PeptiMatrix™
Next generation peptide hydrogels

STANDARD OPERATING PROCEDURE

RNA EXTRACTION FROM CELLS IN PEPTIMATRIX™ HYDROGELS

1. BACKGROUND

This SOP covers 2 separate protocols for extracting RNA from cells encapsulated in hydrogels.

Method A using a commercial kit from Qiagen (RNeasy® Mini Kit) and **Method B** that does not require a kit, using an all-in-one acid guanidinium thiocyanate-phenol solution TRIzol® and isopropanol for the precipitation step.

Depending on the hydrogel functionalisation and kit/reagent availability, you must consider which method is best (see Table 1). The general steps of both protocols include homogenization/lysis of cells or tissues, extraction of RNA, precipitation, and resuspension. The purified RNA is ready for use in downstream applications such as RT-PCR and real-time RT-PCR, cDNA synthesis and microarrays.

Product name	Product code	Method A compatibility	Method B compatibility
PeptiMatrix Core	PMCORE	Y	Y
PeptiMatrix RGD	PMRGD	Y	Y
PeptiMatrix IKVAV	PMIKVAV	N	Y
PeptiMatrix Plus	PMPLUS	Y	Y

Table 1 - Compatibility table of each type of gel functionalisation with both methods of RNA extraction

2. RISK ASSESSMENT

Always follow your organisation's laboratory safety procedures.

Work inside an appropriate microbiology safety cabinet for your cell type. Refer to the **PeptiMatrix Safety Data Sheet (SDS)** for detailed safety, handling, storage, and first aid information relating to the hydrogel components.

If you are working with additional cell lines, media supplements, matrix additives, or other reagents consult the relevant SDS documents for those materials as well.

METHOD A - RNeasy® Mini Kit

A3. MATERIALS

- Certified chemical fume hood
- Cells encapsulated in PeptiMatrix hydrogels (typically combine 3-10x wells in 96 well plate for good yield)
- Microcentrifuge that reaches 8,000 x g
- Vortex
- Molecular biology grade ethanol 100% (e.g. Ethanol, Absolute (200 Proof), Molecular Biology Grade, Fisher BioReagents™ #16685992)
- RNeasy® Mini Kit (Qiagen #74104)
- RNase free water (e.g. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water #11538646)
- RNase free 1.5mL microcentrifuge tubes
- RNase free pipette tips (p10, p200, p1000)
- Pipettes (p10, p200, p1000)
- -80°C freezer
- Ice
- RNase Decontamination Solution (e.g. RNaseZap™ #AM9780 or RNase AWAY™ #10666421)

Optional materials:

- 14.3 M β-mercaptoethanol (β-ME) – if purifying RNA from cell lines rich in RNases, it is recommended to add 10 µl β-ME per 1mL Buffer RLT before use. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.

A4. METHODS

A4.1 Preparation before extraction

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using. RNeasy Mini Spin Columns have a maximum binding capacity of 100 µg RNA and recommend a maximum amount of starting material of 1 x 10^7 cells for efficient lysis to prevent cell debris interfering with the column. If you have more starting material than this, consider splitting sample into 2x spin columns to obtain an optimal RNA yield and purity, then after the final stage pool RNA together or use a further concentration step.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

Carry out all steps in chemical fume hood:

1. Clean chemical fume hood to remove potential ribonuclease enzymes which can rapidly degrade RNA using decontamination solution.
2. Prepare 70% ethanol with 100% ethanol + RNase free water in RNase free centrifuge tube.
3. Prepare a working solution of Buffer RPE concentrate by adding 4 volumes of 100% ethanol as indicated on the bottle.
4. Optional step: If purifying RNA from cell lines rich in RNAses, it is recommended to add 10 µl β-mercaptoethanol (β-ME) per 1mL Buffer RLT before use. Buffer RLT containing β-ME can be stored at room temperature for up to 1 month.

A4.2 RNA extraction

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

Carry out all steps in chemical fume hood:

1. Remove plate from incubator and remove all media carefully, avoiding disturbing the gel.
2. Add 100 µL **RLT Buffer** to each well and pipette up and down vigorously to lyse cells.
3. Combine wells into an RNase free tube and vortex for 1 minute.
4. Add an equal volume of **70% ethanol** and mix well by pipetting.
5. Add volume to an RNeasy Mini Spin Column placed in a Collection Tube and centrifuge at 8,000 x g for 15 seconds.
    - An RNeasy Mini Spin Column can hold up to 700 µL in volume, so if the total volume of gel/RLT/ethanol is over 700 µL, multiple spins can be done. After the first centrifuging, discard the flow through into phenol waste, load another 700 µL and repeat.
6. Once all sample volume has moved through the Spin Column, move the Spin Column to a new RNase free Collection Tube.
7. Add 700 µL **RW1 Buffer** and centrifuge at 8,000 x g for 15 seconds. Discard flow through.
8. Add 500 µL **RPE Buffer** and leave to stand for 1 minute, then centrifuge at 8000 x g for 15 seconds. Discard flow through.
    - Repeat this step 3x times to remove guanidine salts.
9. Add 500 µL **RPE Buffer**, then centrifuge at 8000 x g for 2 minutes. Discard flow through.
10. Centrifuge at 8000 x g for 1 minute to dry membrane.

11. Place spin column into an RNase free tube. Add 30-50 µL RNase free water and leave for 5 minutes.
12. Centrifuge at 8000 x g for 1 minute to elute RNA.
    - If desired, quantify RNA concentration and purity, typically using UV-Vis spectrophotometry (e.g. NanoDrop) or highly selective fluorescent dyes (e.g. Qubit).

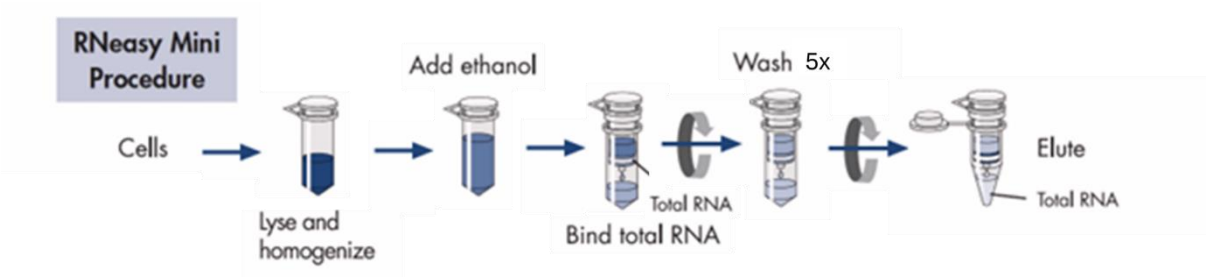

Figure 1 - Schematic of the different steps in RNA extraction Method A (adapted from the RNeasy Mini Handbook)

METHOD B - Trizol® + chloroform method

B3. MATERIALS

- Certified chemical fume hood
- Cells encapsulated in PeptiMatrix hydrogels (typically combine 3-10x wells in 96 well plate for good yield)
- Temperature controlled centrifuge/microcentrifuge that reaches 4°C and 12,000 x g
- Vortex
- TRIzol® reagent (#15596026)
- Chloroform/isoamyl alcohol (49:1) (e.g. Sigma BioUltra Chloroform – isoamyl alcohol mixture 49:1 #25668)
- Molecular biology grade 2-Propanol >=99.5% (e.g. Sigma 2-propanol #I9516)
- Molecular biology grade ethanol 100% (e.g. Ethanol, Absolute (200 Proof), Molecular Biology Grade, Fisher BioReagents™ #16685992)
- RNase free water (e.g. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water #11538646)
- RNase free 1.5mL microcentrifuge tubes
- RNase free 15mL centrifuge tubes
- RNase free pipette tips (p10, p200, p1000)
- Pipettes (p10, p200, p1000)
- -80°C freezer
- Ice
- RNase Decontamination Solution (e.g. RNaseZap™ #AM9780 or RNase AWAY™ #10666421)

B4. METHODS

B4.1 Preparation before extraction

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

Carry out all steps in chemical fume hood:

1. Turn centrifuge/microcentrifuge to 4°C.
2. Clean chemical fume hood to remove potential ribonuclease enzymes which can rapidly degrade RNA using decontamination solution.
3. In chemical fume hood, prepare 75% ethanol with 100% ethanol + RNase free water in RNase free centrifuge tube.

B4.2 RNA extraction

This procedure can be scaled up or down depending on the number of wells you plan to prepare, and which well plate size you are using.

For reference, the steps below describe the process using cells encapsulated in **100 µL of hydrogel** per well plated into a **96-well plate**.

Carry out all steps in chemical fume hood:

1. Remove plate from incubator and remove all media carefully, avoiding disturbing the gel.
2. Add 1 mL **TRIzol®** reagent per 1 x 10^7 cells (~100 µl per well of a 96 well plate).
3. Leave samples for 5 minutes at room temperature to allow dissociation of nucleoprotein complexes.
4. Combine sample into RNase free microcentrifuge/centrifuge tube.
5. Add 0.2 mL **chloroform/isoamyl alcohol per** 1 mL TRIzol®.
6. Shake vigorously by hand for 10 seconds.
7. Incubate sample on ice for 3 minutes.
8. Centrifuge samples for 15 minutes 12,000 x g at 4°C to separate RNA from rest of lysate.
    - The sample will have 3 layers (see Figure 2): clear aqueous layer = contains RNA, fluffy white interphase layer = cell debris, red organic layer = contains phenol and chloroform.

9. Transfer top aqueous layer to new RNase free microcentrifuge/centrifuge tube (be careful to not collect any of the interphase layer).
10. Add an equal volume of **2-Propanol**.
11. Incubate for 1 hour at -20°C or overnight at -80°C for an increased RNA yield.
12. Centrifuge for 20 minutes at 10,000 x g at 4°C.
13. Discard supernatant, leaving a gel-like white pellet of total RNA at the bottom of the tube.
14. Wash the RNA by resuspending the pellet in 0.5-1 mL 75% ethanol and vortexing for a few seconds.
15. Centrifuge for 5 minutes at 10,000x g at 4°C.
16. Discard supernatant (repeat wash step if desired).
17. Resuspend RNA pellet in desired volume of RNase free water.
    - If desired, quantify RNA concentration and purity, typically using UV-Vis spectrophotometry (e.g. NanoDrop) or highly selective fluorescent dyes (e.g. Qubit).

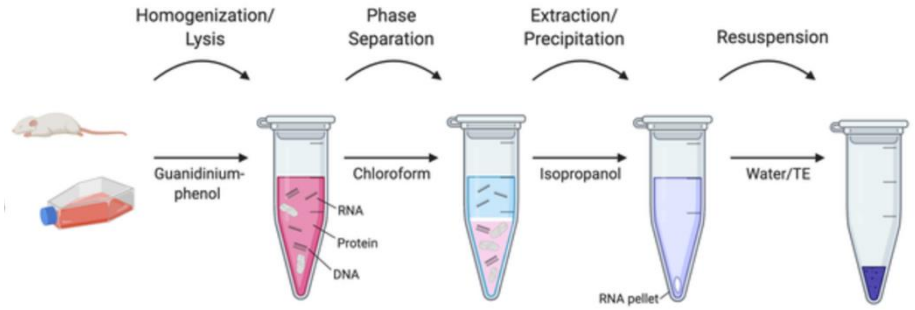

Figure 2 - Schematic of the different steps in RNA extraction (taken from Addgene: https://www.addgene.org/protocols/kit-free-rna-extraction/)

5. DISPOSAL

Dispose of hydrogels containing cells, media, or matrix components according to your local guidelines for biological waste.

Dispose of waste containing phenol, chloroform and β-mercaptoethanol appropriately by referring to institute Biological and Chemical Waste Handling guidelines for safe disposal.

DO NOT add bleach or acidic solutions to the sample waste. RLT buffer (Method A) and Trizol® (Method B) contains guanidine thiocyanate. Guanidine salts can form highly reactive compounds when combined with bleach. If liquid containing these buffers is spilt, clean with suitable laboratory

detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

6. DOCUMENT HISTORY

Version	Date	Summary of Changes
1.0	**05 Feb 26**	First version of customer facing SOP, adapted from internal PeptiMatrix RNA extraction procedures.