LAXCO
MICROSCOPES REDEFINED

Microgenomic Expression Profiling

APPLICATION NOTE #8

ABSTRACT

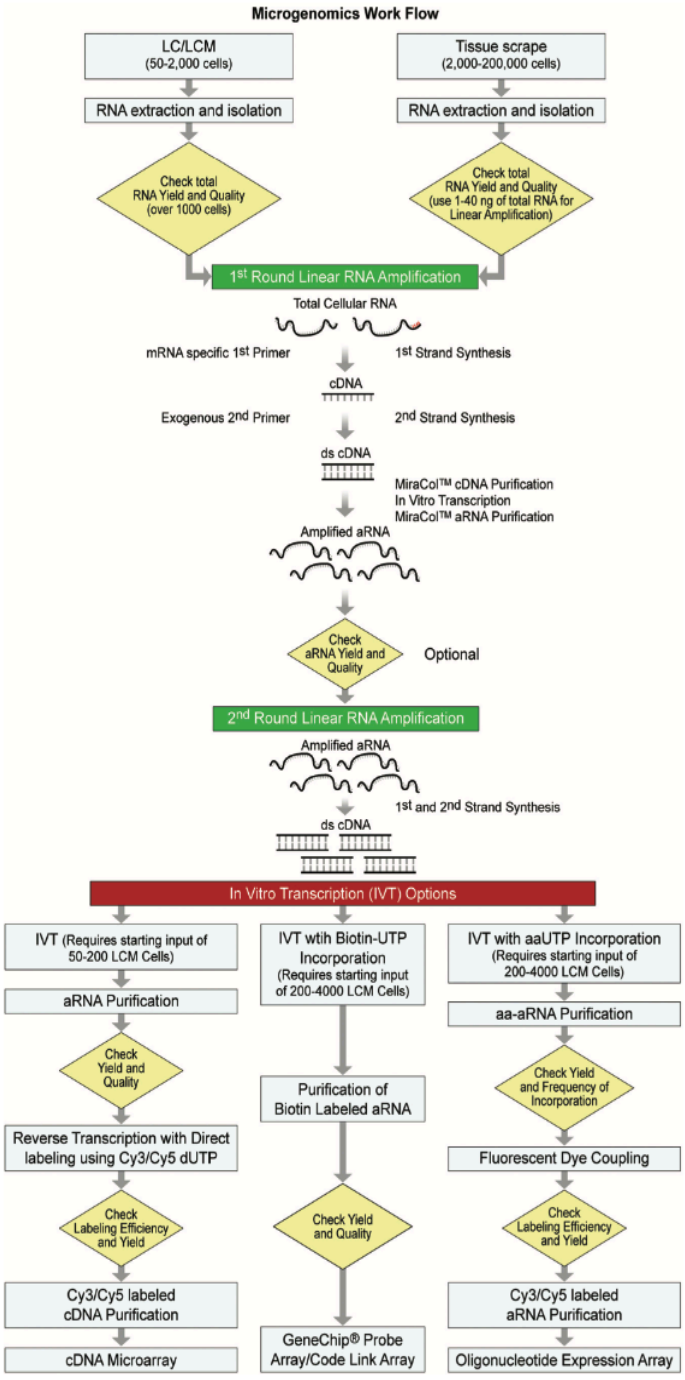
Microgenomic technologies enable expression array analysis from extremely limited cell mass. An integrated system of specialized separation techniques, advanced isolation and amplification protocols, and novel quality assurance checks is used in concert to achieve meaningful expression array data from minute samples with confidence. This application note provides a workflow for laser-cut and laser-capture microdissected pure cell populations through to microarray-based expression analysis using state-of-the-art technologies.

INTRODUCTION

Microgenomic technologies provide the tools necessary to examine expression profiles from samples as limited as a single cell. Laser capture microdissection (LCM) is an advanced separation technology that enables the isolation of desired pure cell populations from heterogeneous tissue samples. LCM utilizes an infrared laser pulse system whichadheres cells of interest to a transparent thermoplastic film, preserving essential cellular and morphological characteristics, while maintaining the integrity of biomolecules such as DNA, RNA, and proteins. UV laser cutting (LC) may also be utilized in conjunction with LCM, allowing for the rapid isolation of larger populations of cells, while still maintaining cellular characteristics critical to downstream analyses, such as gene expression profiling.

Highly efficient RNA isolation kits and linear amplification kits generate microgram amounts of amplified RNA (aRNA) from minute amounts of total RNA, providing sufficient quantity of probe for microarray analysis. Advances in microspectrophotometry allow quantity and purity assessment at several points during probe generation. The retention system of the NanoDrop ND-1000 spectrophotometer uses surface tension to hold 1µL samples in place during spectrophotometric readings. This form of micro-spectrophotometry, in concert with exisitng technologies commonly used for RNA quality assessment, allows for unprecedented process control during microgenomic experiments.

The methods described herein are intended as general guidelines for microarray probe synthesis from limited amounts of RNA (≤2000 cells). Following laser microdissection of the desired cell population, RNA is isolated and then linearly amplified to generate antisense RNA. When the starting cell population is very limited, a second round of linear amplification if necessary in order to have sufficient quantities of aRNA to use for probe synthesis. In the second round of amplification, complimentary DNA (cDNA) is generated through first and second strand synthesis, and then used as template for aminoallyl dUTP incorporation during a second round of RNA amplification inpreparation for fluorescent dye labeling. aRNA quality is typically assessed at critical steps in the workflow using the Agilent bioanalyzer system. Final probe quantity, as well as efficiency of dye incorporation, is determined using the NanoDrop ND-1000 spectrophotometer in preparation for microarray analysis.



METHODS

Special Considerations:

- Minimize degradation by storing RNA at -70°C.
- Frequent freeze-thaw cycles accelerate RNA degradation.
- RNase contamination will cause experimental failure. Adhere to the following recommendations to minimize RNase contamination:
    - Wear disposable gloves and change them frequently.
    - Clean work surfaces, instruments, racks, and reagent bottles with commercially available RNase and DNase decontamination solutions before performing reactions.
    - After putting on gloves, avoid touching surfaces that may introduce RNases onto the glove surface.
    - Use only new, sterile RNase free barrier pipette tips and nonstick RNase free microcentrifuge tubes.
    - Keep thawed RNA on ice until it is needed in the procedure.
- Ensure sample homogeneity for accurate nucleic acid quantitation when using the NanoDrop ND-1000 spectrophotometer.
- Protect fluorescently tagged nucleotide conjugates from long exposures to light.

1. Laser Cut and Laser Capture Microdissection:

Isolate specific cells of interest using laser capture microdissection instrumentation from Laxco according to the manufacturer's protocol.

2. RNA Extraction and Isolation:

Perform RNA extractions and isolations using the Arcturus PicoPure RNA Isolation Kit or the Paradise Reagent System according to the manufacturer's protocol. Expected total RNA yield will vary depending on starting material.

Note: The quantity of total RNA extracted is dependent on several factors, including cell type and tissue quality. Certain cell types, such as monocytes, may yield extremely low RNA quantities, and may fall below the guidelines mentioned here. Please contact Laxco, Inc. Technical Support for questions related to RNA yield.

3. RNA Quantity and Purity Assessment:

This step is optional and may be per formed if the starting cell number is ≥ 1000 cells. Total RNA can be quantitated, and the purity assessed, using the NanoDrop ND1000 Spectro photometer (figure 1a), while the quality of the total RNA can be determined using the RNA 6000 PicoLabChip on the Agilent 2100 bioanalyzer (figure 1b). RNA concentrations < 2ng/µL or 260/280 ratios < 1.6, as determined by NanoDrop ND-1000, may indicate incomplete extraction, inefficient isolation, or copurification of other cellular material. Furthermore, if the 2100 bioanalyzer out put profiles do not exhibit clean 28S and 18S peaks as shown in Figure 1b, it may suggest degradation of the extracted RNA. If problems with RNA quantity and quality are observed, it is recommended before proceeding, to evaluate tissue processing, LCM and RNA extraction procedures, and repeat steps as necessary.

Instrumentation and Kits

PROCEDURE	KITS AND INSTRUMENT
Laser Capture Microdissection	Accuva Cellect Laser Capture Microdissection Instrument
RNA Isolation	PicoPure RNA Isolation Kit (frozen samples) Paradise Reagent System (FFPE samples)
Nuleic Acid Quantitation & Purity Assessment (absorbance ratios)	NanoDrop ND-1000 Spectrophotometer
Nucleic Acid Quality Assessment (fragment analysis)	Agilent 2100 bioanlyzer (PicoChips and NanoChips)
RNA Amplification (Frozen Samples)	RiboAmp HS Amplification Kit (Minimum 100 pg Input RiboAmp® OA Amplification Kit (Minimum 1 ng Input)
RNA Amplification (FFPE Samples)	Paradise Reagent System
Biotin Labeling Kits	ENZO® Bioarray™ HighYield™ RNA Transcription Labeling Kit GeneChip Expression 3' Amplification Reagent for IVT Labeling (Affymetrix)
Amino Allyl Labeling Kit	Fluorescent Linear Amplification Kit (Agilent(
Measuring aaUTP incorporation	Amersham Cy™5 Dyes Perkin Elmer Cy Dyes

Figure 1a. NanoDrop ND-1000 spectrophotometer display output for 2-5ng of total RNA.

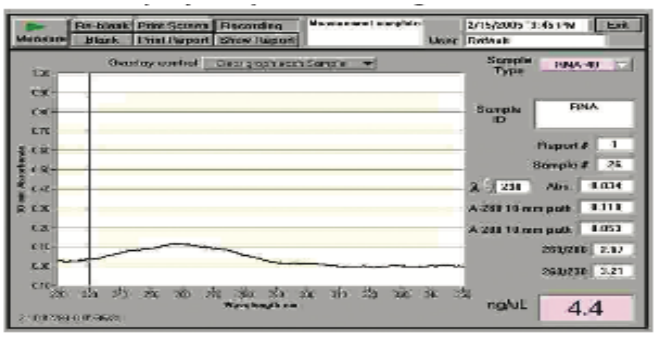
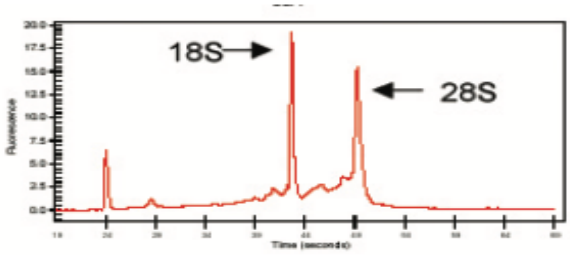


Figure 1b. Bioanalyzer profile of total RNA isolated using Arcturus PicoPure RNA Isolation Kit and run on the Agilent 2100 bioanalyzer using the RNA 6000 Pico LabChip.



4. **First Round RNA Amplification:**
Estimated total RNA quantity is used to determine the appropriate kit for RNA amplification (see Table 1).

5. **Check Yield (optional):**

Note: Checking the yield after one round of amplification is optional and serves the purpose of assessing the success of the first round of amplification only. It is recommended to use all of the material generated after the first round of amplification to process through a second round of amplification in order to generate microgram quantities of aRNA. Yield and quality assessment after the first round of amplification may significantly reduce the end product yield when starting with very limited material.

Quantitate 1 µL of amplified RNA (aRNA) product using the NanoDrop ND-1000 Spectrophotometer according to the manufacturer's specifications.

5.1 Using the Nucleic Acids module of the NanoDrop software, select RNA-40 as the constant for measuring aRNA.

5.2 For optimal results, thoroughly mix and briefly spin down the aRNA sample prior to removing 1 µL from the top of the solution. This step will prevent interference caused by potential residual silica from the column purification procedure.

5.3 The yield can be theoretically calculated based on starting cell input. Example of yield assessment: With a starting cell input of 1000 cells and assuming ~10pg of RNA per cell, one can expect a yield of ~10ng of total RNA. Message content in 10ng of total RNA will be approximately 100-200pg. One round of amplification using the Arcturus Amplification kits will produce 1000-3000 fold amplification, resulting in at least 100-600ng of total aRNA.

6. **First and Second Strand Synthesis (Second Round of RNA Amplification):**
Use the materials and protocols provided in the primary RNA amplification kit to produce cDNA from aRNA made during the first round of RNA amplification.

7. Amino Allyl UTP Incorporation (Second Round of RNA Amplification):
Use the double-stranded cDNA as the template for a second round of amplification with simultaneous labeling using a commercial kit (Fluorescent Linear Amplification Kit, Agilent), then perform 5-(3-aminoal lyl)-UTP (aaUTP) incorporation according to the manufacturer's protocol.

8. **Check Yield:**
Quantitate 1µL of aaUTP aRNA product using the NanoDrop ND-1000 Spectrophotometer according to the manufacturer's specifications.
Under the Nucleic Acids module of the NanoDrop software, select RNA-40 as the constant for measuring the aaUTP aRNA. Expected yield is approximately 30-70µg of total aaUTP aRNA, from a starting input of 10ng of total RNA.

Note: Due to the large amount of aRNA generated after the second round of amplification, a 2 to 4-fold dilution of final aRNA may be required for accurate reading on the ND-1000 spectrophotometer.

9. Check aaUTP Incorporation:

By measuring the amount of aminoallyl that is incorporated into the aRNA, one can better estimate how well the aRNA will label with the dye. Poor incorporation of the amino allyl can result in inefficient labeling which will lead to reduced dye signal and loss of data. Assess the incorporation of aaUTP by checking the 289/260 ratio using the ND-1000. Generally, a ratio of 0.22-0.32 indicates adequate incorporation of aminoallyl. In such instances that the ratio is not within this desired range, the relation of the amino allyl RNA to non-aminoallyl RNA should be noted. A ratio for the amino allyl RNA of at least 0.06 higher than that of the nonaminoallyl RNA is equally acceptable. Using the ND-1000 to assess the incorporation of amino allyl is a useful tool for preventing experimental failure due to poor labeling (See Table 2 for an example of aminoallyl incorporation assessment using the NanoDrop ND-1000).

Table 2. Aminoallyl incorporation rates as measured by the NanoDrop ND-1000. A ratio of 0.2- 0.3 indicates adequate incorporation of aminoallyl.

Template	Sample	A260	A289	A289/ A260	A260/ 280	RNA (ng/µL) (50x dilution)	Yields (µg)
Non-aa-RNA (Control)	C1	1.60	0.28	**0.18**	2.39	63.93	95.90
	C2	1.54	0.29	**0.19**	2.43	62.57	93.86
	C3	0.90	0.17	**0.19**	2.31	36.05	54.08
aa-RNA	a 1	0.86	0.26	**0.30**	1.92	34.29	51.44
	a 2	0.93	0.27	**0.29**	2.04	37.13	55.70
	a 3	0.80	0.25	**0.31**	1.88	31.88	47.82

Table 3. Chart of example FOI data as derived from NanoDrop ND-1000.

Path Length	Input amount (µg)	OD260	OD280	OD550	OD650	Dilution Factor	Elution Volume	aRNA conc. (ng/ul)	Yield (µg)	CY3 FOI	CY5FOI
0.1	15	0.7458	0.362	0.429		1	50	298.32	14.916	31.06195	
0.1	15	0.5412	0.293		0.3411	1	50	216.48	10.824		20.42062

Figure 2a. Agilent 2100 bio analyzer profiles of amino allyl-labeled aRNA.

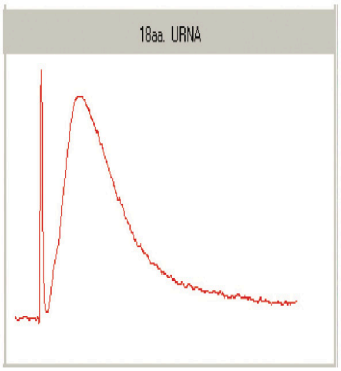


Figure 2b. Agilent 2100 bio analyzer profile of biotin labeled aRNA.

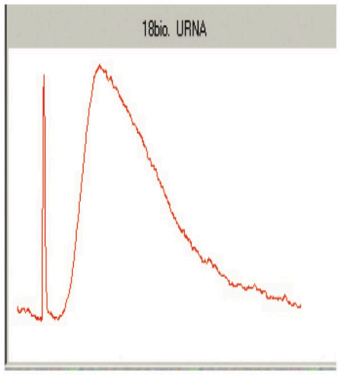
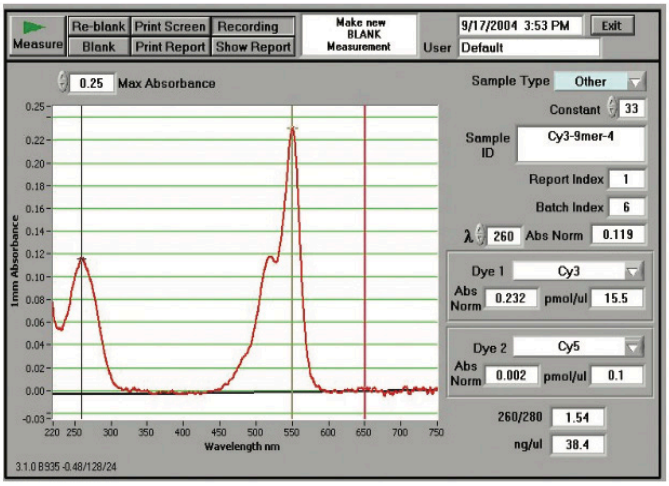


Figure 3. NanoDrop ND-1000 as used for FOI assessment. Cy3 and Cy5 dyes are observed at the expected wavelengths of 550nm and 650 nm respectively.



10. Check RNA Quality:

Assess aaUTP aRNA quality using the RNA 6000 Nano LabChip on the Agilent 2100 bioanalyzer according to manufacturer's protocol (Figures 2a and 2b).

11. Fluorescent Dye Coupling:
Prepare NHS ester dye according to manufacturer's protocol, using one of the following recommended commercial kits:

- Amersham Biosciences Cy Dye Post Labeling Reactive Dyes
- Perkin Elmer Cy Dyes
- Molecular Probes Alexa Dyes.

12. Check Labeling Efficiency:
Measure 1µL of labeled aRNA probe using the NanoDrop ND-1000 Spectrophotometer. Using a sample with suboptimal frequency of incorporation (FOI) may result in low signal on the array, leading to loss of data.

12.1 Using the Microarray module of the NanoDrop software, select RNA-40 as the constant for measuring the aRNA component of the labeled probe. Ensure the appropriate dye type has been selected for measuring the dye component of the labeled probe.

12.2 The guidelines below can be used to determine if the FOI of the fluorescent dye is suitable for microarray hybridization. The example shows the use of Cy3 and Cy5 dyes:

- Measure the absorbance of the fluorescent component of the labeled probe by selecting the appropriate dye from the drop down menu in the Microarray module. In this case, Cy3 and Cy5 are selected which will place the spectrum cursors at the appropriate wavelengths (550 nm and 650 nm respectively).
- Calculate the FOI of Cy3 = (OD550/ 0.15)*(324)/(OD260*40)
- Calculate the FOI of Cy5 = (OD650/ 0.25)*(324)/(OD260*40)
- FOI readings > 20 indicates adequately labeled probe, suitable for array hybridization.
- FOI readings between 15 and 20 are lower than recommended but still useable. The investigator should note that signal issues with the final array data might be due to the lower FOI.
- FOI readings lower than 15 should be treated with caution. These samples may not be suitable for hybridization to microarrays.

13. Perform Expression Array Analysis:
Labeled RNA input will depend on the desired type of expression array platform.

CONCLUSION

The concerted use of several new technologies allows research investigators to perform microgenomic expression profiling from extremely limited cell mass. LCM and LC provide the means of precise cell separation from heterogeneous tissue. Advanced isolation systems and amplification protocols produce sufficient material for microarray probe labeling. Bioanalyzer systems as well as novel microspectrophotometry bring a high level of quality assurance and confidence. The combined use of these various technologies provides a micogenomic work flow from cell separation through expression analysis with unprecedented process control.

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