Document Number:	**SASoM/METHOD/116.v1**
Title:	**Poly(A) RNA isolation using NEXTFLEX® Poly(A) Beads**
Version:	**v1**
Author:	**Peter Mullen**

Effective from:	10/04/2020
Valid to:	09/04/2022

SOP History		
Number	Date	Reason for Change
v1	10/04/20	Original

1.0 Purpose –
This SOP describes the current procedure for carrying out Poly(A) RNA isolation using NEXTFLEX® Poly(A) Beads in Laboratory 248/249 at the St Andrews School of Medicine (SASoM).

2.0 Scope –
This SOP applies to all staff in the SASoM carrying out Poly(A) RNA isolation using NEXTFLEX® Poly(A) Beads in in Laboratory 248 at the St Andrews School of Medicine (SASoM).

3.0 Responsibilities –
All staff performing Poly(A) RNA isolation using NEXTFLEX® Poly(A) Beads in this manner are responsible for ensuring that the methods are followed in accordance with this SOP.

All staff must have read and signed the relevant risk assessment documents before performing this procedure.

4.0 Procedure –

Seeding and treating cells:

Day-1 (Seeding cells): cells will be grown in 12 x T175 flasks until they reach 80% confluence.

Day-5 or 6 (Treating cells): Half the cells will be treated with 7738 (30µM) for 24hrs.

[Number of cells will be about 24 million cells for each of the control and treated cells at the end of the experiment)

Total RNA Isolation: (TRI reagent #AM9738):

1. Cells will be trypsinised, counted, pooled and made as 2 pellets (control and treated).
2. 3 ml of TRI reagent (1 ml for per 5-10 million cells) will be added to each pellet and vortexed immediately.
3. Incubate for 5 min at room temp.
4. (optional) centrifuge at 12000 xg for 10 min at 4°C and transfer the supernatant to a fresh tube.
5. Add 300µl of BCP (100 µL per 1 ml of TRI sol.), vortex and incubate for 5-15 min at room temp.
6. Centrifuge at 12000 xg for 10-15 min at 4°C and transfer the aqueous phase to a LoBind Eppendorf tube.
7. Combine with an equal volume of isopropanol (or 500 µl of isopropanol per 1 ml of TRI)
8. Vortex at moderate speed for 5-10 sec and incubate at room temp. for 5-10 min.
9. Centrifuge at 12000 xg for 8 min at 4°C and discard the supernatant.
10. Wash RNA pellet by adding 750 µl 80% ethanol (Add 1 mL of 75% ethanol per 1 mL TRI Reagent solution) and then centrifuge for 5 min at 12,000g (4 °C).
11. Remove the supernatant without disturbing the pellet. Remove all residual ethanol by centrifuging again briefly and removing the ethanol that collects with a fine tip pipette.
12. Air-dry the pellet for 10 min (3-5 min), resuspend in nuclease-free water (100 µl final volume) by passing the solution a few times through a pipette tip or by vigorous vortexing (The resuspension volume is determined by the size of the RNA pellet. 3–5 mm pellets typically require 300–500 µL. If necessary, increase the resuspension volume or incubate at 55–60°C to completely dissolve the pellet
13. quantify and either store at –80 °C or do poly(A) purification (Store at 4°C for immediate analysis).

[The total RNA isolated with this procedure should have an A260/A280 ratio of 1.8–2.2.]

Poly(A) RNA isolation (NEXTFLEX® Poly(A) Beads 2.0 #NOVA-512911):

We need (i) Total RNA (10 ng – 5 µg) in nuclease-free water - the maximum expected yield from 5µg is 250 ng poly(A) RNA, and (ii) Nuclease-free water as needed.

Step A: NEXTFLEX® Poly(A) Bead Preparation Procedure:

1. Thoroughly resuspend NEXTFLEX® Poly(A) Beads 2.0 by vortexing. Ensure no beads remain settled at the bottom of the bottle or tube.
2. Transfer 100 µL of NEXTFLEX® Poly(A) Binding Buffer 2.0 to fresh microcentrifuge tube. Each sample requires its tube.
3. Add 20 µL of NEXTFLEX® Poly(A) Bead 2.0 to each microcentrifuge tube and mix until homogenous.
4. Place the microcentrifuge tube on the magnet for 30 seconds or until solution becomes clear.
5. Discard the clear supernatant while the microcentrifuge tube remains on the magnet.
6. Remove the microcentrifuge tube from magnet.
7. Resuspend pellet in 50 µL of NEXTFLEX® Poly(A) Binding Buffer 2.0.

Step B: Purification of mRNA from total RNA

8. Bring volume of total RNA sample (10 ng – 5 µg) to 50 µL using nuclease-free water.
9. Each microcentrifuge tube must contain 50 µL of the total RNA sample and 50 µL of the prepared beads.
10. Close the microcentrifuge tube and incubate at 65°C for 5 min, then hold on 4°C.
11. Mix thoroughly until homogenized.
12. Incubate at room temperature for 5 minutes.

First Bead Wash -

13. Place the microcentrifuge tube on the magnetic stand at room temperature for 30 seconds or until the supernatant appears completely clear.
14. Remove and discard clear supernatant taking care not to disturb beads.
15. Remove the microcentrifuge tube from the magnetic stand.
16. Resuspend pellet with 200 µL of NEXTFLEX® Poly(A) Washing Buffer 2.0. Mix thoroughly until homogenized.
17. Place the microcentrifuge tube on the magnetic stand for 30 seconds or until supernatant appears completely clear.
18. Remove and discard clear supernatant taking care not to disturb beads.
19. Remove the microcentrifuge tube from the magnetic stand.
20. Resuspend pellet with 50 µL of NEXTFLEX® Poly(A) Elution Buffer 2.0.

Bead Binding -

21. Close the microcentrifuge tube and incubate at 70°C for 5 min, then hold on 25°C.
22. Add 50 µL of NEXTFLEX® Poly(A) Binding Buffer 2.0 to each sample. Mix thoroughly until homogenized. Do not magnetize.
23. Incubate at room temperature for 5 minutes.

Second Bead Wash –

24. Place the microcentrifuge tube on the magnetic stand at room temperature for 30 seconds or until the supernatant appears completely clear.
25. Remove and discard clear supernatant taking care not to disturb beads.
26. Remove the microcentrifuge tube from the magnetic stand.
27. Resuspend pellet with 200 µL of NEXTFLEX® Poly(A) Washing Buffer 2.0. Mix thoroughly until homogenized
28. Place the microcentrifuge tube on the magnetic stand for 30 seconds or until supernatant appears completely clear.
29. Remove and discard clear supernatant taking care not to disturb beads.
30. Remove the microcentrifuge tube from the magnetic stand.

Final Elution

31. Resuspend pellet with 17µL of NEXTFLEX® Poly(A) Elution Buffer 2.0.
32. Incubate the microcentrifuge tube at 70°C for 5 min.
33. Immediately transfer the hot microcentrifuge tube to a magnet. Do not allow the microcentrifuge tube to cool to room temperature.
34. Transfer 14µL of clear sample to a new microcentrifuge tube. mRNA is now ready for RNA-seq based applications.
35. For total RNA inputs greater than or equal to 1µg, fluorometric analysis using a Qubit fluorometer and electropherogram analysis using a Labchip® instrument is recommended.

[We'll have a maximum of 250ng polyA RNA in 14µL Elution buffer]
[For the nanopore kit we need 500 ng poly A RNA in 9 µL volume]

5.0 Personal protection –

A Howie coat must be worn at all times. Gloves as specified in the appropriate COSHH RA.

6.0 Spillages –

Always clean up any spills immediately after use, only you know what you have spilt and are aware of its hazard. Spillages should be mopped up with paper towel, disinfected with 70% ethanol and finally washed with detergent.

7.0 Training –

All staff should undergo training in this technique before performing the procedure.

8.0 Related documents –

8.1 Risk assessments –
RA20168 - RNA Isolation using TRI Reagent
RA20169 - Poly(A) RNA isolation using NEXTFLEX® Poly(A) Beads.

8.2 SOPs –
SASoM-METHOD-115-RNA Isolation using TRI Reagent

9.0 Approval and sign off –

Author:

Name: Peter Mullen

Position: Research Fellow

Signature: Date:

Management Approval:

Name: Peter Mullen

Position: SOP Administrator

Signature: Date:

QA release by:

Name: Alex MacLellan

Position: QA Manager

Signature: Date:

STANDARD OPERATING PROCEDURE

Please sign below to indicate you have read this S.O.P and understand the procedures involved.

NAME	POSITION HELD	SIGNATURE	DATE