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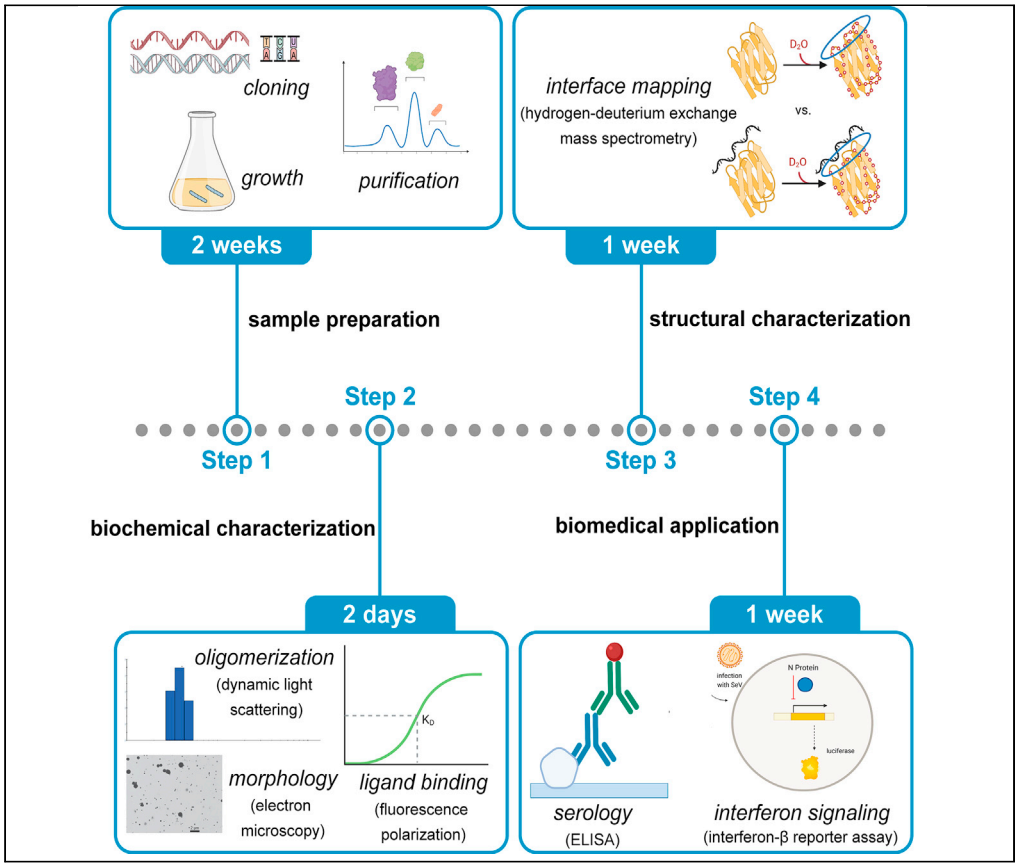
Domain-specific biochemical and serological characterization of SARS-CoV-2 nucleocapsid protein



Nucleocapsid proteins are essential for SARS-CoV-2 life cycle. Here, we describe protocols to gather domain-specific insights about essential properties of nucleocapsids. These assays include dynamic light scattering to characterize oligomerization, fluorescence polarization to quantify RNA binding, hydrogen-deuterium exchange mass spectrometry to map RNA binding regions, negative-stain electron microscopy to visualize oligomeric species, interferon reporter assay to evaluate interferon signaling modulation, and a serology assay to reveal insights for improved sensitivity and specificity. These assays are broadly applicable to RNA-encapsidated nucleocapsids.

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Highlights

Developed biophysical assays to characterize SARS-CoV-2 N oligomerization

Quantified RNA binding using fluorescence polarization

Mapped RNA binding site with hydrogen-deuterium exchange mass spectrometry

Defined clinical insights from serology and interferon reporter assays

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Domain-specific biochemical and serological characterization of SARS-CoV-2 nucleocapsid protein

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SUMMARY

Nucleocapsid proteins are essential for SARS-CoV-2 life cycle. Here, we describe protocols to gather domain-specific insights about essential properties of nucleocapsids. These assays include dynamic light scattering to characterize oligomerization, fluorescence polarization to quantify RNA binding, hydrogen-deuterium exchange mass spectrometry to map RNA binding regions, negative-stain electron microscopy to visualize oligomeric species, interferon reporter assay to evaluate interferon signaling modulation, and a serology assay to reveal insights for improved sensitivity and specificity. These assays are broadly applicable to RNA-encapsidated nucleocapsids.

For complete details on the use and execution of this protocol, please refer to Wu et al. (2021).

BEFORE YOU BEGIN

Human samples

The collection of human plasma must be approved through Institutional Review Boards prior to any studies. Human samples used with these protocols were approved by the Human Research Protection Office at Washington University in St. Louis and the Institutional Review Board of The Hong Kong University and the Hong Kong Island West Cluster of Hospitals.

Construction of expression plasmid

Timing: 5 days

1. Perform PCR to amplify desired SARS-CoV-2 N constructs with corresponding primers.

For duplicate PCR reactions, use:

PCR reactions before adding enzyme	
Volume	Reagents in PCR
62.8 μL	ddH_2O
10 μL	10× KOD buffer #1 or #2 depending on product size
10 μL	DMSO
4 μL	$MgCl_2$ (25 mM)
2 μL	dNTPs (2 mM)
1 μL	gene template (~ 100 ng/μL)
5 μL	forward primer (50 ng/μL)
5 μL	reverse primer (50 ng/μL)

Split into two PCR tubes, add into each tube:

Enzyme needed for each PCR reaction	
Volume	Reagents in PCR
0.1 μL	KOD enzyme

Total reaction volume in each tube should be 50 μL.

Then initiate PCR reaction with following program:

PCR cycling conditions using KOD enzyme			
Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	30 s	30 cycles
Annealing	50°C	8 s	
Extension	72°C	20 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

⚠ CRITICAL: Ensure there are no restriction enzyme sites inside gene of interest. If there are, mutate internal restriction enzyme sites before cloning constructs. Double check that there is no stop codon within the reverse primer prior to use, as the expression vector has a C-terminal tag. Extension time depends on size of the product amplified. Please refer to Millipore Sigma for specific information on KOD polymerase.

2. Run DNA agarose gel (1%) for ~ 25 min at 80 V.
3. Cut out desired DNA bands with visual aid using UV lamp and clean up gel bands with *Promega Wizard® SV Gel and PCR Clean-Up System*.
4. Perform restriction enzyme digestion of expression vector (pET28a-CterTev His6) and amplified gene of interest.

For each digestion reaction, use:

Digestion reaction	
Volume	Reagents in digestion reaction
5 μL	10× NEB buffer 3.1
1 μL	BamHI
1 μL	NdeI
43 μL	Purified PCR products or vector
Incubate at 37°C for 1 h.	

5. For digested vector, remove phosphate group from 5′ end using calf-intestinal alkaline phosphatase for 1 h at 37°C.
6. Run DNA gel and cut out desired DNA bands for vector and gene of interest.
7. Gel purify digested vector and amplified gene of interest.
8. Ligate amplified gene of interest into expression vector.

For each ligation reaction, use

Ligation reaction	
Volume	Reagents in ligation reaction
4 μL	ddH_2O
1 μL	10× ligation buffer
3 μL	digested gene of interest
1 μL	digested expression vector
1 μL	T4 DNA ligase
Incubate at 4°C, 12–18 h.	

9. Transform DH-5α competent cells with ligation reaction and plate onto LB plates with 50 μg/mL kanamycin. Incubate at 37°C for 12–18 h.
10. Pick single colony and inoculate into 10 mL LB supplemented with 50 μg/mL kanamycin.
11. Perform miniprep (*Promega Wizard® Plus SV Minipreps*) and send plasmids for sequence confirmation.

Bacteria growth in LB media

Timing: 2 days

12. Transform 100 μL *E. coli* BL21 (DE3) competent cells with 1 μL of expression plasmid (~ 100 ng/ μL) for each construct by heat shock.
13. Add 900 μL LB media and incubate at 37°C for 1 h.
14. Spin down cell pellet at 6000 × *g* for 10 min, and pipette out 900 μL supernatant.
15. Resuspend remaining media and spread evenly on LB agar plate with 50 μg/mL kanamycin. Incubate at 37°C for 12–18 h.
16. Pick single colony and inoculate into 10 mL of LB media supplemented with 50 μg/mL kanamycin. Culture at 37°C with shaking until media is turbid.
17. Transfer culture into 2 L LB media supplemented with 50 μg/mL kanamycin and culture for 4–5 h.
18. At OD_{600} of 0.6–0.7, induce protein expression with 0.5 mM IPTG after cooling down media on ice for 15 min. Continue to shake at 18°C for 12–14 h.
19. Harvest cells.
    a. Harvest cell culture by centrifugation at 6000 × *g* for 10 min at 10°C.
    b. Using post-induction gel sample, run SDS-PAGE and stain with Coomassie blue to determine expression outcome.

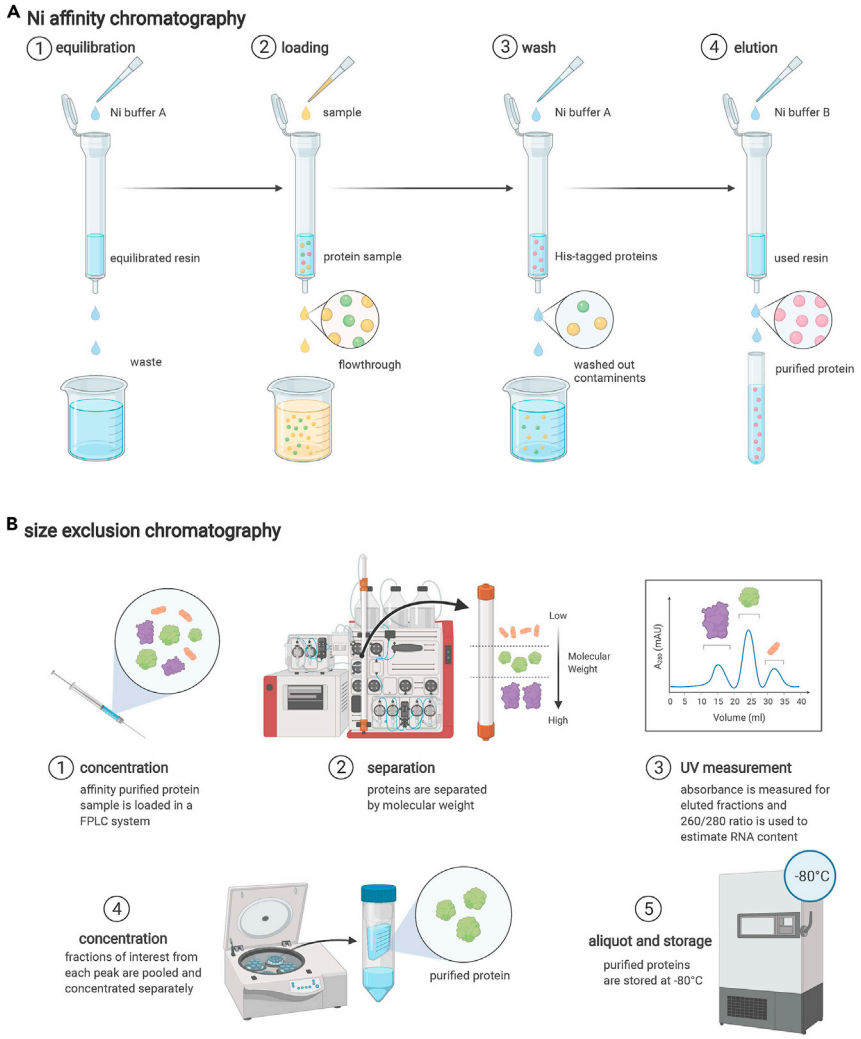

Figure 1. Protein purification of SARS-CoV-2 nucleocapsid

C-terminal tagged nucleocapsid proteins are purified using Ni affinity chromatography (A) and size exclusion chromatography (B). This purification strategy generates reasonably pure proteins for a series of constructs with one day per protein time cost. Adapted from "His-tagged Protein Purification" and "Protein Purification", by Biorender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.

c. Gently discard the supernatant.

d. Resuspend cells in 45 mL lysis buffer containing newly added 2-mercaptoethanol (BME) and protease inhibitors (see materials and equipment for recipe of lysis buffer).

⚠ CRITICAL: For RNA binding nucleocapsids, the concentration of salt is crucial in purification outcome. Higher concentration of NaCl is required for purifying RNA-free nucleocapsids.

Pause point: The harvested cells can be stored at −80°C for years.

Purification of nucleocapsid protein

Timing: 1 day per protein

The C-terminal His tag nucleocapsid proteins are purified using Ni affinity chromatography and size exclusion chromatography (Figure 1).

20. After diluting thawed pellet into 45 mL lysis buffer, pass cells through an Avestin EmulsiFlex-C5 homogenizer without and with pressure three times.
21. Centrifuge sample at 30,000 × *g* at 10°C for 40 min. Gently transfer the supernatant to a clean container.

Note: Before proceeding to chromatography, filter the supernatant with a 1.2 μm filter to remove large particles.

22. Prewash the column with three column volumes of Ni buffer B and three column volumes of Ni buffer A (see materials and equipment for recipe). Apply the supernatant to the Ni-NTA column.
23. Wash out nonspecific binders with three column volumes of Ni buffer A.
24. Elute bound protein with a step gradient of 40% Ni buffer B and a step gradient of 100% Ni buffer B.
25. Perform SDS-PAGE to determine purity.

Note: For nucleocapsids that bind RNA with high affinity and tend to phase separate, low salt conditions are avoided to maximize chance of RNA-free species purification.

26. Concentrate Ni elution to 5 mL using centrifugal filter units (Millpore Amicon® Ultra).
27. Inject concentrated protein into Superdex 200 size exclusion column, pre-equilibrated with size exclusion buffer (see materials and equipment for recipe).
28. Evaluate fraction purity using Coomassie staining of SDS-PAGE.

⚠ CRITICAL: Despite the use of high salt concentration during purification, purified nucleocapsid protein may contain multiple species, including RNA-free and RNA-bound (Su et al., 2018; Wu et al., 2021). To evaluate heterogeneity, measure A260/A280 ratios of samples. A ratio below 0.6 is considered RNA-free.

29. Pool together and concentrate separately fractions within each peak before flash freezing in liquid nitrogen for storage.

⚠ CRITICAL: When aliquoting, use a range of volumes (20, 50, 100, 200, 500 μL) to minimize unnecessary freeze-thaw cycles for experiments requiring varying amounts of protein.

Pre-HDX quality control

Timing: 2–3 h

For successful analysis of proteins by HDX-MS, a homogenous and high purity sample is required. We generally find that in-house expressed and purified protein is higher in quality than commercially available counterparts. Because the instrument and personnel time investments for the HDX-MS experiments and data analysis are considerable, quality control steps are recommended to maximize the likelihood of success. Although biochemical assays may provide coarse-grained information pertaining to the state and identity of the protein sample, using other high resolution mass spectrometry measurements, described below, will be beneficial for quality control in this application.

30. Characterize/confirm sequence and heterogeneity of the protein construct by using denaturing LC-MS.
    a. Denature the protein with low pH (such as 0.1% formic acid), organic (< 5% acetonitrile), reducing agents (such as TCEP), and/or denaturants.

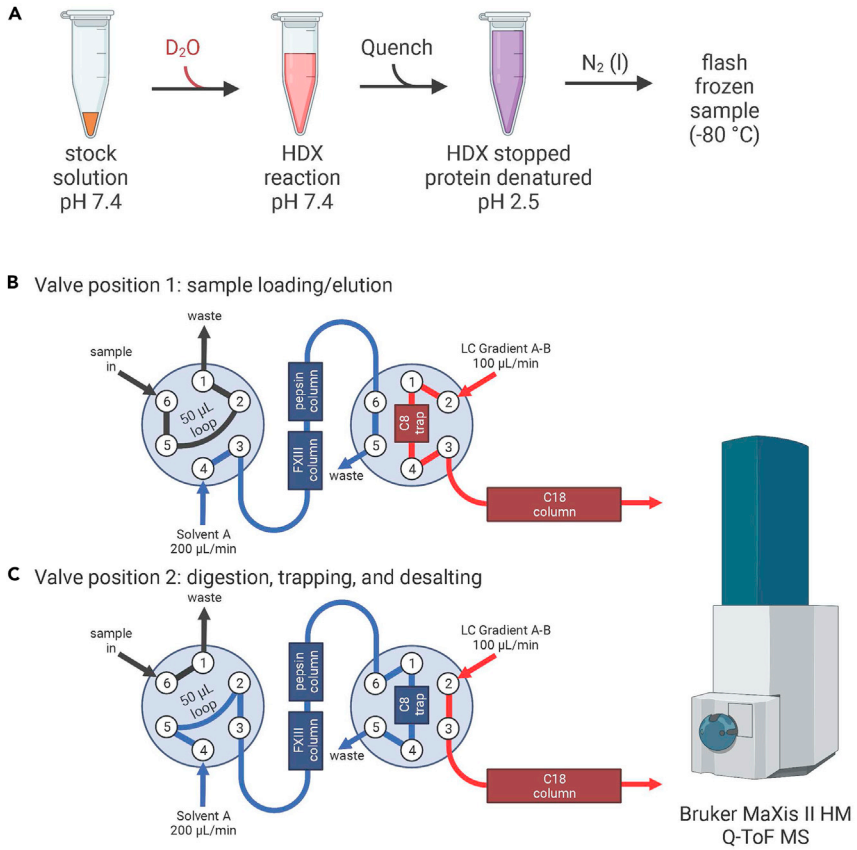

Figure 2. LC-MS diagram

(A) Overview of protein sample submitted to HDX prior to MS analysis.

(B) Sample and LC solvent paths for valve position 1, allowing for sample loading into sample loop or elution from desalting column through protease columns (generating peptides), separated by C18 column, and into the MS.

(C) Sample and LC solvent paths for valve position 2, moving protein from sample loop onto desalting column.

Figure created with Biorender.com.

b. Inject the denatured protein onto an HPLC for desalting and elution into a mass spectrometer (Figure 2).

c. Process data by using deconvolution software (e.g., Intact by Protein Metrics, Inc.) or manually to identify heterogeneity.

⚠ CRITICAL: The differential HDX-MS method described here relies on bottom-up LC-MS/MS analysis to resolve local differences in deuterium uptake. Any heterogeneity (e.g., from mutations, post-translational modifications) will interfere with mapping results and could result in convoluted HDX results reporting on the mixture.

Optional: If the oligomeric state is not clarified by other techniques, characterize oligomeric state and confirm binding using native MS.

LC-MS valve setup

Timing: protease column preparation: 2 days; valve set up: 30 min

Assemble a valve setup for online protease digestion, peptide desalting, analytical separation, and elution into a mass spectrometer (Figure 2).

31. Prepare a protease column(s) for online, post-HDX digestion (Wang et al., 2002).
    a. Immobilize acidic protease(s) on POROS-20AL beads.
        i. Add 20 mg of sodium cyanoborohydride dissolved in 1 mL of 2 M sodium sulfate to a 2 mL solution containing 80 mg of the protease in 50 mM sodium citrate buffer (pH 5), and incubate for 10 min at 18°C–22°C with gentle rocking.

⚠ CRITICAL: Sodium cyanoborohydride is a flammable solid, reactive with water, and exposure can cause serious burns. Pay special attention to the safety data sheet for proper storage and handling.

        ii. To this mixture, add 600 mg of POROS-20AL beads, adjust the rocker speed to suspend the slurry, and incubate 5–10 min at 18°C–22°C.
        iii. Slowly add 2.125 mL of 2 M sodium sulfate (~ 500 μL every 5 min).
        iv. Incubate for 12–16 h at 18°C–22°C with rocking.
        v. Quench the reaction with 1 mL of 1 M ethanolamine and incubate for 2 h at 18°C–22°C.
        vi. Filter the coupled beads and wash with 50 mM citrate buffer (pH 5), 1 M NaCl in citrate buffer (pH 5), 50 mM citrate buffer (pH 5), and water/0.1% formic acid. Resuspend beads in water/0.1% formic acid for packing into a column.

Alternatives: Protease coupled beads are available for purchase.

Note: Once coupled, the beads can be stored at 4°C in water/0.1% formic acid for several months.

    b. Pack beads with an immobilized protease into clean stainless-steel columns (we used 2 mm inner diameter × 20 mm length column).
    c. Wash the packed column for 12–18 h directly into waste with water/0.1% formic acid at 50 μL/min.

Note: With careful use, packed protease columns can be reused for months. After usage, the ends should be capped to keep the beds wet, and the column stored at 4°C. We recommend using a model protein to evaluate the health of the protease column before each use.

Alternatives: Prepacked protease columns are available for purchase.

Alternatives: Instead of online digestion, in-solution protein digestion can be performed with a large excess of proteases, but this increases H/D back-exchange and produces protease peptide fragments congesting the spectra.

32. Incorporate a C8 column for trapping and desalting the digested peptide products.
33. Use a C18 column for analytical separation of the product peptides.
34. Couple the LC apparatus to a mass spectrometer capable of both MS and MS/MS (in this case a Bruker MaXis II HM Q-TOF).
35. During acquisition, maintain the valves, columns, and lines (except the protease column(s)) in an ice bath to reduce back-exchange.

Alternatives: Any mass spectrometer capable of MS and MS/MS can be used for mass analyses.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human IgG secondary Ab labeled with HRP (1:5000)	Southern Biotech	Cat#905209
Bacterial and virus strains		
E. coli BL21(DE3)	Agilent	Cat#200131
SeV	Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY USA	N/A
Biological samples		
Human Plasma, Patient with Confirmed SARS-CoV-2 Infection	Barnes-Jewish Hospital, St. Louis, MO, USA	N/A
Human Plasma, Patient with Confirmed SARS-CoV-2 Infection	Hong Kong University and HK Island West Cluster of Hospitals, Hong Kong, PRC	N/A
Human Plasma, Patient with negative test	Barnes-Jewish Hospital, St. Louis, MO, USA	N/A
Chemicals, peptides, and recombinant proteins		
LC-MS grade formic acid	CovaChem	Cat#PI 85171; CAS 64-18-6
Phosphate buffered saline tablets	Millipore Sigma	Cat#P4417
Deuterium oxide (D, 99.9%)	Cambridge Isotope Laboratories	Cat#DLM-4-100; CAS 7789-20-0;
Urea	Millipore Sigma	Cat#U4883; CAS: 57-13-6
2% Uranyl Acetate	Ted Pella	19481
KOD DNA polymerase	Millipore Sigma	71085
Critical commercial assays		
Dual-Glo luciferase kit	Promega	Cat#E2920
Promega Wizard® SV Gel and PCR Clean-Up System	Promega	A9282
Promega Wizard® Plus SV Minipreps	Promega	A1460
Experimental models: Cell lines		
Human: HEK293T cells	ATCC	CRL-3216
Oligonucleotides		
20nt ssRNA sequence: UUUCACCUCCCUUUCAGUUU	GenScript	N/A
19nt slRNA sequence: GGAAGAUUAAUAAUUUUCC	GenScript	N/A
Recombinant DNA		
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 1-419	This work	N/A
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 44-419	This work	N/A
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 44-369	This work	N/A
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 44-247	This work	N/A
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 44-176	This work	N/A
Plasmid pET28a-CterTEV His6 SARS-CoV-2 N 248-369	This work	N/A
Plasmid pCAGGS MLAV VP35	This work	N/A
Plasmid pCAGGS SARS-CoV-2 N 1-419	This work	N/A
Plasmid pCAGGS SARS-CoV-2 N 44-369	This work	N/A
Plasmid pCAGGS SARS-CoV-2 N 44-176	This work	N/A
Plasmid pCAGGS SARS-CoV-2 N 248-419	This work	N/A
Plasmid pCAGGS SARS-CoV-2 N 248-369	This work	N/A
IFN-β promoter-firefly luciferase reporter plasmid	Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY USA	N/A
pRL-TK Renilla luciferase reporter plasmid	Promega	E2231

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Intact Mass™	Protein Metrics Incorporated	Version 3.11
Byonic™	Protein Metrics Incorporated	Version 3.11
Byologic™	Protein Metrics Incorporated	Version 3.11
DataAnalysis v 4.4	Bruker Daltonics	Version 4.4
HDExaminer	Sierra Analytics Incorporated	Version 2.5.1
Origin	OriginLab	Version 7
PRISM	GraphPad	Version 7
Dynamics Software	Wyatt	Version 7
Gen5 Software	BioTek	Version 3
Other		
ZORBAX Eclipse XDB C8 trap column (2.1 × 15 mm)	Agilent Technologies	Cat#975700-936
XSelect CSH C18 XP (130 Å, 2.5 µm, 2.1 × 50 mm)	Waters Corporation	Cat#186006101
MaXis II 4G Q-TOF	Bruker Daltonics	N/A
MaXis II HM Q-TOF	Bruker Daltonics	N/A
EmulsiFlex-C5 homogenizer	Avestin	N/A
Glow-Discharged Copper Grid, 200 mesh	Ted Pella	01840-F
JEM-1400 plus TEM	JEOL	N/A
Phosphor-Scintillated 12-bit CCD Camera	AMT	XR111
DynaPro-PlateReader II	Wyatt Technologies Corporation	N/A
Cytation5 Plate Reader	BioTek	N/A

MATERIALS AND EQUIPMENT

Lysis buffer		
Reagent (stock concentration)	Final concentration	Amount (mL) for 1000 mL
Tris-HCl pH 7.5 (2 M)	20 mM	10
NaCl (5 M)	1 M	200
Imidazole (2 M)	20 mM	10
2-mecaptoethanol (BME) (14.3 M)	5 mM	0.35
Antipain (1 mg/mL)	1 µg/mL	1
Benzamidine (1 mg/mL)	1 µg/mL	1
Leupepsin (1 mg/mL)	1 µg/mL	1
Pepstain (0.5 mg/mL)	1 µg/mL	2
ddH_2O	n/a	774.65
Total	**n/a**	**1,000**
Store at 4°C for up to 6 months. Add protease inhibitors and BME right before use.		

Ni buffer A		
Reagent (stock concentration)	Final concentration	Amount (mL) for 1,000 mL
Tris-HCl pH 7.5 (2 M)	20 mM	10
NaCl (5 M)	1 M	200
Imidazole (2 M)	20 mM	10
2-mecaptoethanol (BME) (14.3 M)	5 mM	0.35
ddH_2O	n/a	779.65
Total	**n/a**	**1,000**
Store at 4°C for up to 6 months. Add BME right before use.		

Ni buffer B		
Reagent (stock concentration)	Final concentration	Amount (mL) for 1,000 mL
Tris-HCl pH 7.5 (2 M)	20 mM	10
NaCl (5 M)	1 M	200
Imidazole (2 M)	500 mM	250
2-mecaptoethanol (BME) (14.3 M)	5 mM	0.35
ddH_2O	n/a	539.65
Total	**n/a**	**1,000**
Store at 4°C for up to 6 months. Add BME right before use.		

Size exclusion buffer		
Reagent (stock concentration)	Final concentration	Amount (mL) for 1,000 mL
HEPES pH 7.5 (1 M)	25 mM	25
NaCl (5 M)	500 mM	100
Glycerol (80%)	5%	62.5
TCEP (0.5 M)	2 mM	4
ddH_2O	n/a	808.5
Total	**n/a**	**1,000**
Sterilize with 0.22 μm filter. Store at 4°C for up to 6 months.		

DLS buffer		
Reagent (stock concentration)	Final concentration	Amount (mL) for 1,000 mL
HEPES pH 7.5 (1 M)	25 mM	25
NaCl (5 M)	150 mM	30
Glycerol (80%)	5%	62.5
TCEP (0.5 M)	2 mM	4
ddH_2O	n/a	878.5
Total	**n/a**	**1,000**
Sterilize with 0.22 μm filter. Store at 4°C for up to 6 months.		

RP-LC Solvent A		
Reagent	Final concentration	Amount
HPLC Grade water	99.9% (%v/v)	999 mL
MS-Grade Formic Acid	0.1%	1 mL
Total	**n/a**	**1 L**
Store at 18°C–22°C for no longer than 1 month.		

RP-LC Solvent B		
Reagent	Final concentration	Amount
HPLC Grade water	19.9% (%v/v)	199 mL
HPLC Grade acetonitrile	80%	800 mL
MS-Grade Formic Acid	0.1%	1 mL
Total	**n/a**	**1 L**
Store at 18°C–22°C for no longer than 1 month.		

HDX H_2O buffer		
Reagent	Final concentration	Amount
1 × Phosphate buffered saline tablet	10 mM Phosphate Buffer 137 mM NaCl 2.7 mM KCl pH 7.4	1 tablet
HPLC Grade Water	n/a	200 mL
Total	**n/a**	**200 mL**
Store at 4°C for no longer than 1 year.		

HDX D_2O buffer		
Reagent	Final concentration	Amount
1 × Phosphate buffered saline tablet	10 mM Phosphate Buffer 137 mM NaCl 2.7 mM KCl pH 7.0 (pD 7.4)	1 tablet
deuterium oxide (99.9%)	n/a	200 g
Total	**n/a**	**200 mL**
Aliquot and store frozen (−20°C) for no longer than 1 year.		

HDX Quench buffer		
Reagent	Final concentration	Amount
Urea (8 M)	4 M	25 mL
1×PBS	5 mM Phosphate Buffer 68.5 mM NaCl 1.35 mM KCl pH 2.4	25 mL
Total	n/a	**50 mL**
Aliquot and store frozen (−20°C) for no longer than 1 year		

⚠ CRITICAL: Adjust the pH with concentrated HCl or NaOH to minimize incorporation of exchangeable hydrogen into the deuterated buffer. Alternatively, you can use DCl or NaOD diluted with D_2O. Ensure recording the pH of the final solution.

⚠ CRITICAL: The kinetic minimum for protein backbone amide H/D exchange is at pH ~2.5 (Narang et al., 2020); after quenching the exchange, HDX should be minimized, and back exchange with the solvent minimized. Test the pH of a 2:3 mixture of HDX H_2O buffer:HDX quench buffer solution to ensure the pH is ~2.5 and record the value.

Note: The appropriate pH is also dependent upon the protease activity. Some sacrifices to H/D back-exchange may be necessary if alternative proteases to pepsin are used.

Alternatives: A variety of quench buffers may be prepared for optimizing the digestion conditions.

MS data acquisition parameters		
Method node	Parameter	Setting
MS and MS/MS		
Mode	Spectra File Type	save line and profile spectra
	Ion Polarity	positive
	Mass Range	50–4000 m/z
	Rolling Average	Off

(*Continued on next page*)

Continued		
Method node	Parameter	Setting
	Spectra Rate	2.00 Hz
ESI Source	End Plate Offset	500 V
	Capillary	4500 V
	Nebulizer	2.0 Bar
	Dry Gas	5.0 L/min
	Dry Temp	250 C
Tune Tab	Transfer Funnel 1 RF	400.0 Vpp
	Transfer isCID Energy	0.0 eV
	Transfer Multipole RF	400.0 Vpp
	Quadrupole Ion Energy	3.0 V
MS/MS		
Auto MS/MS	Precursor Ion List	include
	Mass Range	300.00–4000.00
	Width	± 0.5
	Cycle Time	3.0 s
	Exclude after	1 spectrum
	Release after	2.00 min
	Reconsider Precursor if Curr./Prev. Intens.	checked
	Reconsider Precursor if Curr./Prev. Intens.	3
MS/MS Preference	Preferred Mass Range (checked)	300.00–2200.00
	Width	± 0.5
	Charge State Range	1–6
	Exclude Singly	unchecked
	Exclude Unknown	unchecked
Acquisition (Precursor	MS Spectra Rate	2.00 Hz
Acquisition Control)	MS/MS Low (per 1000 sum.)	25000 cts, 2.00 Hz
	MS/MS Low	57175 cts.
	MS/MS High (per 1000 sum.)	250000 cts., 5.00 Hz
	MS/MS High	571750 cts.
	MS/MS Total Cycle Time Range	3 s
	MS/MS Absolute Threshold	1144 cts.

CID collision energy list

Type	Mass	Width	CE	Charge state
Base	300	3	21	3
Base	300	3	26	2
Base	300	3	34	1
Base	500	4.8	28	3
Base	500	4.8	34	2
Base	500	4.8	39	1
Base	1,000	6	36	3
Base	1,000	6	40	2
Base	1,000	6	52	1
Base	2,000	9	40	3
Base	2,000	9	45	2
Base	2,000	9	55	1

STEP-BY-STEP METHOD DETAILS

Dynamic light scattering (DLS)

Timing: 2 h

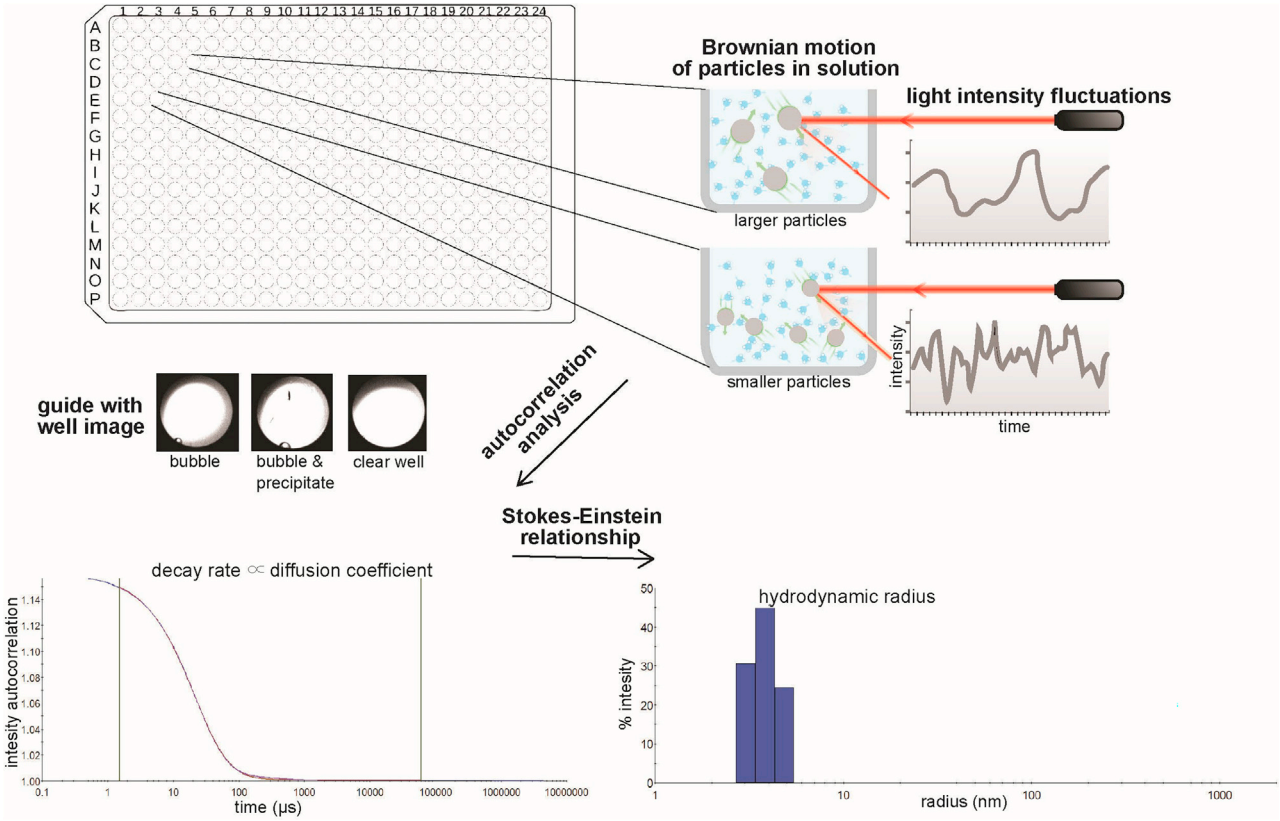

Figure 3. Dynamic light scattering assay for size characterization

Brownian motion of particles produces dynamic light scattering signals. These intensity fluctuations contain useful information and diffusion coefficients from autocorrelation analysis that can be converted to size.

DLS measures hydrodynamic properties of purified RNA-free nucleocapsid protein to monitor oligomerization using a Wyatt DynaPro PlateReader-III. This assay is useful to characterize particles with a wide range of hydrodynamic radii from 1 nm to 1000 nm (Figure 3) (Stetefeld et al., 2016; Su et al., 2018).

1. Thaw purified protein on ice.
2. Filter DLS buffer with a 0.22 µm filter.
3. Dilute protein solution into 2 mg/mL. Prepare 120 µL of sample for each protein, sufficient for technical triplicates. With Corning® Low Volume 384-well Clear Flat Bottom plate (Corning 3540), each well requires 35 µL of sample.

   Note: To adjust salt concentration, prepare buffer without salt for a simple calculation. First calculate the amount of buffer without salt needed for dilution, then dilute with DLS buffer to target for desired protein concentration.

   ⚠ CRITICAL: Centrifuge sample for 5 min at 4°C using a tabletop centrifuge at top speed to remove aggregates.

4. Load 35 µL sample into each well.

   Note: Before loading, check for presence of dust in wells. Cover plate with aluminum foil whenever possible when not loading to reduce likelihood of dirt getting inside.

⚠ CRITICAL: While loading, place pipette tip on the bottom of well and pipet slowly. Avoid generating bubbles. Gently shake plates if bubbles are trapped inside and remove bubbles by pipetting. It is important to get rid of bubbles before experiments.

5. Set up experiment for acquisition.
   a. Correlation function high cutoff set to 60 ms.
   b. Acquire well images set to Yes.
   c. DLS Acq time set to 5 s.
   d. DLS Number Acq set to 10.
   e. Auto-attenuation time limits set to 60 s.
6. When analyzing data, access Regularization Graph and use triplicates to reach consensus.
   a. Remove peaks with less than 1% mass.
   b. Align triplicates to check consistency for each major peak.
   c. Determine statistics of measured radius across triplicates.
   d. Repeat with newly prepared sample to check consistency across runs.

Note: DLS is extremely sensitive to dirt and bubbles. Use well images to check sample conditions and repeat when inconsistency arises. Please refer to Wyatt for additional guidance on instrument specific protocols.

Fluorescence polarization (FP) assay

Timing: 1 day

Timing: 2 h for step 7

Timing: 1 h for step 13

The FP assay uses fluorescently labeled RNA and titrates increasing concentrations of protein for affinity estimation based on polarization change of fluorescence label upon binding (Figure 4). Due to the high sensitivity of this technique, as low as 1 nM FITC-labeled RNA can be used, making it a useful probe for high affinity binding system (Liu et al., 2017; Su et al., 2018).

7. Prepare protein titration series.
   a. Determine highest concentration point and fold of dilution. For nM affinity binding, a 2.5 fold dilution series with 12 concentration points starting with 10 μM as highest concentration and 0.42 nM as lowest concentration covers the entire binding curve. This step usually requires optimization.

Note: FITC labeled RNA at 1 nM concentration in this assay setup results in reasonable signal. Optimize RNA concentration with a known RNA binder so Z-factor is above 0.5. $Z = 1 - \frac{3(\sigma(p)+\sigma(n))}{\mu(p)-\mu(n)}$, μ and $\sigma$are means and standard deviations of positive (p) and negative (n) controls.

   b. Prepare 120 μL of highest concentration point for the entire dilution series (each concentration point needs a volume of 25 μL and duplicates need 60 μL). Binding buffer is same as DLS buffer has 150 mM NaCl.
   c. Pipette 90 μL binding buffer into each of 11 Eppendorf tubes. Take out 60 μL from highest concentration tube and mix well into the next concentration point. Repeat 11 times until entire dilution series are prepared.

8. Prepare labeled RNA stock.
   a. Calculate amount of RNA needed for each duplicate run. 700 μL at 2 nM (1 nM final concentration after mixing with protein) are prepared.
   b. Cover tubes with aluminum foil to protect from light.

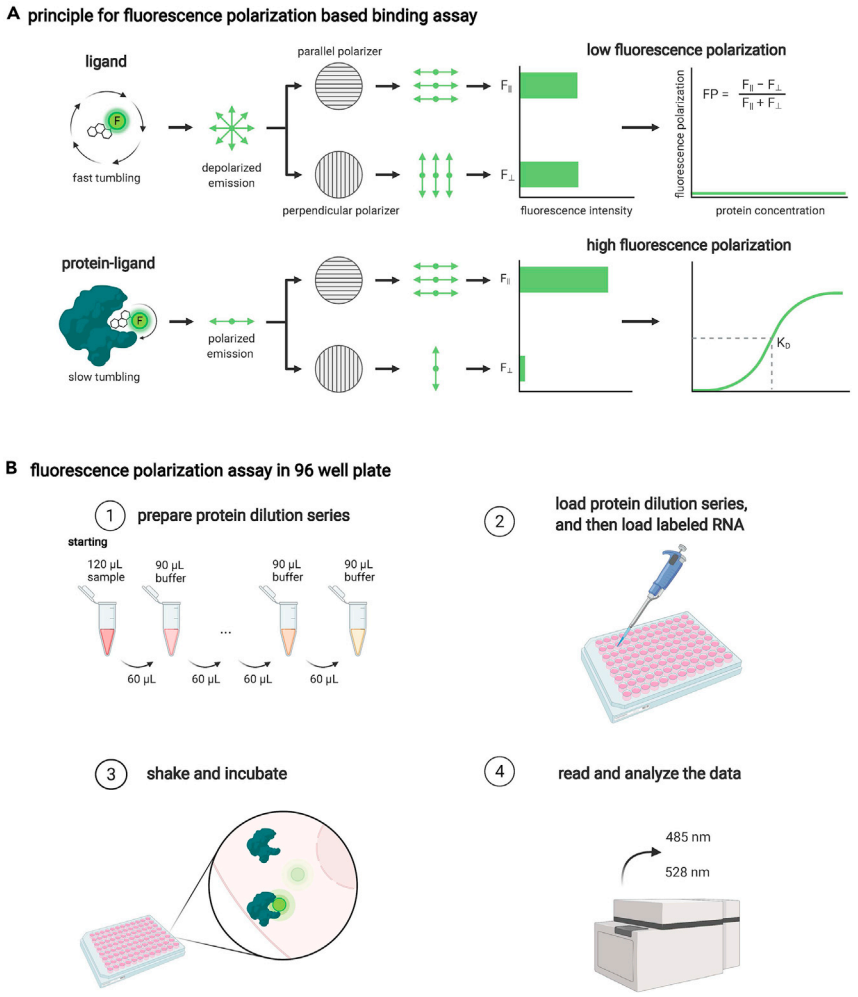

Figure 4. Fluorescence polarization assay for nucleocapsid-RNA binding

(A) Principle for fluorescence polarization based RNA binding assay. Fluorescence labeled RNA molecules tumble fast and do not lead to preferential orientation. This results in depolarized emission that can be recorded with equal parallel and perpendicular fluorescence intensity. This corresponds to low FP values. After binding with protein, labeled RNA tumble slowly resulting in residual orientation that can be recorded with increased fluorescence polarization. By titrating protein concentration, the varying FP values will yield a binding curve that can be fit for a K_D.

(B) Fluorescence polarization assay in 96 well plate. First, a protein dilution series is prepared. Depending on binding mechanism which results in different curve shape, choose a suitable dilution factor to cover both unbound and bound side of baselines. A 2.5 fold dilution usually is a good starting point for a 12 point dilution series. After the dilution series are prepared, load protein first into wells and then labeled RNA. Cover solution containing labeled RNA with aluminum foil whenever possible to reduce quenching. A 10 min shake and incubation is recommended before reading with plate reader. Adapted from "Fluorescence Polarization Assay", by Biorender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.

9. Load protein dilution series into wells of a 96 well plate (Corning 3650). Each titration series is in one row. For example, duplicate runs of one experiment will take row A and B.
10. Load 25 µL of RNA into each well.
11. Load buffer and RNA only controls in row C.

Optional: Load multiple controls to estimate uncertainty associated with detection.

12. Read with Cytation 5 Cell Imaging Multi-mode Reader.

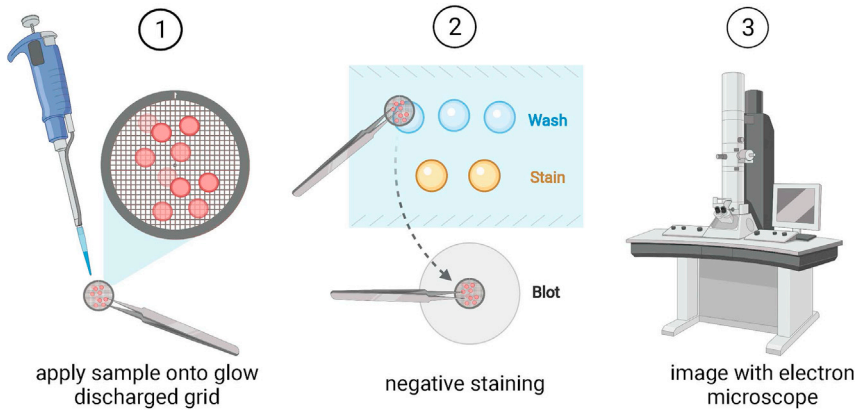

Figure 5. Negative-stain electron microscopy

Freshly thawed proteins samples are loaded onto glow-discharged grids. After blotting with filter paper, grids are washed in water before stained with 2% UA solution. Air dried grids are then imaged with electron microscope. Adapted from "EM", by Biorender.com (2020). Retrieved from https://app.biorender.com/biorender-templates.

a. Set excitation to 485 nm and emission to 528 nm with a bandpass of 20 nm.
b. Set read height to 8.5 mm.

Note: Optical probe height has a considerable effect on the signal to noise ratio. Therefore, read height may need optimization for best performance.

13. Fit K_D using Origin.
    a. Determine FP values using Gen5 Analysis software. More information on analysis using Gen5 Analysis can be found on the BioTek website.
    b. Averages and standard deviations for titration points are calculated in Excel.
    c. Binding curves are fit using Origin. See quantification and statistical analysis for details (Liu et al., 2017; Su et al., 2018). Alternative software such as GraphPad PRISM can also be for analysis.

Note: Each experiment containing duplicates are repeated at least three times. Statistics about K_D values are reported across these technical repeats.

Negative-stain electron microscopy

Timing: 1 day

Timing: 30 min per grid (including loading and imaging) for step 22

This step describes preparation of nucleocapsid-RNA negative-stain sample for electron microscopy (EM) (Figure 5).

14. Glow discharge carbon-coated copper grids (Ted Pella, 01840-F).
    a. Place grids onto a glass slide with carbon film side facing up.
    b. Put the glass slide on sample stage of Leica EM ACE600.
    c. Glow discharge for 45 s with following settings: Current (12 mA), Air (2.0E-1 mbar), Working Distance (50 mm), and Tilt (0°C).
15. Apply 2 μL of sample at 1 mg/mL diluted in PBS to each grid. Wait for 1 min.
16. Blot the grid with filter paper for 3 s.
17. On a piece of parafilm, pipette three droplets of ddH_2O and two droplets of 2% uranyl acetate (UA) solution. Each droplet has a volume of 20 μL.

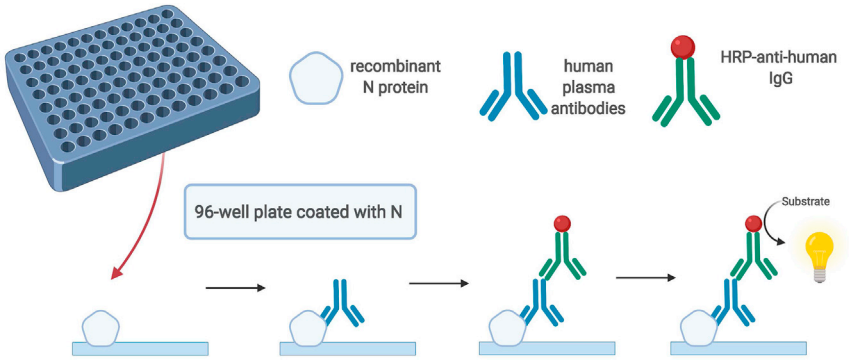

Figure 6. ELISA overview

A 96-well plate is coated with N-protein fragments. The plate is subsequently blocked with protein and exposed to patient sera. A secondary antibody, an anti-human IgG conjugated to horseradish peroxidase (HRP) is then added to the plate for read-out. Created with Biorender.com.

⚠ CRITICAL: Though the external radiological and chemical hazards of UA are relatively low, it is acutely toxic if swallowed or inhaled and may cause damage to organs through prolonged or repeated exposure. Review Safety Data Sheet and use with precautions.

Prepare fresh 2% UA from 4% UA stock solution and spin for 1 min at top speed using a tabletop centrifuge. Prepare 4% UA stock every 6 months.

18. Face the grid onto the ddH_2O droplet to wash for 30 s and blot the grid for 3 s.
19. Repeat two more times with ddH_2O.
20. Repeat two more times with 2% UA.
21. Airdry the grid before storing in a grid box.

⚠ CRITICAL: Make sure grids are completely dry before imaging.

22. Image grids with electron microscope. Each negative-stained grid is loaded onto the side-entry holder of JEOL JEM-1400plus Transmission Electron Microscope operating at 120 kV and recorded with an AMT XR111 high-speed 4K × 2K pixel phosphor-scintillated 12-bit CCD camera at 3,000 to 40,000 magnification.

Enzyme-linked immunosorbent assay (ELISA) for human plasma

Timing: 2 days for step 23

This section outlines how to perform an ELISA with patient plasma samples to screen for serological responses (Figure 6).

23. Recombinant N proteins were coated on 96 well flat bottom immunosorbent plates at a concentration of 500 ng/mL in 100 μL coating buffer (PBS with 53% Na_2CO_3 and 42% $NaHCO_3$) at 4°C for 12–18 h.
    a. An additional plate was coated with a non-specific protein mixture to measure background binding of each sample.
        i. Non-specific protein mixture consisted of PBS with 5% fetal bovine serum (FBS).
24. Diluted plasma samples (1:100) were bound for 2 h.
    a. Samples were diluted in dilution buffer (PBS with 0.05% Tween-20 and 0.1% FBS) for a total volume of 200 μL.

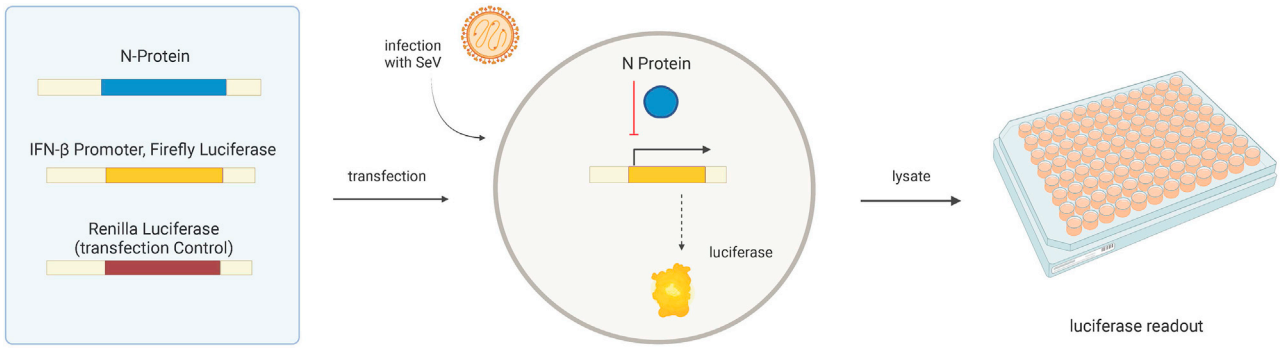

Figure 7. IFN-β reporter assay

HEK-293T cells are transfected with a plasmid containing an IFN-β promoter-firefly luciferase reporter, a control plasmid containing a Renilla luciferase reporter, and an expression plasmid for one of the studied N-protein variants. Subsequently, cells are infected with Sendai virus (SeV), which stimulates the Interferon signaling pathway. N-protein variants inhibit the Interferon response pathway. Following transfection and infection, cells are lysed, and luciferase activity evaluated. Created with Biorender.com.

25. The 96 well plate was rinsed with 150 μL of wash buffer (PBS with 0.1% Tween-20).
26. Both 96 well plates were exposed to an anti-human IgG secondary antibody labeled with HRP (Invitrogen) diluted at 1:5000 in 20 mL dilution buffer (PBS with 0.05% tween-20 and 0.1% FBS).
27. Plates were read-out with R&D substrate (Cat#DY999) as per manufacturer's recommendation on a Wallac spectrophotometer. Incubation was between 18°C–22°C for 20 min while protecting from light. The absorbance was detected at 450 nm.

⚠ CRITICAL: Patient plasma samples carry risk of pathogens, not limited to hepatitis B (HBV), hepatitis C (HCV), and human immunodeficiency virus (HIV). Therefore, proper PPE must be utilized when handling any samples (Twitchell, 2003).

IFN-β promoter reporter assay

Timing: 3 days

This assays measures the ability of transfected proteins of interest to influence an interferon response induced by Sendai virus (SeV) (Figure 7) (Messaoudi et al., 2015).

28. Seeding HEK-293T cells.
    a. HEK-293T cells were prepared from a stock solution of cells.
        i. Cells were split between 70%–90% confluency.
    b. HEK-293T cell media was aspirated from the flask.
    c. Cells were exposed to EDTA-Trypsin for 5 min.
    d. DMEM + 10% was added to the cell flask.
    e. Trypsinized cells, split 5 × 10^4 into a 96 well plate.
29. 24 h later, HEK-293T cells (5 × 10^4) were co-transfected using Lipofectamine 2000 with:
    a. 25 ng of an IFN-β promoter-firefly luciferase reporter plasmid
    b. 25 ng of pRL-TK Renilla luciferase reporter plasmid
    c. 125, 12.5, and 1.25 ng of the indicated viral protein expression plasmid
30. 24 h after transfection, cells were mock-treated or treated with SeV (15 hemaglutination units/mL).
31. 18 h post-treatment or post-infection, cells were lysed.
32. Cell lysates were analyzed for luciferase activity using a Dual-Luciferase reporter system.
    a. The Promega Dual-luciferase system utilizes enzymatic substrates for both Renilla luciferase and Firefly Luciferase.

b. Preparation of Buffers.
   i. Resuspend the Luciferase Assay Substrate in Luciferase Assay Buffer II.
   ii. Dilute the stock solution of Stop & Glo® 50x to 1x with Stop & Glo® Buffer.
c. Read-out of 96-well plate.
   i. Add 20 μL of cell lysates prepared above.
d. Assays were performed in triplicate.

33. Viral protein expression was confirmed by Western blot analysis.

⚠ CRITICAL: It is important that all reagents used in cell culture are kept sterile (Coté, 1998). Prior to readout with the luciferase, verify that the LAR II and the Stop & Glo® Reagents have been warmed to 18°C–22°C. Luciferase activity is highly variable (Repele and Manu, 2019), and it is recommended that an auto-injector is used for injection of reagents.

Differential HDX-MS structural characterization of RNA binding

Timing: Mapping: 1 day; HDX-MS: 1 day per protein state

Hydrogen-deuterium exchange mass spectrometry (HDX-MS) provides sensitive, high-resolution measurement of solvent accessible surface area and changes in protein dynamics (Masson et al., 2019). We and others successfully used HDX-MS to characterize host-pathogen interactions (Pei et al., 2021; Raghuvamsi et al., 2021), monitor individual host and viral protein dynamics and interactions (Batra et al., 2021; Su et al., 2018; Courouble et al., 2021), map antibody-antigen interactions (Huang et al., 2018; Chen et al., 2019), and determine binding sites of DNA/RNA with proteins (Long et al., 2017). This protocol outlines a method to pinpoint viral protein RNA binding.

34. Perform pre-HDX mapping.
    a. To ~ 100 pmol of the protein of interest (2 μL), dilute 10:1 with H2O buffer (+ 18 μL).
    b. Dilute 2:3 with a selected quench buffer.

Note: For more dilute protein samples, scale up the volumes if the ratios are retained.

Optional: Flash freeze quenched solutions for ease in the HDX-MS analysis.

    c. Inject into the LC-MS apparatus for digestion, desalting, and LC-MS/MS analysis
       i. Digest and desalt the protein with 200 mL/min solvent A using valve position 2 (Figure 2) for 3 min (or 3 trap column volumes).
       ii. Analytically separate and elute the digested protein fragments by using valve position 1 (Figure 2) and an organic gradient (solvent A – solvent B; for an example gradient, see below).

Example linear gradient for reversed-phase peptide elution

Time (min)	% A	% B
0	95	5
0.5	95	5
11	50	50
13	0	100
13.5	0	100
15	95	5

Note: Include a high organic pulse (i.e., 100% solvent B) at the end of the LC gradient to decrease carryover from run to run.

Note: The LC gradient may need to be adjusted for differences in protein hydrophobicity or to improve temporal separation of peptides/chromatogram congestion.

d. Process the LC-MS/MS results to optimize digestion efficiency using Byonic and Byologic (see quantification and statistical analysis below for details) to compare:
   i. Protein coverage
   ii. Peptide length
   iii. Peptide abundance
   iv. Count of identified peptides
   v. Redundancy

Note: Peptides with a 5–20 amino acid length are recommended for HDX-MS. Redundancy can be measured numerically as a function of the average number of overlapping peptides across all residues and tabulated in the suggested comparison. We also recommend visual inspection of redundancy in the context of protein coverage.

e. Repeat with a range of quench conditions and protease columns/column orientations (Cravello et al., 2003) (see Figure 8A for an example of digestion optimization logic). Optimization of temperature, quench incubation time, addition of other chaotropic reagents can be done.
f. Repeat the mapping experiment with the chosen conditions, but with added RNA to confirm the RNA is not affecting the digestion.

Pause point: As long as mapping conditions (i.e., MS settings and protease choice) are replicated during the HDX-MS experiment, HDX-MS can be completed at any time assuming sample integrity. Proteins can typically be stored for months at −80 °C.

Note: If the protein contains disulfide bonds, a reducing agent should be added to the quench. Reduction of disulfide bonds may necessitate incubation at elevated temperature for 1–3 minutes. Incubation, especially at elevated temperatures, increases H/D back-exchange; care should be taken to minimize back exchange.

Note: Denaturation using other chaotropic agents such as guanidine HCl may be used to better denature the protein. The efficacy of such denaturants on improving protein digestion is protein-dependent.

⚠ CRITICAL: Screening of mapping conditions defines the feasibility and resolution of the HDX-MS experiment. Carefully optimize the mapping results before proceeding with HDX-MS. For an example of mapping logic, see table below and Figure 8A.

Example screening of quench and digestion conditions

	Denaturant (starting conc.)	Protease column (s)	Coverage (%)	Average length (aa)	ID'd peptide count
1	3 M Urea	Pepsin	100%	28.2 (± 0.8)	341 (± 7)
2	3 M Urea	FXIII	100%	20.86 (± 0.04)	577 (± 3)
3	3 M Urea	FXIII-Pepsin	100%	18.3 (± 0.4)	550 (± 20)
4	3 M Urea	Pepsin-FXIII	100%	20.18 (± 0.04)	556 (± 4)

35. Equilibrate unbound and bound stock solutions. Protein/ligand concentrations and buffer conditions should be consistent across all HDX samples. A higher concentration stock should be generated for both bound and unbound states such that 2 µL of stock diluted with 18 µL D2O buffer yields the same final protein concentration.

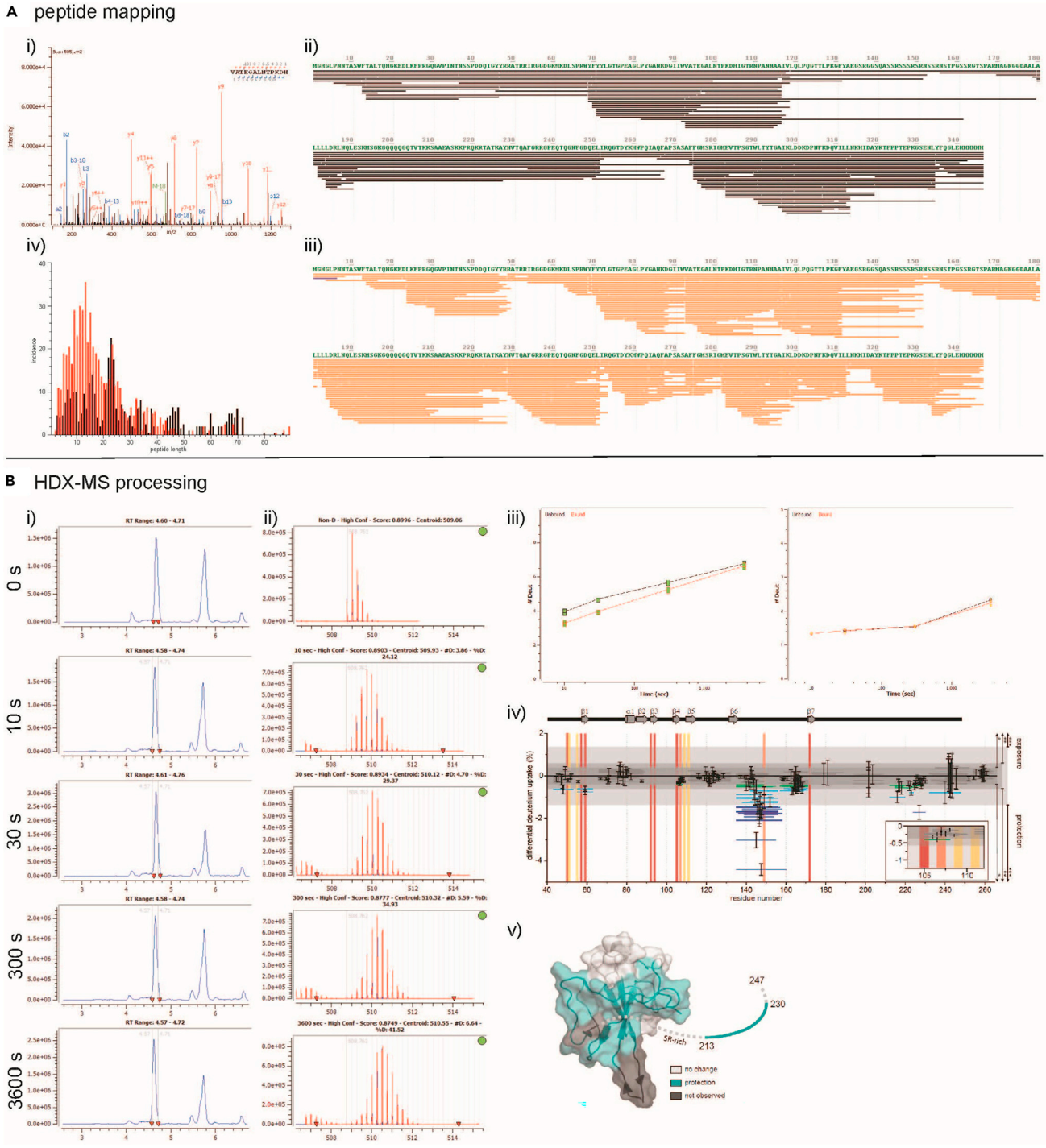

Figure 8. HDX expected outcome

(A) (i) Representative MS^2 spectrum of positively identified peptide from the mapping experiment, interpreted from b- and y- ion fragments that provide sequence information. Blue, N-terminal-facing notches in the peptide sequence indicate identification of b-ions; red, C-terminal-facing notches in the peptide sequence indicate identification of y-ions. A screenshot of data from Byologic software. Peptide map spanning SARS-CoV-2 N protein NTD-LKR-CTD showing coverage from digestion by pepsin (ii, black) and pepsin-FXIII columns (iii, orange). iv) Comparison of effect of protease selection on peptide length. In general, pepsin (black) generates longer peptides than pepsin-FXIII columns in series (red).

(B) (i) LC extracted ion chromatograms, indicated by red triangle bookends, for a peptide submitted to incubation in D_2O for 0 (nondeuterated control), 10, 30, 300, 3600 s (top to bottom), demonstrating consistent elution times with time. The mass spectra (ii) associated with these peaks demonstrate increased deuteration as a function of time. (iii) Graphing the centroid shift allows for the comparison of unbound (black) and the RNA-bound protein

Figure 8. *Continued*
(red), indicating protection (left) or no change (right) for peptide generated by pepsin-FXIII digestion in the bound state. (iv) A Woods' plot includes cumulative differences from all time points for the peptides to be viewed in one image, with statistics ($p < 0.1$, *; $p < 0.05$, **; $p < 0.01$, ***), enabling an overall/global view/overview of changes in HDX across the entire protein sequence. Changes in deuteration upon RNA-binding mapped on a known structure of N protein NTD (PDB: 6M3M) (v). Meaningful decreases shown in blue. Raw data screenshots from PMI Byos and HDExaminer softwares. Woods' plot and N protein NTD structure (iv and v) from Wu et al. (2021).

a. Prepare the unbound protein stock solution (~25 μM).
b. Prepare the bound protein stock solution (~25 μM) with 1:1 RNA.

Optional: The amount of RNA added may be increased to maximize the percentage of protein bound to RNA.

Note: Prepare and acquire the non-deuterated control and all HDX time points in at least duplicate.

36. Prepare the non-deuterated control (i.e., 0 s).
    a. To 2 μL of the unbound protein stock, add 18 μL H_2O buffer.
    b. Stop HDX with addition of 30 μL quench solution.
    c. Flash freeze, and store solution at −80°C.

37. Prepare HDX labeled protein (Figure 2A).
    a. To 2 μL of unbound protein stock on ice, add 18 μL D_2O buffer (to maximize HDX at 90% D).
    b. Incubate at range of times logarithmic: 4 time point: 10, 30, 300, 3600 s.

Optional: More time points may be added (e.g., 60, 900, 14400 s) to define better HDX kinetics.

    c. Quench HDX with addition of 30 μL quench solution for final volume of 50 μL (same as sample loop).
    d. Flash freeze in liquid nitrogen.

38. Repeat all with the bound protein stock solution.

⚠ CRITICAL: All times should be controlled! Deviations in timing can lead to differential back-exchange extents and poor precision. Deviations in observed LC elution times result in more manual adjustment of extraction window during processing.

⚠ CRITICAL: HDX-MS is sensitive to fluctuations in temperature, pH, and ionic strength. All buffer conditions should be controlled, and all reagents should be pre-equilibrated at the temperature they will be used to ensure no unwanted deviations across the experiment.

Alternatives: Inject immediately if protein aggregates upon flash freeze.

Pause point: Store flash frozen HDX samples at −80°C for up to 1 month.

39. Perform LC-MS analysis of the HDX labeled protein states.
    a. Thaw flash frozen HDX samples at 37°C for 30 s just prior to injection (consistency is essential).
    b. Inject the protein and analyze as noted for mapping above for MS analysis (without MS/MS).

Note: Deuteriums are prone to scrambling, especially with collision induced dissociation. Other dissociation techniques can be used for fragmentation without scrambling (Ferguson et al., 2007).

40. To reduce carryover, wash sample loop with at least 3 volumes (i.e., 150 μL for a 50 μL loop) with water/0.1% formic acid, acetonitrile + 0.1% formic acid, and again water + 0.1% formic acid between injections.
41. To monitor carryover extents, run a blank between samples.
    a. H_2O blank: 20 μL H_2O buffer + 30 μL quench.
    b. D_2O blank: 2 μL H_2O buffer + 18 μL D_2O buffer + 30 μL quench.

EXPECTED OUTCOMES

Negative-stain EM is particularly useful to visualize oligomeric species with higher resolution of the electron microscope compared to the light microscope. For nucleocapsids bound to RNA, it is common to observe loose coils where oligomers of nucleocapsids are linked with copurified RNA as well as more condensed form helical assembly, depending on specific constructs and solvent environment. Due to inherent conformational flexibility, oligomers of nucleocapsid protein can adopt different forms, such as rings and tubes of different lengths. In the case of SARS-CoV-2 N, upon RNA binding and dilution into 150 mM NaCl, we observed additional species of N-RNA which phase separates into liquid droplets of μm size. These droplets are a separate phase from solvent and lack internal details. They might be relevant to RNA packaging.

HDX-MS mapping of the protein-RNA binding system with appropriate selection of protease and quench conditions generates a set of peptides spanning the protein (Figure 8A). Positively identified peptides are selected prior to exchange based on high quality MS2 spectra whereby fragment ions essentially sequence the peptide (Figure 8Ai). The efficacy of mapping may be checked by peptide coverage maps to afford quick comparison (e.g., pepsin vs. FXIII-pepsin, Figures 8Aii and 8Aiii, respectively). Because 5–20 amino-acid peptides are optimum for maximizing HDX-MS resolution, peptide length as a metric should be compared across mapping conditions (Figure 8Aiv). Taken together, coverage and peptide length are two metrics for optimization of HDX-MS conditions.

Differential HDX-MS of a two-state system measures meaningful differences for peptides containing residues involved in RNA binding (Figure 8B). Timing of the HDX-apparatus for repeatability of LC conditions should generate chromatograms with consistent elution times. Specifically, the extracted ion chromatogram (XIC/EIC) of an identified peptide should show maintenance of elution time, shape, and relative abundance across samples undergoing differential HDX (Figure 8Bi). If the exchange kinetics are appropriately measured in the HDX experiment, the extracted mass spectrum for the LC peak will demonstrate an increase in deuteration, whereby the centroid will shift with time (Figure 8Bii). Kinetic plots, representing peptide deuteration as a function of incubation time will show changes in deuteration for the protein *bound state* relative to the *unbound state* for those peptides that include RNA binding sites (decreased HDX) and/or changes in backbone flexibility/hydrogen bonding (increased and/or decreased HDX) (Figure 8Biii, left). Conversely, there will be no meaningful difference in HDX for the two states for those peptides covering regions of the protein not affected by RNA binding (Figure 8Biii, right). The cumulative difference (defined in quantification and statistical analysis section) for all kinetics plots are plotted across the protein sequence, allowing the user to visualize dynamics for the entire system (Figure 8Biv). Finally, meaningful differences in deuteration are represented on known structures to draw conclusions about differential H-D kinetics in the context of structure (Figure 8Bv).

QUANTIFICATION AND STATISTICAL ANALYSIS

Curve fitting of fluorescence polarization binding data

Timing: 1 h

Titrations curves of varying protein receptor concentration were fitted using the following user defined equation in Origin.

$$F = (Fb - Ff) \times \frac{(KD + L + R) - \sqrt{(KD + L + R)^2 - 4 \times R \times L}}{2 \times R}$$

Fb is fluorescence polarization value when FITC-ssRNA is saturated with protein receptor and Ff is fluorescence polarization value when FITC-ssRNA is alone. KD is the dissociate constant. L and R are total RNA and protein concentration, respectively.

Alternatives: Origin was used for curve fitting. However, any other software, e.g. GraphPad Prism, that is capable of nonlinear curve fitting can be used.

Processing pre-HDX mapping results using Protein Metrics software

Timing: 2 h

Alternatives: Several LC-MS/MS peptide identification processing softwares are available (e.g., Mascot, PEAKs, Proteome Discoverer), and any that identifies peptides from MS/MS analysis and reports the LC retention time for the peptide can be used.

1. Process each LC-MS/MS mapping datafile using Byonic™.
   a. Import raw data and FASTA sequence files.
   b. Search data using *non specific* [*sic*] *(slowest) digestion specificity*, as is appropriate for nonspecific digestion of proteins with Pepsin/FXIII proteases.
   c. Check newly-generated Byonic file for protein coverage by using peptides with good MS^2 fragmentation.

2. Import Byonic™ results into Byologic®.
   a. Use the *Byonic Import* function to search directly Byonic data for LC retention window information.
   b. Validate MS2 spectra for good fragmentation and appropriate assignment to LC peaks.

⚠ CRITICAL: If data quality or coverage are poor, do not move on; consider evaluating additional mapping conditions.

3. Export parameters for comparison of the quench and digestion conditions (Figure 8A).

Note: Consider, given that peptides containing 5–20 amino acid residues are most useful for HDX, evaluating and selecting optimum quench and digestions conditions.

4. Export a csv file for all peptides with the chosen quench and digestion conditions.
5. Within the csv file, remove all columns except sequence, XIC apex (or retention time), charge, and score. This is the working mapping file for processing HDX results.

Processing HDX-MS results using HDExaminer

Timing: 3 days

Alternatives: Several HDX processing software packages are available (e.g., HDX Workbench, HX Express) and can be used.

6. Start new project from the protein FASTA sequence.
7. Import the peptide list from the mapping results created in Byologic in step 5, previous section.
8. Import the raw LC-MS datafiles; adjust incubation times and percent deuterium to correspond with each data file.

9. For each peptide, adjust LC extraction windows to cover the same retention time across all time-points. Watch for contaminating ions in the *m/z* window, as they perturb the fitting for *m/z* centroid calculation (and resulting deuteration values).

   Note: Care should be taken to ensure the same extraction window for each peptide for all time points and all states (i.e., unbound/bound).

   Note: Some peptides should be deleted from the analysis pool on basis of quality of extracted ion chromatogram and/or MS of isotopologues. Export peptide pool results.

Woods' plot generation and statistical analysis

Timing: 1 h

10. Calculate cumulative differences in HDX (Figure 3A) as the sum of all differences in D at all time points:

$$Summed\ \Delta HX = \sum(D_{t,\ bound}) - \sum(D_{t,\ unbound})$$

where D_t is the deuterium uptake in Da at each time point for the respective state (unbound or bound).

   Note: *Positive* summed *ΔHX* values indicate the HDX for a given peptide is *higher* in the bound state than the unbound (i.e., binding increased solvent accessibility or decreased amide hydrogen bonding). *Negative* summed *ΔHX* values indicate the HDX for a given peptide was *lower* in the bound state than the unbound state (i.e., binding decreased solvent accessibility or increased amide hydrogen bonding).

11. Calculate the error of the HDX measurement for each differential peptide result as 3 times the propagated error from the standard deviations at each time point and for both states.

12. Calculate global HDX significance limits.
    a. Calculate the pooled standard deviation for the entire dataset for each state:

$$s_{pooled} = \sqrt{\frac{\sum s^2}{N}}$$

where *s* is all standard deviations across the dataset and N is the number of data points (the number of timepoints * the number of peptides).

   Note: Peptides for which standard deviations were not calculated at any time point should be excluded from the dataset.

    b. From the pooled standard deviations of both states, calculate the standard error of the mean (SEM):

$$SEM_{\overline{\Delta HX}} = \sqrt{\frac{s^2_{p,unbound}}{n_{unbound}} + \frac{s^2_{p,bound}}{n_{bound}}}$$

where n is the number of experimental technical replicates (in this case 2 for both).
    c. Using the SEM and t-values for a two-tailed Student's t-distribution, $p < 0.1$, 0.05, and 0.01, and 2 degrees of freedom (for duplicates of two states, 2 + 2 - 2), calculate the confidence interval or global *ΔHX* significance limit(s):

$$\pm CI_{\Delta\overline{HX}} = t \times SEM_{\Delta\overline{HX}}$$

Peptide HDX differences are only considered statistically significant if the propagated error does not cross 0 and the HDX differences exceed the global significance limit(s).

13. Generate HDX summary and HDX data tables.

LIMITATIONS

HDX-MS modifies amide hydrogens on the entire protein surface as a function of solvent accessibility to probe changes in backbone protein dynamics and hydrogen bonding occurring the in higher order structure. Owing to the risk of back-exchange necessitating expedited sample handling and special considerations for low pH digestion, regions of the protein in critical regions of interest may not digest well, and the user may wish to probe solvent accessibility of specific protein side chains.

TROUBLESHOOTING

Problem 1

Broad peaks in size exclusion chromatography (purification of nucleocapsid protein, step 27).

Potential solution

Since only one affinity tag column is used before size exclusion chromatography, contaminants might be a problem to cause broad peaks in size exclusion chromatography. Due to the RNA binding and aggregation-prone nature of N, especially for longer constructs, it is crucial to maintain high salt concentration in lysis and Ni column steps. This helps remove nonspecific binding. In addition, when choosing molecular weight cutoff of concentrators, it is better to use concentrators of highest molecular weight cutoff to improve purity. In addition, keep everything on ice and purify as fast as possible to minimize degradation.

Problem 2

Introduction of bubbles while loading (dynamic light scattering, step 4).

Potential solution

Avoiding generating bubbles in the sample in the first place. This can be done by sample centrifugation. While loading, touch the bottom of the well, then slowly release sample inside while pulling up pipette tip. Leave the last bit of sample and do not pipette everything out. Practice loading with buffer and BSA protein sample first. If bubbles are introduced into wells, gently tap the plate to let bubbles float to surface and remove bubbles with pipette tip.

Problem 3

Mapping digestion shows missing sections of protein (i.e., low coverage), poor peptide redundancy, low peptide signal (differential HDX-MS structural characterization of RNA binding, step 34).

Potential solution

These are indications that mapping requires further optimization before starting HDX experiments. One or both of the following may be required: (1) adjust quench solution components (e.g., chaotropic reagent, salt concentration, reducing agent). (2) Incorporate additional protease on-line with original protease or use alternative protease or preparation.

Problem 4

No meaningful differences in H-D are observed between two protein states (differential HDX-MS structural characterization of RNA binding, step 37).

Potential solution

HDX is reporting on all states of the target protein in solution. If complex formation is incomplete, differences in HDX are diluted. To ensure more protein binding, the user can increase the relative concentration of the RNA. It may be possible that the time range of the experiment is not the same as the protein dynamics/kinetics of HDX. When both protein states are highly exchanged across all timepoints, decrease the temperature for HDX (e.g., perform on ice); for cases where both protein states undergo slow exchange with time, increase the HDX temperature. It is also possible that back exchange is occurring during separation and measurement. Ensure that minimal time is involved for measurement and that sample is kept at low T. Check the pH of the final solution after quench to ensure a value corresponding nearly to the kinetic minimum at pH 2.6.

Problem 5

Peptide XIC elution times drift, making HDX-MS data processing more time consuming (differential HDX-MS structural characterization of RNA binding, step 39).

Potential solution

Consistent timing of sample injection, start of LC gradient, and operation of MS file acquisition are critical to maintain a consistent measurement of XIC elution time. We recommend the use of a hand-held timer to set alarms reminding the user to operate each piece of equipment in manual mode. Given that peptide elution times are also a function temperature; ensure constant temperature in the HDX-MS apparatus by maintaining an ice water slush for the duration of the experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Gaya Amarasinghe (gamarasinghe@wustl.edu).

Materials availability

Plasmids in this study are available with a completed Materials Transfer Agreement Request for these reagents by submitting to Dr. Gaya Amarasinghe (gamarasinghe@wustl.edu).

Data and code availability

The protocol includes all data sets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

C.W., G.K.A., and D.W.L. conceived the overall project. M.L.G. provided the MS facilities for HDX. All authors were integral to the design and execution of the study. C.W., A.J.Q., A.B.M., N.D.W., G.K.A., and D.W.L. wrote the initial draft with significant input and editing from all authors.

DECLARATION OF INTERESTS

M.L.G. is an unpaid member of the scientific advisory board of Protein Metrics and GenNext Technologies, two companies commercializing protein footprinting software and tools.

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