

# Measuring expression levels of small regulatory RNA molecules from body fluids and formalin-fixed, paraffin-embedded samples

Running Title: miRNA measurements from patient derived samples

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## Summary

MicroRNAs are involved in the regulation of various pathophysiological processes such as immune regulation and cancer. Next-generation sequencing methods enable us to monitor their presence in various types of samples but we need flexible methods for validating datasets generated by high throughput methods.

Here we describe the detailed protocols to be used with our MiRNA Primer Design Tool assay design system. The presented methods allow the flexible design of the oligonucleotides needed for the RT-qPCR detection of any variant of small regulatory RNA molecules from virtually any species. This method can be used to measure miRNA levels from formalin-fixed, paraffin-embedded (FFPE) samples and various body fluids. As an example, we show the results of the hsa-miR-515-3p, hsa-miR-325, and hsa-miR-155 quantification using a specific UPL probe (Universal Probe Library) and a stem-loop RT-qPCR assay. The small nucleolar RNA RNU43 is used as endogenous control for normalization of the results. Urine from healthy pregnant women and FFPE samples from patients diagnosed with colorectal cancer and treated with antibody-based anti-EGFR monotherapy were used as samples.

Keywords: miRNA, RT-qPCR, stem-loop, urine, FFPE, cancer

## 1. Introduction

MicroRNAs (miRNAs) are short 18-25 nucleotide-long, single stranded, non-coding RNA molecules (1). MiRNAs act as post-transcriptional regulators of gene expression through induction of translational repression or RNA decay (2). MiRNAs play an important role in a wide range of normal physiological and pathological processes (3) (4). Aberrantly expressed miRNAs contribute to the development of various human disorders including cancers, neurodegenerative disorders, and autoimmune diseases (5) (6).

Unlike mRNAs, miRNAs are highly stable and well preserved RNA molecules in formalin-fixed paraffin-embedded (FFPE) samples and different body fluids including plasma, saliva and urine (7) (8) (9). The high stability as well as their disease-specific expression pattern renders miRNAs suitable for biomarkers in molecular diagnostic applications. Recently, miRNAs have been investigated as potential diagnostic and prognostic markers in patients suffering of cancer and autoimmune diseases by using samples of various origin, like FFPE, plasma, and urine (10) (11) (12) (13). Interestingly, a miRNA expression profile is equally useful for identifying tissue origin of tumors, subtype classifications of different cancers, prediction of clinical outcome, and selection of patients for targeted therapy (14) (15) (16) (17) (18) (19).

Stem-loop RT-qPCR is a widely used technique for detection and quantification of mature miRNAs (20). This method includes two steps: 1. miRNA specific stem-loop RT primer-based reverse transcription, and 2. quantification of product using conventional TaqMan<sup>TM</sup> assay with miRNA specific TaqMan<sup>TM</sup> probe and forward primer. This method enables us to quantify mature miRNA expression levels from nanograms of total RNA (20). A simplified setup of stem-loop RT-qPCR uses a general stem-loop primer specific probe from the Universal Probe Library (UPL, Roche Applied Science) for quantification of RT products without loss of specificity and efficiency (21). More recently, we developed a novel UPL probe-based stem-loop RT-qPCR assay design software (MiRNA Primer Design Tool, freely available online at: <http://genomics.dote.hu:8080/mirnadesigntool/>) that enables anyone to design the stem loop and specific primers needed for the assay. The MiRNA Primer Design Tool was validated in various species and experimental conditions (22). The method broadens the application of the stem-loop primer-based miRNA quantitation to virtually any species or variant of small regulatory RNA molecules.

Here we present a detailed protocol for a UPL probe-based stem-loop RT-qPCR assay. We used this method to quantify hsa-miR-515-3p, hsa-miR-325, and hsa-miR-155 using the small nucleolar RNA RNU43 as a normalization control. Human urines from healthy pregnant donors as well as FFPE samples from patients diagnosed with colorectal cancer were analyzed using the method described here.

## 2. Materials

### 2.1. Urine samples

Urine samples were collected from pregnant women during a prospective study in the Department of Obstetrics and Gynecology, University of Debrecen, Hungary. Samples were collected according to rules and regulations of the University of Debrecen, Medical and Health Science Center, with the approval of the local ethics committee (file number:

DEOEC RKEB/IKEB 3092-2010). All patients were informed about the purpose of sample collection and signed an informed consent form.

## 2.2. FFPE samples

FFPE samples were obtained during routine diagnostic procedures for histological and molecular analysis performed in the Department of Pathology, University of Debrecen. All samples had KRAS mutant genotype confirmed by capillary sequencing and were divided into two groups of five patients according to disease-free survival after anti-EGFR therapy (good and poor responders). Samples were collected according to rules and regulations of the University of Debrecen, Medical and Health Science Center, with the approval of the local ethics committee (file number: RKEB/IKEB 3856-2013).

## 2.3. Reagents and equipments

1. The High Pure miRNA Isolation Kit is used to prepare small RNA (Roche Applied Science). Before miRNA isolation prepare the following buffers:

- 20% Binding Buffer: for each sample, mix 80  $\mu$ l of Binding Buffer (from the High Pure miRNA Isolation Kit) with 320  $\mu$ l of nuclease-free water (NFW) in a sterile, nuclease-free 15 ml centrifuge tube. (*see Note 1*).
- Wash Buffer: if the High Pure miRNA Isolation Kit is freshly opened, prior of the first use, add 40 ml absolute ethanol.
- Tissue Lysis Buffer: 100  $\mu$ l Paraffin Tissue Lysis Buffer, 8  $\mu$ l 20% SDS, and 40  $\mu$ l Proteinase K working solution per sample.

2. Transcriptor First strand cDNA Synthesis Kit (Roche Applied Science)

3. First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Applied Science)

4. Sodium dodecyl sulfate (SDS)

5. Xylene (Sigma-Aldrich)

6. Absolute ethanol

7. Nuclease-free water (NFW, Lonza)

8. Microcentrifuge with centrifugal force of 15,000  $xg$

9. Microcentrifuge tubes (1.5 ml)

10. 0.2 ml PCR strip (Axygene)

11. Eppendorf Thermomixer comfort

12. Microcentrifuge Combi Spin FVL-2400N

13. Agilent 2100 Bioanalyzer and a Small RNA Chip (Agilent Technologies)

14. Applied Biosystems 2720 Thermal Cycler

15. Roche LightCycler 480 QPCR System

16. Roche LightCycler 480 compatible qPCR plates

17. The assays used for the QPCR measurements were designed by using the MiRNA Primer Design Tool at: <http://genomics.dote.hu:8080/mirnaesigntool/>. (see **Note 2**).

### 3. Methods

#### 3.1 Sample preparation and requirements

Collected urine samples should be stored temporarily at 4 °C. Samples are centrifuged at 3,000 xg for 10 min to remove cellular debris. The supernatant is transferred to 1.5 ml pre-labeled screw tubes (sterile), and stored at -75 to -80 °C.

Regarding your FFPE samples: use only tumor blocks with malignant cell content higher than 30%. A dedicated pathologist should evaluate the tumor cell content of the paraffin embedded blocks. For each sample to be measured by QPCR, miRNA isolation should be carried out from five pieces of 4 µm-thick FFPE tumor slices and their adjacent non-tumorous tissues.

#### 3.2. Small RNA/miRNA preparation protocol from urine samples

Urine samples can be frozen and stored at -70°C until isolation process. Samples should be thawed slowly and kept on ice (see **Notes 3-7**).

1. Under a chemical fume hood, mix 150 µl of urine with 312 µl of 20% Binding Buffer in a 1.5 ml microcentrifuge tube (see **Note 1**). Mix the solutions by vortexing.
2. Pour the mixture into a High Pure filter tube.
3. Place your filter tube into a clean 1.5 ml microcentrifuge tube.
4. Centrifuge at 15,000 xg for 1 min at room temperature. Collect the flow-through in a clean 1.5 ml microcentrifuge tube.
5. Add 200 µl Binding Enhancer to the flow-through, and mix well by vortexing. This fraction contains your microRNAs.
6. Add the mixture to a new High Pure filter tube.
7. Centrifuge at 15,000 xg for 1 min.
8. Discard the flow-through.
9. Add 500 µl of Wash Buffer and centrifuge columns at 15,000 xg for 1 min.
10. Repeat the washing steps 8 and 9.
11. Transfer High Pure Filter columns into a new collection tube and centrifuge at 15,000 xg for 2 min to completely dry the filter fleece.
12. Place High Pure filter tube in a new 1.5ml microcentrifuge tube.



13. Add 50  $\mu$ l of Elution Buffer to the top of the filter and wait for 1 min.
14. Centrifuge at 15,000 xg for 1 min to elute the purified miRNAs.
15. Store samples below -20°C.
16. For miRNA isolation from higher amount of urine, *see Note 8*.

### 3.3. miRNA preparation from FFPE samples

Tumor slices from FFPE (formalin-fixed, paraffin-embedded) blocks can be obtained by microtome dissection and should be collected in 1.5ml microcentrifuge tubes as shown in Figure 1. Five slices of 4  $\mu$ m thickness should be combined in one microcentrifuge tube (*see Note 9*). For nucleic acid isolation, Xylene deparaffinization is used with subsequent washing by absolute ethanol. The deparaffinization steps should be performed 4-5 times to remove all the paraffin from samples. All steps of deparaffinization are recommended to be done under a chemical fume hood; solutions can be stored at room temperature.

(Figure 1.)

Perform miRNA isolation using the Roche High Pure miRNA Isolation Kit as it follows.

#### Day 1: Deparaffinization

1. Add 1 ml Xylene to each sample and incubate for 5 min at 56°C in a thermomixer at 800 rpm.
2. Centrifuge the samples for 2 min at 15,000 xg.
3. Discard supernatant containing xylene (*see Note 10*).
4. Add 1 ml absolute ethanol.
5. Vortex for 10 seconds.
6. Centrifuge for 2 min at 15,000 xg.
7. Discard supernatant containing absolute ethanol with traces of xylene.
8. Repeat steps 4-7 at a minimum of two times.
9. If tumor mass is larger than 30% of the slice, repeat steps 1-3 at least two times, then proceed to steps 4-7 and repeat ethanol washing for four times.
10. Dry the samples at 56°C for 15 min with the centrifuge tube lid open to rid of residual ethanol (*see Note 11*).
11. Add Tissue Lysis Buffer solution.
12. Vortex and incubate at 55 °C overnight.

#### Day 2: miRNA isolation

13. Centrifuge samples for 5 min, 15,000 xg at room temperature.
14. Transfer supernatant to a new microcentrifuge tube.
15. Add 325  $\mu$ l 20% Binding Buffer working solution and vortex briefly.
16. Add 120  $\mu$ l Binding enhancer and vortex briefly.
17. Pipette mixtures into High Pure filter columns.
18. Centrifuge at 15,000 xg for 1 min and collect the flow-through. This fraction contains your miRNAs.
19. Add 205  $\mu$ l Binding Enhancer to flow-through and vortex.

20. Pipette mixtures onto a new High Pure filter column.
21. Centrifuge at 15,000 xg for 1 min and discard the flow-through.
22. Add 500 µl Wash Buffer and centrifuge at 15,000 xg for 1 min and discard the flow-through.
23. Add 300µl Wash Buffer and centrifuge at 15,000 xg for 1 min and discard the flow-through.
24. Centrifuge at 15,000 xg for 2 min to completely dry the filter fleece.
25. Place the High Pure filter tube into a fresh 1.5 ml microcentrifuge tube, and then add 50 µL Elution Buffer and incubate for 1 min at room temperature.
26. Centrifuge for 1 min at 15,000 xg.
27. The flow-through contains the isolated miRNA, which can be stored at -20°C.

### 3.4. RNA quality control and yield

For detection of miRNA content of the isolated samples and for quality control, use an Agilent Bioanalyzer 2100 System (Agilent Technologies) with a Small RNA Agilent Chip.

Perform the run according to the instructions of the manufacturer.

The report will contain the amount of RNA purified and the percentage of miRNA in the purified fraction. Sensitivity of the Agilent microchip kit is 50 pg/µl for the 40 bp fraction. Representative results of the analysis are shown on Figure 2. (*see Notes 12-17*)

The yield of isolated miRNAs is donor dependent. Table 1 represents the concentrations of miRNAs from human urine samples.

(Figure 2.)

(Table 1)

### 3.5. Quantification of miRNA by qPCR

For quantification of specific miRNAs, the first step of cDNA synthesis is followed by PCR amplification using the Real Time Quantitative method (qPCR). Design primers using the UPL probe-based stem-loop quantitative PCR assay design software located at: <http://genomics.dote.hu:8080/mirnaesigntool/> (*see Notes 2, 18, 19*).

#### 3.5.1. cDNA Synthesis:

##### **Option A: recommended for miRNA with low or unknown expression levels**

Transcribe miRNAs using the Transcriptor First strand cDNA Synthesis kit and a final stem-loop primer concentration of 50 nM. Use miRNA templates diluted to 10 ng/µl.

1. Prepare the following Annealing Mix:

- |                               |       |
|-------------------------------|-------|
| • miRNA [10 ng/µl]:           | 1 µl  |
| • Stem-loop RT primer [1 µM]: | 1 µl  |
| • NFW                         | 11 µl |

• Total: 13  $\mu$ l/reaction  
If processing more than 8 samples, prepare a mastermix with a minimum of 10% excess (*see Note 20*). Mix components in a 0.2 ml PCR strip by pipetting up and down five times and spin briefly in a Combi Spin microcentrifuge.

2. Incubate samples for 10 min at 65 °C in an Applied Biosystems 2720 Thermal Cycler (*see Notes 3-7*).

3. For each reaction, prepare the RT Mix as follows:

• 5x reaction buffer:	4 $\mu$ l
• RNase inhibitor [40 U/ $\mu$ l]:	0.5 $\mu$ l
• 10 nM dNTP mix:	2 $\mu$ l
• <u>RT enzyme:</u>	<u>0.5 <math>\mu</math>l</u>
• Total	7 $\mu$ l/well

4. Add the RT Mix to the Annealing mix in the 0.2 ml PCR strip, vortex and spin briefly (*see Note 21*). Perform the RT reaction on a thermocycler using the following thermal profile:

- 50 °C 60 min
- 85 °C 5 min
- 4 °C  $\infty$

### **Option B: recommended for miRNA with relatively high expression levels**

A more rapid protocol can be used to reverse transcribe miRNAs with high expression level. This method uses the First Strand cDNA synthesis kit for RT-PCR (AMV) that has shorter hands-on time but lower sensitivity (higher average crossing point (Cp) values); therefore it should be used only for miRNAs with relatively high expression levels. On Figure 3 the performance of the two different options is shown.

(Figure 3.)

1. If processing more than 8 samples, prepare a mastermix containing all components of the reaction listed below except your miRNA sample (*see Note 20*). Prepare separate mastermix for the measured miRNA and the normalizer miRNA. It is advisable to measure the normalizer and the target miRNA on the same qPCR plate from the same batch of reagents. (*see Note 3-7*). In this setup, a separate annealing step is not needed. (*see Note 21*)

Mix the following components per sample:

• miRNA [10 ng/ $\mu$ l]:	1 $\mu$ l
• 25 mM MgCl <sub>2</sub> :	4 $\mu$ l
• 10X buffer:	2 $\mu$ l
• stem-loop RT primer [100 $\mu$ M]:	0.2 $\mu$ l
• RNase inhibitor:	1 $\mu$ l
• RT Enzyme:	0.8 $\mu$ l
• <u>NFW:</u>	<u>11 <math>\mu</math>l</u>

- Total: 20  $\mu$ l/well
2. Perform the RT reaction on a thermocycler using the following thermal profile:
    - 25 °C 10 min
    - 42 °C 60 min
    - 99 °C 5 min
    - 4 °C  $\infty$

### 3.5.2. qPCR amplification

1. Dilute reverse transcribed miRNA two-fold with NFW (*see Note 3-7*).
2. Prepare qPCR mix in triplicate for each RT reaction. Components for one single qPCR reaction are:
 

• NFW:	10.28 $\mu$ l
• 25 mM MgCl <sub>2</sub> (Fermentas):	2 $\mu$ l
• 10X buffer (Fermentas, MgCl <sub>2</sub> free buffer):	1.6 $\mu$ l
• 2.5 mM dNTP (Fermentas):	0.8 $\mu$ l
• miRNA specific Forward primer [100 $\mu$ M]:	0.06 $\mu$ l
• Universal Reverse primer [100 $\mu$ M]:	0.06 $\mu$ l
• UPL Probe 21:	0.1 $\mu$ l
• Taq Polymerase [5U/ $\mu$ l] (Fermentas):	0.1 $\mu$ l
Total:	15 $\mu$ l/well
3. Mix 5 $\mu$ l of the two-fold diluted reverse transcribed miRNA with 15 $\mu$ l qPCR mix /reaction. Perform all qPCR measurements in triplicate. (*see Notes 20, 22-24*)
4. Run the qPCR amplification in a thermo cycler using the following temperature profile:
  - 95 °C 3 min
  - 95 °C 10 sec
  - 58 °C 30 sec
  - 72 °C 1 sec
  - repeat steps 2 to 4 for 45 cycles
  - 40 °C 10 min
5. Collect data from fluorescence signal at step 3 in “Single mode”. (*see Notes 25-29*)

We quantified miR-155 using this protocol and RNU43 as endogenous control. Our results correlate with previously published results (23) and suggest that in colorectal cancer increased levels of miR-155 might predict a better overall survival (OS) and disease-free survival (DFS) after treatment with anti-EGFR therapy. Results of the measurements are presented in Figure 4. Due to the low number of samples (five independent samples per group), the presented results are not statistically significant and further investigation is needed.

(Figure 4.)

### 4. Notes

1. Guanidine-thiocyanate in Binding Buffer may form toxic gases; therefore, perform the

dilution step under a chemical fume hood

2. Preparatory steps for the protocol:

- Select your target miRNA sequence from mirBase (<http://mirbase.org/>) or from your next-generation sequencing dataset. (E.g. hsa-mir-155: UUA AUGCUAAUCGUGAUAGGGGU). Use the identified sequence to design your assay at: <http://genomics.dote.hu:8080/mirnadesigntool/>

Useful information and details on how to use the software have been collected and stored at our dedicated website: <http://genomics.med.unideb.hu/en/research-interest/mirna-research>. The MiRNA Primer Design Tool can be used freely under GNU/GPL license and installed on local servers from the following location: <https://github.com/lbalintatmedunidebhu/mirnaprimerdesigntool>

- During selection of your target, perform a sequence search in the miRBase database to identify microRNAs with similar sequences. If similar sequences on the 3' end are present, evaluate your assays for differences in the other regions and consider using alternative methods for detection.
  - Order your stem loop oligo by HPLC purification. All other oligos used can be of standard desalted purification.
3. Always work with samples kept on ice.
  4. Work in RNase-free conditions.
  5. Always wear gloves.
  6. Use only nuclease-free water.
  7. Use RNase-free plasticware.
  8. A higher amount of body fluid can be used if the miRNA concentration is too low. It is important to keep the same proportion of urine sample and Binding Buffer. For example, add 920  $\mu$ l Binding Buffer to 450  $\mu$ l urine, and repeat the binding steps to the column (steps 5 to 8).
  9. During microtome dissection of the FFPE blocks, carefully clean tweezers with nuclease-free water and 70% ethanol to exclude ribonucleic acid transfer and contamination between samples.
  10. Xylene should be discarded according to the local rules of waste management. Xylene is an organic solvent and should be treated as a chemically hazardous waste.
  11. To remove all the supernatant during deparaffinization, additional centrifugation steps can be performed.
  12. The Agilent small RNA kit contains a highly viscous gel and, therefore, it is essential to pipette carefully.
  13. If your sample contains significant amounts of tRNA then you will observe a 60 nt band in chromatogram of the Agilent Bioanalyzer run (24).
  14. Specific miRNA concentrations can be measured only by an Agilent Bioanalyzer miRNA microchip. Due to the lower sensitivity, Nanodrop or Qubit systems are not appropriate.
  15. If starting with a sample isolated from cells or tissues, the initial material should be between 5 ng and 50 ng of total RNA per RT reaction. This value stands for total RNA isolated by Trireagent. The concentration of total RNA mentioned here was measured by Nanodrop or Qubit systems due to the fact that beside the miRNA-s it contains all the other RNA fractions of a cell.
  16. When using a specific miRNA isolation protocol, this starting material can be much lower and should be optimized for each sample type.
  17. Conventional, non miRNA optimized column-based RNA purification methods are not suitable for microRNA purification since they are optimized for larger RNA molecules and the low molecular weight fraction of miRNA-s are not bound to the column.

18. Universal Reverse Primer and the UPL probe 21 can be used with all your assays designed with the previously presented miRNA Primer Design Tool.
19. Each assay should be tested on a sample known to contain your miRNA of interest.
20. For the PCR step, if processing more than 8 samples, prepare a Mastermix. Always calculate the volumes for your mastermix with a minimum of 10% excess.
21. Perform a No Amplification Control (NAC) by leaving out the RT (reverse transcription) enzyme. Your NAC control should be at least a 3.33- $C_p$  higher value (meaning lower signal) than your control. Some background noise is acceptable but it should be less than 10% of your signal (3.33  $C_p$  would mean 10% signal with an optimal PCR efficiency).
22. You can test efficiency of your qPCR by performing a qPCR on a dilution series of 10-fold dilutions steps that cover five orders of magnitude.
23. At the qPCR step, always perform a No Template control (NTC) by replacing your reverse transcribed sample with nuclease-free water.
24. qPCR step should be performed in triplicate (three technical replicates).
25. Normalizers should be carefully selected by measuring their expression levels across several samples used in your experiment. Usually small nucleolar RNAs (snoRNAs) and nuclear RNAs like U6 can be used after careful consideration.
26. Normalization should be performed with the delta delta  $\Delta\Delta C_T$  method (25).
27. If working with body fluids, you can normalize your sample to a relevant marker that reflects the changes in the concentration of the body fluid e.g. total protein concentration, density.
28. If working with body fluids, the purification step can be controlled by adding spike-in artificial microRNA molecules to the sample.
29. Synthetic RNA molecules can be used for carefully monitoring the efficiency of your reactions and to generate standard curves. If using synthetic RNA molecules as positive controls for your reaction, order them separately from your assay components in order to avoid cross contamination at the manufacturing site. Dissolve them under a chemical hood. Keep them in separate locations compared than your assays, reagents and samples. The danger of contaminating your reagents with traces of the amplicon is extremely high.

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## Figure legends

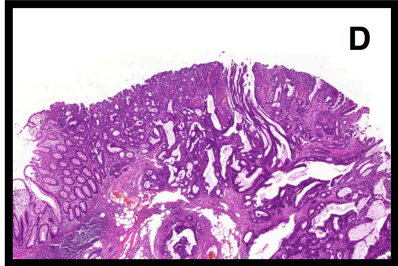
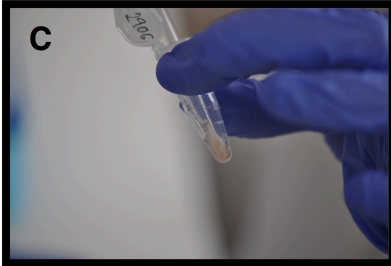
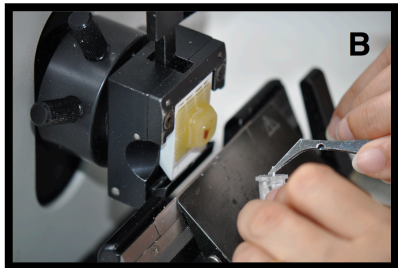
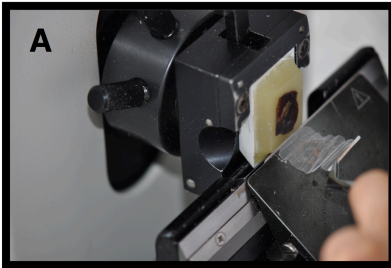
**Figure 1.** FFPE sample preparation for deparaffinization. A. FFPE blocks were sectioned with microtome. B and C. Individual slices were removed from the microtome and placed into 1.5 ml microcentrifuge tubes. D. Marginal area of a moderately differentiated colorectal adenocarcinoma with a tubular architecture and mucin production showing invasion through the muscular layer of the bowel wall (HE staining, 20 fold magnification). The normal colorectal epithelium is visible on the left side of the picture. The importance of the histological selection relies on accurate identification and enrichment of tumor tissue in FFPE samples applied for molecular testing.

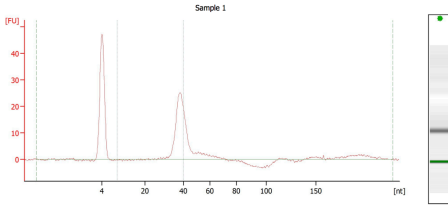
**Figure 2.** Assessment of urinary miRNA concentration and quality using the Agilent 2100 bioanalyzer and associated Small RNA Agilent Chip.

**Figure 3.** Comparison of two different RT-QPCR methods. Ten nanograms of miRNA were reverse transcribed by Transcriptor First Strand cDNA Synthesis kit (Transcriptor) or First Strand cDNA Synthesis kit for RT-PCR (AMV) according to Kit protocols. Cp values of the RT-qPCR measurements for hsa-miR515-3p and hsa-miR-325 from urine sample of one representative donor are shown. Error bars represent the SD value of the qPCR triplicates.

**Figure 4.** Relative expression of miR-155 normalized to RNU43 in colorectal adenocarcinoma. miR-155 expression profile of 5-5 good and poor responder patients with short and long disease-free survival period after anti-EGFR monotherapy.

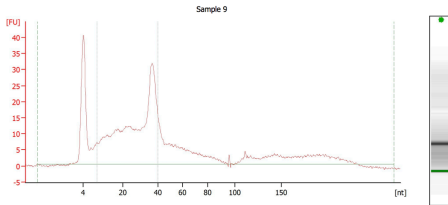






**Overall Results for sample 1 :** Sample 1

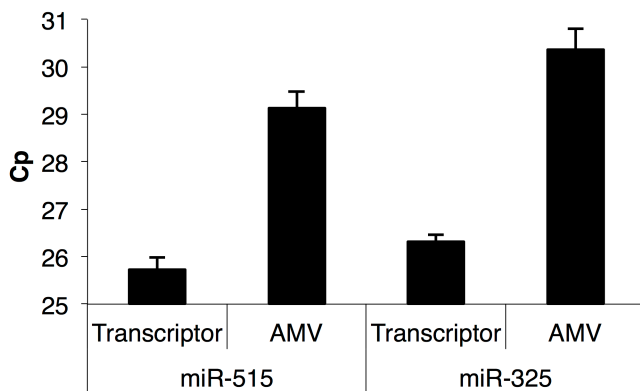
Small RNA Concentration [pg/ul]:	451.1	Result Flagging Color:	<span style="background-color: green; color: black;">████████</span>
mRNA Concentration [pg/ul]:	234.6	Result Flagging Label:	52 % mRNA; Concentration: 234.60 pg/ul
mRNA / Small RNA Ratio [%]:	52		



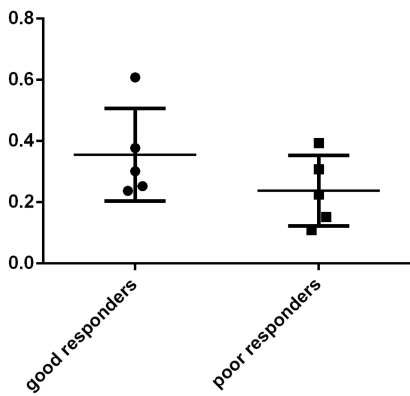
**Overall Results for sample 9 :** Sample 9

Small RNA Concentration [pg/ul]:	2,544.8	Result Flagging Color:	<span style="background-color: green; color: black;">████████</span>
mRNA Concentration [pg/ul]:	1,569.3	Result Flagging Label:	62 % mRNA; Concentration: 1569.30 pg/ul
mRNA / Small RNA Ratio [%]:	62		

## Cp values of miRNA QPCR measurements after different kits used for RT reaction



Relative expression of  
miR-155 normalized to RNU43



**Table1:** miRNA content of urine samples.

<b>Sample name</b>	<b>miRNA/Small RNA</b>	<b>Concentration pg/<math>\mu</math>l purified RNA</b>
mi312/450 $\mu$ l	20%	3115,8
mi312/150 $\mu$ l	30%	1043,6
mi313/450 $\mu$ l	39%	1553,7
mi313/150 $\mu$ l	40%	823,4
mi314/450 $\mu$ l	28%	454,2
mi314/150 $\mu$ l	53%	474,6
mi315/450 $\mu$ l	41%	144,7
mi315/150 $\mu$ l	28%	7,7
mi5/450 $\mu$ l	54%	573,5
mi5/150 $\mu$ l	34%	117,2

Sample name column indicates the volume of urine used for miRNA purification..