Science Lab
by Leica Microsystems

Workflow & Protocols: How to Use a Leica Laser Microdissection System and Qiagen Kits for successful RNA Analysis

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Laser Microdissection (LMD) allows isolating individual cells or chromosomes and is a well established technique for sample preparation prior downstream analysis of the nucleic acid content via PCR or sequencing techniques. Here we describe the successful combination of the Leica Microsystems LMD system and Qiagen kits for purification of nucleic acids even from little amounts. The presented workflow and protocols provide a basis for successful LMD applications without any loss of nucleic acid quantity and remaining the RNA integrity during the process underlining the high quality of the products.

Material – lab inventory

Leica LMD system

Fig. 1

- Cryostat (e.g. Leica CM1850 UV)
- Pipet 10–1,000 µl
- Pipet 2–20 µl
- Centrifuge for tubes
- Tweezer
- Tweezer and brush for Cryostat

Material – chemicals and consumables

Qiagen RNeasy Micro kit

Fig. 2

- A bag of 50 ml Falcon tubes
- Pure Ethanol (EtOH), molecular biology grade
- Molecular biology grade water
- Cresyl Violet
- Aluminium foil

- 0.5 or 0.2 ml thin walled PCR tubes with flat cab suitable for the LMD stage collection holder
- Pipet tips (for 1 ml pipet and 20 µl pipet)
- Syringe with sterile filter tip
- Ice
- Blade for cryotome
- Parafilm
- Leica LMD slides (e.g. 4 µm PEN frames)
- Silica bags
- Kimwipes

Preparation upstream

1 % cresyl violet (CV) solution in 100 % Ethanol (EtOH): add 5 mg cresyl violet powder into a 50 ml Falcon tube and add 100 % EtOH to fill 50 ml. Should be prepared 1 week prior usage. But Falcon in fridge, cover with aluminium foil (cresyl violet is light sensitive) and shake every day gently.

EtOH row for fixation and wash steps:

- 2 times 75 % EtOH: fill 37.5 ml EtOH into 50 ml Falcon tube and add molecular biology grade water to 50 ml
- Place one 75% EtOH Falcon into –20 °C freezer
- 95 % EtOH 47.5 ml EtOH into 50 ml Falcon tube and add molecular biology grade water to 50 ml
- 100 % EtOH 50 ml EtOH into 50 ml Falcon tube
- Ethanol row in Falcon tubes can be (re-)used for up to 3 days.

> **Important note:**
> - Seal Falcons with Parafilm for storage → this will avoid EtOH from evaporation!
> - Place LMD slides for 10 min under UV-C light → acivate the membrane for better section adhesion and sterilize the surface!
> - Place 0.2 or 0.5 ml tubes with lid open under UV-C light for at least 30 min → sterilize and eliminate any RNA & RNase!
> - Prepare three 50 ml Falcon tubes with some silica bags in the conus → The silica will keep the slides and section dry, working with RNA means humidity and moisture are your enemy because those could activate RNases!

Cryosectioning

Place block with Cryosample into Leica Cryostat directly from –80 °C and let it equilibrate for at least 30 min at –19 °C.

Fig 3.: Tissue block in a Leica CM1850 UV cryotome at –19 °C ready to cut.

After Equilibration prepare section of a suitable thickness (thickness should be chosen according diameter of cells of interest, for this test 10 µm sections were prepared).

Place one (curled) section directly from cryo into a 0.5 ml tube and immediately add 350 µl RLT buffer (content of Qiagen Micro RNeasy kit). This will serve as positive control for quality and quantity.

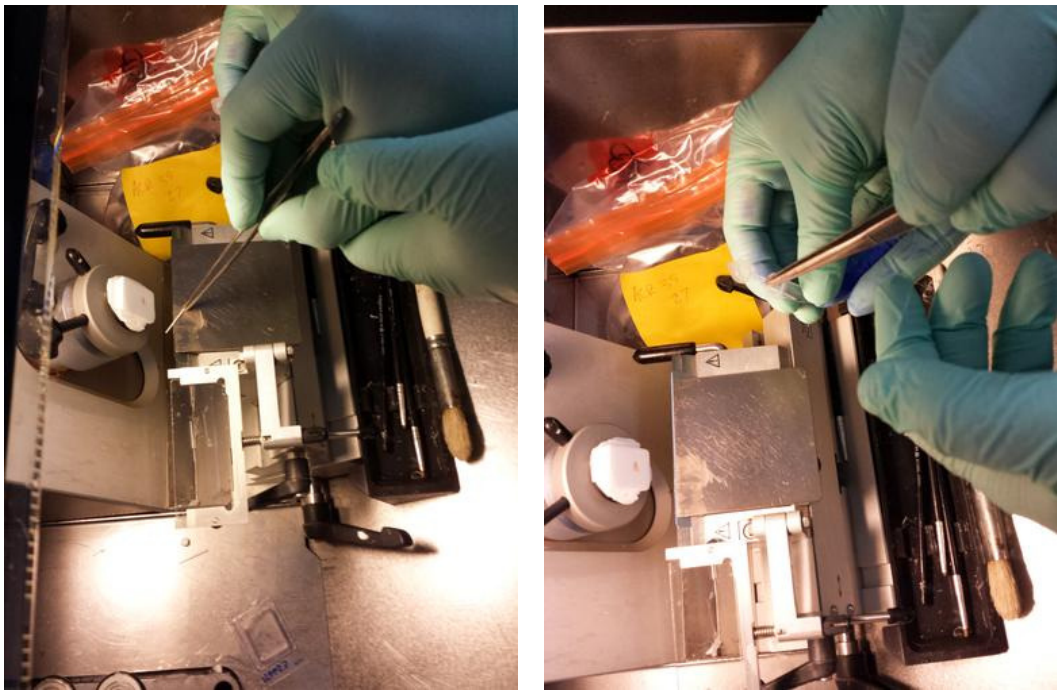


Fig. 4: Curled section collected with cold tweezers direct into a sterile tube cap. 350 µl RLT buffer are added immediately to protect RNA from degradation.

Fig. 5: Section preparation and mount on a PEN frame slide. The frozen section will easily melt to the membrane which comes from room temperature.

Put the slide with the section into the Falcon with 75 % EtOH solution at –20 °C for 2 min (brief fixation). After 2 min briefly air dry the slide and store it in a 50 ml Falcon with some silica bags in the conus (to keep it dry).

Repeat this mounting with another section and slide, but instead of storage add some drops of 1 % cresyl violet solution with the syringe through the sterile filter tip. Let the staining solution for 1 min on the section. Afterwards wash of the cresyl violet by dipping the slide briefly into 75 % EtOH, then 95 % EtOH and finally 100 % EtOH. Briefly air dry the slide and store it in a 50 ml Falcon with some silica bags in the conus (to keep it dry).

Repeat the whole process with another section and slide.

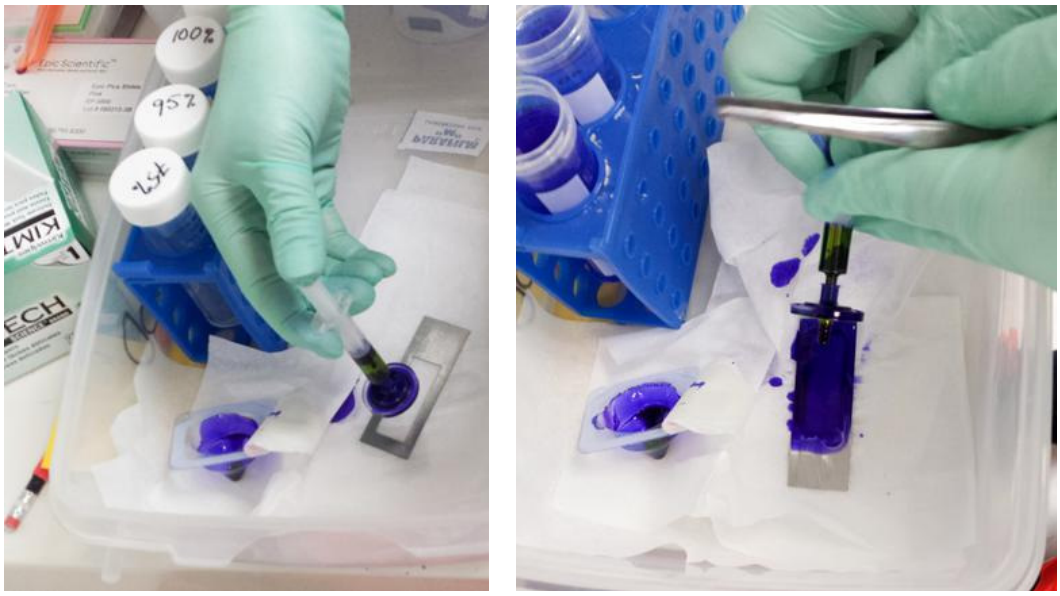


Fig. 6: Cresyl violet is applied with a syringe through a sterile filter covering the section on the slide for 1 min.

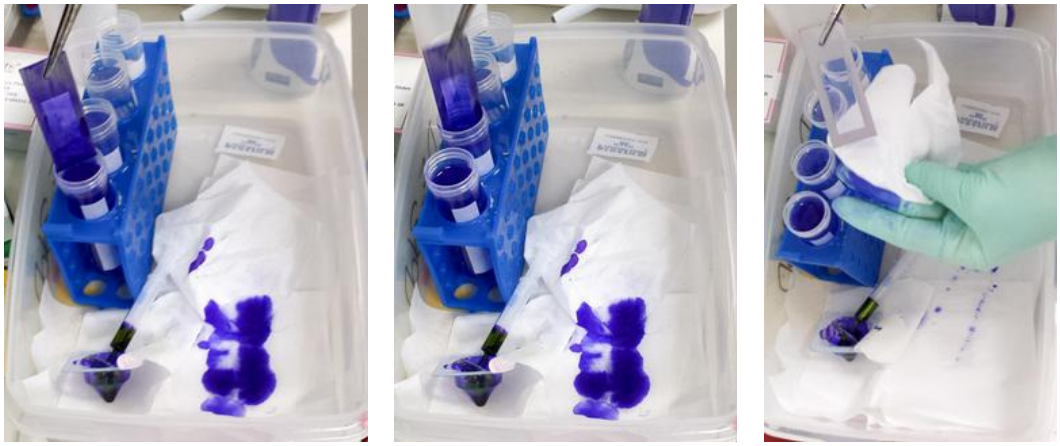


Fig. 7: Washing steps after staining: the slide is dipped into the ascending EtOH row to wash off remaining cresyl violet.

> **Important note:**
> - Store slides in Falcon tubes with some silica bags in the conus and seal those with Parafilm → the silica will keep the slides and section dry, sealing prevents from environmental moisture!

Store all tubes and Falcons with slides on ice. Let the slides equilibrate for 15 min at room temperature next to the LMD system in the sealed Falcon prior usage.

LMD test slides

You should now have:

- 1 section in a tube with 350 µl RLT buffer (positive control)
- 1 section mounted on a slide and fixed with EtOH stored in a 50 ml Falcon with Silica bags
- 2 slides with mounted sections fixed with EtOH and stained with CV, stored in a 50 ml Falcon with Silica bags

Fig. 8: Buffer, tubes, slides in Falcons and 75 % EtOH from the freezer on ice.

The slide with the unstained section is used as another control. Therefore directly digest it from the membrane with RLT buffer and transfer it into a 0.5 ml tube (total amount of RLT buffer must be 350 µl, do not use all of it for digestion from the slide as it will rinse off the slide!).

One of the fixed and stained slides is used as another control. Therefore directly digest the section from the membrane with RLT buffer and transfer it into a 0.5 ml tube (total amount of RLT buffer must be 350 µl, do not use all of it for digestion from the slide as it will rinse off the slide!).

Leica LMD7000 for RNA analysis

The remaining fixed and stained slide is applied for LMD. Therefore load the slide in the sample holder and load a 0.5 ml tube in the collection holder. Select the position of the loaded collector and mark the whole section for dissection with the LMD software using the desired magnification. Start the laser and control after dissection that all section are collected into the collection tube. In case needed recut section using Move + Cut to ensure all are collected.

Unload the collection tube and carefully close the lid and briefly spin down the dissectates. Open the lid and add 350 µl RLT buffer (up to 65 µl RLT buffer can be added to the collection cap before cutting).

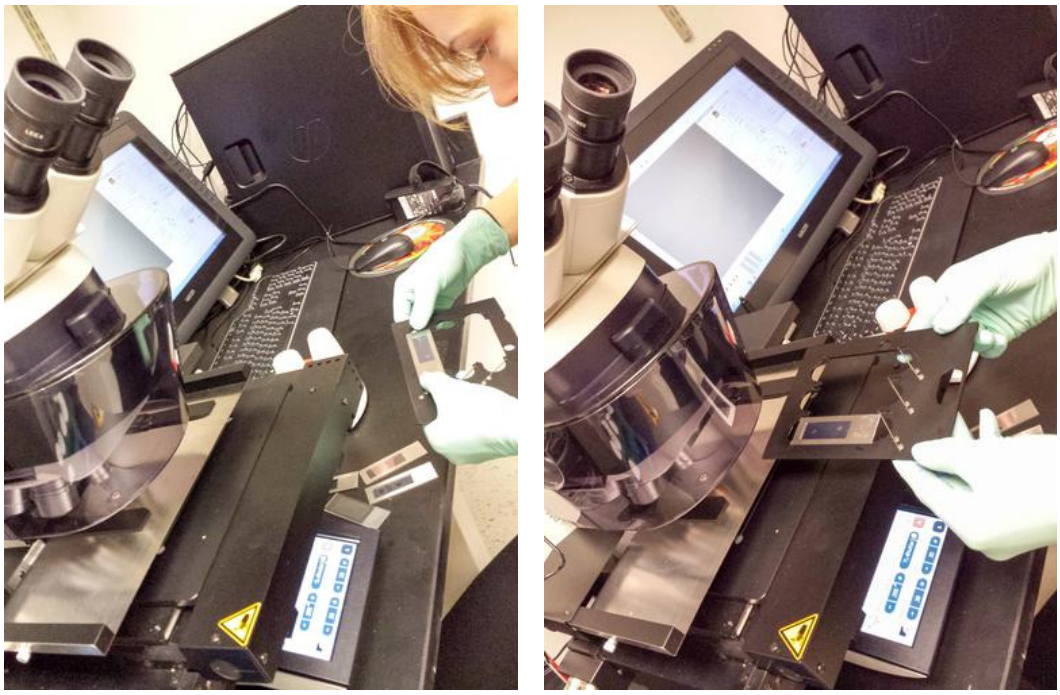
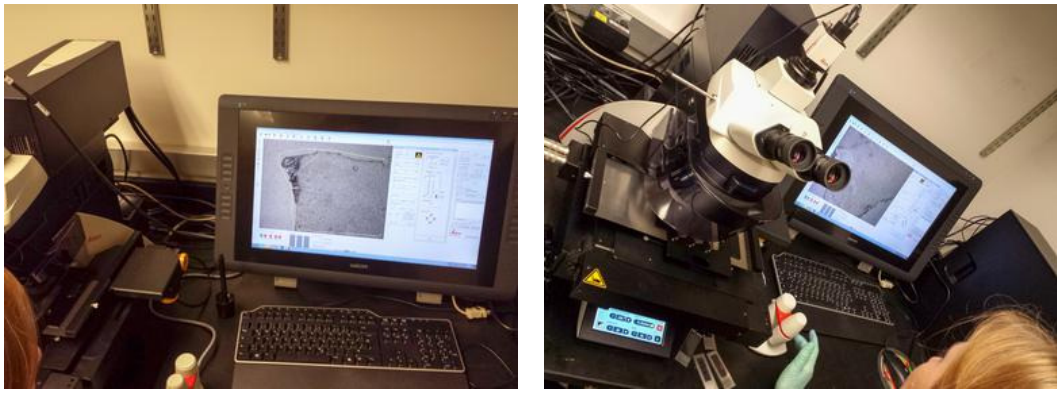


Fig. 9: PEN frame slide with fixed and stained section is loaded to the LMD system.

Load another collection cap and dissect empty membrane close to the area where the section was collected (roughly same area size in µm²). Unload the collection tube and carefully close the lid and briefly spin down the dissectates. Open the lid and add 350 µl RLT buffer (up to 65 µl RLT buffer can be added to the collection cap before cutting). This tube will serve as a LMD negative control later on.



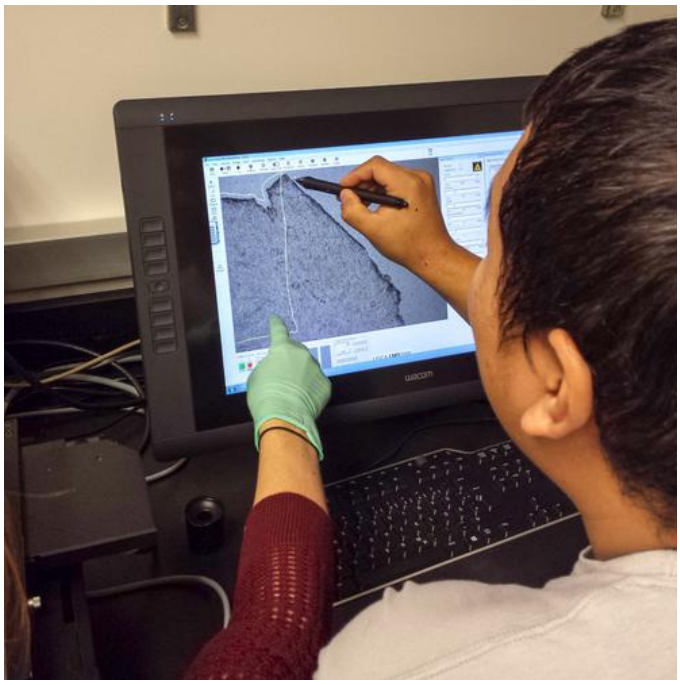


Fig. 10: Sections are dissected piece by piece at chosen magnification into a sterile tube cap.

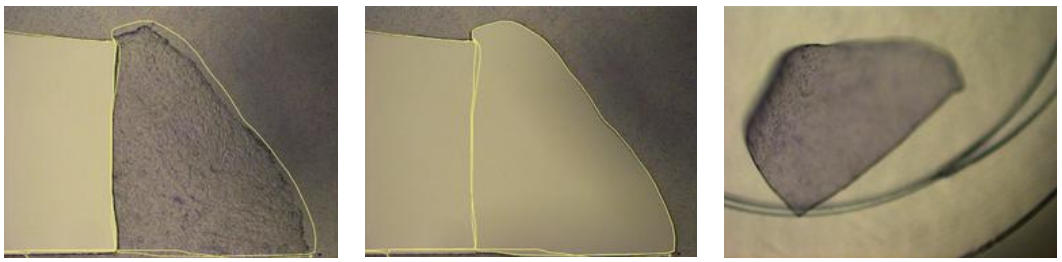


Fig. 11: Marked region and dissection result are easily visualized: left cutting line, middle, dissected area, right dissectate in the collection tube cap.

Important note:

- Up to 65 µl RLT buffer can be directly applied to the collection tube cap to protect the content of the dissectate from degradation directly after capture

Fig. 12: Dissectates collected with a low magnification are visible with the naked eye as well (example of dissectates achieved using 5x objective).

Preparation and processing downstream laser microdissection

- Add 44 ml to the bottle with RPE and check the checkbox that EtOH is added.
- Prepare a solution of 70 % EtOH: add 35 ml 100 % EtOH to a 50 ml Falcon and add 15 ml molecular biology grade water.
- Prepare a solution of 80 % EtOH: add 40 ml 100 % EtOH to a 50 ml Falcon and add 10 ml molecular biology grade water.

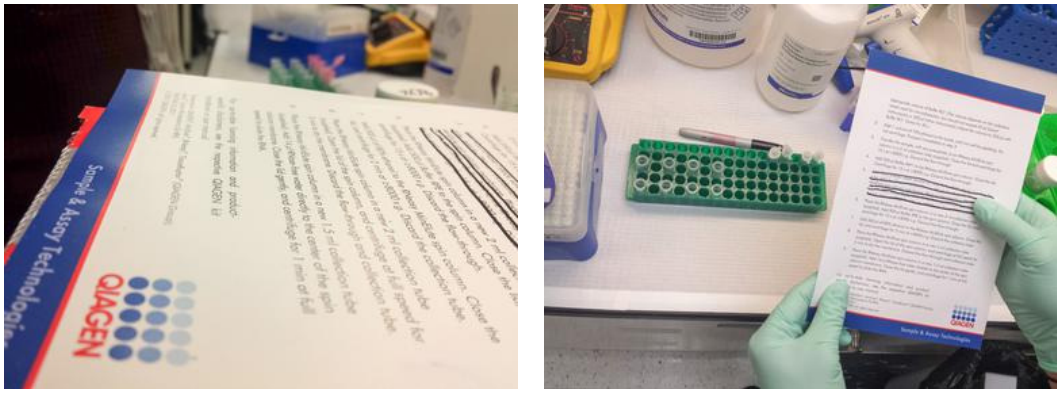


Fig. 13: A slight modified protocol for RNA extraction using the Qiagen RNeasy® Micro kit was used.

Extract RNA using the Qiagen Micro kit with the following slightly modified protocol:

1. Have all tubes ready.

2. Add 350 µl 70% EtOH to each tube (in case transfer into another tube of suitable volume before adding 350 µl 70 % EtOH), carefully mix with pipet and directly transfer to red spin column.

Important note:

350 µl should be split to 100 and 250 µl to not exceed the volume of the 0.5 ml tube. Mixing can be done directly on the column.

3. Spin 15 s at 10,000 rpm.

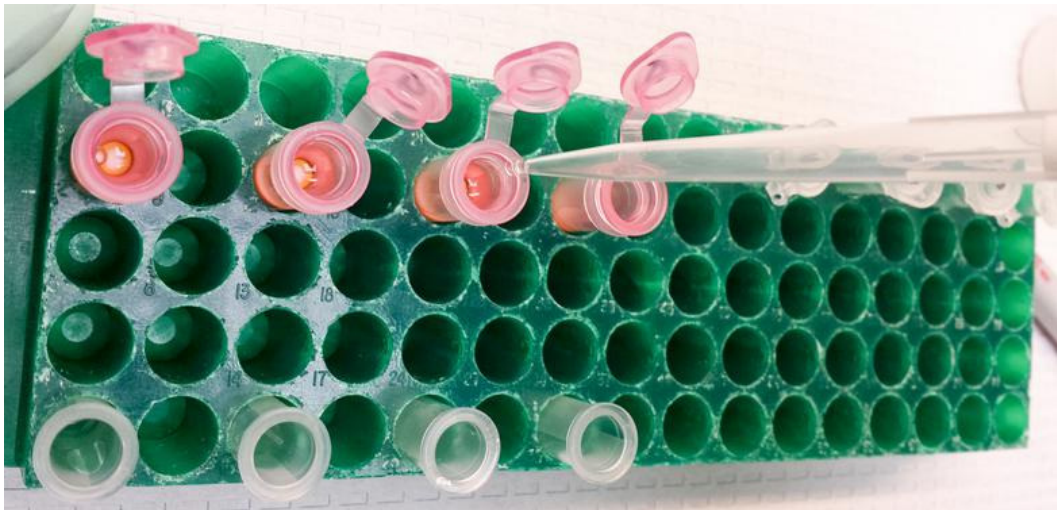


Fig. 14: Buffer is applied to the Qiagen RNeasy® spin column.

4. Discard the flow through and add 350 µl RW1 to wash column
5. Spin 15 s at 10,000 rpm.

Fig. 15: Columns in the centrifuge ready to go.

6. Discard the flow through, place the column to a fresh 2 ml tube and add 500 µl RPE (EtOH added before) to wash column
7. Spin 15 s at 10,000 rpm.
8. Discard the flow through and add 500 µl 80 % EtOH to wash column
9. Spin 2 min at 13,000 rpm

10. Discard flow through and place the column to a fresh 2 ml tube
11. Spin 5 min with max. speed and lids open to dry the silica membrane of the column
12. Elute in 14 µl water: carefully pipet 14 µl RNAse free water into the middle of the column membrane and place the column to a fresh and labelled 1.5 ml tube

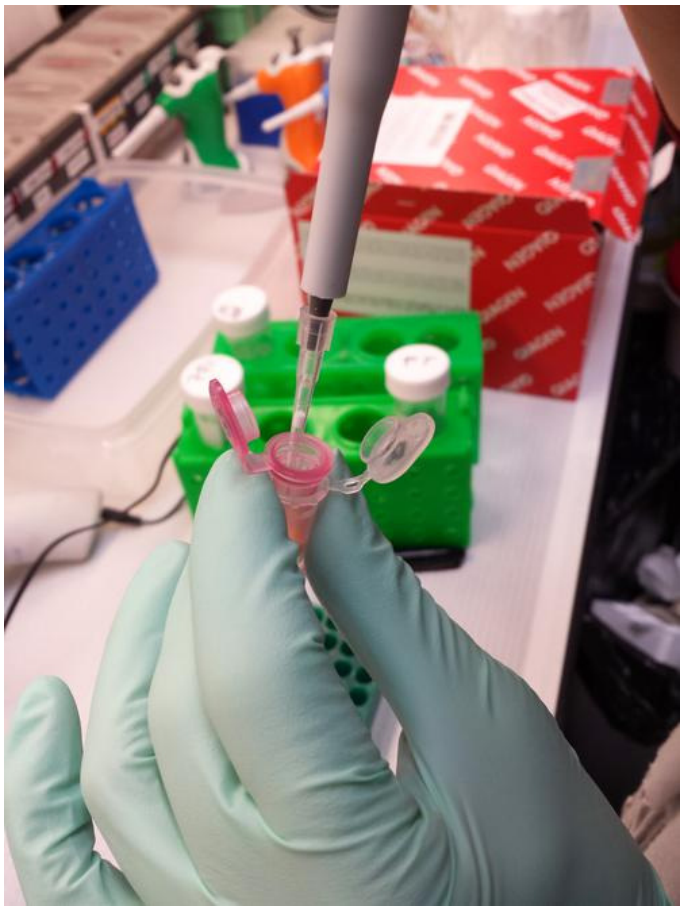


Fig. 16: 14 µl RNase free water are gently applied into the middle of the column for eluation of the extracted RNA.

13. Spin 1 min at 13,000 rpm
14. Carefully pipet flow through back into the middle of the column membrane
15. Spin 2 min at 13,000 rpm

The eluate should be stored at –80 °C or if possible immediately used for evaluation of quality and quantity.

Quick protocol summary

Samples:

A – sample picked directly from cryo (overall positive control)
B – sample fixed, picked by pipet (positive control, effect of EtOH fixation)
C – sample fixed and stained, picked by pipet (positive control, effect of EtOH fixation and staining)
D – sample fixed and stained, collected by LMD (effect of LMD)
E – empty membrane collected beside sample (negative control LMD process/contaminations)
F – RLT buffer negative control downstream purification

RNA extracted with Qiagen Micro kit:

1. Add 350 µl RLT buffer
2. Add 350 µl 70 % EtOH, mix with pipet and directly transfer to column
3. Spin 15 s, 10,000 rpm
4. Discard flow through and wash with 350 µl RW1
5. Spin 15 s, 10,000 rpm
6. Discard flow through and wash with 500 µl RPE (EtOH added before)
7. Spin 15 s, 10,000 rpm
8. Discard flow through and wash with 500 µl 80% EtOH
9. Spin 2 min, 10,000 rpm
10. Discard flow through
11. Spin 2 min, max. speed with lids open to dry membrane
12. Elute in 14 µl water*
13. Spin 1 min, full speed
14. Use eluate and place it back to column
15. Spin 1 min, full speed

Results

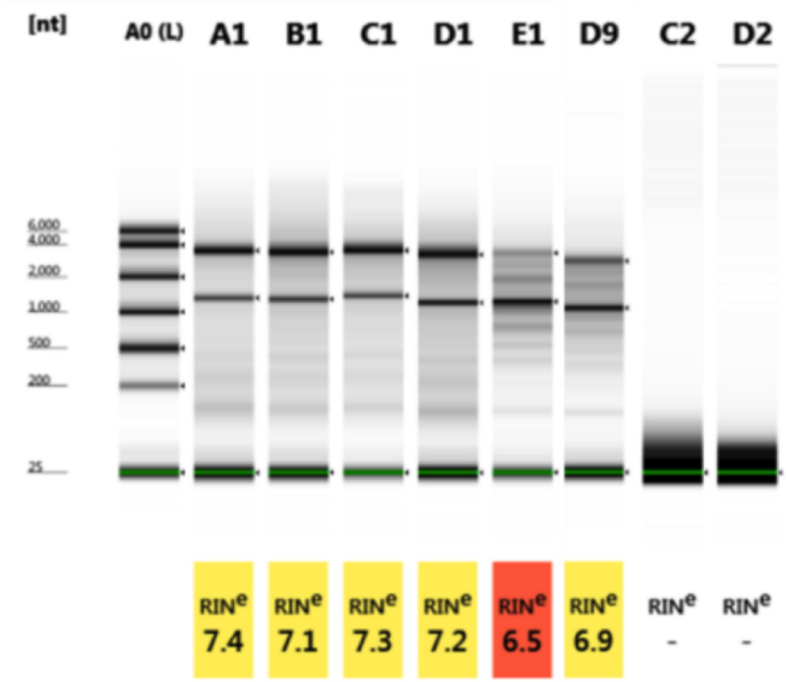


Fig. 17

Well	RIN*	28S/18S (height)	28S/18S (area)	Conc. (ng/µl)	Sample description	Alert	Observations	Total RNA area	rRNA area
A0	–	–	–	137			Ladder	–	–
A1	7.4	1.4	1.9	69.1	A			2.79	0.61
B1	7.1	1.3	1.9	84.5	B			3.41	0.76
C1	7.3	1.4	2.0	127	C			5.13	1.26

Well	RIN*	28S/18S (height)	28S/18S (area)	Conc. (ng/µl)	Sample description	Alert	Observations	Total RNA area	rRNA area
D1	7.2	1.0	2.0	83.6	D			3.38	0.78
E1	6.5	0.4	0.4	110	E			4.44	0.98
D9	6.9	0.7	0.9	67.3	D			2.68	0.59
C2	–	–	–	7.36	K	!	RNA concentration outside recommended range for RINe	0.30	–
D2	–	–	–	6.57	L	!	RNA concentration outside recommended range for RINe	0.27	–

Sample name	Material	Date	Time	A260 concentration (ng/ul)	A260 (10 mm)	A280 (10 mm)	A260/A280	A260/A230
blank_6	RNA	11.12.2014	######	0	0	0	–	–
A	RNA	11.12.2014	######	32.42	0.81	0.41	1.98	0.13
B	RNA	11.12.2014	######	48.18	1.2	0.58	2.07	0.1
C	RNA	11.12.2014	######	71.01	1.78	0.89	2	1.51
D	RNA	11.12.2014	######	48.32	1.21	0.54	2.24	0.03
E	RNA	11.12.2014	######	64.65	1.62	0.73	2.2	0.06
D	RNA	10.12.2014	######	41.79	1.04	0.47	2.23	0.03
K	RNA	11.12.2014	######	1.72	0.04	0.02	1.97	0.02
L	RNA	11.12.2014	######	0.74	0.02	0.01	2.91	0.02

Quality

All samples (A–D) with different treatments have almost the same quality (RIN 7.1–7.4). No influence on EtOH fixation, staining and Leica LMD dissection detectable.

Cryo-storage of slide in a Parafilm-sealed 50 ml Falcon tube at–80 °C overnight, gently thawed step-wise for 20 min in –20 °C freezer, 20 min in 4 °C fridge and 15 min at room temperature prior re-opening the Falcon tube led to comparable results after Leica LMD dissection as well (sample E1&D9, RIN 6.9 and 6.5).

All negative controls were empty.

Quantity

All samples (A–D) with different treatments have almost the same quantity, only sample C (fixed, stained and picked directly from slide) shows higher quantity then positive control (sample A). No influence from EtOH fixation, staining or Leica LMD dissection detectable.

Cryo-storage of slide in a Parafilm-sealed 50 ml Falcon tube at –80 °C overnight, gently thawed step-wise for 20 min in –20 °C freezer, 20 min in 4 °C fridge and 15 min at room temperature prior re-opening the Falcon tube led to comparable results after Leica LMD dissection as well (sample E1 and D9, slightly higher quantity after storage).

All negative controls were empty.

Different measurement methods led to different results à common inaccuracy of measurements.

To summarize, these data clearly indicate that the workflow doesn't affect the RNA quality or quantity.

Acknowledgement

I would like to thank Dr. Randolph-Habecker (http://sharedresources.fhcrc.org/profile/randolph-habecker-julie) for hosting the LMD workshop.