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50 Notes from RNA extraction to Microarray

Reference:

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50 Notes from RNA extraction to Microarray

1. Wear gloves at all times. This is important to avoid RNA degradation and to avoid contact between β-mercaptoethanol-containing solutions and skin. In addition, when possible, try to work in a laminar flow cabinet.
2. β-Mercaptoethanol will crystallize when storing the RLT[1] lysis buffer. This will result in a drop in concentration. Make sure to store tubes with β-mercaptoethanol-containing RLT upright at all times to avoid excessive crystallization. In addition, buffer RLT can contain precipitations. Make sure to redissolve the precipitate.
3. cDNA binding buffer may form a precipitate if stored below room temperature. If a precipitate is visible, redissolve it by warming the solution to 37°C for up to 10 min and vortexing vigorously. Cool to room temperature before use.
4. Use an empty box of pipette tips as hybridization chamber, preferably a box that is impermeable to light. These boxes usually consist of two parts separated by a surface containing holes in which the pipette tips are placed. Use the lower part to pour in the moisturizing buffer. At 47°C, the moisturizing buffer will evaporate through the holes and the hybridization chamber will remain moist for optimal hybridization conditions. Use the upper part to place the microarray slide during hybridization. One pipette box can hold up to three microarray slides, but for safety, do not use more then two slides and make sure the slides do not touch. In this way, the microarray slides will not be in contact with each other or with the moisturizing buffer. If this happens, capillarity might cause the hybridization buffer to be withdrawn from underneath the coverslip on the microarray.
5. When the aluminum cryotube is appropriately chilled, you will hear a sizzling noise. Wait until you hear this noise to interrupt the chilling process.
6. In general, not all tissue pieces are pulverized. This does not mean that you will not have enough RNA. When a piece of tissue does not completely pulverize, do not wait until it is completely pulverized because this can result in RNA degradation. It is better to work as quickly as possible. When using a piece of tissue with appropriate dimensions, you will generally have enough RNA left.
7. When mixing the pulverized tissue with the β-mercaptoethanol-containing RLT buffer, most of the tissue will be at the bottom of the RNAse-free tube, whereas the lysis buffer will be on top. It is important to mix the tissue powder and lysis buffer as quickly as possible. Therefore, while the lysis buffer

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RLT: RNA Lysis Buffer (QIAGEN)

is on top of the tissue powder, try to apply as much pressure as possible on the tissue powder by pushing the RNAse-free pestle into the tissue powder. In this way, the tissue powder will resolve more quickly.

8. When homogenizing the sample by passing it through a needle, be careful. By applying pressure to the syringe, your sample might be ejected forcefully from the needle into the RNAse-free tube. This can result in the loss of substantial amounts of the tissue homogenate.
9. Try not to transfer any tissue debris. In our experience, tissue debris can seriously impair the function of the RNeasy Mini spin columns, resulting in decreased yields of RNA. Therefore, first transfer a substantial portion of the supernatant (e.g., 500 μL) and then use finer pipette tips to transfer the remaining supernatant in smaller aliquots. In this way, the pellet remains largely undisrupted.
10. Try not to transfer any DNA precipitate to the RNeasy Mini spin columns. In our experience, the DNA precipitate seriously impairs the functioning of the RNeasy Mini spin columns, again resulting in decreased yields of RNA. Therefore, gently aspirate the sample while gently turning the tube. In this way, the DNA precipitate will stick to side of the RNAse-free 1.5-mL tube. This requires some expertise.
11. Do not transfer more than 650 μL into the spin columns at any point. Otherwise, when closing the tube, this will result in overflow.
12. Adjust the centrifugation times by taking into account the time a centrifuge needs to attain its programmed speed. This applies to every centrifugation step throughout the protocols.
13. Make sure the spin columns are completely empty after every centrifugation step. Otherwise repeat the step.
14. When eluting the RNA, place the pipette tip as close to the silica gel membrane as possible, approximately near the center of the membrane, without touching the membrane. Then forcefully eject the RNAse-free water onto the membrane. In this way, the water will be efficiently distributed over the entire membrane, resulting in a more efficient elution.
15. Alternatively, one can also elute the RNA using 30 μL RNAse-free water two times without reusing the first elute. In this way, the yield will be higher (approximately 15–30%) but the final concentration will be lower. For genomewide gene expression profiling using cDNA microarrays, one typically prefers high concentrations to high yield.
16. When filling the UVettes, place the pipette tip in a bottom corner and gently fill the UVette while holding the UVette slightly skewed. In this way, the UVette will be gradually filled

without the creation of air bubbles. Always check for air bubbles in the UVette before performing the measurements. If air bubbles are present, use a small pipette tip and very gently try to aspirate them. In addition, check for potential damage to the UVettes (scratches on the optical surfaces). If necessary, clean the optical surfaces.

17. When inserting the UVette into the BioPhotometer, make sure that the path of the light beam is in the same direction as the UVette shaft.
18. Choose an appropriate dilution. If you have a low volume of sample, you might want to increase the dilution factor in order to avoid spilling too much sample during the RNA quantification procedure. However, make sure the UVettes are always adequately filled (at least 50 µL for each UVette). Otherwise, the measurements will be incorrect.
19. The relation between the absorbance of 1 unit at 260 nm and 40 µg RNA/mL is valid only in water. The relation is based on an extinction coefficient calculated for RNA in water. When using a different diluent, make sure to adjust you calculations.
20. The filtered RNA gel matrix has to be used within 1 month of preparation. The gel–dye mix should be used within 1 week after its preparation and should be stored at 4°C when not used for more than 2 h. Protect the gel–dye mix from light, because when exposed to light the dye will degrade, which will reduce the signal intensity.
21. Although only 1 µL of sample is needed to run an RNA lab chip, heat-denature 2 µL. This will ensure you have at least 1 µL of sample left to run the RNA lab chip. Before starting heat-denaturation, spin the samples quickly to make sure the entire sample is at the bottom of the tubes.
22. Check that the chip priming station is in the right position for the RNA 6000 lab chips. The base plate should be at position “C” and the syringe clip should be at the topmost position.
23. To avoid air bubbles in the well of the lab chips, insert the pipette tip to the bottom of the well when dispensing the liquid.
24. Prior to dispersing the gel–dye matrix in the microchannels, using the syringe on the chip priming station turn over the lab chip. It is then fairly easy to see the air-filled microchannels. After dispersing the gel–dye matrix in the microchannels, turn the chip over to visually check appropriate dispersion of the gel–dye matrix in the microchannels. Normally, the microchannels should be less visible because they are filled with gel–dye matrix. If the microchannels remain (partially)

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air-filled, repeat the dispersion process using the syringe and plunger.

25. To disperse the RNA 6000 Nano Marker, use the technique of reverse pipetting. Usually a pipette has three “stops”: one stop to aspirate the appropriate volume, a second stop to remove any leftover from the pipette tip after ejecting the liquid, and a third stop to remove the pipette tip from the pipette. With reverse pipetting while aspirating, push the pipette to the second stop, and while ejecting push the pipette to the first stop. In this way, you will disperse the appropriate volume without creating air bubbles.
26. Do not touch the Agilent 2100 BioAnalyzer during an assay, as this can result in “ghost peaks”. These are false peaks in the electropherogram, which are not related to any RNA content. In fact, try not to touch the table during an assay.
27. If the electropherogram of the ladder is not as expected, one might check if the syringe is blocked. If this happens, the dispersion of the gel–dye matrix can be impaired, resulting in poor results. Normally, each RNA 6000 Nano LabChip kit is provided with an addition syringe.
28. If the electropherogram demonstrates a huge amount of fluorescence after the last peak (28S peak), DNA will be present in your sample . In this case it might be interesting to perform a DNA digestion step during RNA isolation.
29. Add the enzymes at the very end when preparing a master mix. Leave it as long as possible on ice. It is even preferable to make the master mix without the addition of the enzymes. Then add the master mix without the enzymes to all samples. Add the enzymes to the samples individually when they are already in the incubator.
30. When handling more than one sample, prepare the master mix by multiplying the given amounts of the individual components by the amount of samples. Add 5% of the final volume for each component in the master mix, to account for pipetting errors.
31. Be very careful when handling RNAse-H. The best option would be to reserve a part of the bench specifically for working with RNAses. In addition, leave RNAse-H on ice as long as possible.
32. It is important to cool down the thermal cycler block to 16°C before adding the reaction tubes, because subjecting the reactions to temperatures >16°C will compromise aRNA yield.
33. Some cDNA filter cartridge membranes can be broken. This can impair the cDNA purification step. Pay close attention to this fact before using a cDNA filter cartridge. In addition,

the membrane might be loose, which also impairs the cDNA purification step.

34. Infrequently, the volume will be less than 14 µL; if this is the case, add Molecular Biology Grade water to bring each sample to 14 µL. Mix well and store at –20°C.
35. The in vitro transcription reaction can best be performed overnight. Be sure not to incubate for longer than 14 h.
36. Make sure the incubator is at 37°C when incubating the sample. Allow some preheating time. The amount of time depends on the type of incubator used.
37. Check the drying process at regular intervals (every 5–10 min). Do not overdry your sample. When no drops are visible at the edges of the tube, the sample is usually dry enough.
38. Cy3 and Cy5 dyes are very sensitive to light. Try to shield them as much as possible when working with them. Use an aluminum foil to shield them from direct light when working (e.g., pipetting). Place the solutions containing Cy3 or Cy5 dye or labeled amino ally aRNA in a drawer, shielded with aluminum foil when incubating.
39. The Cy3 and Cy5 dyes are dissolved in DMSO. Be cautious when handling them. Use at least one pair of gloves; if possible use a second pair of gloves on top of the first pair.
40. When using a heat block to dry the pellet or to fragment and denature the labeled amino allyl aRNA, gently pour water into the holes of the heat block. This will improve the contact between the tube and the warm surface, resulting in faster and better results, particularly when denaturating the labeled amino allyl aRNA.
41. When forcefully pipetting up and down the hybridization solution, white foam should form. This is very important.
42. When applying a volume of 38 µL of hybridization solution onto the coverslip, aspirate 40 µL, and when ejecting do not try to remove the excess hybridization solution from the pipette tip by pushing the pipette to the second stop. This will create air bubbles.
43. If air bubbles persist, use a small pipette tip to gently remove them by pushing them outward to the edge of the drop of hybridization solution.
44. Place the microarray in the hybridization chamber horizontally. If not, the hybridization solution will pile up at one edge of the microarray, resulting in poor hybridization at the opposite edge.
45. When acquiring the images, adjust the settings of the photomultiplier tubes of the scanner in such a way that the overall intensities in both channels (e.g., Cy3 and Cy5) are approxi-

mately equal. This is important for the normalization steps later on.

46. When scanning the microarray slide, it is possible that there is quite a lot of background. Repeating the wash steps performed after the hybridization and prior to the scanning can solve this problem.
47. Avoid repeating the scanning too much. This will result in substantial photobleaching, particularly for the Cy5 dye.
48. While performing the microarray hybridization and washing steps, avoid exposing the labeled amino allyl aRNA to light. Always try to shield the tubes containing the labeled amino allyl aRNA using an aluminum foil.
49. The assumption that all spots on a cDNA microarray are circular is wrong. Other more advanced segmentation procedures exist. The choice for a segmentation procedure is completely user dependent.
50. The choice for background correction is completely user dependent. Background correction is usually carried out to acquire a better estimate of the signal fluorescence intensity, but it will also introduce substantial variance. The best option is to use a global background correction method instead of subtracting a locally estimated background for each spot.

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