# METHODS FOR ISOLATION OF RNA AND/OR DNA FROM FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE SAMPLES

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There are numerous reports in the literature describing methods for extraction of RNA and/or DNA from formalin-fixed paraffin-embedded (FFPE) tissue samples. This Technical Bulletin references a small number of these reports and includes MRC recommendations. The methods addressed here include paraffin removal, proteinase K digestion, and subsequent RNA and/or DNA extraction. Instruments, tubes and solutions should be RNase-free whenever RNA isolation is the final goal.

## PARAFFIN REMOVAL

This step isn't necessary if samples are only formalin-fixed and not embedded in paraffin.

### Xylene Removal of Paraffin

Numerous articles list versions of this method with slight alterations. What follows is a generally accepted method which can be further altered for specific applications. Before chemical deparaffinization, remove excess paraffin with a blade when possible. Use a polypropylene microcentrifuge tube. Procedures are performed at room temperature.

1. Add 1 ml of xylene; vortex and store for 10 min; centrifuge at 14,000 g x 10 min; remove supernatant.
2. Repeat.
3. Add 1 ml of 100% ethanol; vortex and store for 10 min; centrifuge at 14,000 g x 10 min; remove supernatant.
4. Repeat.
5. Add 1 ml of 80% ethanol; vortex and store for 10 min; centrifuge at 14,000 g x 10 min at; remove supernatant.
6. Repeat.
7. Add 1 ml of 50% ethanol; vortex and store for 10 min; centrifuge at 14,000 g x 10 min at; remove supernatant.
8. Repeat.
9. Add 1 ml of DEPC-treated water and store overnight at 4C.

### Hemo-De Removal of Paraffin

Loudig et. al. (2007) used Hemo-De as an alternative to avoid using xylene. Krafft, et. al. present a similar procedure. Use a polypropylene microcentrifuge tube. Procedures are performed at room temperature unless otherwise noted.

1. Add 0.5 ml of Hemo-De; vortex and store on an agitator for 10 min; centrifuge at 12,000 g x 10 min; remove supernatant.
2. Repeat 2 more times.
3. Add 1 ml 100% ethanol; vortex and store for 10 min; centrifuge at 12,000 g x 10 min; remove supernatant.
4. Repeat 2 more times.
5. Add 1 ml 95% ethanol; vortex and store for 8 min on ice; centrifuge at 12,000 g x 10 min; remove supernatant.
6. Repeat 2 more times.
7. Wash pellet with 1.0 ml of PBS; centrifuge at 12,000 g x 10 min; remove supernatant.
8. Rehydrate pellet in 200 µl of PBS with 6.5 µl of RNase-Out (Invitrogen) for 90 min on ice.

### Abbreviated Protocol Using Octane and Methanol

Fredricks and Relman (1999) provide an abbreviated protocol for paraffin removal. Use a polypropylene microcentrifuge tube. Procedures are performed at room temperature. (We found this method to work well at MRC.)

1. Add 1.0 ml octane; vortex for 10 sec at maximum speed; store at room temperature for 10 min with periodic mixing.
2. Add 0.1 ml methanol; remix by vortexing for 10 sec at maximum speed.
3. Centrifuge the sample at 7,000 g x 2 min; remove the upper octane layer.
4. Remove the remaining methanol.
5. Invert the tube to drain excess solution and remove remaining solution with a micropipette tip.
6. Do not vacuum dry the sample. The sample is ready for proteinase K digestion or RNA/DNA isolation.

## PROTEINASE K DIGESTION

Many investigators follow paraffin removal with proteinase K digestion prior to RNA or DNA isolation. As with deparaffinization protocols, there are numerous reports and manuals describing proteinase K digestion. Investigators may vary the length of digestion as well as digestion solution parameters and amount of enzyme. After deparaffinization, sample rehydration with PBS may improve results of subsequent RNA or DNA isolation. Loudig, et. al., Kotorashvili, et. al., Ghatak, et. al. And Krafft et. al.

present different examples of proteinase K digestions used for FFPE specimens. Proteinase K digestion isn't required when using TRI Reagent® or RNAzol® RT for RNA isolation but it will increase yields. Please see below for an example of proteinase K digestion in the presence or absence of DNAzol® Reagent.

**RNA ISOLATION USING TRI REAGENT® OR RNAZOL® RT**

In reports referenced below, investigators performed proteinase K digestion of samples prior to acid guandine thiocyante - phenol extractions for RNA isolations. At MRC we performed TRI Reagent® extractions to obtain RNA from deparaffinized samples without enzymatic pre-treatment according the procedure described here.

1. After removal of paraffin and any remaining solution, add 0.5 - 1.0 ml of TRI Reagent® or RNAzol® RT to the sample.
2. Supplement with 1 - 3 µl of Polyacryl Carrier (PC 152).
3. Homogenize completely until the sample is suspended in the reagent.
4. Remix periodically for 15 - 60 minutes. Heat samples as required to increase yield. Do not exceed 55 C. Time and heat may improve RNA recovery depending the application and must be determined in the investigator's laboratory.
5. Follow the TRI Reagent® or RNAzol® RT protocol as written for RNA isolation.

**DNA ISOLATION**

**DNA Isolation using TRI Reagent®**

Following RNA isolation, recover the DNA pellet from the remaining organic phase according to the manufacturer's protocol. Kotorashvili, et. al. and Ghatak, et. al. describe proteinase K digestion of the DNA pellet prior to DNA precipitation steps. Otherwise, follow the DNA isolation procedure in the TRI Reagent® protocol.

**DNA Isolation using DNAzol® Reagent.**

1. Suspend or homogenize a deparaffinized sample in 1.0 ml of DNAzol®.
2. Add 20 µl of proteinase K (20 mg/ml stock solution) and incubate at room temperature overnight.
3. Optionally, add 4 µg of RNase for the last hour of the incubation.
4. Centrifuge at 10,000 g x 10 min x 4 C. Remove the supernatant.
5. Precipitate the DNA and wash the pellet as described in the DNAzol® protocol.
6. For DNA yields less than 5 µg, add 1 - 3 µl of Polyacryl Carrier (MRC PC 152) during precipitation.
7. To perform proteinase K digestion prior to DNAzol® extraction: Resuspend the sample in 30 - 50 µl of buffer containing 50 mM Tris-HCl pH 7.5 - 9.0, 1 mM CaAcetate, 0.5% SDS and 1.0 µl proteinase K (20 mg/ml stock solution). Incubate overnight at 50 - 55 C or for 3 hours at 65 C. Add RNase as above prior to adding 1.0 ml of DNAzol. Resuspend the sample in DNAzol® and centrifuge to remove remaining particulate matter. Precipitate DNA and wash the pellet according to the DNAzol® protocol.

**DNA Isolation using DNAzol® Direct**

This protocol works well with small FFPE tissues. Use 30 µl of DNAzol® Direct per mg of FFPE tissue. A thermal cycler works well for heating of samples. Templates up to about 300 bp amplify without any additional steps or pretreatment.

1. Remove excess paraffin with a blade. Add to 30 - 50 µl of DNAzol® Direct to the sample.
2. Heat the sample at 95 C for 10 min to melt the paraffin and solubilize the DNA.
3. Immediately vortex the hot sample to break the tissue and disperse the sample in the reagent.
4. Allow the sample to cool until the paraffin solidifies on top of the DNAzol® Direct lysate.
5. Break through the solid paraffin with a pipet tip to remove the lysate and transfer it to a clean tube.
6. Use the DNAzol® Direct lysate as template in a PCR reaction without any further treatment.
7. To increase the DNA concentration of the final product, use less DNAzol® Direct per sample volume.
8. To increase the yield when isolating small fragments, (a) increase the temperature and time of incubation up to a maximum of about 110 C and 15 minutes, (b) add 2 - 4 µl of 1M KOH to the incubation, (c) deparaffinize the sample , or (d) perform proteinase K digestion.
9. To increase the yield of larger fragments, increased time, temperature or KOH may degrade the product. Paraffin removal and proteinase K digestion will increase the yield of larger fragments. Use the octane/methanol procedure described above for paraffin removal. Then resuspend the sample in 27 µl of digestion buffer described in the DNAzol® procedure step 7. Heat dried samples for 10 minutes at 95 C to rehydrate and briefly centrifuge to collect the condensate. Add 3 µl of proteinase K (10 mg/ml stock solution) and mix. Incubate for 1 - 3 hours until digestion is complete. Centrifuge at 12,000 g for 5 minutes at room temperature. Mix 1 volume of the supernatant with 10 volumes of DNAzol® Direct by vortexing. Use 1 - 5 µl as template in a 50 µl PCR reaction volume.

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