DAHLIA Biosciences

RNA Flow Assays

User Guide

Introduction

Dahlia RNA Flow Assays enable multi-color single-cell RNA analysis via flow cytometry at high throughput and accuracy. The workflow can be completed in as little as 6 hours, with an additional 2 hours if antibody co-staining is performed. Applications include protein and RNA co-detection, scRNAseq validation, cytokine profiling, and RNA-based cell enrichment.

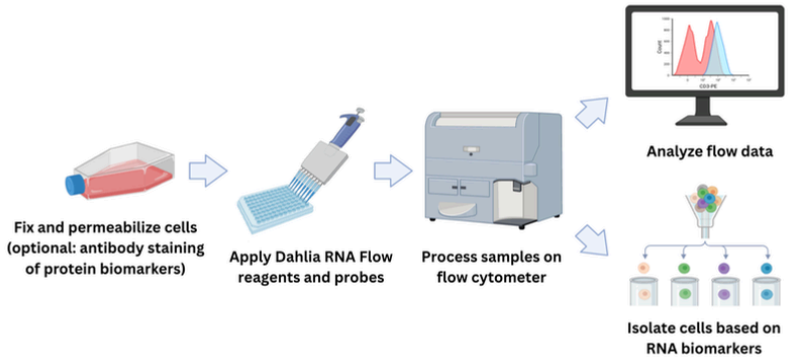
Workflow

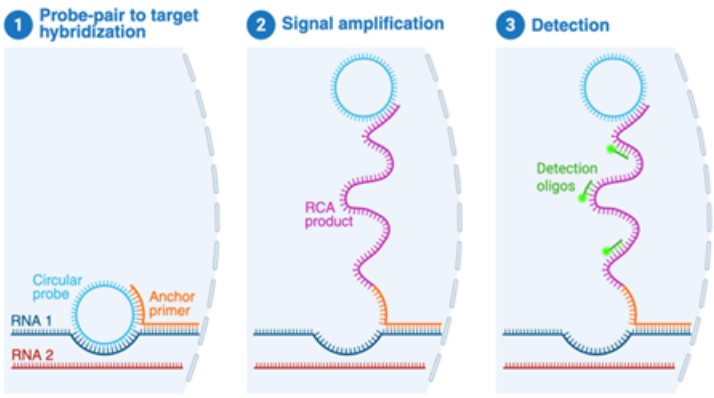
Step 1. Single-cell suspensions are fixed and permeabilized. As an optional step, surface protein markers can be co-stained with antibodies.

Step 2. The Dahlia RNA Flow Assay hybridization, amplification, detection, and accompanying wash steps are performed.

Step 3. Samples are then processed on a conventional or spectral flow cytometer.

Step 4. Further analysis or cell sorting is performed based on target RNA expression.





Assay Principle

1. Probe hybridization. The circular probe (CP) and anchor primer (AP) hybridize proximal to each other on the target RNA sequence, enabling the initiating primer sequence on the AP to hybridize to the CP.

2. Signal amplification. The target sequence is amplified via rolling circle amplification (RCA).

3. Detection. Fluorescent detection oligos are hybridized to the RCA product and the cells are processed by flow cytometry. Multiplexing of 3 unique RNA targets is currently supported.

Supplied by Dahlia

Note
All reagents are shipped frozen on dry ice. Place in appropriate storage conditions upon receipt.

Product	Components	Storage
RNA Flow Assay Kit (DB-RFK-01-SM, DB-RFK-01-LG)	Cell Resuspension Buffer (CRB)	4°C
	2.5X Probe Hybridization Buffer (PHB)	4°C
	10X RNAse Inhibitor (RI)	-20°C
	Wash Buffers A, B, and C	4°C
	5X Amplification Mix	-20°C
	Amplification Enzyme	-20°C
	Detection Buffer	4°C

Product	Components	Storage
RNA Flow Probe Set	10X RNA Target-Specific Probe Set	-20°C
	5X Detection Probe Complexing & Dilution Buffer	4°C
	Label Oligo (Cy5, Cy3, or FITC)	-20°C
	Target-Specific Detection Oligo	-20°C

Other Required Materials

Reagent/Consumable	Source	Part Number
96-well V-Bottom Plates	USA Scientific	1833-9610
16% Paraformaldehyde	Electron Microscopy Sciences	15710
Methanol	Any	N.A.
Formamide	Any	N.A.
Nuclease-Free Water	Any	N.A.
10X PBS	Any	N.A.
Adhesive Aluminum Plate Foils	Any	N.A.
0.2 mL PCR Strip Tubes	Any	N.A.
1.5 mL Eppendorf Tubes	Any	N.A.

Other Required Materials (optional: Antibody Co-staining)

Reagent/Consumable	Source	Part Number
BS3 crosslinker	ThermoFisher Scientific	A39266
Cell Staining Buffer (CSB)	BioLegend	420201
Fluorophore-conjugated antibodies Notes: Choose fluorophores resistant to methanol and formamide	Any	N.A.

Equipment

Equipment	Purpose	Specifications
Flow Cytometer	Analyze or sort cells	FITC: Blue laser, Laser line: 488 nm, Ex: 498 nm, Em: 517 nm Cy3: Yellow laser, Laser lines: 532, 561 nm, Ex: 554 nm, Em: 566 nm Cy5: Red laser, Laser lines: 633, 647 nm, Ex: 647 nm, Em: 665 nm
PCR Thermal Cycler	Incubate 96-well V-bottom plate during probe hybridization, washes, amplification, and detection probe hybridization	Heated lid temp can be adjusted from 37°C to 95°C
Heat Block (1.5 mL)	Warm up frozen 10X RNAse Inhibitor at 65°C for 15 min.	
Plate Centrifuge (Swinging Bucket)	Spin down 96-well V-bottom plate during washes	

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RNA Flow Assays

Protocol

Info

This protocol is validated using 96-well V-bottom plates. To discard supernatant by flicking from the wells, the motion is done very quickly and intentionally. See page 9 for more detailed instructions. Alternatively, aspiration with a 12-channel pipette or plate aspirator may be used without disrupting the pellet.

Step 1: Reagent Preparation & Storage

1. Remove from 4°C and leave at Room Temperature (RT) for at least 15 min:
   a. Formamide (F)
   b. Cell Resuspension Buffer (CRB)
   c. 2.5X Probe Hybridization Buffer (PHB)
   d. Wash Buffer A (WBA)
2. Remove 10X RNAse Inhibitor (10X RI) from -20°C. Heat at 65°C on 1.5 mL tube Heat Block for 15 min. Vortex and spin down.

Important

Add 10X RI immediately to CRB and 2.5X PHB after it has warmed to 65°C for 15 min. Do not place on ice.

Danger

Formamide is a teratogen, irritant, and possible carcinogen. See Safety Data Sheet from vendor for complete information.

3. Mix CRB + 10X RI sufficient for the number of tests in the experiment and leave at RT (see table below).

CRB + RI	Reagent	Volume per test	Volume per 25 tests (+10% overage)	Volume per 100 tests (+10% overage)
	CRB	180 µL	4.95 mL	19.8 mL
	10X RI	20 µL	550 µL	2.2 mL
	Total	200 µL	5.5 mL	22 mL

4. Mix Probe Hybridization Master MIX sufficient for the number of tests in the experiment and leave at RT (see table below).

PROBE HYBRIDIZATION MASTER MIX	Reagent	Volume per test	Volume per 25 tests (+10% overage)	Volume per 100 tests (+10% overage)
	2.5X PHB	40 µL	1.1 mL	4.4 mL
	Water	10 µL	275 µL	1.1 mL
	10X RI	10 µL	275 µL	1.1 mL
	Formamide	10 µL	275 µL	1.1 mL
	Total	70 µL	1.925 mL	7.7 mL

5. Set up **Probe Hybridization Reactions** in 0.2 mL PCR strip tubes.
   a. Aliquot 70 µL of Probe Hybrdization Mater Mix per test
   b. Add 10 µL of 10X RNA Flow Probe Set for each RNA target (up to 3)
   c. Add water such that total volume is 100 µL (0 µL for 3-plex, 10 µL for 2-plex, 20 µL for singleplex)
6. Mix Wash Buffer A + Formamide sufficient for number of tests in the experiment and leave at RT (see table below).

WASH BUFFER A + FORMAMIDE	Reagent	Volume per test X 2 washes	Volume per 25 tests (+10% overage)	Volume per 100 tests (+10% overage)
	Wash Buffer A	240 µL	6.6 mL	26.4 mL
	Formamide	160 µL	4.4 mL	17.6 mL
	Total	400 µL	11 mL	44 mL

Step 2a: Cell Preparation

7. Harvest cells needed needed for the number of tests in the experiment into 15 mL conical tubes. Note: 250,000 cells after fix/perm is required per test. Therefore, we recommend the starting cell counts below to account for loss during cell handling, fix/perm, and antibody staining steps.
   a. 1 x 10^6 cells per test (RNA detection only)
   b. 2 x 10^6 cells per test (Antibody co-staining)
8. Pellet cells by centrifugation (**500g, 3 min, RT**) and remove supernatant.
9. Resuspend cells with **1X PBS** (500 µL per 1 x 10^6 cells).
10. Pellet cells by centrifugation (**500g, 3 min, RT**) and remove supernatant.
11. Resuspend cells with **1X PBS** (100 µL per 1 x 10^6 cells), and leave on ice.
12. Prepare the following and keep on ice:
    a. **1.6% (wt/vol) PFA in 1X PBS** (500 µL per 1 x 10^6 cells)
    b. **Methanol** (150 µL per 1 x 10^6 cells)
    c. **1X PBS** (500 µL per 1 x 10^6 cells)
13. For antibody co-staining protocol, proceed to step 15.
14. For RNA detection only protocol, proceed to step 27.

Step 2b: Surface Antibody Co-staining (Optional)

15. Aliquot 2 x 10^6 cells per test into 1.5 mL tubes.
16. Pellet cells by centrifugation (**500g, 3 min, RT**) and remove supernatant.
17. Resuspend and stain the cells in 100 µL of **Cell Staining Buffer** with appropriate dilutions of fluorochrome-conjugated antibodies for 30 min. on ice.
18. Centrifuge (**500g, 3 min, RT**) and discard supernatant.
19. Resuspend cells in 200 µL of **CSB**, centrifuge (**500g, 3 min, RT**), and discard supernatant.
20. Resuspend cells in 200 µL of **1X PBS**, centrifuge (**500g, 3 min, RT**), and discard supernatant.
21. Prepare 5mM **BS3 crosslinker solution** immediately prior to use.
    a. Resuspend 2 mg of **BS3 crosslinker** in 700 µL of **1X PBS**. Sufficient for 13 samples. Scale up preparation as needed for the total number of samples.
22. Resuspend the cells in 50 µL of **BS3 crosslinker solution**.
23. Mix immediately and leave on ice for 30 min, agitating the cells every 10 min.
24. Add 200 µL of **1X PBS**.
25. Combine samples stained with the same antibodies (if applicable) into 15 mL conical tubes, and leave on ice.
26. Proceed to step 27.

Step 2c: Cell Fixation and Permeabilization

27. Centrifuge (**500g, 3 min, RT**) and remove supernatant.
28. Resuspend the cells in **1.6% (wt/vol) PFA in 1X PBS** (500 µL per initial 1 x 10^6 cells input). Pipette mix.
29. Incubate cells for 10 min at RT.
30. Centrifuge (**800g, 3 min, RT**) and discard supernatant
31. Loosen the pellet by flicking the tube and add **ice-cold methanol** (150 µL per 1 x 10^6 cells). Quickly cap lid and vortex.
32. Incubate cells for 10 min on ice.
33. Take an aliquot of each unique cell type and count cells.
34. Proceed to target probe hybridization step or pause by storing fixed cells in **methanol** at -80°C.

Step 3: Target Probe Hybridization

35. Aliquot sufficient number of fixed/perm cells (250,000 fixed/perm cells per test) stored in **methanol** needed for the number of tests in the experiment into 15 mL conical tubes.
36. Centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
37. Resuspend cells with **CRB + RI** (200 µL per 250,000 fixed/perm cells)
38. Aliquot 200 µL of cells resuspended in **CRB + RI** into wells of 96-well V-bottom plate.
39. Centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
40. Resuspend cells with 100 µL of **Probe Hybridization Reactions** (prepared in Step 1). Pipette mix.
41. Seal the plate with a foil seal, place in the **PCR Thermal Cycler** and incubate at 45°C for 60 min. Note: Set lid temp to 40°C. Do not set lid temp to 95°C.
42. After 60 min probe hybridization, centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
43. Resuspend cells with 200 µL of **Wash Buffer A + Formamide**.
44. Centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
45. Repeat steps 43 and 44.
46. Resuspend the cells in 200 µL of **Wash Buffer B.**

47. Seal the plate with a foil seal, place in the **PCR Thermal Cycler** and incubate at 40°C for 20 min. Note: Set lid temp to 40°C. Do not set lid temp to 95°C.
48. During 20 min Wash B incubation, make Amp Master Mix sufficient for the number of tests in the experiment and leave on ice (see table below).

AMPLIFICATION MASTER MIX	Reagent	Volume per test	Volume per 25 tests (+10% overage)	Volume per 100 tests (+10% overage)
	5X Amplification Mix	20 µL	550 µL	2.2 mL
	Water	79 µL	2.173 mL	8.69 mL
	Enzyme	1 µL	27.5 µL	110 µL
	Total	100 µL	2.75 mL	11 mL

Step 4: Signal Amplification and Detection

49. Resuspend the cells in 100 µL of **Amplification Master Mix.**
50. Seal the plate with a foil seal, place in the **PCR Thermal Cycler** and incubate at 30°C for 60 min. Note: Set lid temp to OFF. Do not set lid temp to 95°C.
51. During the 60 min amplification step, set up 200X detection probe complexing reactions in 0.2 mL PCR strip tubes (see table below).

200X Detection Probe	Reagent	Volume
	5X Detection Probe Complexing & Dilution Buffer	2 µL
	Water	5.8 µL
	Label Oligo (Cy5, Cy3, FITC)	1.2 µL
	Target-Specific Detection Oligo	1 µL
	Total	10 µL

52. Place in **PCR Thermal Cycler.** Incubate at 85°C for 3 min. Incubate at 65°C for 3 min. Incubate at 25°C for 3 min. Note: Set lid temp to 95°C.
53. Dilute 200X Detection Probes 20-fold to 10X concentration by diluting in 1X Detection Probe Complexing & Dilution Buffer.
54. Set up **Detection Probe Reactions** in 0.2 mL PCR strip tubes. Keep on ice in the dark until ready to add to cells.
    a. Aliquot 60 µL of Detection Probe Buffer per sample
    b. Add 10 µL of 10X Detection Probes for each RNA target (up to 3)
    c. Add water such that total volume is 100 µL.
55. When amplification is complete, centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
56. Resuspend the cells in 100 µL of **Detection Probe reactions.**
57. Seal the plate with a foil seal, place in the **PCR Thermal Cycler** and incubate at 37°C for 30 min. Note: Set lid temp to 37°C. Do not set lid temp to 95°C.
59. Centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
60. Resuspend the cells in 200 µL of **Wash Buffer C**.
61. Centrifuge the plate (**800g, 3 min, RT**) and discard supernatant.
62. Resuspend cells in 100 µL of **Wash Buffer C** and analyze by flow cytometry.

Dahlia RNA Flow

Quick Reference Protocol

CELL PREP	☐ Prepare and keep on ice: 1.6% PFA in 1X PBS, methanol, and 1X PBS. ☐ Harvest and count cells. Aliquot 2M cells per test into 1.5 mL Eppendorf tubes.
AB STAIN **(Optional)**	☐ Centrifuge cells (500g 3 min RT) and remove supernatant. ☐ Incubate cells in Antibody mix for 30 min on ice. ☐ Centrifuge (500g 3 min RT) and remove supernatant. ☐ Resuspend cells in 200 µL CSB. Centrifuge (500g 3 min RT) and remove supernatant. ☐ Resuspend cells in 200 µL 1X PBS. Centrifuge (500g 3 min RT) and remove supernatant. ☐ Resuspend cells in 50 µL BS3 crosslinker. Incubate 30 min on ice + agitation. ☐ Add 200 µL 1X PBS and transfer cells to 1.5 mL Eppendorf tubes ☐ Centrifuge (500g 3 min RT) and remove supernatant.
FIX/PERM	☐ Incubate cells in 1 mL of 1.6% PFA for 10 min at RT. ☐ Centrifuge (500g 3 min RT) and remove supernatant. ☐ Add 300 µL methanol and vortex. Incubate for 10 min on ice. ☐ Proceed or store cells at -80°C.
REAGENT PREP	☐ Warm up Formamide, CRB, and 2.5X PHB at RT for 15 min. ☐ Set the heat block to 65°C. Place RNAse Inhibitor in heat block for 15 min. ☐ Make CRB + R, PHB, Wash Buffer A + Formamide, and keep at RT. ☐ Make Probe Hybridization reactions.
PROBE HYB	☐ Centrifuge cells in methanol (800g 3 min RT). ☐ Resuspend cells with 200 µL CRB + RI and transfer to 96-well V-bottom plate. ☐ Centrifuge (800g 3 min RT) and remove supernatant. ☐ Resuspend cells with 100 µL Probe Hybridization reactions. ☐ Place the 96-well plate in the PCR Thermal Cycler. ☐ Run program: Incubate at 45°C for 60 min. ☐ Centrifuge (800g 3 min RT) and remove supernatant.
WASHES	☐ Resuspend cells with 200 µL Wash Buffer A + F. Centrifuge (800g 3 min RT) and remove supernatant. Repeat. ☐ Incubate in 200 µL Wash Buffer B at 40°C for 20 min. ☐ Make Amplification mix and keep on ice. ☐ Centrifuge cells (800g 3 min RT) and remove supernatant. ☐ Resuspend cells in 200 µL Wash Buffer C. Centrifuge cells (800g 3 min RT) and remove supernatant. Repeat.
AMPLIFI-CATION	☐ Incubate in 100 µL Amplification Master Mix at 30°C for 60 min. ☐ Centrifuge cells (800g 3 min RT) and remove supernatant. ☐ Make Detection Probe reactions and keep in dark.
DETECTION PROBE HYB	☐ Incubate in 100 µL Detection probe reactions at 37°C for 30 min. ☐ Centrifuge cells (800g 3 min RT) and remove supernatant. ☐ Resuspend cells with 200 µL Wash Buffer C. ☐ Centrifuge cells (800g 3 min RT) and remove supernatant. ☐ Resuspend cells in 100 µL Wash Buffer C and process cells by flow cytometry.

Dahlia RNA Flow Assays

Supplemental Procedures & Suggestions

Plate Washing/Incubation/Centrifugation

- When centrifuging live cells use settings: 500 x g, 3 minutes, RT.
- When centrifuging fixed cells use settings: 800 x g, 3 minutes, RT.
- When flicking supernatant: all motions are done very quickly and intentionally - invert plate completely so it is parallel to the flat surface below, move plate forcibly up and down on a z axis, and return plate back face up.
- When washing plate wells, pipette up and down with 200 µL of reagent described in the step, lay foil seal over wells and press down firmly (swiping motion over the plate), centrifuge, carefully peel back foil seal, and then flick supernatant over a chemical hazard labeled bin in the chemical fume hood.
- When incubating, place foil seal on top of plate and seal firmly. Seal plate for every incubation and wash.
- Replace foil after each step to avoid cross contamination with condensation.

Recommended Plate Layout (to avoid cross contamination)

- To help with washing, prepare tip boxes with alternating spaces

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample		Sample		Sample		Sample		Sample		Sample	
B												
C		Sample		Sample		Sample		Sample		Sample		Sample
D												
E	Sample		Sample		Sample		Sample		Sample		Sample	
F												
G		Sample		Sample		Sample		Sample		Sample		Sample
H												

Technical Guidelines

- Do not use kit beyond expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- Detection probes are light sensitive and must be always protected from light. Use foil or opaque plate seals for these incubation steps.
- For accuracy, use a multichannel pipette and reagent reservoir when possible.
- Incomplete washing can affect results and increase background. Please follow all wash steps.
- Detection Probes complexed for each experimental run will be discarded after use.
- RNAse Inhibitor is sensitive to temperature. Limit freeze/thaw cycles to two. If one tube of RNAse Inhibitor is not used to completion after first thaw, only use it once more. During reagent preparation, keep RNAse Inhibitor at 65°C. As RNAse Inhibitor degrades it will turn into a dark black color rather than army green.
- Always allow formamide to reach RT before opening.
- Pipette slowly to avoid cell loss in tip.
- Keep samples at 4°C if they cannot be read by flow immediately upon completion of protocol. Samples can be analyzed up to 1 week after completion of detection step.
- It is highly recommended to include replicates and positive/detection only negative controls in each experiment.

Technical Support

Info

Please visit https://www.dahliabio.com/ for more information

Technical support: support@dahliabio.com