

Supplementary Table S1

Comparison of current work with seminal published studies using conventional northern and liquid hybridization assays.

A Comprehensive Analysis of Northern versus Liquid Hybridization Assays for mRNAs, small RNAs, and miRNAs using a Non-radiolabeled Approach

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Supplementary Table S1. Comparison of current work with seminal published studies using conventional northern and liquid hybridization assays.

Research Article	Ahmad et al., (this study)	Li F et al., 2016 (36) <i>Int J Mol Sci</i> 17(9).	Huang et al., 2014 (51) <i>Rice</i> 7(1), 26.	Li X et al., 2014. (47) <i>RNA</i> 20(2), 252-9.	Li X et al. 2012 (41) <i>Methods.</i> 58(2), 151-5.	Wang et al. 2010 (32) <i>Int J Mol Sci.</i> 11(9), 3138-48.	Kim et al. 2010 (35) <i>Nucleic Acids Res</i> 38(7), e98.
Paper Title	A comprehensive analysis of northern <i>versus</i> liquid hybridization assays for mRNAs, small RNAs, and miRNAs using a non-radiolabeled approach	Liquid hybridization and solid phase detection: a highly sensitive and accurate strategy for microRNA detection in plants and animals	A non-radioactive method for small RNA detection by northern blotting	A convenient system for highly specific and sensitive detection of miRNA expression	Detecting miRNAs by liquid hybridization and color development	Rapid and Accurate detection of plant miRNAs by liquid northern hybridization	A sensitive non-radioactive northern blot method to detect small RNAs
Organism	Animal (mouse HC11 cells)	Plant (rice) Animal (mouse tissue & human blood)	Plant (rice)	Animal (rat cells/tissues)	Animal (rat tissues)	Plant (rice, maize, wheat, tobacco, etc.)	Animal (human MCF-7 cells)
Conventional /Liquid Hybridization	Liquid and Conventional	Liquid and Conventional	Conventional	Liquid and Conventional	Liquid	Liquid	Conventional
Target RNA	mRNAs small RNAs (5S & U6) miRNAs	miRNAs	miRNAs siRNAs	miRNAs	miRNAs	miRNAs	miRNAs
RNA amount tested	Total RNA NB: 25-2.5 ug LH: 1.0-0.25 ug	Small RNA NB: NA LH: 1 ug	Total RNA 1-20 ug Small RNA 0.5-10 ug	Total RNA NB: 20 ug LH: 1ug Small RNA LH: 4 ng	Small RNA 8 ng	Small RNA 1 ug	Total RNA 3-10 ug

Level of sensitivity	<p>RNA NB: < 3.12 ug for mRNA; <3.12 ug for small RNAs; undetectable for miRNAs LH: <0.312 ug for mRNA; <0.312 ug for small RNAs; <250 ng for miRNAs Probes: <i>mRNA:</i> 3 pmol (50 pmol/ml) <i>small RNA & miRNAs:</i> 0.3 pmol (10 pmol/sample)</p>	<p>Small RNA NB: NA LH: 1ug Probe – <i>DIG-CDP Star:</i> 0.01–0.25 fmol <i>Biotin-CDP Star:</i> 0.005–0.1 fmol <i>Biotin-luminol:</i> 0.05–0.5 fmol</p>	<p>Total RNA: 1ug Small RNA: 1ug Probe: 50 pmol/ml</p>	<p>RNA NB: 20 ug total RNA LH: 1 ug total RNA LH: 4 ng small RNA</p>	<p>Small RNA 8 ng Probe: 25 fmol</p>	<p>Small RNA 1 ug Probe: 0.1 pmol</p>	<p>Total RNA 3 ug Probe: 0.05 fmol</p>
Technique (Dot/PAGE)	PAGE	PAGE	PAGE	Dot blot	Dot blot	Dot blot & PAGE	PAGE
Probe type	<p>For mRNA: 5' biotinylated dsDNA probe For small & miRNAs: 5' biotinylated oligo probe</p>	5' DIG probe or biotinylated probe	5' or 3' Biotin - labelled probe completely complementary	Hairpin DNA probes and 5'-biotinylated or γ - ³² P-labeled oligonucleotides	5' biotinylated probes	FITC-labelled probe complementary probe	DIG-labelled LNA or ³² P-labeled LNA probes
Probe amount	<p>NB: 3 pmol (50 pmol/ml) LH: 0.3 pmol (10 pmol/sample)</p>	0.1 pmol	50 pmol/ml	<p>NB- 5 pmol LH- 20 fmol to 4 pmol</p>	20 pmol	<p>0.1 pmol (PAGE) 0.01 pmol (Dot)</p>	<p>0.05-0.5 nM (0.2 nM) Difficult to detect 2 nM</p>
Enzyme used for detection	Horseradish peroxidase	Alkaline Phosphatase or Horseradish peroxidase	Horseradish peroxidase	Alkaline Phosphatase	Alkaline Phosphatase or Horseradish Peroxidase	Florescence detection No Enzyme Needed	Alkaline Phosphatase

Crosslinking method	LH - UV crosslinking (254 nm; 700 μJoules) for 5-7 mins & baked at 80°C for 2 hrs NB - UV crosslinking (254 nm; 700 μJoules) for 5-7 mins & EDC-treated at 60°C for 2 hrs	UV Crosslinking (254 nm; 15–20 KJ/cm ²) for 5–9 mins	UV crosslinking (254 nm; 1,200 μjoules) for 20 mins & dried at 50°C for 30 mins	UV crosslinking (254 nm; 3,000 μjoules) for 90 secs	UV crosslinking (254 nm; 3,000 μjoules) for 90 secs	No Blotting	EDC crosslinking at 60°C for 1-2 hrs
Detection type	Chemiluminescence	Chemiluminescence	Chemiluminescence	LH: Colorimetric NB: Phosphor imaging	Colorimetric	Fluorescence imaging	Phosphor imaging
Detection method/ Instrument	X- ray film detection Sapphire and Typhoon Biomolecular Imagers	ChemiDoc XRS System	Chemiluminescent nucleic acid detection kit X-ray film detection	Color development X-ray film detection	Color development	CitaBlue conversion screen in a Chemi Doc XRS System	Phosphor image screen used in the ChemiDoc-IT Imaging System
Substrate/ Reagent	Luminol based: ECL: X-Ray film detection & Typhoon biomolecular imager Femto: Sapphire biomolecular imager	Luminol CDP Star	Luminol	BCIP/NBP	BCIP/NBP or DAB or ECL Prime	Florescence detection. No substrate needed.	CSPD
Incubation time for detection	Luminol based: ECL – 2 min Femto - 10 secs	30 mins	5 mins	X-ray film detection: 12 hrs BCIP/NBP: 2 mins	BCIP/NBP: 30 secs DAB: 5 secs ECL Prime: 1 min	NA	20 mins

Exposure time	5-30 secs	20 mins	Auto-exposure	5 secs – 1 min	5 sec – 15 mins	Auto-exposure	Autoexposure DIG = 1 min ³² P = 24 h
Conclusions	Development of cost-effective, sensitive, and rapid biotin-based northern and liquid hybridization assays for mRNAs, small RNAs, and miRNAs	The development of Liquid Hybridization Solid Phase Detection (LHSPD) technique for analysis of miRNAs in plants and animals	Development of the Northern blot method by using biotin-labeled probes	An improved version of liquid hybridization and color development technique (LHCD) with signal amplification using bridged-PCR (named LH-CD or aLHCD)	Development of liquid hybridization and color development (LHCD) technique that uses signal amplification of avidin-biotin complex (ABC) for detection of miRNAs	Establishment of the Liquid Northern hybridization technique using fluorescently labeled oligonucleotide probes for small RNA detection	Development of a new northern blot-based protocol (LED) using digoxigenin (DIG)-labeled, LNA-probes and EDC cross-linking technique for small RNA detection

Supplementary File 1

Recipe of Buffers and Solutions Used

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1% Formaldehyde-agarose (FA) gel

Agarose = 1.5 g
10x MOPS = 15 ml
RNase free H₂O = 135 ml
37% formaldehyde = 2.7 ml
EtBr = 5 µl (of 10 mg/ml)

15% Urea-acrylamide gel (15 ml)

40% acrylamide = 5.63 ml
Urea = 7.2 g
10X TBE = 1.5 ml
Warm solution at 55°C until urea dissolve.
Bring to room temperature and add:
H₂O = up to 15 ml
20% APS = 200 µl
TEMED = 10 µl

Streptavidin blocker solution (500 ml)

1X Casein stock (50 ml)
1% Casein/ TBS = 5 ml
20% SDS = 1.25 ml
1X TBS = 43.75 ml

Blocker preparation

1X Casein-TBS = 50 ml
20% SDS = 12.5 ml
10% TBS = 43.75 ml
H₂O = 394 ml

Blocker washing (500 ml)

1x TBS = 487.5 ml
20% SDS = 12.5 ml

Native-PAGE non-denaturing gel (25 ml)

40% acrylamide = 7.5 ml
10X TBE = 2.5 ml
H₂O = 15 ml
20% APS = 200 µl
TEMED = 10 µl

Native RNA/DNA dye (10 ml)

Glycerol = 5 ml
1M EDTA = 0.3 ml
H₂O = 4.7 ml
Xylene cyanole = 5-10 mg
Bromophenol blue = 5-10 mg

Native (Non-denaturing) MOPS gel (100 ml)

Agarose = 1 g
10X MOPS = 10 ml
H₂O = up to 100 ml
EtBr = 5 µl (10 mg/ml)

Denaturing RNA loading buffer

Formamide = 9.5 ml
0.25M EDTA = 500 µl
Xylene cyanole = 5-10 mg
Bromophenol blue = 5-10 mg

20xSSC (500 ml)

Sodium Chloride = 87.65 g
Sodium Citrate = 44.1 g
H₂O = up to 500 ml

Modified Church buffer (500 ml)

Sodium hydrogen phosphate = 25.56 g
Sodium dihydrogen phosphate = 8.4 g
0.5 M EDTA = 1 ml
20% SDS = 175 ml
H₂O = up to 500 ml

Low Stringency buffer (100 ml)

20X SSC = 10 ml
20X SDS = 0.5 ml
H₂O = 89.5 ml

High Stringency buffer (100 ml)

20X SSC = 0.5 ml
20X SDS = 0.5 ml
H₂O = up to 99 ml

0.2 M EDC crosslinking buffer (12 ml)

Methylimidazole = 122.5 µl
H₂O = 10 ml
Adjust pH to 7.5-8.5 using HCl (~25 µl)
EDC = 0.375 g
H₂O = up to 12 ml

Liquid hybridization buffer (10 ml)

10 mM EDTA = 200 µl
1M NaCl = 3 ml
1M Sodium Phosphate (pH = 8.0) = 300 µl
H₂O = up to 10 ml

1M Sodium Phosphate buffer pH 8 (stock)

Add 1.42 g NaHPO_4 in 10 ml H_2O (Solution A)
Add 1.38 g NaH_2PO_4 in 10 ml H_2O (Solution B)
Mix 4.66 ml of Solution A + 340 μl of Solution B

10X TBE

Tris = 108 g
Boric acid = 55 g
0.5M EDTA = 40 ml (pH = 8)

TE buffer; 10 mM Tris/1 mM EDTA (100 ml)

1 M Tris-HCl = 1 ml (pH = 7.4)
0.5 M EDTA = 0.2 ml
 H_2O = up to 100 ml

Membrane stripping buffer

SDS = 0.5g
Water= 100ml
Boil and immerse membrane in it.

Supplementary File 2
Full gel images for Figures 4, 6, 12, & 13

**A Comprehensive Analysis of Northern *versus* Liquid
Hybridization Assays for mRNAs, small RNAs, and miRNAs
using a Non-radiolabeled Approach**

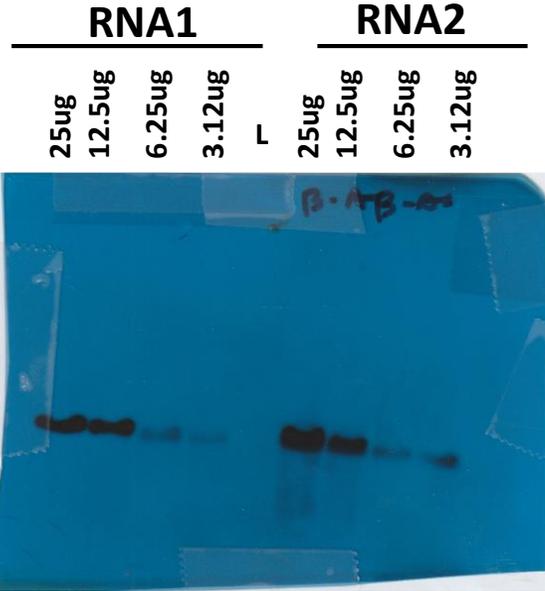
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Figure 4

Conventional Northern

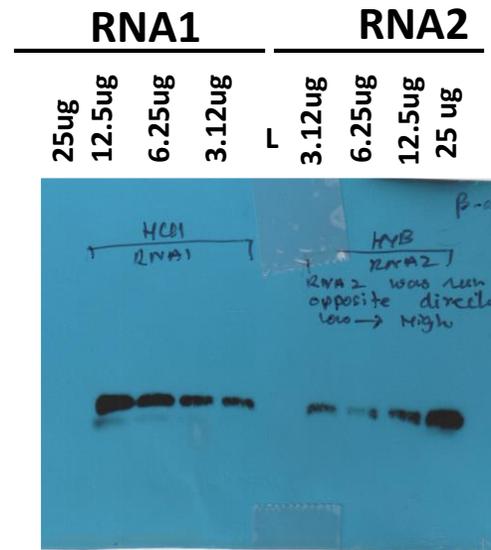


Beta-actin full image

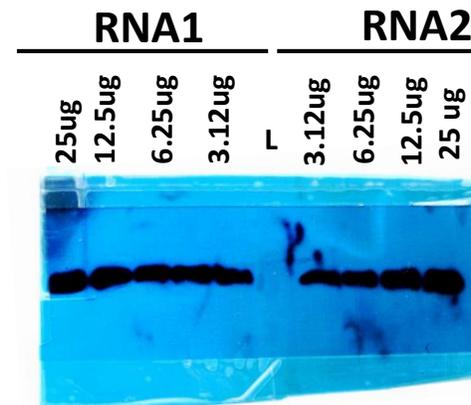


c-Myc full image

Liquid Hyb Northern



Beta-actin full image



c-Myc full image

RNA2 in these images was run from lower concentrations to high. It was flipped in Figure 4(d) for the manuscript.

Figure 6

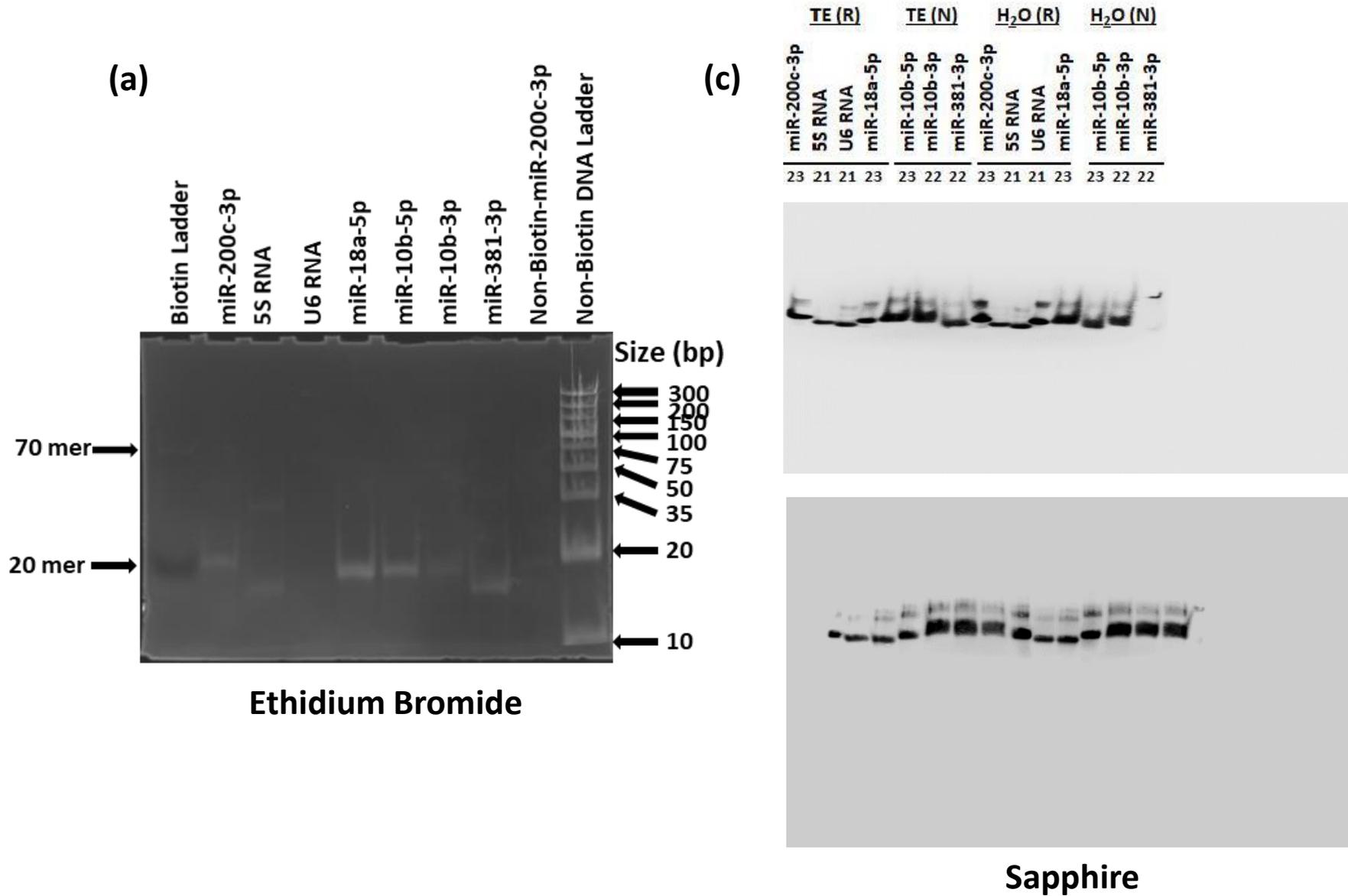


Figure 12

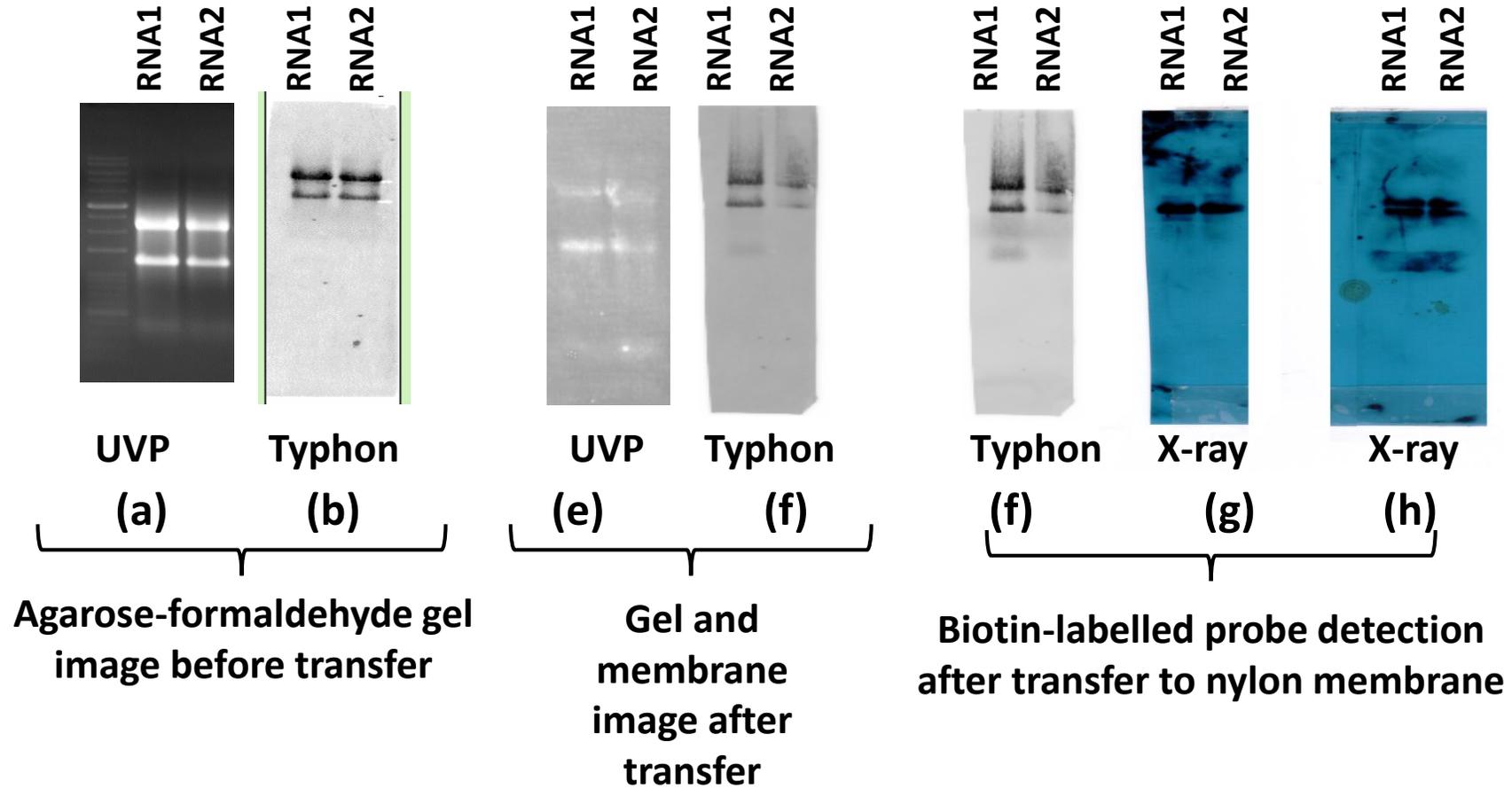
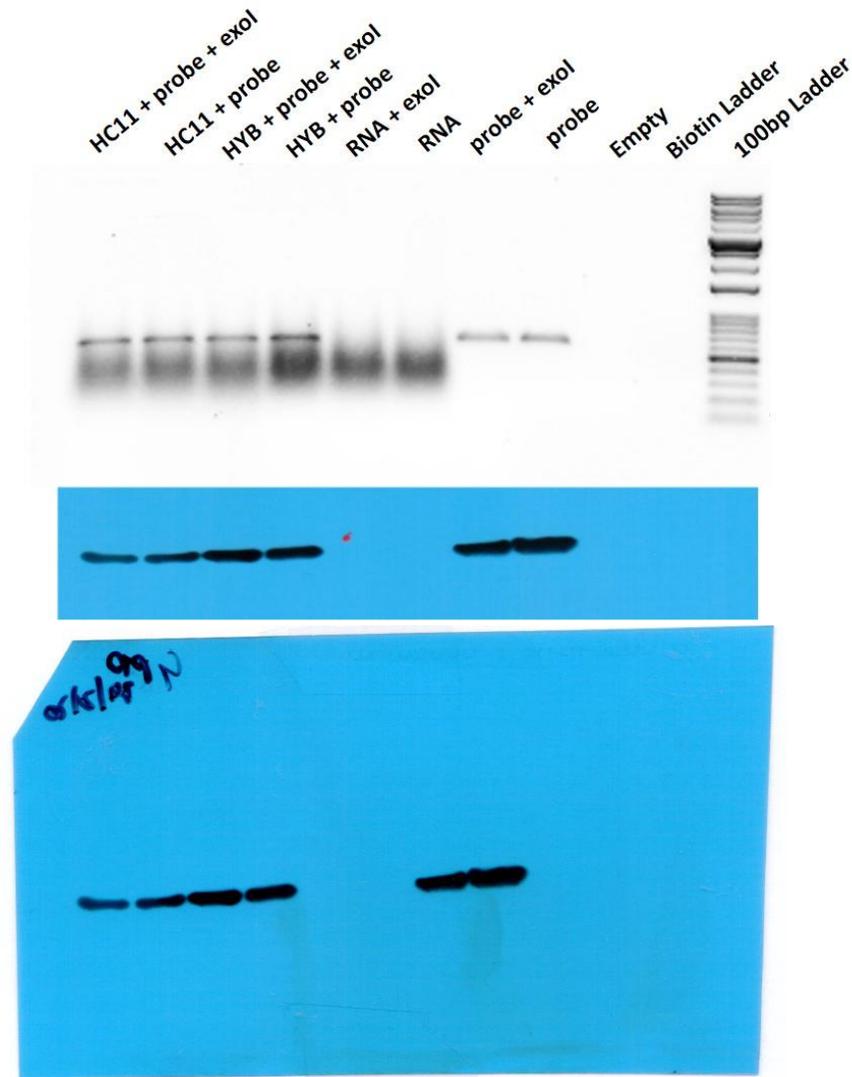
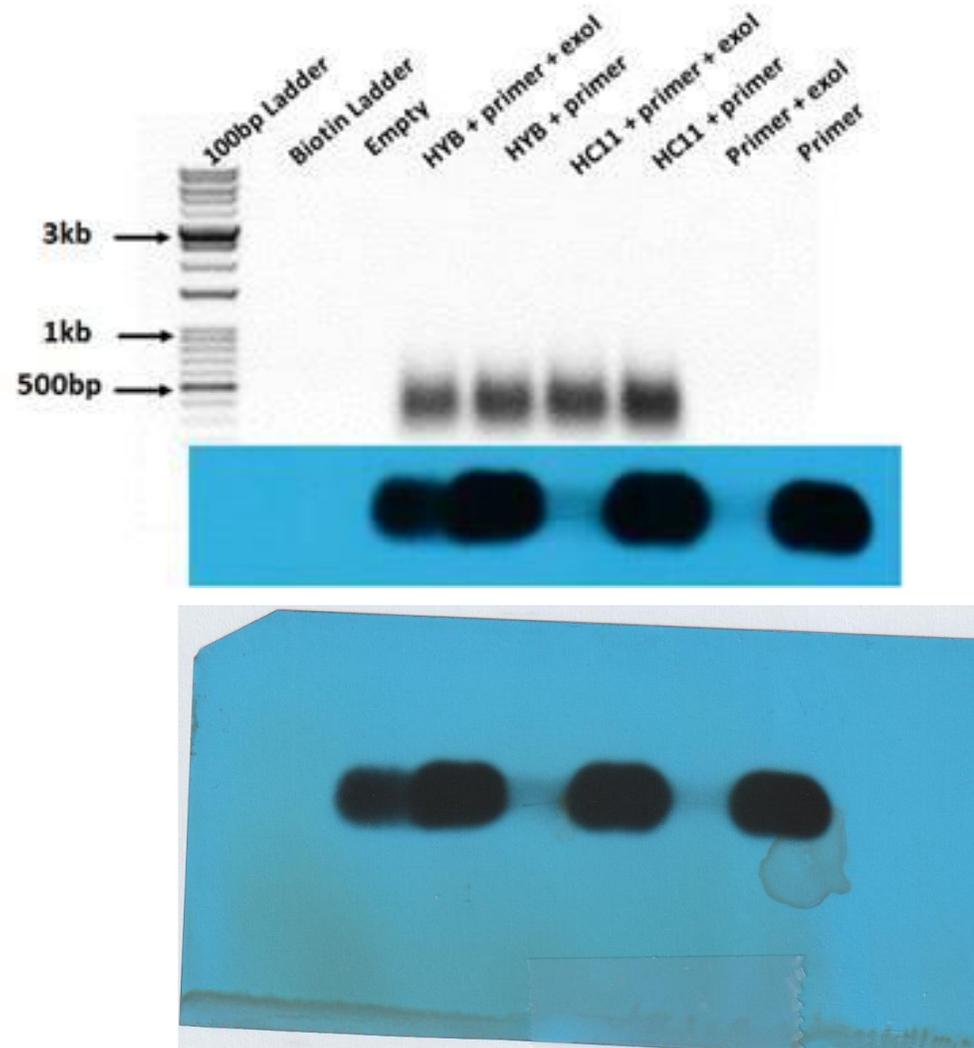


Figure 13



Full gel image



Full gel image