Cat. No: 10270-106 or equivalent) to a final concentration of 10%.
3. Stripped medium for LNCaP cells to reduce androgens prior to treatment: RPMI-1640 without phenol red (Biowhittaker®, Lonza, Cat.No: BE12-918F) supplied with 10% charcoal dextran-stripped (CDS) FBS (Gibco®, Life Technologies™, Cat. No: 12676-029) and 2 mM l-glutamine (Gibco®, Life Technologies™, Cat. No: 25030-081).
4. Androgen treatment medium for LNCaP cells: RPMI-1640 without phenol red (Biowhittaker®, Lonza, Cat.No: BE12-918F) supplied with 10% charcoal dextran-stripped (CDS) FBS (Gibco®, Life Technologies™, Cat. No: 12676-029), 2 mM l-glutamine (Gibco®, Life Technologies™, Cat. No: 25030-081), and 1–100 nM dihydrotestosterone or synthetic androgen (DHT or R1881, Sigma-Aldrich, Cat.No: 730637 and R908, respectively) (*see* **Note 2**).

2.2 Reagents for Total-RNA Isolation

1. Trizol®-Reagent (Ambion, Life Technologies, Cat. No: 15596-018).
2. Chloroform.
3. Isopropyl alcohol.
4. 75% Ethanol (in DEPC-treated water).
5. RNase-free water.
6. Centrifuge and rotor capable of reaching up to 12,000 × *g*.
7. Polypropylene microcentrifuge tubes.

2.3 qRT-PCR Reagents

The most important aspect when studying small-RNA expression is to obtain reliable and specific detection of the RNA of interest. TaqMan® MicroRNA Assay (Thermo Fisher Scientific, Waltham, MA) allows the detection and quantification of specific miRNAs in 1–10 ng of total RNA and can distinguish the mature form from its precursor. The assay consists of preformulated primer and probe sets designed to detect and quantify mature miRNAs. The primers are available for the majority of the miRNAs included in the miRBase database, making the technology ideal for both high-throughput expression studies and validation of expression data acquired from microarray or sequencing platforms. The materials required for miRNA qRT-PCR using TaqMan® MicroRNA Assay are the following:

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems® 4366596).
2. TaqMan® Universal PCR Master Mix (Applied Biosystems® 4324018).

3. TaqMan® MicroRNA assay(s) (specific for the miRNAs of interest).
4. qRT-PCR thermal cycler.
5. qRT-PCR data analysis software.

2.4 Microarray Reagents

miRNA microarrays provide a cost-effective and high-throughput method for studying miRNA expression in cell lines, allowing the detection of all the known mature miRNAs as included in the database miRBase (http://mirbase.org/). Agilent Technologies (Santa Clara, CA) provides glass slides, each of them containing eight arrays of 60,000 probes per array and hybridization kit for the preparation of the sample to be used in the experiment. The reagents and materials required for Agilent miRNA microarray are the following:

1. miRNA Complete Labeling and Hybridization Kit (Agilent 5190-0456).
2. MicroRNA Spike-In Kit (Agilent 5190-1934, optional).
3. Gene Expression Wash Buffer Kit (Agilent 5188-5327).
4. Human miRNA Microarray slide, Release 19.0, 8x60K (Agilent G4872A-046064).
5. Microarray Scanner (Agilent G4900DA, G2565CA or G2565BA).
6. Agilent G4450AA Feature Extraction software 9.5 or later when Spike-In is not used or Feature Extraction 10.7.3 when Spike-In is included in the experiment.
7. Agilent Scan Control software, version A. 7.0 or later.
8. Hybridization Chamber (Agilent G2534A).
9. Hybridization Chamber gasket slides 8 microarrays/slide (Agilent G2534-60014).
10. Hybridization oven with 20 rpm capability and temperature set at 55 °C (Agilent G2545A).
11. Nuclease-free 1.5 mL microcentrifuge tubes.
12. Magnetic stir bar (×2) and magnetic stir plate.
13. Slide-staining dish, with slide rack (×3).
14. Circulating water baths or heat blocks set to 16 °C, 37 °C, and 100 °C.
15. Vacuum concentrator with heater.

3 Methods

Note that cell culture conditions vary for each cell type, and this protocol has been adjusted especially for LNCaP cell line. Maintain cells in a cell incubator (37 °C, 5% CO_2). Grow LNCaP cells in 60 or 100 mm polystyrene tissue culture dishes depending on the number of cells (the amount of RNA) required for the following methods.

3.1 Hormonal Treatment of Androgen-Sensitive LNCaP Cells

1. Divide LNCaP cells in 1:3 ratio after reaching 80% confluence (appr. 5×10^4 cells cm^2) directly into stripped growth medium onto two equal dishes (*see* **Notes 1**, **3**, and **4**).
2. Let the cells grow undisturbed in a cell incubator for 3–4 days.
3. Check the viability of the cells.
4. Carefully remove the strip medium and replace with androgen treatment and control (vehicle) medium (*see* **Note 2**).
5. Let both dishes be undisturbed for 24 h.
6. Collect total RNA from both androgen treatment and control dish as explained in next paragraph.

3.2 Isolation of Total RNA

1. Remove growth medium from culture dish.
2. Add 6 mL TRIzol® Reagent directly onto the cells in the 100 mm culture dish (*see* **Note 4**).
3. Work under hood and lyse the cells directly in the culture dish by pipetting up and down several times (*see* **Note 5**).
4. Collect and incubate the homogenized sample in an RNase-free polypropylene microcentrifuge tube for 5 min at room temperature (*see* **Note 6**).
5. Add 1.2 mL of chloroform.
6. Shake the tube vigorously by hand for 15 s.
7. Incubate for 2–3 min at room temperature.
8. Centrifuge the sample at 12,000 × *g* for 15 min at 4 °C. After centrifugation the mixture will separate into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase (~50% of the total volume and includes the total RNA).
9. Remove the aqueous phase of the sample by angling the tube and pipetting the solution out (*see* **Note 7**).
10. Place the aqueous phase into a new RNase-free tube.
11. Add 3 mL of 100% isopropanol to the aqueous phase.
12. Incubate at room temperature for 10 min.
13. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
14. Remove carefully the supernatant from the tube.
15. Wash the pellet, with 6 mL of 75% ethanol (*see* **Note 8**).
16. Vortex the sample briefly, and then centrifuge the tube at 7500 × *g* for 5 min at 4 °C.
17. Carefully discard all the supernatant with a fine tip pipet taking care of preserving the integrity of the RNA pellet (*see* **Note 9**).
18. Air-dry the pellet for 5–10 min, at room temperature or 37 °C, or as long as the pellet starts to turn slightly transparent (*see* **Note 10**).

19. Resuspend the RNA pellet in 50–100 μL RNase-free water by pipetting the solution up and down several times and measure the concentration and 260/280 (*see* **Note 11**) ratio with, e.g., Nanodrop 2000 spectrophotometer. Check the integrity of the RNA by agarose gel electrophoresis.
20. Check the success of the androgen stimulation if needed by measuring expression of the androgen-regulated *KLK3* gene (*PSA*) of vehicle-treated (0 nM DHT) and DHT-treated RNA samples (*see* **Note 12**).

3.3 Microarray

In order to achieve a successful microarray hybridization it is important to assess the integrity of the RNA samples, either by agarose gel electrophoresis or by using an automated quantitation and quality control instrument such as Agilent Bioanalyzer 2100. The protocol for Agilent miRNA microarray supports only total RNA as starting material; therefore it is not advised to use purified miRNAs or small RNA fractions.

The first step of the protocol consists in labeling the total RNA and optionally the spike-in solutions, which serve as process controls to help distinguish biological data from experimental errors. Two spike-in solutions are available, called labeling spike-in and hybridization spike-in, and they are used as controls for the labeling and hybridization steps, respectively. The spike-in solutions are prepared as follows:

1. Dilute the spike-in stock solution 1:100 by adding 2 μL of stock solution into 198 μL of dilution buffer provided with the spike-in kit to obtain the first dilution (*see* **Note 13**).
2. Dilute the first dilution 1:100 to obtain the second dilution as described above.
3. Dilute the second dilution 1:100 to obtain the third dilution.

The labeling of the RNA is performed according to the following steps:

4. Dilute the total RNA to obtain a final concentration of 50 ng/μL in nuclease-free water. Add 2 μL of RNA (100 ng) to a 1.5 mL polypropylene tube.
5. Prepare the calf intestinal alkaline phosphatase (CIP) master mix by mixing 0.4 μL of 10× calf intestinal phosphatase buffer, 1.1 μL of labeling spike-in solution (third dilution, **step 3** above), and 0.5 μL of calf intestinal phosphatase per reaction (it is advisable to add 10–20% extra volume to compensate pipetting losses).
6. Add 2 μL of CIP master mix to each sample to obtain a total reaction volume of 4 μL, gently mixing by pipetting.
7. Incubate the samples at 37 °C in a circulating water bath or heat block for 30 min. This step will dephosphorylate the 3′-end of the original RNA, to allow for subsequent labeling.

8. Denature the samples (this allows the breaking of any secondary structure in the starting material) by adding 2.8 μL of 100% DMSO to each sample and then incubating at 100 °C in a circulating water bath or heat block for 5–10 min. Immediately transfer the samples on ice-water bath after denaturation to ensure that the RNA remains properly denatured.
9. Prepare the ligation master mix (this step will add a labeled cyanine3-pCp to the 3′-end of the dephosphorylated RNA) by mixing 1.0 μL of 10× T4 RNA ligase buffer, pre-warmed at 37 °C and cooled to room temperature, 3.0 μL of cyanine3-pCp and 0.5 μL of T4 RNA ligase per reaction. Add 4.5 μL of ligation master mix to each sample, mix gently by pipetting, and incubate at 16 °C for 2 h in a circulating water bath.

   After ligation, the samples can be optionally purified using MicroBioSpin 6 Columns (Bio-Rad 732-6221).

   After ligation or purification, the labeled RNA must be completely dried, using a vacuum concentrator for up to 3 h at 45–55 °C.

   The next step consists in hybridizing the labeled RNA to the microarray slide. The hybridization reaction is prepared as follows:
10. Resuspend the dried samples into 17 μL of nuclease-free water (18 μL if spike-in solution is not used).
11. Prepare the hybridization mix by adding 1.0 μL of hybridization spike-in solution (third dilution), 4.5 μL of 10× GE blocking agent, and 22.5 μL of 2× Hi-RPM hybridization buffer to each sample for a total volume of 45 μL.
12. Incubate the hybridization mix at 100 °C water bath or heat block for 5 min, immediately followed by 5 min on ice-water bath.
13. Prepare the hybridization assembly chamber and dispense the volume of sample in the gasket well of the gasket slide. It is advised to avoid introducing air bubbles at this step.
14. Place the microarray slide with the active side of the slide (where the probes are bound) facing the gasket slide.
15. Close the hybridization assembly and verify the absence of air bubbles by gently turning the assembly and observing the volume inside the gasket wells.
16. Hybridize the slide(s) in the hybridization oven at 55 °C for 20 h, at a rotational speed of 20 rpm.

    The next step consists in washing the slides in gene expression wash buffers 1 and 2 (*see* **Note 14**). The wash buffer 2 must be pre-warmed overnight in a slide-staining dish to 37 °C before use. The wash protocol consists of three steps:

17. The microarray slide is detached from the gasket slide in wash buffer 1.
18. The microarray slide is washed for 1 min at room temperature in wash buffer 1 in a slide-staining dish on a magnetic stirrer with continuous agitation.
19. The slide is subsequently washed in wash buffer 2 at 37 °C for 1 min as described above.

    To minimize the impact of environmental oxidants it is advised to scan the slide immediately after washing. The scanner is connected to a computer and controlled via Agilent Scan Control software. The scanner generates .tiff image files for each scanned slide and the position and identity of each spot on the image is then quantified and qualified via Agilent feature extraction software which converts the intensity of the signal in each spot into a numerical value, normalized against background signal, and organized in a .txt file. The .txt file can be subsequently analyzed to obtain expression values for each miRNA in the array slide and samples can be compared to experimental controls to obtain biologically significant data.

3.4 qRT-PCR

For optimal qRT-PCR performance, prepare the reaction in a dedicated area to avoid contamination from artificial templates and keep all the reagents on ice (*see* **Note 15**). Thaw the reagents on ice and vortex and centrifuge them briefly to properly resuspend them. Calculate the number of reactions needed for the amount of samples to be used.

The first step is the reverse transcription of the total RNA. The reverse transcription enzyme (reverse transcriptase) will convert the miRNA(s) of interest into cDNA, using specifically designed RT-primer(s).

1. Prepare the RT master mix as shown in the following list for a total reaction volume of 7 μL per sample (consider adding 10–20 % extra volume to compensate pipetting losses):
   (a) 0.15 μL 100 mM dNTPs
   (b) 1.00 μL MultiScribe™ Reverse Transcriptase, 50 U/μL
   (c) 1.50 μL 10× Reverse transcription buffer
   (d) 0.19 μL RNase inhibitor, 20 U/μL
   (e) Nuclease-free water 4.16 μL
2. Add 1–10 ng of total RNA in a total volume of 5 and 3 μL of reverse transcription miRNA-specific primer per reaction, per miRNA, for a total final volume of 15 μL per reaction. Each reaction can be prepared in a 0.5 mL polypropylene tube of well of a 96-well plate.
3. Seal the tube(s) or well plate(s) and mix thoroughly by inversion of the solution, followed by a brief centrifugation to bring

the solution to the bottom of the tube or well. Incubate the tube(s) or well plate(s) on ice for 5 min and subsequently transfer to the thermal cycler, using the following parameters to program the reaction:

(a) 16 °C, 30 min

(b) 42 °C, 30 min

(c) 85 °C, 5 min

At this point, the reaction can be stored in the freezer (−15 to −25 °C) until the next step.

4. The second step is the actual PCR amplification of the cDNA prepared in the first step. It is recommended to perform each reaction in triplicate to ensure optimal reliability and to include non-template controls (NTC) to evaluate background signal and nonspecific amplification.

   Prepare the qRT-PCR master mix as in the following list for a total reaction volume of 18.67 μL per individual sample (add 10–20% extra volume to compensate for pipetting losses):

   (a) 1.00 μL TaqMan® microRNA Assay (20×)

   (b) 10.00 μL TaqMan® Universal PCR Master Mix II (2×)

   (c) 7.67 μL Nuclease-free water

5. Add 1.33 μL of reverse transcription product to each sample, for a total final volume of 20 μL. The reverse transcription product must be diluted 1:15 before qRT-PCR as to avoid concentrated reverse transcription by-products to interfere with the PCR amplification. Prepare each reaction in a 0.5 mL tube or well of a 96-well reaction plate. Seal the tube(s) or plate(s) and mix by inversion, followed by brief centrifugation. Load the tube(s) or plate(s) in the thermal cycler, using the following parameters for the reaction program:

   (a) 50 °C 2 min

   (b) 95 °C 10 min

   (c) 95 °C 15 s

   (d) 60 °C 60 s

   (e) Repeat **steps 3** and **4** for 40 cycles

   When quantifying miRNA expression levels, variation in the amount of starting material, sample preparation, and RNA extraction, as well as in reverse transcription efficiency, can introduce errors. Therefore, it is highly recommended to normalize the raw expression values to endogenous control genes to correct for potential biases. The endogenous control needs to be accurately validated and its expression needs to be relatively constant and abundant in the particular sample set used in the experiment (*see* **Note 16**).

4 Notes

1. LNCaP cell line is heterogeneous, and may thus lose its AR expression when cultured for extended periods of time in vitro. Use fresh ATCC clone or check the AR status with conventional qRT-PCR and/or western blot.
2. EtOH/DMSO concentration (solvent) of the ready medium should be less than 0.001%.
3. Start the procedure soon after reaching 80% cell confluence to avoid excessive clumping of the cells.
4. This protocol has been adjusted for 100 mm dish. If using smaller or larger plates adjust the volumes of reagents needed, or follow the manufacturer's guidelines.
5. Pipet the cells up and down as long as the Trizol® reagent is clear, free from fibrous-like filaments, to ensure proper lysis of the cells.
6. After this step, the homogenized sample can be stored at −20 °C for up to 1 week before proceeding. Ensure proper thawing and incubation at RT when continuing later with the isolation protocol.
7. Avoid collecting any of the interphase or organic layer into the pipette when removing the aqueous phase.
8. The RNA can be stored in 75% ethanol for at least 1 year at −20 °C.
9. Repeat the centrifugation or increase the centrifugation speed if needed to avoid detaching of the RNA pellet.
10. Do not allow the RNA to dry completely or use vacuum centrifuge, because fully dried RNA can lose its solubility.
11. In order to ensure accurate results, a ratio of ~2.0 is generally required to guarantee good purity of the RNA. If the ratio is significantly lower, it may indicate the presence of protein, phenol, or other contaminants that negatively affect the efficiency of microarray and qRT-PCR.
12. If necessary, before proceeding to MicroArray ensure the success of the androgen stimulation by assaying the well-known, strongly androgen-regulated *KLK3* (PSA) transcript, using conventional qRT-PCR; *see* Waltering et al. [21].
13. The first dilution of the spike-in can be stored at −80 °C for future use, although it is advisable to limit the freeze/thaw cycles to a maximum of two.
14. Add 2 mL of 10% Triton X-102, provided with the wash buffers, to both wash buffers 1 and 2 before use.

15. Particular care must be exercised as to avoid excessive exposure of TaqMan® microRNA Assay to light, as this might affect the fluorescent probe.
16. An example of reference gene validation is shown by Scaravilli et al. [20] in a prostate cancer clinical sample dataset. The authors compared qRT-PCR expression data of a subset of selected miRNAs with previously generated microarray and small RNA deep-sequencing data. The qRT-PCR results were normalized with four commonly used reference genes (RNU6B, RNU44, RNU24, and RNU48). The RNU6B-normalized expression results were the most consistent with microarray hybridization and deep-sequencing data. Moreover, the authors analyzed the individual expression of RNU44, RNU24, and RNU48 in the same set of samples, using RNU6B as a reference gene, confirming significant deregulation of all three genes in cancer samples, compared with the normal controls.

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