Development and validation of a commercial dsRNA (J2 based) ELISA for the quantification of double-stranded RNA in biological preparations.

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ABSTRACT

mRNA vaccines have quickly gained ground after both BioNTech and Pfizer gained Emergency Use Approvals for their mRNA based SARS-CoV-2 vaccines. The development of a successful mRNA vaccine requires both stability and purity. After in vitro transcription, purification processes must be performed for removal of DNA template as well as contaminants like the highly immune stimulatory double-stranded RNA (dsRNA), which can induce fatal immune responses. dsRNA is typically removed using HPLC methods, though ELISA is used to measure the presence and therefore additional purification requirements for a successful vaccine. Currently, only one ELISA for estimation of dsRNA is commercially available - a qualitative assay in a reagents-box format, that requires the scientist to coat, optimize and validate their own assay for estimation of dsRNA. This allows for human error and variability in detection. Additionally, since the ELISA available are qualitative, the scientist is required to potentially run the same sample through purification multiple times until a satisfactorily purified sample is received with guess estimates and no benchmarks on impurity levels.

The objective was to develop a new, sensitive, easy to use and pre-validated sandwich assay for measurement of dsRNA, using J2 antibodies, the gold standard in the industry for detection of dsRNA contaminants. This would ensure standardization of results, and a quantitative result would allow scientists to have a better understanding of the purity of their sample, and therefore the efficiency of their purification method.

The development of the assay involved coating microplates with antibodies specific to dsRNA, followed by sample incubation and a subsequent enzyme-based detection system. We optimized various parameters, including antibody concentration, blocking conditions, and incubation time, to enhance assay sensitivity, dynamic range, and reproducibility. Validation studies using known dsRNA standards demonstrated excellent linearity and accuracy. The simplicity, sensitivity, and specificity of this assay make it an essential tool in virology research and diagnostics and as an in-process tool for mRNA vaccine development and management.

INTRODUCTION

mRNA vaccines have quickly gained ground after both BioNTech and Pfizer gained Emergency Use Approvals for their mRNA based SARS-CoV-2 vaccines (Polack et al. 2020; Baden et al. 2021). With the effectiveness of these mRNA based vaccines not just against the wild type variants but also against subsequent variants, many mRNA vaccines are now under R&D and pre-clinical stages.

The development of a successful mRNA vaccine requires both stability and purity. After in vitro transcription, purification processes must be performed for removal of DNA template as well as contaminants like double-stranded RNA (dsRNA). dsRNA is recognized by multiple viral RNA sensors in vitro – specifically PRRs (TLR3, MDA-5, and RIG-I) and induces the secretion of type I interferons (Alexopoulou et al. 2001; Botos et al. 2009) which upregulates the expression and activation of protein kinase R and 2'-5'-oligoadenylate synthetase (OAS), leading to the inhibition of translation and the degradation of cellular mRNA and ribosomal RNA, respectively. Therefore, these highly immune stimulatory dsRNA must be removed from mRNA preparations for the development of a successful vaccine. The most common method of removing dsRNA effectively is via HPLC or other chromatographic methods like FPLC. However, purifying IVT mRNA via HPLC yields only about 50%. Vaccine preparations at a scalable level must be analysed to quantify any potential dsRNA that may have been left behind (Baiersdörfer et al. 2019). Accurate quantification of dsRNA plays a vital role in the process of identifying and removing these dsRNA contaminants.

Currently, either HPLC or commercially available ELISA are used for the detection of dsRNA. However, the currently available dsRNA ELISA require complete reagent preparation, plate coating and optimization on the user end. All validation of the assay must also be performed by the user to ensure accuracy and robustness in results. Krishgen's objective was to develop a sensitive and optimized direct sandwich assay that was validated on the manufacturer end for reproducibility and accuracy. Rather than the currently available assays that use an indirect sandwich assay method, Krishgen aimed to develop a more sensitive ELISA. Removal of manual methods and thus, reduction in chances of error are particularly important since the mRNA vaccines with incorrect values of dsRNA contamination may be fatal for patients.

The objective was to develop a sensitive, easy to use and pre-validated sandwich assay for measurement of dsRNA, using J2 antibodies, the gold standard in the industry for detection of dsRNA contaminants. The J2 anti-dsRNA IgG2a monoclonal antibody (Schönborn et al. 1991) has become the gold standard in dsRNA detection. It was used initially for the study of plant viruses, but since the seminal paper of Weber et al. in 2006, where J2 was used to show that all the positive strand RNA viruses tested produced copious amounts of dsRNA in infected cells, this antibody has been used extensively in a wide range of systems, as documented in over 200 scientific publications. Recently, J2 has also been used to monitor the removal of dsRNA from in vitro synthethized mRNA preparations that may have potential use in gene therapy (Kariko et al., 2011). J2 has been used successfully in various immunocapture methods, such as ELISA. The Anti-dsRNA monoclonal antibody J2 was observed to recognise double-stranded RNA (dsRNA) provided that the length of the helix is greater than or equal to 40 bp.

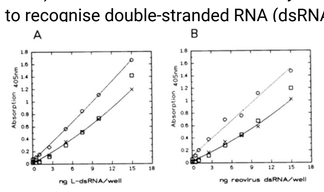


Figure 1. From Schönborn et al, 1991. Results of the comparison of dsRNA binding to dsRNA specific MAB in the presence and in the absence of DNA, rRNA or tRNA by sandwich ELISA. L-dsRNA (A) or reovirus dsRNA (B) were used as antigens in the absence or in the presence of 100 jg/well of herring sperm DNA (IA and IB), 16S + 23S rRNA from E. coli (-0-, 1A) or E. coli tRNA (-0-, IB). The sandwich ELISA was carried out as described in the paper's Material and Methods section and the absorption was measured 2 h after substrate addition. DNA, tRNA and rRNA showed no or only very low binding to the antibodies and they did not reduce the sensitivity of dsRNA detection. The results indicate that the apparent affinity of the anti-dsRNA antibodies to ssRNAs (and to DNA) may be several orders of magnitude lower than that to dsRNA. This study formed the basis of the J2 antibody becoming gold standard in dsRNA detection.

The principle of the assay was based on the use of two double-stranded RNA (dsRNA)-specific monoclonal antibodies in a direct sandwich format which allows sensitive and selective detection of dsRNA molecules (>=40 bp), independent of their nucleotide composition and sequence.

METHODS

Anti-dsRNA monoclonal antibody J2, a gold standard for dsRNA detection, was used as the coating / capture antibody. This was coated overnight onto Corning CoStarTM microwell plates using a proprietary coating solution and blockers for long term immobilization and stability of the antibody. Both antibodies are mouse monoclonal antibodies (IgG2a kappa/IgM kappa). The Standard used was an in-vitro synthesized dsRNA of 142 bp in a lyophilized form. It was run at six dilutions to form the standard curve of the kit. A low molecular weight Poly(I:C) dsRNA sequence was used as the positive control. Anti-dsRNA monoclonal antibody K1 was conjugated to HRP using an in-house conjugation protocol and was used as the detection antibody. The assay scheme is depicted in Fig. 2.

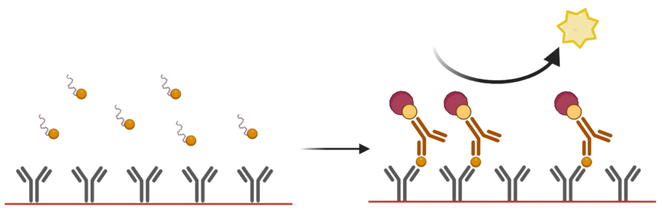


Figure 2: dsRNA assay principle: a direct sandwich assay

Various incubation and wash steps were used to optimize removal of unbound proteins at various steps. The substrate solution of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and incubated. The enzyme reaction was terminated by stop solution dispensed into the wells turning the solution from blue to yellow. The optical density (OD) of the solution read at 450 was directly proportional to the specifically bound amount of dsRNA present in the sample. Absorbances were read on a Tecan Safire2.

Each assay step was optimized for optimal noise-to-signal ratio and working range using a checkboard experiment format. This included running the standards in duplicates in various diluents, at various coating and detection conjugate levels, and other variables. The optimized kit was then validated using the guidelines set by the ICH M10 (USA FDA / EMA). This included determining precision, sensitivity, stability and robustness. Repeatability was determined using ten replicates of the same extract in one assay. Intra-assay reproducibility was evaluated by analyzing ten extracts of the same sample in one assay. Inter-assay reproducibility was determined analyzing three extracts of the same sample in three independent assays. Additional optimization and spiking experiments were performed for minimal %CV and relative error. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low, medium and high concentrations, run in duplicates. Robustness was estimated by introducing deliberate changes in the established procedure in the same experiment. The Limit of Detection (LOD) was estimated as the average concentration of ten replicates of the zero standard plus three. Finally, an accelerated stability test was conducted by keeping various temperature sensitive parameters at 37°C and calculating deterioration via %CV. Other in-house and regulatory validation processes were also compeleted. Calculations and statistical analysis were performed using the GraphPad Prism Software v5.

RESULTS - METHOD OPTIMIZATION

The sandwich ELISA was optimized for dsRNA concentration and buffer composition of coating and detection antibodies, washing buffer composition, as well as incubation temperature and time of the different steps of the assay to give a commercially acceptable assay that surpasses current industry standards. The final protocol was set for 210 minutes, with two incubation steps. Optimal noise:signal ratio and sensitivity formed the pillars of the validation. The ELISA was designed for 0 - 200 ng/ml as assay range during optimization, with standards 3.125, 6.25, 12.5, 25, 50, 100 and 200 ng/ml.

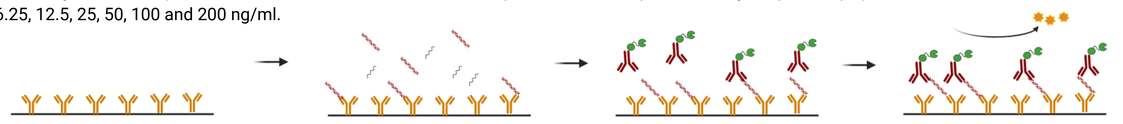


Figure 3: The final optimized protocol for the assay. *Samples or standards are added to the pre-coated well and incubated.* *After a wash, detection antibody is added and incubated.* *After another wash, TMB and Stop solutions are added and results are read.*

***Limit Of Detection*:** It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. Over 10 runs in duplicates, the LOD was found to be 1.5 ng/ml.

***Standard Lyophilization*:** To ensure stability of the standard over the 12 month expiry, it was lyophilized using in-house proprietary solutions and methods. Validation was performed for the quality of lyophilization over various lots to ensure that it provides robust and reliable results for each run. In each complete run, acceptable recovery results were considered when between 8-12%CV only. The final concentration of the lyophilized standard was set at 1000 ng/ml, which was diluted by the user to the required standard range.

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.144	--	--
3.125	0.377	3.2	103.7
6.25	0.601	5.9	94.3
12.5	1.113	12.9	103
25	1.701	25.1	100.5
50	2.229	47.9	95.7
100	2.666	108.4	108.4
200	2.822	189.1	94.5

Table 1: Results obtained from one of the 142bp dsRNA standards lyophilized, reconstituted and then run as full range standards. This table also shows recovery obtained by spiking standards, within 90-110%.

RESULTS - ASSAY VALIDATION

***Limit Of Detection*:** It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. Over 10 runs in duplicates, the LOD was found to be 1.5 ng/ml.

***Specificity*:** The assay works on the sandwich ELISA principle and uses the J2 (IgG2a kappa) mouse monoclonal antibody to dsRNA as the capture antibody. The J2 antibody is the gold standard for dsRNA detection, with an extensive study conducted by J. Schönborn et. al (1991) acting as the primary specificity study for J2 antibody users globally. To increase specificity and incorporate different types of samples, some of which may not be as well captured by the J2 antibody, we included a K1 dsRNA antibody as the detection antibody. Anti-dsRNA monoclonal antibody K1 recognises double-stranded RNA (dsRNA) provided that the length of the helix is greater than or equal to 40 bp as well. The detection is highly specific: dsRNA can be detected in nucleic acid extracts in the presence of 1,000-10,000-fold excess of other nucleic acids.

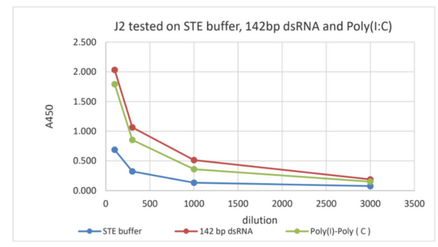


Figure 4: The J2 antibody was tested for its affinity to both the 142bp dsRNA standard as well as the Poly (I:C) control. The antibody showed great affinity for both, with the positive control showing the most affinity, ensuring accurate quality control results.

Additionally, to ensure specificity, the J2 anti-dsRNA antibody was subjected to an affinity study with the 142bp dsRNA standard and the poly (I:C) control for better optimization of the final assay. The lyophilized antibody was run after immediately after a simple reconstitution, while the 142bp dsRNA and the Poly (I:C) were run neat. The antibody showed great affinity for both the samples, with greater affinity for the Poly (I:C), which was then used as the positive control for the kit (represented in Figure 4).

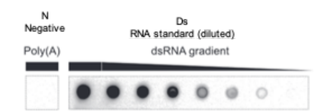


Figure 5: Dot Blot Assay for dsRNA Standard Used

Precision: It is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (31.25 ng/ml), medium (250 ng/ml) and high (2000 ng/ml) concentrations. Additionally, when running the complete standard range as well in duplicates, %CV within and between plates was under 10%, ensuring robust precision and reproducibility.

	Lot 1	Lot 2	Lot 3		
Standard Concentration (ng/ml)	Mean Absorbance	Mean Absorbance	Mean Absorbance	% Standard Deviation	%CV
0	0.124	0.112	0.126	0.8	6.4
3.125	0.406	0.404	0.414	0.5	1.2
6.25	0.646	0.654	0.651	0.4	0.7
12.5	1.088	1.063	1.103	2.0	1.9
25	1.676	1.682	1.692	0.8	0.5
50	2.189	2.259	2.213	3.6	1.6
100	2.614	2.585	2.570	2.2	0.9
200	2.786	2.887	2.837	5.1	1.8

Table 2: Three lots of complete standards were run on three different days to observe the deviation in absorbance. They were run in duplicates following the protocol, and mean absorbance was noted. Standard deviation was calculated by subtracting difference between wells on each day for each standard, and the average was then noted. In all three lot runs, satisfactory recoveries were observed, and statistical results showed low standard deviation between wells, and minimal co-effecient of variation.

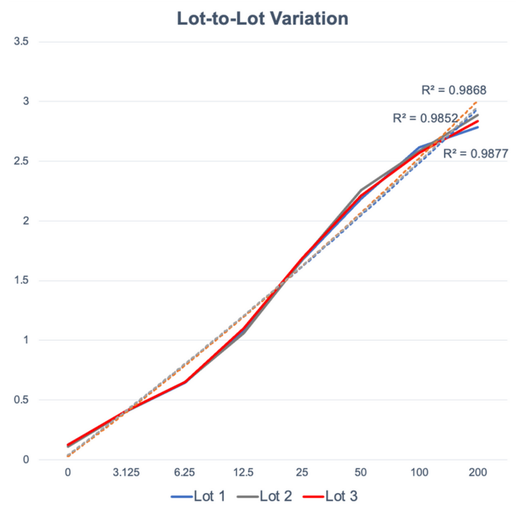


Figure 6: Graphical representation of Table 1. Three lots were mapped on graphpad prism and a best curve trendline of 2nd order polynomial was fit. For each lot, the the R2 of 0.98 or higher, the acceptable standard for bioassays as per EMA / FDA guidelines.

***Recovery*:** It is used to determine whether analyte detection can be affected by the difference between diluent used for preparation and the experimental sample matrix. It is an important technique for analyzing the accuracy of the dsRNA ELISA. The recovery of this assay was assessed by comparing observed vs. expected values based on non-spiked and/or neat (undiluted) samples across several lots of samples. Various dilutions of spiked samples were run in duplicates and concentration was interpolated. Next, the percentage of recovery was calculated. For the three lots (n=5) run for recovery analysis, each lot provided satisfactory results. with recovery between 90-110%. Please refer to table 2 for an example of this run.

Sample	Dilution	Expected ng/ml	Observed ng/ml	% Observed/ Expected
A	undiluted	---	200.0	---
	1:2	100.0	96.0	96%
	1:4	50.0	47.5	95%
	1:8	25.0	23.25	93%
B	undiluted	---	100.0	---
	1:2	50.0	53	106%
	1:4	25.0	28.5	114%
	1:8	12.5	14.25	114%
C	undiluted	---	50.0	---
	1:2	25.0	23.5	94%
	1:4	12.5	11.5	92%
	1:8	6.25	6.0	96%

***Linearity of Sample Dilutions (Parallelism)*:** It refers to the extent in which a spike or natural sample's dose response is linear and in the desired assay range. Three samples were diluted in duplicates with the optimized sample diluent. Results (mean) in ng/mL are shown below in Table 4 and Figure 3.

High Dose Hook Effect: It is a reduction in measured signal that occurs in the presence of very high concentrations. Over several duplicate runs, the ELISA kit did not experience a high dose hook effect when it was tested up to a dsRNA concentration of 1000 ng/ml.

Table 3: Three samples were run at neat + three dilutions to observe dilutional linearity. Each sample, at each dilution point, offered accurate values, with the % recovery (percent observed concentration / expected concentration) was between the acceptable 90 - 110%.

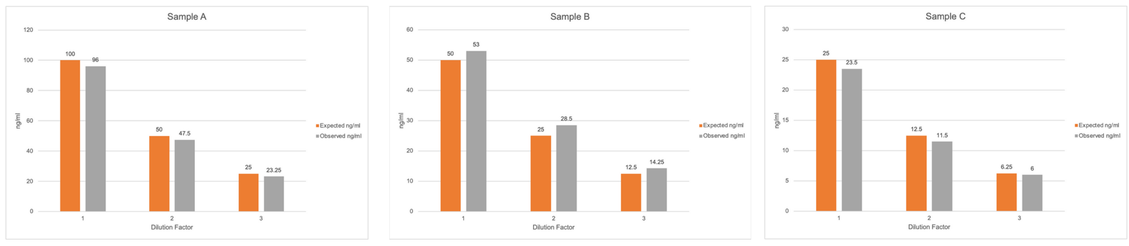


Figure 7 shows the three samples (A, B and C) at the three dilution points (1:2, 1:4 and 1:8). The orange bars show expected concentration and the grey bars represent observed or interpolated concentration of that same sample.

***Accelerated Stability*:** Accelerated stability testing increases the rate of degradation and physical change of components by using exaggerated storage conditions as part of the formal stability testing program. Three dsRNA kits from the same lot were subjected to a fourteen day accelerated stability study, with one critical component from each kit at stored at 37 degrees Celsius. The entire standard range was run on days 1, 2, 4, 6, 11 and 14 as per the protocol, meant to represent the stability of the kit over a period of 12 months. Inter- and intra- assay precision and recovery was analysed for each lot at each run. Satisfactory results were obtained from the accelerated stability studies under the acceptable 20% CV over all standards across all types of runs (detection conjugate, standard and plate).

CONCLUSION

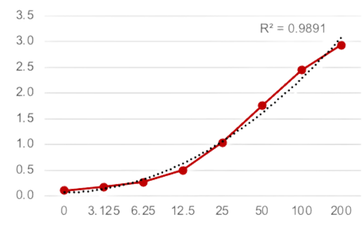
Upon completion of the validation process of the assay as per both internal and regulatory standards, we report that the KRIBIOLISA dsRNA ELISA kit successfully and accurately detected dsRNA contaminants with high accuracy. The ELISA was designed for 0 - 200 ng/ml as assay range and achieved a sensitivity of 1.5 ng/ml. The sensitivity of the assay developed was better than most competitors, allowing for smaller concentrations of dsRNA contaminants to be detected and reducing purification times. The pre-coated and validated format also allows for better precision and recovery due to the commercial standardization of the kit.

The developed KRIBIOLISA dsRNA ELISA kit is an accurate, simple, rapid and cost-effective method to detect dsRNA contaminants. We recommend using the ELISA to detect viral dsRNAs or large natural or synthetic dsRNAs of non-viral origin in nucleic acid extracts, as well as to detect the presence of undesired dsRNA molecules in artificially synthesized (m)RNA preparations.

Standard Concentration (ng/ml)	Absorbance A	Absorbance B	Mean Absorbance	% Standard Deviation	%CV
0	0.123	0.125	0.124	0.2	1.5
3.125	0.394	0.419	0.406	1.8	4.3
6.25	0.646	0.645	0.646	0.1	0.1
12.5	1.100	1.075	1.088	1.8	1.6
25	1.618	1.734	1.676	8.2	4.9
50	2.122	2.255	2.189	9.4	4.3
100	2.627	2.602	2.614	1.8	0.7
200	2.816	2.756	2.786	4.3	1.5

Table 4: Typical results from the KRIBIOLISA dsRNA ELISA run.

- Developed as a direct sandwich assay format, the first of its kind, and comes with a pre-coated plate and pre-optimized reagents, with a 210 minute protocol time.
- Use of anti-dsRNA K1 antibody in conjunction with the J2 antibody offers specificity.
- It is well validated and performs within required precision parameters, as demonstrated over many lots of kits and testing.
- Final assay range was set at 0 - 200 ng/ml, with a sensitivity of 1.5 ng/ml.
- Offers robust inter- and intra- assay precision of <12% CV each.
- Ships at room temperature or 2-8 degrees Celsius, owing to lyophilized standards.
- Provides 90% - 110% recovery and shows dilutional linearity.



A typical graph that is included with each kit as part of the certificate of analysis. It represents the lot characteristics and expected graph.

REFERENCES

Corbett KS, Edwards DK, Leist SR et al. SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. Nature 586(7830), 567–571 (2020).

Pardi, N., Hogan, M., Porter, F. et al. mRNA vaccines — a new era in vaccinology. Nat Rev Drug Discov 17, 261–279 (2018). https://doi.org/10.1038/nrd.2017.243

Kariko K, Muramatsu H, Ludwig J, Weissman D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res. (2011) 39:e142. doi: 10.1093/nar/gkr695

Zhang Cuiling, Maruggi Giulietta, Shan Hu, Li Junwei, Advances in mRNA Vaccines for Infectious Diseases. Frontiers in Immunology, 10 (2019), 10.3389/fimmu.2019.00594

Lukacs (1997) Detection of sense: antisense duplexes by structure-specific anti-RNA antibodies. In: Antisense Technology. A Practical Approach, C. Lichtenstein and W. Nellen (eds), pp. 281-295. IRL Press, Oxford

Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol. 2006 May;80(10):5059-64. doi: 10.1128/JVI.80.10.5059-5064.2006. PMID: 16641297; PMCID: PMC1472073.

Richardson SJ, Willcox A, Hilton DA, Tauriainen S, Hyoty H, Bone AJ, Foulis AK, Morgan NG. Use of antisera directed against dsRNA to detect viral infections in formalin-fixed paraffin-embedded tissue. J Clin Virol. 2010 Nov;49(3):180-5. doi: 10.1016/j.jcv.2010.07.015. Epub 2010 Aug 21. PMID: 20729142.

Field AK, Davies ME, Tytell AA. Determination of Antibodies to Double-Stranded RNA by Enzyme-Linked Immunosorbent Assay (ELISA). Proceedings of the Society for Experimental Biology and Medicine. 1980;164(4):524-529. doi:10.3181/00379727-164-40909

Motomichi Fujita, Koji Adachi, Michiaki Nagasawa,Development of a homogeneous time-resolved fluorescence assay for detection of viral double-stranded RNA, Analytical Biochemistry, Volume 566, 2019, doi:10.1016/j.ab.2018.10.021.

Yoshichika Kitagawa, Eiji Okuhara, Anti-poly(I)-poly(C) antibody bound to cellulose and its use in the specific separation of double-stranded RNAs, Analytical Biochemistry, Volume 115, Issue 1, 1981 https://doi.org/10.1016/0003-2697(81)90531-5.

J. Schonborn, J. Oberstraß, E. Breyel, J. Tittgen, J. Schumacher, N. Lukacs, Monoclonal antibodies to double-stranded RNA as probes of RNA structure in crude nucleic acid extracts, Nucleic Acids Research, Volume 19, Issue 11 11 June 1991, Pages 2993–3000, https://doi.org/10.1093/nar/19.11.2993

Gabriel CJ (1986) J Virology Methods 13, 279-283

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