Highly-efficient miRNA Isolation using the Agencourt FormaPure and RNAdvance Cell v2 Kits and Biomek Automated Extraction Methods

Bee Na Lee, Ph.D.
Senior Application Scientist, Beckman Coulter Life Sciences, Brea, CA, USA

INTRODUCTION

MicroRNAs (miRNAs) are small ribonucleic acids with an approximate size of 22 nucleotides that is found abundantly in mammals as well as in other organisms. These non-coding RNAs are known to play an important role in many biological processes, although the precise molecular function of miRNAs in mammals is yet to be defined. More than 1,000 unique sequences of miRNA have been identified in the human genome, and it is estimated that miRNAs could target and regulate over 60% of human genes. In human cells, miRNA complementarily binds to the 3' un-translational regions (3'UTRs) of messenger RNA and this binding plays a significant role in gene silencing and transcriptional/translational regulation in cellular responses involved in developmental processes, signal transduction, cancer and disease control.

Recently, biomedical research has placed great importance on characterizing miRNA to further understand its biological functions, and to determine how it can be used as a biomarker for cancer development and prognosis, as well as insight into how it might be used as a therapeutic target for cancer treatment. With the high demand and fast growth of miRNA research, it is necessary to develop an efficient and robust miRNA purification method to enhance downstream application assays. However, the sample preparation process for extracting miRNA from cells and tissues can be very challenging because most commercial nucleic acid extraction kits and protocols are inefficient when recovering small nucleic acids such as miRNAs.

In this application note, we describe an efficient and robust miRNA extraction method using Agencourt SPRI (Solid Phase Reverse Immobilization) technology and Biomek automation. The SPRI procedure is an easy, rapid, high yield, and automation-friendly nucleic acid purification method that does not require organic solvents, centrifugation or filtration steps. This method uses carboxyl-coated magnetic particles that reversibly bind nucleic acids in the presence of binding buffers and crowding reagents.

Typically, there are three basic steps in the extraction/purification procedure. In the first step, nucleic acids are immobilized onto the magnetic SPRI beads, leaving contaminants in solution. In the second step, contaminants are removed and nucleic acids are washed, after a magnetic field is used to pull the micro-particles out of solution. Contaminants are aspirated and nucleic acids are thoroughly washed with molecular biology-grade ethanol. In the third step, purified nucleic acids are easily eluted from the micro-particles under aqueous conditions, which provide maximum flexibility for downstream applications. This application note describes two methodologies: (1) miRNA extracted from cell culture using the Agencourt RNAdvance Cell v2 Kit and (2) miRNA extracted from FFPE (formalin-fixed, paraffin-embedded) samples using the Agencourt FormaPure Kit.

MATERIALS AND METHODS

miRNA Extraction using the RNAdvance Cell v2 Method

Frozen HeLa cells (50,000 cells per microplate well) were thawed at room temperature for 10-15 minutes. A 63µL lysis proteinase K solution was added to each well and incubated for 30 minutes. The cell lysate was transferred into the wells of a microplate (Abgene) containing 330µL of freshly-made binding solution (80µL Bind Buffer and 250µL of Isopropanol (American Bioanalytical, part number AB07015-01000). The cell lysate was incubated with binding solution for 5 minutes; the microplate was placed on the Agencourt SPRIPlate 96 Ring Super Magnet plate (Beckman Coulter Life Sciences, part number A32782) for 5 minutes to settle the beads. Supernatant containing contaminants and unwanted waste material was aspirated and removed from the microplate wells. The beads were washed once with 300µL of fresh 85% ethanol (American Bioanalytical, part number AB00138-01000), the microplate was placed back on the magnetic plate and the ethanol was aspirated and removed. Samples were allowed to air dry on the magnetic plate for 10 minutes, miRNA was eluted with 40µL of nuclease free water and the sample plate was placed back on the magnetic plate for 2 minutes. Eluted nucleic acids were transferred into a clean plate. If desired, steps for DNase treatment can be introduced before the elution step to remove genomic DNA as it was described in the RNAdance Cell v2 protocol (Beckman Coulter Life Sciences, part number A47942).

FFPE miRNA Extraction using the FormaPure Method

Lysate from FFPE samples was prepared according to the Agencourt FormaPure protocol (Beckman Coulter Life Sciences, part number A3342). 200µL of the FFPE lysate was transferred into a 96-Well Riplate-2.2 mL (Word Wide Medical Products, part number 99181000), 150µL of Bind 1 buffer and 20µL of Bind 2 buffer containing beads plus 800µL of Isopropanol were added into the lysate, mixed by aspirating and dispensing with a pipette five times and incubated at 55° C for 5 minutes. The microplate was placed onto the Super Magnet plate (Beckman Coulter Life Sciences, part number A32782) for 5 minutes, the supernatant was slowly aspirated and discarded, the sample plate was removed from the magnetic plate and the beads were washed with 750µL of freshly made 85% ethanol by pipette mixing five times. Beads were settled on the magnetic plate again for another 5 minutes. Clear supernatant was carefully removed. The sample plate was air dried for about 10 minutes, and the miRNA and total nucleic acid were eluted with 40µL of nuclease free water.

Taqman MicroRNA Assay

For the TaqMan let-7c assay (Life Technologies, part number 4427975, assay ID 000399), 40µL of extracted samples containing miRNA and total RNA were collected from each well. The total RNA quality was measured by a Bioanlyzer 2100 (Agilent Technologies, data not shown) and the concentration of the sample was measured by a NanoDrop 2000 (Thermo Fisher Scientific). Either 50ng or 20ng of nucleic acid samples were used for the TaqMan MicroRNA assays. The assays were performed according to the manufacturer's protocol with the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, part number 4366596) and TaqMan Universal Master Mix (Life Technologies, part number 4440038). The RT and PCR reaction set up is described in the tables below. The RT and PCR cycling conditions followed the instructions of the manufacturer's protocols.

RT Reaction Set Up for a Single Reaction

Component	Volume/15µL reaction
dNTP mix (100mM total)	0.15 µL
Multiscribe RT enzyme (50U/µL)	1.00 µL
10x RT Buffer	1.5 µL
RNase Inhibitor (20U/µL)	0.19 µL
Primer	3.0 µL
Nuclease free water	4.16 µL*
RNA	5.0 µL
Total	15.0 µL

*Adjust the water volume according to the usage of the RNA volume.

PCR Set Up for a Single Reaction

Component	Volume/20ÐµL reaction
Primer and Probe	1.0 µL
Master Mix	10.0 µL
Nuclease free water	7.67 µL
cDNA	1.33 µL
Total	20.0 µL

RESULTS

Optimization of the Binding and Rebinding Buffer Conditions in Recovery of the 22 nt Oligonucleotides using SPRI Chemistry

The current RNAdvance Cell v2 protocol was designed for cell culture total RNA purification. The binding buffer conditions are optimal for capturing larger nucleic acids, and any nucleic acid fragments smaller than 50 nucleotides are usually removed during the washing steps. Our initial test for miRNA isolation using the Agencourt RNAdvance Cell v2 protocol monitored the recovery of the 22 nt oligonucleotides through various binding and rebinding buffer conditions. Figure 1a shows that the yield of the 22 nt oligonucleotides can be enhanced by increasing the isopropanol volume in the binding and rebinding buffer solutions. For each test condition, 1µg of oligonucleotide DNA was used as the input sample and eluted in 40µL of nuclease free water, estimating a yield of 25ng/µL of oligonucleotide DNA if the recovery rate reaches 100%. The recovery yield of the 22 nucleotide DNA fragment was then measured by Nanodrop 2000 (data not shown) and ethidium bromide staining using E-Gel® EX 4% agarose electrophoresis (Life Technologies, part number G4010-04) (Figure 1a). The binding buffer condition (lane 9) that showed the highest yield of the 22 nt oligonucleotides recovery was then selected for HeLa cell miRNA purification. The initial experiments were performed manually. To demonstrate that the Biomek automation platform can be used for miRNA purification, the manual purification method was transferred onto the Biomek automation platform with RNAdvance Cell v2 method (Beckman Coulter Life Sciences, part number A47946). Figure 1b shows that the Biomek automation method produced a comparable yield to the manual isolation method using the 22 nt oligonucleotides as the test sample. The total time required for 96 samples on the Biomek was less than 90 minutes.

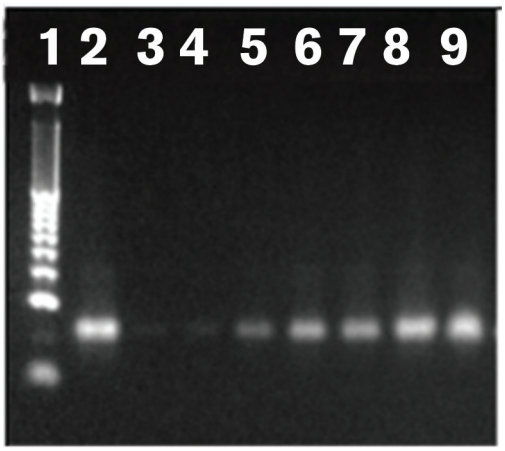


Figure 1a: Yields of the 22 nt oligonucleotides using different binding conditions.

1µLof sample was loaded in each lane. Lane 1 was the 10bp DNA size marker ladder (Life Technologies, part number 10821-015), lane 2 was the control sample (25ng of the 22 nt oligonucleotides), lanes 3-9 were loaded with 1µL of the 22 nt oligonucleotides that were recovered from different binding conditions, with lane 9 having the highest amount of isopropanol in the binding and rebinding steps.

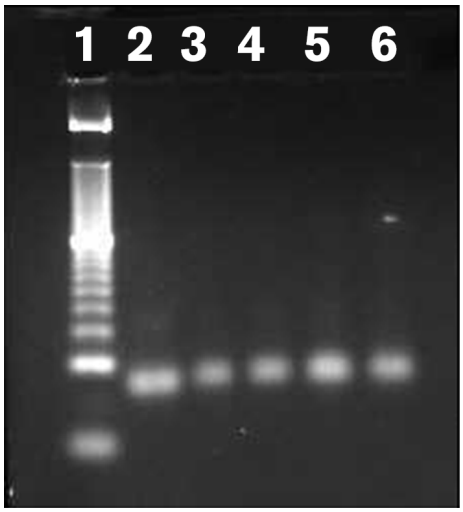


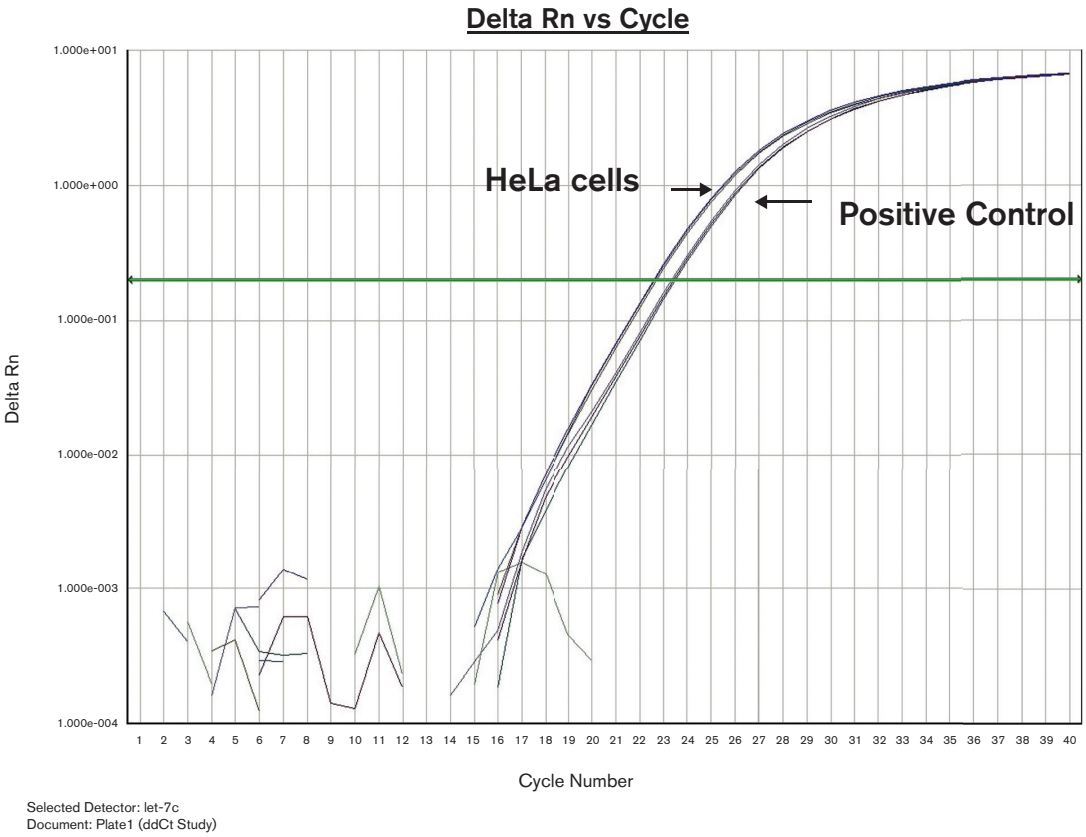
Figure 1b: The yield from the manual extraction method is comparable with the automated extraction method using the 22 nt oligonucleotides.

1µL of sample was loaded in each lane. Lane 1 was the 10bp DNA size marker ladder (Life Technologies), lane 2 was the control sample (25ng of the 22 nt oligonucleotides), lanes 3-4 were DNA recovered from the manually-extracted method and lanes 5-6 were DNA recovered from the Biomek extraction method.

Examine the let-7c Expression in HeLa Cells

miRNA from a 96-well HeLa cell culture plate were extracted using RNAdvance Cell v2 96 MC Biomek method running on the Biomek FXP (Beckman Coulter Life Sciences, part number A47946). The binding condition was modified by increasing the isopropanol volume from 95µL to 250µL. The ethanol wash step was modified from 200µL of 75% ethanol to 300 µL of 85% ethanol. A 40µL of nuclease free water was used as the elution buffer for each well. To determine whether miRNA was successfully extracted from the HeLa cells, let-7c TaqMan MicroRNA assay (Assay ID 000379) was performed (let-7c is a known miRNA that is expressed in almost all cell types). Figure 2 shows the overlaid amplification plots of the let-7c assay in triplicate of HeLa cell extracted RNA (50ng) and the let-7c positive control RNA from placenta tissue (50ng) (Ambion, part number AM7950). The minus RT reaction and non template control samples showed no amplification, suggesting that the amplification resulted from the extracted miRNA. The average threshold cycle value (Ct) for HeLa cells was at the 23rd cycle whereas the average Ct value of the positive control was at the 24th cycle. The results suggest that the expression level of let-7c in HeLa cells is higher than the positive control placenta tissue.

Figure 2: TaqMan qPCR profiling of the let-7c expression in HeLa cells.



miRNA from a 96-well HeLa cell culture was extracted using the Biomek 96 multi-channel method. The Taqman qPCR amplification plots showed that the expression of let-7c is more abundant in HeLa cells.

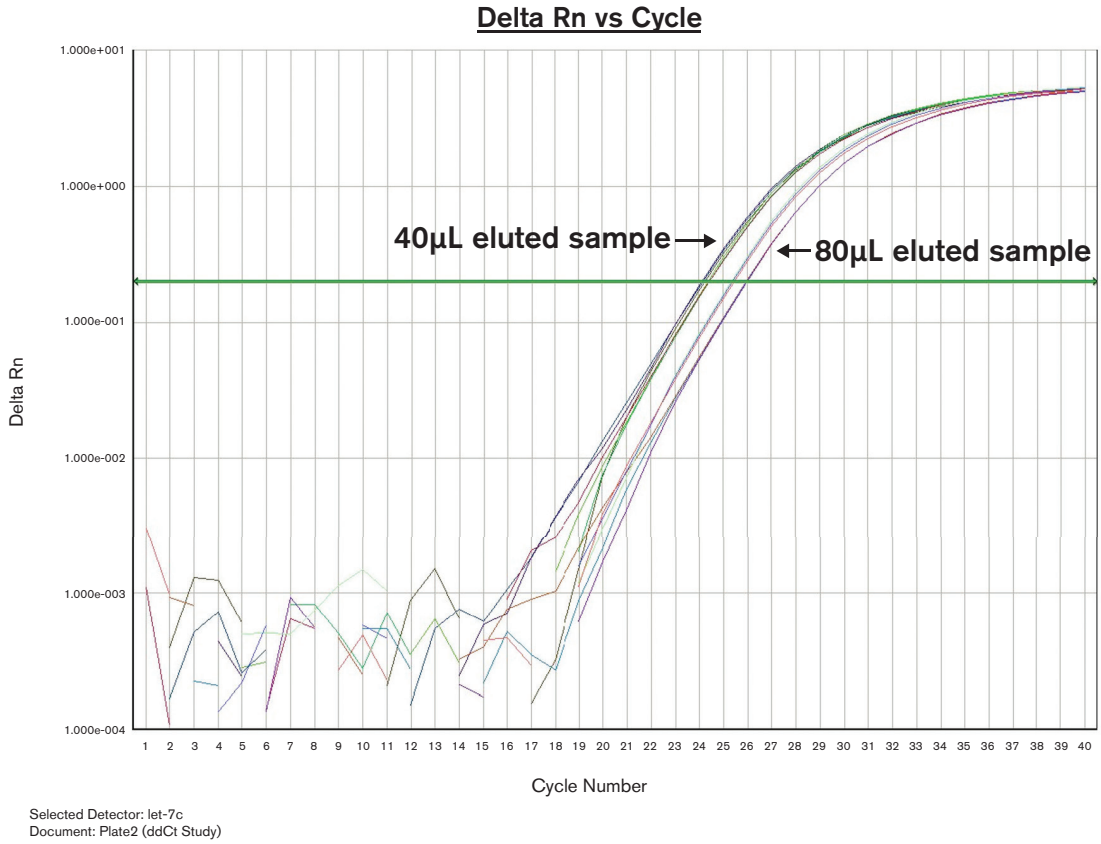
Examine the let-7c Expression in FFPE Samples

miRNA and total RNA from FFPE samples were extracted using the FormaPure Biomek Span 8 method running on the Biomek FXP (Beckman Coulter Life Sciences, part number A35556). Either a 40µL or 80µL of nuclease-free water was used for elution. 5µL of the extracted FFPE samples were used for the let-7c TaqMan MicroRNA assay. Figure 3 shows the overlaid let-7c amplification plots in the triplicate of FFPE samples from two different experiments. The results showed that the average Ct value was at the 24th cycle when eluted with 40µL of elution buffer and the average Ct value was at the 25th cycle when eluted with 80µL of elution buffer.

Examine the let-7c Expression in FFPE Samples

miRNA and total RNA extracted from FFPE samples were extracted using the FormaPure Biomek Span 8 method running on the Biomek FXP (Beckman Coulter Life Sciences, part number A35556). Either a 40µL or 80µL of nuclease-free water was used for elution. 5µL of the extracted FFPE samples were used for the let-7c TaqMan MicroRNA assay. Figure 3 shows the overlaid let-7c amplification plots in the triplicate of FFPE samples, from two different experiments. The results showed that the average Ct value was at the 24th cycle when eluted with 40µL of elution buffer and the average Ct value was at the 25th cycle when eluted with 80µL of elution buffer.

Figure 3: TTaqMan qPCR profiling of the let-7c expression in FFPE samples.

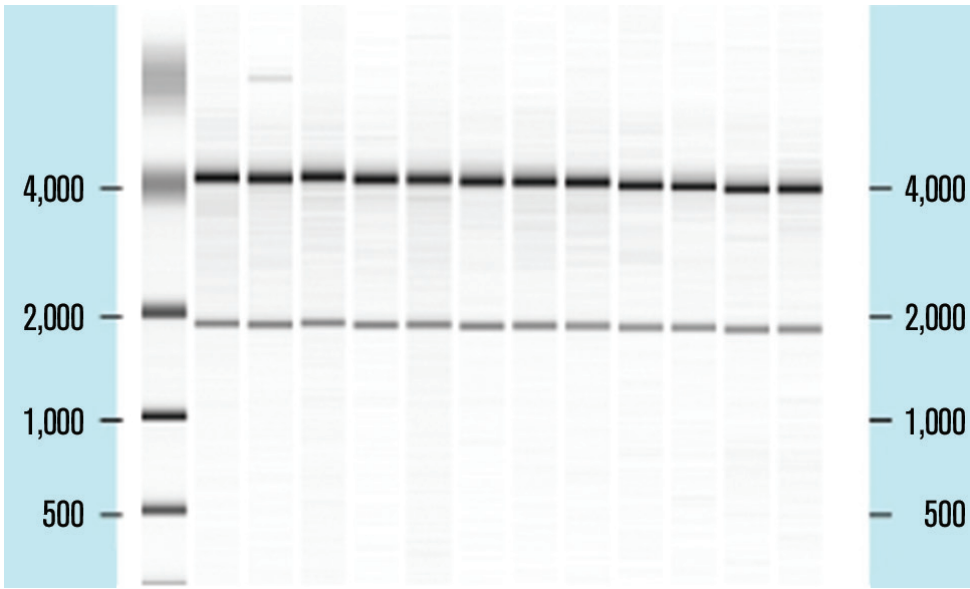


miRNA from FFPE samples was prepared using the Biomek Span8 method. This figure shows the amplification plots of multiple FFPE samples. Samples that were eluted with 80µL of nuclease-free water showed an average amplification Ct value at 25 cycles and the samples that were eluted with 40µL of nuclease-free water showed an average amplification Ct value at 24 cycles.

Recovery of the miRNA Did Not Compromise the Yield of the Total RNA

To determine how much of the total RNA yield was affected when changing the binding buffer conditions, total RNA quality was measured using the Bioanalyzer 2100 (Agilent Technologies, Figure 4), and the concentration of the nucleic acids was measured by NanoDrop 2000 spectrophotometer (data not shown). The NanoDrop reading showed the average concentration from multiple runs of experiments was at 20ng/µL, the average RNA integrity number (RIN) was at 9.0, and the ratio of OD260/OD280 was above 2.0 (data not shown). This result suggested that the total RNA yield using the miRNA extraction method is similar to the total RNA isolation method; therefore, modification of the binding buffer and washing conditions did not significantly affect the total RNA yield.

Figure 4: Total RNA quality prepared from HeLa cells



1µL of sample was loaded in each lane. Lane 1 was the RNA ladder, lanes 2-12 are the RNA samples prepared from HeLa cells uing the RNAdvance Cell v2 Kit and Biomek FX^{P}

SUMMARY

This study showed that changing the buffer conditions in the binding and rebinding steps is sufficient to recover the smaller nucleic acids and miRNA from cell culture or FFPE samples using the Agencourt RNAdvance Cell v2 and Agencourt FormaPure kits, respectively. Yield from the manual extraction is comparable to the automated extraction method. The procedure can be accomplished in less than 90 minutes on a Biomek FX^{P} platform using the automated method for 96 samples. Importantly, the recovery of miRNA using modified binding buffer conditions did not compromise the yield of the total RNA. Therefore, this efficient and robust SPRI Biomek automated extraction method is suitable for performing both miRNA and total RNA purification in a single process.

This process provides the capability to compare miRNA and gene expression profiles within the same sample, which allows users to save time and cost for sample preparation.

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