Optimizing dsRNA Removal with High-Throughput Filter Plate Screening and Avipure® dsRNA Clear Affinity Resin

Application Note

Nathaniel Clark, PhD. nclark@repligen.com

Introduction

Filter plate screening is an efficient method for finding optimal pH and salt conditions for the removal of dsRNA byproducts from transcribed RNA. AVIPure® dsRNA Clear removes dsRNA from all ssRNA feeds, including mRNA, circRNA, and saRNA.

Many conditions can be screened with relatively small quantities of RNA. Yield of RNA is measured with UV260, and dsRNA removal can be measured with immuno-dot blots, ELISAs, immuno-northern blots, or other analytical methods.

The results of filter plate screening can establish initial conditions for subsequent column runs. Multiple constructs can be screened to identify those which are most effectively polished with AVIPure dsRNA Clear.

Buffers

AVIPure dsRNA Clear performs well in the high salt buffers commonly used for oligo-dT affinity column binding. These solutions consist of a buffering agent (e.g., Tris, HEPES, MES, phosphate, or carbonate), a salt (0.3 – 1.5 M sodium chloride), and a metal chelator (1 – 5 mM EDTA).

RNA

Typical RNA concentrations are from 0.25 to 2 mg/mL, but higher and lower concentrations can be tested, if compatible with the analytics. RNA concentrations relevant to the bioprocess operations should be evaluated. With low RNA concentrations, the impact of non-specific binding to plasticware is greater, and apparent recoveries may be lower.

Overview

The high-throughput filter plate assay is ideal for quickly determining the optimal pH and salt concentration for both dsRNA removal and RNA solubility. The conditions identified in the filter plate assay are the starting point for subsequent small-scale column runs. PD-scale AVIPure dsRNA Clear columns are available in OPUS® MiniChrom® columns from 0.2 to 10 mL, and OPUS ValiChrom® columns up to 50 mL.

Selective binding of dsRNA occurs in a batch-binding experiment with a small quantity of loose resin and a sample of ssRNA feed. After a brief (30 – 60 minute) incubation period, the dsRNA-depleted flow through is collected by centrifugation. Samples are then analyzed for mRNA recovery by UV260 absorbance, and for dsRNA removal by immuno-dot blot, ELISA, immuno-northern blot, or other assays.

The level of dsRNA byproducts in a given RNA sample can vary by several orders of magnitude; therefore, a wide range of load challenges (grams of RNA/L_{resin}) may still provide satisfactory dsRNA removal. A challenge of 10 mg RNA/$mL_{Column\ Volume\ (CV)}$ is a good starting point for initial testing. Depending on the feed, the resin may be able to treat significantly more than 10 mg RNA/mL_{CV}, but, in the case of a low dsRNA feed, loading the column to a 10% breakthrough capacity might require loading hundreds of column volumes and long processing times.

A simple way to evaluate dsRNA clearance at different challenges is to vary the resin:RNA ratio (Table 1). Reducing the challenge, either by increasing the volume of resin or decreasing the RNA concentration, will indicate whether the capacity for dsRNA was exceeded for a given sample (Table 1). A challenge that results in a satisfactory dsRNA clearance provides a starting point for column runs. Note that because column runs are dynamic and filter plate assays are static, results may differ between the two because of varying phase ratios of sample to resin.

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Table 1. Varying Load Challenge by Adjusting Resin Volume

Resin Volume, mL	RNA Concentration, mg/mL	RNA Volume, mL	Challenge, mg RNA/mL$_{res}$
0.0025	1	0.1	40
0.005	1	0.1	20
0.010	1	0.1	10
0.020	1	0.1	5
0.005	0.5	0.1	10
0.010	0.5	0.1	5

Optimal conditions for each RNA sample must be determined empirically. Generally, dsRNA removal is enhanced with high salt (>0.25 M NaCl) and pH values ≥7.2 (Figure 1). However, the salt concentration must be balanced against the impact of high salt on RNA solubility, which depends on the length and sequence of the RNA, temperature, pH, divalent cations, and other components of the sample matrix. Though 0.5 – 0.75 M NaCl works for most RNAs tested, the effect of salt on RNA solubility should considered for each individual RNA.

The approach described here can simultaneously test for dsRNA clearance and RNA solubility. The RNA(s) of interest are diluted 1:1 with a 2X concentrated buffer stock. One aliquot of the RNA is mixed with AVIPure dsRNA Clear resin, and one aliquot is added to an empty filter plate well. The empty filter plate well serves as a control for dsRNA removal and measures the solubility of the RNA in a given buffer. If the RNA precipitates in the buffer, the precipitates will be removed by the 0.22 µm filter, and the recovered sample will have lower UV260 absorbance, thus providing information on RNA solubility.

Purified RNA suspended (or diluted) in the oligo-dT binding buffer should be used to purify the RNA. Oligo-dT binding buffers have high salt (>0.5 M), and compatibility with the specific RNA should already be established. Processing purified RNA in this manner should provide the highest dsRNA clearance (>2 log reduction of dsRNA). Some RNAs precipitate slowly (over ~15 – 60 minutes) in high salt buffers after initially appearing clear and passing through a 0.22 µm filter. This precipitation reduces the yield of RNA in the flow through and increases the RNA that elutes in 6 M guanidine hydrochloride. Salt concentration should be optimized to minimize precipitation.

The quality of the RNA sample is an important factor in successful processing. Samples that display a single crisp band on a gel or capillary electrophoresis (CE) trace give the best results (i.e., >2 log reduction of dsRNA). Samples that are smeary, degraded, or display multiple bands may have lower dsRNA clearance. For the best results, optimize the in vitro transcription (IVT) parameters until a feed has a single, sharp RNA band.

Materials:

1. Filter plates with hydrophilic, low protein binding, PVDF membranes (MilliporeSigma, MSGVS2210, or similar).

Note: *Include controls to ensure the filter plate does not bind RNA, as some filter plates display significant RNA binding.*

2. UV-transparent plates (Corning® 3635, or similar).
3. Wide-bore pipette tips (Rainin 30389241, or similar).
4. Vacuum manifold (Pall 5017 Multi-well Plate Vacuum Manifold, or similar).
5. Plate shaker (Eppendorf® Thermomixer® 2231001127, or similar).
6. Centrifuge equipped with a swinging-bucker microplate rotor.
7. Western blot processor (Next Advance BlotBot®, or similar).

Protocol

Note: *Protocol room temperature should be consistent with bioprocessing room temperature. RNA solubility is influenced by temperature and placing RNA/salt samples on ice can lead to excessive precipitation.*

1. Buffer preparation
    a. Formulate a grid screen of salt vs pH using a solution of salt (e.g., 5 M NaCl) and 1 M buffer stocks, and 1 – 5 mM EDTA. To enable multi-channel pipetting, formulate the buffers in a deep-well block.

Note: *The buffers and salts tested are presented in Table 2**. However, this is presented only for illustration and is not suggested to be the most relevant set of conditions for your experiment. A very wide range of pH and sodium chloride is evaluated in this example. More narrow ranges of pH and salt or the testing of different buffers, salts, or additives may be more appropriate.*

2. Sample preparation
    a. Use mRNA, circRNA, or saRNA in water or a low concentration buffer (e.g., Tris EDTA, 1 mM citrate, etc.). The RNA should be twice the desired final concentration to enable a 1:1 dilution with buffer stocks.
    b. Mix RNA 1:1 with 2X buffer stocks (Table 2). Prepare at least 200 µL for each condition (one resin test, one control well).

Tip*: prepare 10% extra volume to allow for pipetting errors and losses. If replicates are desired, prepare additional sample. Alternatively, the RNA and buffers can be mixed directly in the filter plate (as described in Section 4 below).*

    c. To test for non-specific filter plate binding, prepare an additional aliquot to pipette directly into the UV-transparent 96-well plate.
3. Resin preparation
    a. The resin is supplied as a 50% slurry in 18% ethanol.
    b. Dilute slurry to 10% using 18% ethanol (1 volume 50% slurry +4 volumes 18% ethanol).

Note: *Using a 10% slurry allows for more precise dispensing than a 50% slurry.*

    c. Use wide-bore tips for pipetting.
    d. Pour resin into a multi-channel pipetting reservoir and shake gently before pipetting to minimize settling. Use a multi-channel pipettor with repeating mode to dispense appropriate volume of slurry into wells. Dispense quickly to minimize resin settling in the pipette tips which could lead to uneven aliquoting.
        i. With a 10% slurry, dispense 25 µL to assay 2.5 µL resin/well, 100 µL to assay 10 µL resin/well, etc.
    e. Remove ethanol supernatant with vacuum or centrifugation.
    f. Add 100 µL of buffer or water to each well. Incubate plate for 1 – 5 minutes on shaker and remove solution with vacuum or centrifugation. Repeat 1 – 2 additional times.
4. Sample preparation and addition.
    a. In the example shown in Table 2, the same buffers are added to columns 1 – 6 and 7 – 12 of the filter plate to achieve two replicates per buffer. A multichannel pipette with 6 tips attached was used to dispense 50 µL aliquots of 2X buffers to columns 1 – 6, then 7 – 12, repeating this step for each row of buffer stocks.
    b. After adding 50 µL buffer, 50 µL of the RNA solution was dispensed using a multi-channel pipette basin and a 12-channel pipette to achieve the target working buffer concentration.

Table 2. Example Plate Map for Concentrated Buffers to Achieve Two Replicates per Buffer

	Column	1	2	3	4	5	6	7	8	9	10	11	12
All contain 5 mM EDTA	Row	0.1 M MES pH 6.5	0.1M HEPES pH 7.2	0.1 M Tris pH 8	0.1M Tris pH 9	0.1 M Na-carbonate pH 9.5	0.1 M Na-carbonate pH 10	0.1 M MES pH 6.5	0.1M HEPES pH 7.2	0.1 M Tris pH 8	0.1M Tris pH 9	0.1 M Na-carbonate pH 9.5	0.1 M Na-carbonate pH 10
NaCl, M	A)	0	0	0	0	0	0	0	0	0	0	0	0
	B	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	C	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
	D	1	1	1	1	1	1	1	1	1	1	1	1
	E	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
	F	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	G	2	2	2	2	2	2	2	2	2	2	2	2
	H	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5

5. Static-binding phase
    a. Cover the filter plate containing RNA plus buffers with a lid and place on a mixer. Place an empty plate below the filter plate to prevent damage and contamination. Affix the plates to the shaker with two rubber bands. An Eppendorf Thermomixer or similar set to 1,100 rpm can be used to mix the samples.
    b. After 30 – 60 minutes of shaking, place the filter plate over a full-area 96-well UV-transparent plate, and collect the RNA by centrifuging the plate for 1 minute. If liquid remains in the filter plate, extend the centrifugation time to 5 minutes. If liquids remain after an extended centrifugation interval, the filters are likely plugged by RNA precipitates (suggesting that the specific condition is not compatible with the RNA).

Note: *Depending on the RNA concentration and plate geometry, the sample absorbance could be higher than the linear range of the plate reader. For this reason, ½ area UV plates should be avoided.*

6. Analysis
    a. Measure absorbance at 260 nm with a plate reader and pathlength correction mode. RNA concentrations in mg/mL can be calculated with the following conversion factor: 1 mg/mL RNA = 25 OD260
        i. If overflow signals appear, a dilution plate may be required, or the pathlength can be reduced by removing a portion of the sample.
    b. To measure non-specific binding to the filter plate, pipette aliquots of the input RNA directly into the 96-well UV-transparent plate. Compare the absorbance of this sample to the control well containing the same sample. Use a second UV plate if there are no empty wells or modify the plate layout to accommodate these controls.
    c. Examine the RNA concentrations in the control wells to determine if significant precipitation occurred in the selected conditions. Look for trends of concentration as a function of salt and pH.
    d. Examine the pathlength values to determine if any filter plate wells were clogged by precipitated RNA. Ideally the pathlengths should be consistent (+/- 10%). Look for trends of decreased pathlength values with salt and pH as this may indicate where significant precipitation is occurring.

Note: *Filter plate clogging could also be due to insoluble buffer components.*

    a. Immuno-dot blot
        i. For a qualitative analysis of dsRNA removal, spot 2 µL of each sample directly from the UV plate onto a dry, positively charged nylon membrane (Cytiva P/N 10416294 or similar).

ii. If the volumes required are >2 µL, a vacuum dot blot manifold (e.g., Bio-Rad) can be used to apply large volumes to each spot. Assemble the device with a membrane and add 100 µL water or buffer to each well, then pipette the desired mass of RNA and apply gentle vacuum until the wells are empty.
iii. Place membrane in blocking buffer for 2 – 6 hours (match blocking time to antibody incubation time)
iv. Incubate with 1:10,000 dilution of J2-HRP antibody in blocking buffer (Novus NBP3-11395H or similar) for 2 – 6 hours
v. Wash 5 times, 15 minutes each, with 1x TBST buffer

Note: *The dot blot can be processed using standard western blotting reagents, instruments, and protocols, if a western blotting workflow is already established, it should be compatible with the dot-blot.*

vi. Add chemiluminescent detection substrate (Thermo P/N PIA38555, or similar) and image blot with gel-doc system. If the experiment includes different RNA samples containing varied dsRNA levels, capturing images with different exposure times may be necessary to capture the information. Either set up the instrument for multiple exposure times (e.g., 1, 2, 5, 10, 30, 60, 180 seconds), or set the region of the image used for autoexposure calculation to the darkest control wells, capture an image, then change the autoexposure region to an area with lighter control wells. Examine the images and manually adjust brightness/contrast to ensure the capture is appropriate for all samples.

b. ELISA
   i. Quantify dsRNA levels (Vazyme DD3509EN-01). Due to the wide range of dsRNA levels, analyze a range of dilutions to ensure that some dilutions fall on the standard curve. Matrix effects are not observed with common buffers such as those shown in Table 1, and samples can be assayed without desalting.
   ii. AVIPure dsRNA Clear reduces dsRNA by 2 – 3 logs in some instances. However, these very low levels can still be quantified by ELISA. Smaller dilutions will be required for samples with very low dsRNA, to ensure they fall on the standard curve.
c. Immuno-northern blot
   i. The immuno-northern blot (Figure 2) can provide insights into dsRNA removal by showing the size distributions of dsRNAs before and after incubation with AVIPure dsRNA Clear resin. This technique may provide insights into the nature and type of dsRNA produced during transcription and be a useful tool in optimizing the transcription step.
   ii. See related manuscript for detailed protocols on the immuno-northern technique (Reference 1).

Interpretation of Results

Double stranded RNA levels can vary by several orders of magnitude in different RNA feeds. Levels of 0.01% – 1% are frequently encountered, and after purification with AVIPure dsRNA Clear, the levels can be reduced by 1 – 3 logs, depending on the RNA and processing conditions. A wide range of dsRNA levels must be considered in analysis of these experiments, as a series of ELISA dilutions, or dot blot image exposures that work for one set of samples will be too high or low for another set, leading to many samples being above or below the limits of quantitation.

The immuno-dot blot is useful for qualitative screening, as it can process many samples with little hands-on time. Trends in dsRNA removal as a function of salt, pH, additives, or other variables can be quickly identified. Though the dot blot can be used for quantitative tool, a dsRNA ELISA assay is recommended for quantitation. The ELISA is reliable, easy to standardize, and provides consistent inter-assay results. Additionally, matrix effects are not observed with the buffers in Table 2 and the Vazyme dsRNA ELISA kit; therefore, no buffer exchange or desalting step is required prior to analysis.

Example Data

Firefly luciferase (fLuc) was prepared with in vitro transcription (NEB T7 Quick E2050S) and purified with lithium chloride precipitation. Though this sample is easy to produce using the control template provided with many IVT kits, the dsRNA removal is usually low, ~3-10-fold reduction. The reasons for this are not clear but may be related to the partial effectiveness of the lithium chloride precipitation. There may be IVT components that interfere with dsRNA removal. A stock of 0.5 mg/mL fLuc in water was mixed 1:1 with the buffers in table, to a final concentration of 0.25 mg/mL. 10 µL of resin was added to each well for a final

challenge of 2.5 mg RNA/mL_{resin}. The buffers covered a wide range of sodium chloride concentrations (0 – 1.25 M), and pH values (6.5 – 9.5).

Analysis of the RNA concentrations with UV260 absorbance (Table 3) revealed 75% RNA recovery for most conditions ([AVIPure FT concentration]/[control well concentration]*100). A chase wash was not performed, as the objective was optimizing dsRNA removal with pH and salt. Inclusion of a chase may increase the RNA recovery. When the experiment was repeated using a 0.2 mL column at pH 7.2 with 0.625 M NaCl, the recovery was 90%.

The RNA concentrations in the control wells decreased in the 1.25 M NaCl condition, suggesting that that some RNA was precipitating in the buffer, and, therefore, 1.25 M NaCl was considered too high for subsequent column runs.

The experimental wells (containing AVIPure dsRNA Clear) indicate that recoveries are highest at pH 9.5 and pH 10. However, there is a risk of loss of RNA integrity due to base-catalyzed hydrolysis at elevated pH values. Several RNAs have been successfully purified at pH 9 in filter-plate and column formats without loss of integrity with post-column neutralization. The addition of a neutralization buffer (e.g., 20 – 40% 1 M acetate, pH 5) to either the UV collection plate (filter plate assays), or the fraction tubes (columns runs) will minimize the time the RNA spends at elevated pH. In this case, good dsRNA reduction was observed at pH 7.2 – 8, and more alkaline buffers were unnecessary.

A qualitative dot blot indicated how effective dsRNA removal was at different pH and salt concentrations (Figure 1). 2 µL of each flow through sample was spotted on the membrane, for a load of approximately 0.5 µg RNA/spot. The blot revealed that for all pH values, dsRNA removal was enhanced by NaCl. Concentrations of 0.38 M NaCl and higher had the faintest spots. dsRNA removal was also enhanced by increasing the pH from pH 6.5 to 7.2 and above. At 0 and 0.25 M NaCl, dsRNA removal clearly improved at pH 9, 9.5, and 10, but with higher NaCl (0.38 – 1.3 M), there was no indication that the higher pH values removed more dsRNA.

Considering the precipitation in 1.25 M NaCl, and dot blot results, a condition of 10 mM tris, pH 8, 0.5 M NaCl, and 0.5 mM EDTA was selected. A total of 5 purification cycles were run on a 0.2 ml MiniChrom dsRNA Clear column (Figure 3). The feed was 0.51 mg/mL fLuc with 1.4% dsRNA content by ELISA. The challenge was 10.2 g fLuc/L_{res}, at a flowrate 0.4 mL/min for a 30 second residence time. Based on dot blot analysis of cycles 1 and 5, the dsRNA was effectively removed under these conditions. RNA recovery was between 86 and 91%, and mass balances were close to 100% for all 5 runs.

Notes

1. The mRNA yields are typically lower in filter plate experiments than in columns runs. In the example presented, the mRNA recovery was 75% in filter plate (Table 3, well 2C), but 90% in column runs (Figure 3). This may be due to non-specific binding to the surfaces of the filter plate. At lower RNA concentration (e.g., <0.1 mg/mL) the recovery may be lower due to non-specific binding to plasticware.
2. A neutralization buffer is recommended if the pH is greater than 8. One option is 20 – 40 % (v/v) 1 M acetate buffer (pH 4 – 5), but compatibility with the RNA of interest and the final pH value should be tested.
3. If dsRNA clearance optimization is the primary objective of a filter-plate assay, to save time, stop the experiment after collecting the flow through samples. If the resin and plate will be reused, perform all steps in Table 3. Depending on the objective (e.g., recovery optimization, sanitization testing, or resin cycling studies), modify the protocol as needed.

Table 3. RNA concentration in filter-plate flow-through (mg/mL), calculated from absorbance at 260 nm

		AVIPure dsRNA Clear						empty control wells						
	pH:	6.5	7.2	8	9	9.5	10							
[NaCl]	+fLuc	1	2	3	4	5	6	7	8	9	10	11	12	
0	A	0.20	0.20	0.21	0.23	0.24	0.24	0.30	0.27	0.28	0.27	0.27	0.28	mg/ml
0.5	B	0.18	0.20	0.20	0.21	0.21	0.21	0.27	0.26	0.26	0.25	0.26	0.26	mg/ml
0.75	C	0.16	0.20	0.21	0.20	0.23	0.24	0.27	0.27	0.26	0.25	0.27	0.29	mg/ml
1	D	0.19	0.19	0.19	0.20	0.19	0.22	0.26	0.25	0.25	0.25	0.27	0.26	mg/ml
1.25	E	0.18	0.18	0.20	0.19	0.22	0.23	0.26	0.24	0.25	0.25	0.24	0.34	mg/ml
1.5	F	0.22	0.20	0.21	0.22	0.24	0.25	0.25	0.26	0.24	0.26	0.27	0.26	mg/ml
2	G	0.19	0.19	0.20	0.21	0.21	0.24	0.26	0.25	0.26	0.25	0.25	0.25	mg/ml
2.5	H	0.22	0.19	0.20	0.21	0.22	0.24	0.21	0.22	0.22	0.24	0.20	0.24	mg/ml

Figure 1. Immuno-dot blot of luciferase filter plate experiment. dsRNA is detected with the J2 mAb

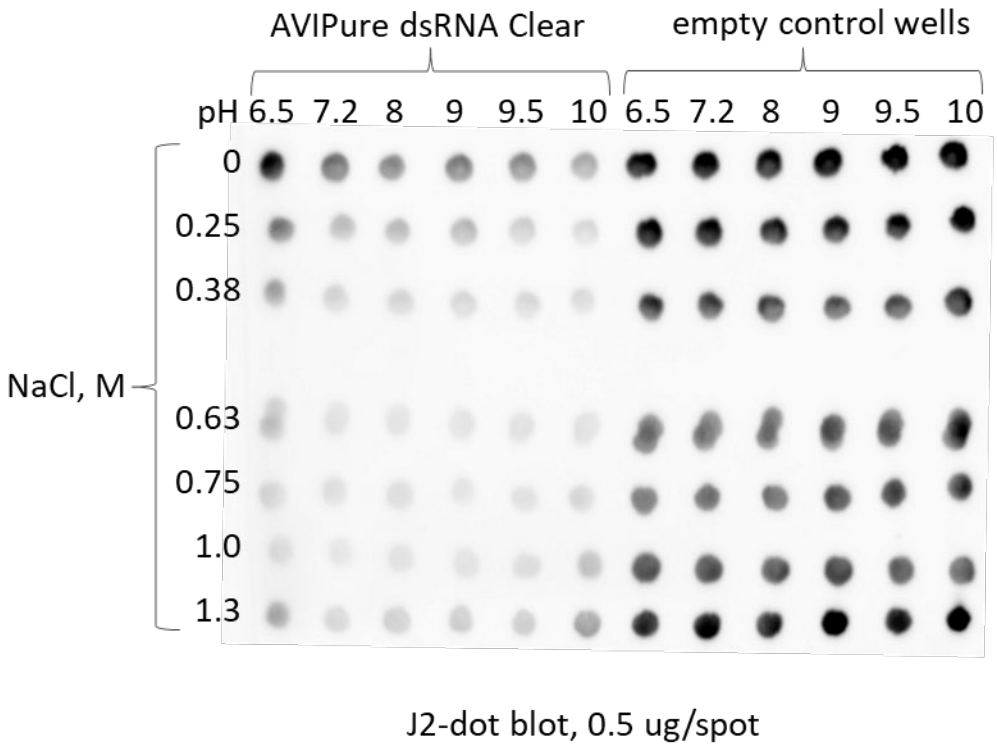
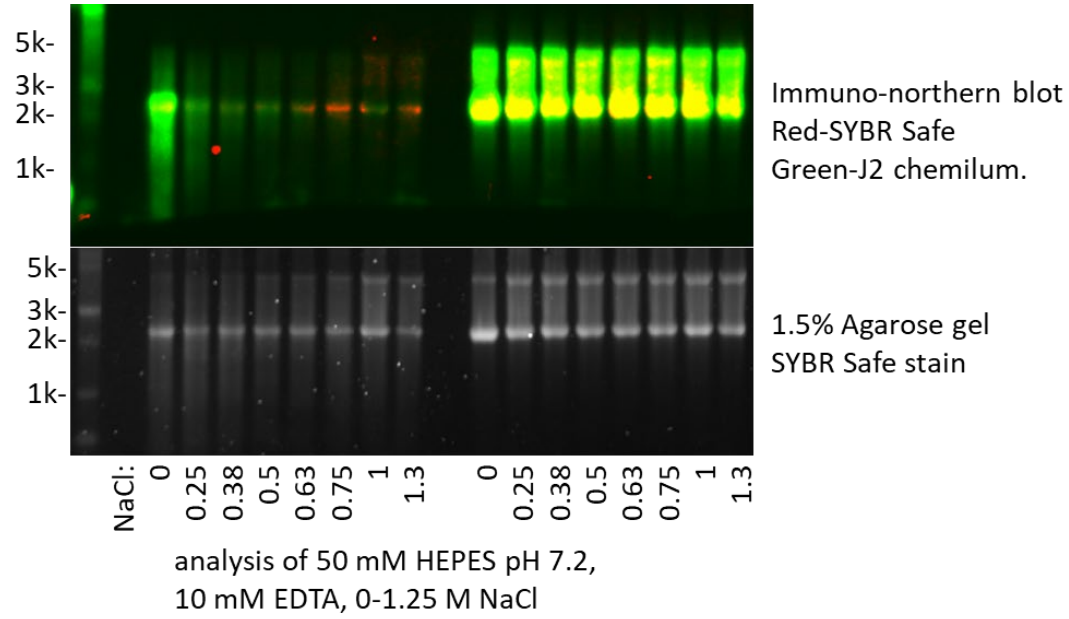
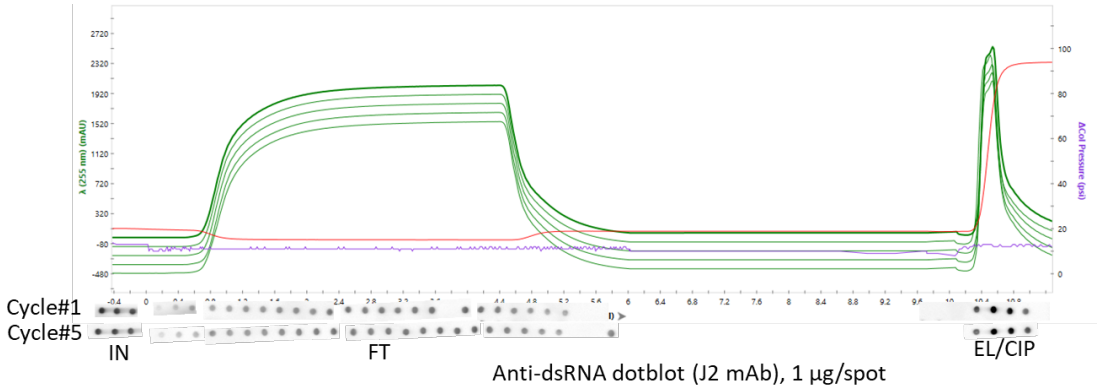


Figure 2. Immuno-northern blot of the pH 7.2 input and flow-through samples



Green: J2 chemiluminescence signal corresponding to dsRNA, Red: SybrSafe total RNA stain.

Figure 3. Immuno-northern blot of the pH 7.2 input and flow-through samples



	Run 1	Run 2	Run 3	Run 4	Run 5
% FT	88%	87%	86%	91%	90%
% Elut	13%	11%	10%	10%	10%
% mass balance	102%	98%	96%	101%	100%

Reference

1. Clark NE, Schraut MR, Winters RA, Kearns K, Scanlon TC. An immuno-northern technique to measure the size of dsRNA byproducts in in vitro transcribed RNA. Electrophoresis. 2024;45(17-18):1546-54.

Customer Service

Repligen Corporation
41 Seyon Street
Building 1, Suite 100
Waltham, MA 02453

customerserviceUS@repligen.com

(781) 250-0111

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