Optimization of total RNA extraction from Human Post-mortem Brain Tissue

Introduction

The success of gene expression applications will depend on the isolation and purification of intact cellular RNA from human post-mortem brain tissue. The integrity of RNA can be affected by biological and clinical factors which include the agonal state of the individual, post-mortem delay and the method and duration of storage of brain tissue before it is used for RNA isolation. Most of these are factors are beyond our control, but we can take further steps to ensure that we retrieve the best quality RNA from the available material. These will include preventing further degradation of the cellular RNA by endogenous and exogenous RNases by proper handling of tissue before and during experiments as well as using a protocol that is most appropriate for post-mortem brain and proper storage of the extracted RNA. In order to achieve these objectives we provide what we consider to be an optimal procedure, including several options. We also provide some assessment of advantages and disadvantages of a number of optional methods.

Initial preparation

In addition to the method used, a policy of avoiding RNase contamination is crucial to the successful extraction of good quality RNA. The following guidelines in general will help in avoiding contamination of your RNA sample by exogenous RNases.

- Always wear clean lab coat and gloves during the RNA isolation procedure and when handling materials and equipment involved in the method. Be aware of contaminating surfaces and equipment and change gloves whenever necessary. It is good practice to wear a clean pair of gloves before taking out tubes, eppendorfs etc. to be used in the RNA isolation procedure
- If possible have a separate work area, equipment, pipettes, tubes etc. for RNA work
- Before starting work, clean the lab bench, pipettors and other equipment with RNase decontamination solutions/ wipes (eg. RNase Zap) and rinse well with nuclease-free water. Clean up the work area after use
- Use molecular biology grade ethanol, solutions and other reagents in the isolation procedure

BrainNet Europe II

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Content

- All tubes and containers including those used for homogenising tissue, storing tissue and microcentrifuge tubes should be RNase-free. Only use RNase-free filtered pipette tips. Wherever possible use sterile disposable plastics. If using glassware, decontaminate by baking at 250°C overnight

Equipment

- Cryostat or medical chisel and hammer
- Biological safety cabinet
- Refrigerated laboratory centrifuge for 50ml and 15ml falcon tubes and speed up to 6500rpm.
- Rotor-stator homogeniser
- Gel electrophoresis equipment
- UV spectrophotometer/ Nanodrop spectrophotometer/ Agilent 2100 Bioanalyser

Recommended sterile disposable labware and solutions

It is recommended that you use disposable nuclease free labware whenever possible as this will reduce the risk of contamination by RNases

- Sterile 50ml and 15ml falcon tubes
- Nuclease free microcentrifuge tubes and tubes for storage of RNA
- Sterile Petri dishes, sterile disposable scalpels and plastic forceps for dissecting and handling tissue
- Sterile disposable pipettes, pastettes and nuclease-free filtered pipette tips
- Commercially available nuclease free water or DEPC treated water and molecular biology grade Ethanol
- Virkon to decontaminate labware coming into contact with brain tissue

PROTOCOL FOR EXTRACTING TOTAL RNA FROM POST-MORTEM BRAIN TISSUE

Fresh Tissue

At the time of post-mortem dissection, select the regions of interest and section them into approximately 0.5-1.0 cm cubes. These can either be used immediately for RNA extraction or snap frozen for future use. If using fresh tissue, dissect, weigh and put the tissue immediately into the lysis solution, homogenise and carry on with the rest of the protocol.

Frozen Tissue

When using frozen tissue it is very important that you do not let the tissue thaw at any stage in the procedure till it is homogenised in the lysis solution. Therefore, retrieve, carry and keep your samples on dry ice all the time and cool the tubes, Petri dishes or any other container on dry ice before putting your samples in them. Continue with the procedure as quickly as possible.

Retrieving brain tissue from the freezer

- Mark the container you are using to put the tissue in, with the sample ID
- Get dry ice in polystyrene box with lid and put the empty container on dry ice
- Use RNase free forceps (cleaned with RNase Zap and RNase free water or sterile disposable plastic) to pick out the tissue block and place it in the empty cooled container on dry ice and keep it frozen

Dissection of frozen brain tissue

We have tried out three different ways of dissecting samples from frozen tissue blocks and all three methods have given good results with subsequent RNA extractions. (Always have the tubes and containers ready labelled and cooled on dry ice to put in the dissected tissue). If you are dissecting multiple samples, take out one sample at a time and leave all the others on dry ice.

If you do not wish to proceed immediately with the RNA extraction procedure, store the dissected tissue as mentioned under storage for each method.

DNA Preservation

1. The simplest method is to use a medical chisel and hammer to break up the tissue. Put a brass plate in a large box of dry ice. Place the block of frozen tissue in an appropriately sized polythene bag (freezer food bags/biological sample bags which can be sealed), place it on the cooled brass plate and chip off the necessary amount of tissue with the chisel and hammer.

Advantages: Easy, time saving and inexpensive; more tissue left for future use; can even be used for brain tissue stored as slices

Disadvantages: Difficult to separate grey and white matter

Storage: -70 to -80° C (2ml cryotubes recommended)

2. The tissue blocks can be dissected in the cryostat at -16° C to-15° C (clean the cryostat before use). Fix the tissue on the cryostat chuck and cut a small number sections until grey and white matter are clearly visible. Cut thick sections (Using a clean disposable scalpel and forceps dissect out the tissue.

Advantages: We consider this to be the best method if you need to dissect precise areas and separate grey and white matter. It has the added advantage that sections can be taken at the same time for immunohistochemistry

Disadvantages: Time consuming, and a lot of tissue can be lost

Storage: -70 to -80° C (2ml cryotubes recommended)

3. Dissecting the frozen tissue after thawing in RNAlater-ICE (Ambion). RNAlater-ICE is a solution in which snap frozen tissues can be soaked until they thaw and become easy to dissect at room temperature without loss of RNA integrity. The tissue is placed in an appropriate volume of RNAlater-ICE pre-chilled at - 70° or -80° C and left at -20° C for at least 16hrs before the dissections are carried out once the tissue has become soft.

It is recommended by the manufacturer that the frozen tissue should not be larger than 0.5cm in the smallest dimension and 10 volumes of RNAlater-ICE compared to the sample mass (eg. 2.5ml for a 250mg sample) should be used.

We have soaked whole blocks of tissue weighing between 2-8g in 25ml of RNAlater-ICE for 7-10 days (tissue can be left in RNAlater-ICE at -20° C for up to 6 months with no loss of RNA integrity) and obtained good results with RNA extractions. Make sure you use a wide enough container (Sterilin -125CP- 60ml containers are recommended) and that the tissue is fully immersed and left for a sufficient length of time for the solution to fully permeate the tissue. After transition in the solution, tissue can be dissected at RT for up to 30 minutes without affecting the RNA. However, we recommend that you dissect the tissue in a sterile Petri dish or similar container on a tray of dry ice to minimise the risk of degradation if there is a time delay. Make sure that you do not add any of the RNAlater-ICE solution to the homogenising solution.

Advantages: Easy to manipulate the tissue and no risk of RNA degradation due to the tissue thawing

Disadvantages: Expensive and due to the blue colour of the solution it is not easy to distinguish between grey and white matter. In addition, the left over tissue can't be used for any other procedure

Storage: -20° C in an appropriate volume of RNAlater-ICE (2ml cryotubes recommended)

Weigh the tissue

After dissecting the tissue using one of the above methods, weigh the tissue as quickly as possible, and put back on dry ice. (Remember that small tissue samples thaw quite fast). To minimise the risk of thawing, weigh the empty tubes first and leave them on dry ice before starting the dissections. Add the dissected tissue to the tube and weigh. Determine the volume of lysis reagent depending on the weight of the tissue. The volume of Qiazol lysis reagent should be at least 10 times the weight of tissue sample (eg. 1ml for up to 100mg of tissue)

Homogenising brain tissue using a rotor stator homogeniser

Good recovery of RNA depends on the efficient disruption and homogenisation of the brain tissue. Best results are obtained when using a rotor-stator homogeniser which simultaneously disrupts and homogenises the brain tissue in the presence of the lysis buffer. There are several types of rotor-stator homogenisers some of which are from the following suppliers

- Polytron : Kinematica AG (We use this instrument)
- Tissue – Tearor: Bio-Spec Products

- Ultra Turrax: IKA Analysentechnik
- OMNI: OMNI International Inc.
- Tissuemizer: Tekmar Inc.

As foaming occurs during homogenisation, it is important to use a tube large enough to accommodate this, depending on the size of the probe diameter of the homogeniser and the volume of lysis buffer used. Homogenisation of brain tissue should be carried out in a biological safety cabinet. Clean the homogeniser with 100% ethanol and nuclease-free/DEPC treated H2O before starting and repeat the process after each sample and finally rinse with ethanol after use.

The isolation of total RNA from up to 500mg of post-mortem brain tissue

After evaluating several reagent kits and protocols available for RNA isolation, we have found that the Qiagen RNeasy lipid tissue kit is best for brain tissue as it gave good yield and quality from both grey and white matter tissue samples. We have chosen the 'midi' kit as it allows extraction of sufficient quantities of RNA for several applications as well as for different methods of testing. Additionally, the weight of tissue can vary within a large range and therefore is easier to dissect without having to stick to a strict weight limit. We strongly feel that for a process of standardisation, a kit based method with an easy to follow protocol is the best option.

Equipment, reagents and consumables

- Refrigerated centrifuge (capable of 6500rpm) for 50ml and 15ml falcon tubes
- Rotor-stator homogeniser
- Qiagen RNeasy lipid tissue midi kit (catalogue# 74804)
- Qiagen RNase-free DNase set (catalogue# 79254)
- Molecular biology grade ethanol
- RNase free water
- 70% Ethanol
- Chloroform
- Sterile 50ml and 15ml falcon tubes
- Nuclease free 1.5ml and 0.5ml microcentrifuge tubes

- RNase free tubes for storage of RNA
- The lysis solution, buffers and RNase free water for elution are provided with the kit

It is recommended that up to a maximum of four samples are processed at any one time.

Before starting the homogenisation cool the refrigerated centrifuge to 4°C. Clean the homogeniser with 100%Ethanol and Nuclease-free/DEPC treated H2O

This basically follows the manufacturer's protocol with a few additions we found useful.

1. Place the weighed tissue (up to 500mg) in 5ml of Qiazol lysis solution (contains phenol and guanidine thiocyanate) in a 50ml falcon tube and homogenise immediately at maximum speed for 30 seconds. (The volume of Qiazol can vary, but has to be at least 10 times the weight of tissue; eg. 1ml for 100 mg).

2. Place tube containing homogenate at RT for 5 min to promote dissociation of nucleoproteins.

3. Centrifuge at 5000g for 10min at 4°C to separate the cellular debris.

4. Using a clean pastette, transfer the supernatant into a clean 15ml falcon tube.

5. Add 1ml (20% of the volume of Qiazol) chloroform to the tube, replace the cap securely and shake it by hand vigorously for 15 seconds. Place tube at RT for 2-3 minutes.

6. Centrifuge at 6500rpm for 25min at 4°C for phase separation. During this centrifugation and have one labelled 15ml falcon tube and one Qiagen midi spin column-collection tube assembly ready for each of the samples. Once the centrifugation is complete, set the centrifuge temperature at 20-25°C for the next centrifugation step.

7. Carefully transfer the colourless upper aqueous phase to a new 15ml falcon tube, avoiding the material that has collected at the interphase. Leave the tube on ice if the centrifuge has not reached 20°C, and add ethanol only after the centrifuge has reached this temperature. Add 1 volume of`70% ethanol (equal to the volume of transferred aqueous phase) and mix thoroughly by vortexing (do not centrifuge). Proceed immediately to the next step.

8. Transfer 4ml of sample into the spin column, close tube gently and centrifuge for 5min at 3000-5000g at 20-25°C. Discard flow through.

[The flow through is not compatible with acidic solutions or bleach. If spilt clean with laboratory detergent and water and then with 1 %(v/v) sodium hypochlorite]

9. Repeat step with remainder of the sample.

For highly sensitive applications such as RT-PCR, an additional DNase step with the recommended RNase free DNase set can be performed at this stage. For other applications, follow the rest of the protocol.

10. Add 4ml Buffer RW1(contains a guanidine salt and ethanol) to the column, close gently and centrifuge for 5min at 3000-5000g to wash the column and discard flow through [not compatible with bleach]

11. Add 2.5ml Buffer RPE (contains ethanol) to the spin column, close gently and centrifuge for 2min at 3000-5000g and discard flow through.

12. Repeat step with centrifugation for 5min. Discard flow through, put the spin column back on the collection tube and centrifuge at 5000g for 1 minute to completely dry the silica membrane and prevent ethanol carry-over.

13. To elute the RNA, transfer column to a new collection tube, pipette appropriate amount of RNase free water (we recommend a volume of 150 µl for up to 500mg of tissue) directly onto the membrane and let stand for 1min. Centrifuge for 3min at 3000-5000g.

14. Repeat elution step (with an equal volume of water) into the same collection tube.

15. Reserve an aliquot of each sample in a 0.5ml RNase-free microcentrifuge tube stored at -20°C to check the quality and quantity. To avoid multiple freeze-thaw cycles and contamination, store the rest of the sample as aliquots at -80°C for long term use.

Check the quality and quantity of RNA by one or a combination of the following methods

- Gel Electrophoresis
- UV spectrophotometer
- Nanodrop spectrophotometer
- Agilent Bioanalyser

The best currently available piece of equipment to check RNA is the Agilent 2100 Bioanalyser. In the absence of this, a combination of gel electrophoresis and a spectrophotometric method should be used.

Gel Electrophoresis: Formaldehyde-agarose gel

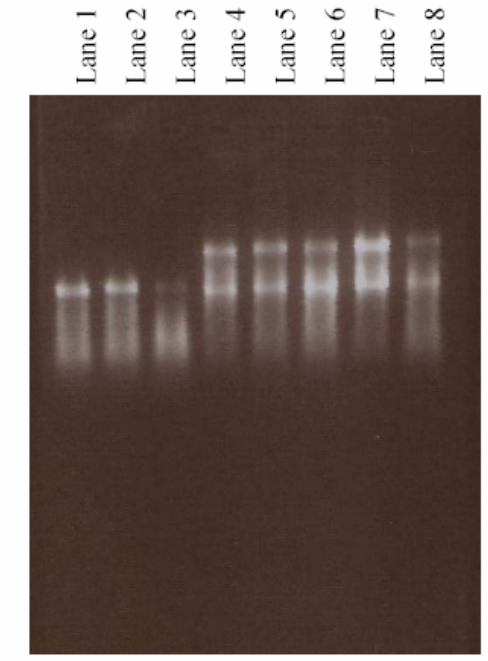
The most common method used to assess the integrity of total RNA is electrophoresis of an aliquot of the RNA sample under denaturing conditions using agarose/formaldehyde gels and the MOPS buffer system with ethidium bromide staining.

Intact total RNA run on a denaturing gel will have sharp, clear 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact. Partially degraded RNA will have a smeared appearance, will lack the sharp rRNA bands, or will not exhibit the 2:1 ratio of high quality RNA. Completely degraded RNA will appear as a very low molecular weight smear.

Drawbacks: Generally, at least 200 ng of RNA must be loaded onto a denaturing agarose gel in order to be visualized with EtBr. The appearance of the RNA bands will be affected by electrophoresis conditions, amount of RNA and saturation of EtBr. It is not possible to get an accurate concentration of the RNA.

Figure 1. Formaldehyde agarose gel electrophoresis with EtBr staining showing the varying quality of RNA from different samples.

Lane 7 – intact total RNA; Lanes 4, 5, 6 and 8 – slight degradation; Lanes 1and 2 – partial degradation; Lane 3 – totally degraded RNA



UV spectrophotometer

The concentration and purity of RNA can be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. To ensure significance, A260 readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A260=1 ,40µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. When measuring RNA samples, be certain that cuvettes are RNase-free. Use the buffer in which the RNA is diluted to zero the spectrophotometer. Usually a 1 in 100 dilution of the RNA sample is analysed.

The ratio between the absorbance values at 260 and 280 nm gives an estimate of.

RNA purity. A ratio of ~2.0 is generally accepted as "pure" for RNA. However, we have observed an A260/A280 ratio of ˜ 1.6 for good quality RNA (dilution of the sample could be the reason for this as we observed with the Nanodrop, see below). If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Drawbacks: Contamination of cuvettes can lead to false values. It is not possible to either visualise the RNA or detect DNA contamination.

Nanodrop spectrophotometer

The Nanodrop ND-1000 UV-Spectrophotometer enables accurate analysis of extremely small (1-2 ml) samples with remarkable reproducibility. It eliminates the need for cuvettes and capillaries and no dilutions are needed for most samples. It is easy to set up and the measurements can be done within 10 seconds.

We analysed non-diluted samples against, 1/5, 1/10 and 1/100 dilutions of the same samples and observed that, whereas the non-diluted samples gave A260/A280 ratios between 1.9 -2.0, once diluted, these same samples gave ratios between 1.5-1.6 as shown in the table below.

Sample ID	Date	Time	ng/µl	260/280
1	06/12/2004	11:59	2139.59	2.09
1;5	06/12/2004	12:14	533.09	1.94
1;10	06/12/2004	12:16	252.57	1.92
1;100	06/12/2004	12:19	26.14	1.6
1;100	06/12/2004	12:22	26.66	1.62
1;100	06/12/2004	12:29	26.56	1.57

Drawbacks: Inability to visualise the quality of RNA and cost of instrument.

Agilent Bioanalyser

The Agilent 2100 Bioanalyser (Agilent Technologies) integrates the quantitation of RNA samples with quality assessment in one quick and simple assay. As little as 1µl of 25ng/µl is required per analysis, which simultaneously assesses RNA integrity, concentration, and purity. Data can be displayed as an electropherogram, a gel-like image, and in tabular format.

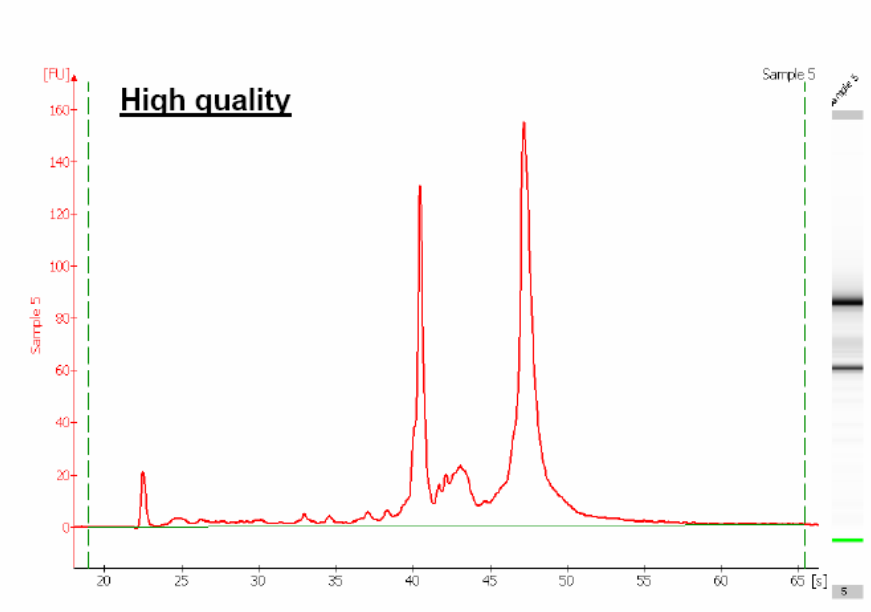


Figure 2. Electropherograms and corresponding gel images obtained with the Bioanalyser showing the quality of RNA

Drawbacks The Agilent 2100 Bioanalyser is an expensive piece of equipment (15,000 Euros)

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