Electronic Supplementary Information

Rapid electrotransfer probing for improved detection sensitivity in in-gel immunoassays

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Table S1: Fabrication conditions for polyacrylamide gels.

Reagent	Sizing Gel	20%T PA gel	4%T PA loading gel
30%T, 29:1C stock acrylamide/bis-acrylamide; No. A3574, Sigma-Aldrich	7% w/v final acrylamide concentration	20% w/v final acrylamide concentration	4% w/v final acrylamide concentration
10X Tris-glycine; No. 1610734, Bio-Rad	10% v/v	10% v/v	10% v/v
Rhinohide™; No. R33400, Invitrogen	4.66% v/v	4.66% v/v	4.66% v/v
100mM N-[3-[(3-Benzoylphenyl)-formamido]propyl] methacrylamide in DMSO; No. PAL0603, PharmAgra Labs	3% v/v (3mM)	--	--
Donkey anti-Rabbit IgG AlexaFluor® 647; No. A31573, Invitrogen	--	--	0.2 mg/mL
Degas and Sonication Time	5 min	5 min	5 min
Initiators	--	--	--
APS; No. A36778, Sigma-Aldrich	0.08% w/v	0.08% w/v	0.08% w/v
TEMED; No. T9281, Sigma-Aldrich	0.08% v/v	0.08% v/v	0.08% v/v
Polymerization Time	60 min	60 min	60 min
Gel Dimensions (x-y-z)	10mm-10mm-1mm	10mm-10mm-1mm	14mm-14mm-1mm

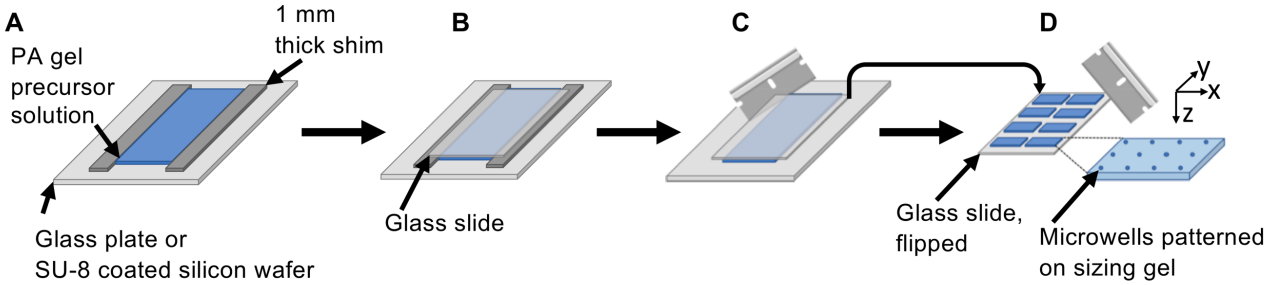

Figure S1: PA gel fabrication assembly. (**A**) To assemble the fabrication mold for PA gels, two 1 mm thick shims were affixed with adhesive onto either an SU-8 coated silicon wafer (for sizing gel fabrication) or a glass plate (for 20%T PA gel fabrication). PA gel precursor solution was cast to the fabrication mold by pipetting the solution between the shims. (**B**) A glass slide was placed on top of the shims without trapping air bubbles in the precursor solution. (**C**) After the gel polymerized (60 min), the gel was released from the fabrication mold by first removing the shims by hand, then sliding a razor between the glass slide and the bottom mold surface (silicon wafer or glass plate), and finally using the razor to gently pry the glass slide away from the silicon wafer or glass plate. To prevent gel surface adhesion, the glass surfaces were treated with Gel Slick® and the SU-8 features were coated with dichlorodimethylsilane. (**D**) The glass slide and gel were removed from the fabrication mold and flipped (glass slide on a flat surface, gels on top of glass slide). The glass slide was used as a support to trim the gels (x-y-z dimensions: 10-10-1 mm). Microwells were patterned on a x-y face of the sizing gel from the SU-8 micropost features (Microwell diameter: 32 µm. Microwell depth: 40 µm. Microwell center-center spacing: 100 µm.). The 20%T gel was not patterned with any features, as the glass plate was featureless.

Table S2: Buffer compositions.

Electrotransfer Buffer	1X RIPA Buffer
10% v/v 10X Tris-glycine; No. 1610734, Bio-Rad	10% v/v 10X Tris-glycine; No. 1610734, Bio-Rad
0.5% v/v Triton X-100; No. X100, Sigma-Aldrich	0.5% w/v Sodium Dodecyl Sulfate; No. L3771, Sigma-Aldrich
89.5% v/v MilliQ Water	0.25% w/v Sodium Deoxycholate; No. D6750, Sigma-Aldrich
--	MilliQ Water: 79.8% v/v

Table S3: Fabrication conditions for probe loading gels

Reagent	Probe Loading Characterization Gel	OVA Immunoprobing: Primary Probe Loading Gel	OVA Immunoprobing: Secondary Probe Loading Gel
Ultrapure Low Melting Point Agarose; No. 16520050, Invitrogen	1.5% g/mL (dissolved in 1X Tris-glycine)	1.5% g/mL (dissolved in 1X Tris-glycine)	1.5% g/mL (dissolved in 1X Tris-glycine)
Antibody Probes	0.2 mg/mL Donkey anti-Rabbit IgG AlexaFluor® 647; No. A31573, Invitrogen	Diffusive probing: 0.1 mg/mL Rabbit anti-OVA IgG; No. Ab181688, Abcam Electrotransfer probing: 0.0075 - 0.1 mg/mL Rabbit anti-OVA IgG; No. Ab181688, Abcam	Diffusive probing: 0.2 mg/mL Donkey anti-Rabbit IgG AlexaFluor® 555; No. A31572, Invitrogen Electrotransfer probing: 0.015 - 0.2 mg/mL Donkey anti-Rabbit IgG AlexaFluor® 555; No. A31572, Invitrogen
Gelation Time	5 min on ice pack	5 min on ice pack	5 min on ice pack

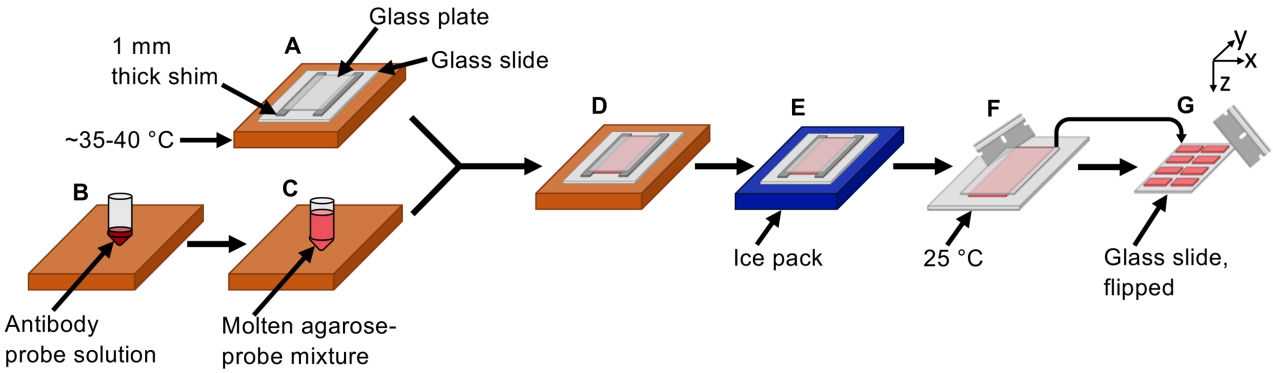

Figure S2: Agarose probe loading gel fabrication assembly. (**A**) To assemble the fabrication mold for the loading gel, first, two 1 mm thick shims were affixed with adhesive onto a glass plate. Then, a glass slide was affixed with adhesive onto the two shims. The fabrication mold was then placed on a hot plate and warmed to ~35-40 °C. (**B**) An Eppendorf tube was warmed on a hotplate and brought to ~35-40 °C. Antibody probe solution was added to the heated Eppendorf tube. (**C**) Molten agarose was added to the antibody probe solution, and mixed by pipetting. (**D**) To cast the loading gel to the fabrication mold, the molten agarose gel-probe mixture was pipette between the glass slide and glass plate. Enough molten agarose-probe mixture was pipetted to fill the volume encased by the glass slide, glass plate, and shims, without introducing air bubbles. (**E**) To gelate the loading gel, the fabrication mold was transferred to an ice pack for the molten agarose-probe mixture to cool for 5 min. (**F**) After gelation, the fabrication mold was removed from the ice pack, and placed on a 25 °C surface. The gel was released from the fabrication mold by first removing the shims by hand, then sliding a razor between the glass slide and glass plate, and finally using the razor to gently pry the glass slide away from the glass plate. (**G**) The glass slide and loading gel were removed from the fabrication mold and flipped (glass slide on a flat surface, loading gel on top of glass slide). The glass slide was used as a support to trim the loading gels (x-y-z dimensions: 14-14-1 mm).

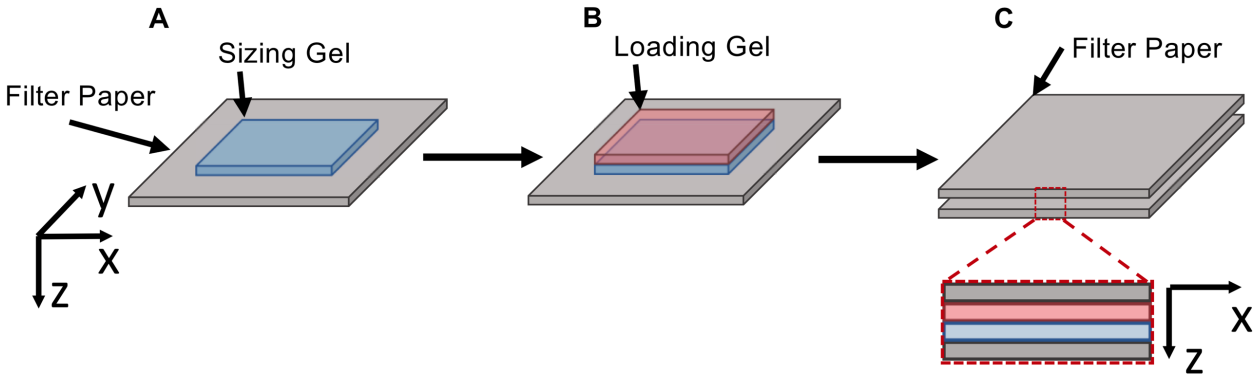

Figure S3: Filter paper – gels – filter paper sandwich assembly for electrotransfer probe loading. (**A**) A sizing gel was placed on a western blot filter paper piece. (**B**) A loading gel was placed on the sizing gel without trapping air bubbles between the two gels. The loading gel was aligned in the x-y plane to entirely overlap the sizing gel. (**C**) A second western blot filter paper piece was placed on top of the loading gel without perturbing the alignment of the loading and sizing gels.

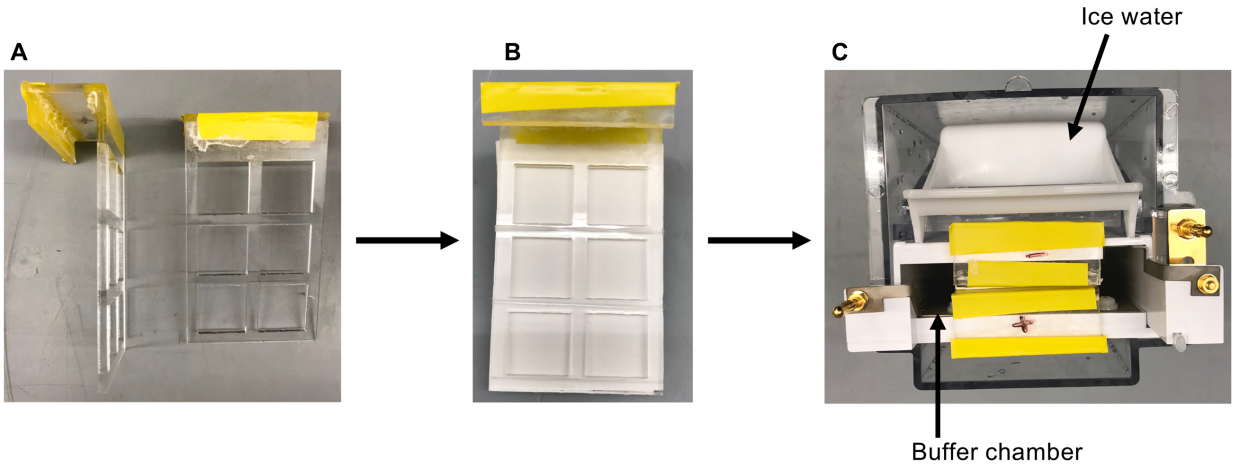

Figure S4: Acrylic clamp assembly. (**A**) The trace for the acrylic clamp face in contact with the filter paper have the following dimensions: 55 mm width x 85 mm height. Window cut outs have the following dimensions: 20 mm squares, 3 x 2 array, 5 mm edge-to-edge spacing between square windows. The windowed component of the clamp was super glued to an acrylic rectangle (55mm wide x 30 mm long) so that the assembled gel holder could rest on the brim of the electrotransfer system buffer chamber. (**B**) The clamp was designed with capacity for up to 6 gel sandwiches to be housed simultaneously. The clamp is compressed by using plastic elastomers along the frame of the windows. (**C**) The acrylic clamp rests on the brim of the electrotransfer buffer chamber, and the gels sandwiches are submerged in buffer volume.

Table S4: Electrotransfer conditions in probe loading and unloading

Electrotransfer Probing Process	Electric Field Strength	Electrotransfer Time
Probe Loading	8 V/cm	11.5 min
Probe Unloading	12 V/cm	15 min

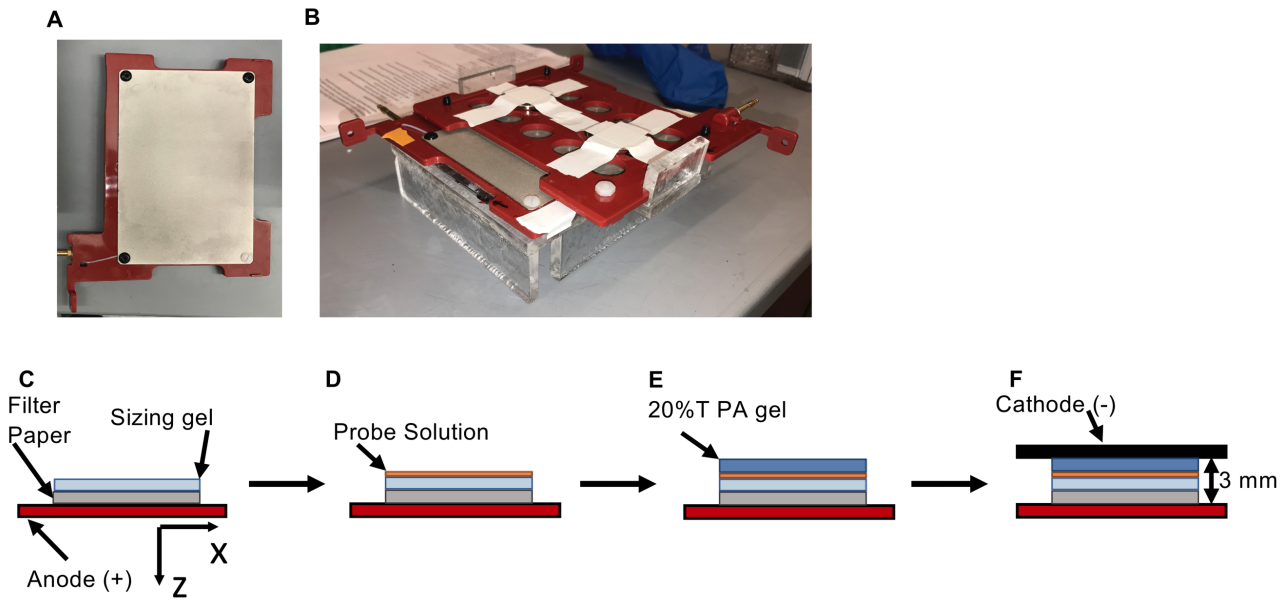

Figure S5: The semi-dry electrotransfer system was used to electrophoretically inject antibody probe from free-solution into a sizing gel for probe electromigration characterization. (**A**) The system is composed of two electrode plates surrounded by plastic alignment casings. (**B**) The system was assembled by aligning the two electrode plates and magnetically bringing the plates into contact. To prepare the sample between the electrode plates (**C**) first, a piece of filter paper was placed on the anode (filter paper x-y-z dimensions: 10-10-1 mm; filter paper x-y face placed in contact with anode; filter paper equilibrated in Electrotransfer Buffer for 5 min). (**D**) Then, a sizing gel was placed on the filter paper. The mircowell patterned face of the sizing gel was faced upwards and was gently blotted dry with a Kimwipe. Next, 5 µL of 5 µM Donkey anti-Rabbit IgG AlexaFluor® 647 solution (in 1X Tris-glycine) was pipette on the microwell patterned face of the sizing gel. The microwells acted as a reservoir for probe solution to facilitate electrophoretic injection. (**E**) After pipetting the Ab probe solution, a 20%T PA gel was blotted dry (gel fabrication described in Table S1) and placed on the sizing gel. (**F**) Two, 3 mm shims were placed around the filter paper – gel assembly, and the cathode was placed on top. The electrode plates were magnetically compressed.

Table S5: Diffusive transfer times in probe loading and unloading

Diffusive Transfer Probing Process	OVA Immunoprobing: Primary & Secondary Probe	Loading and Unloading Characterization
Probe Loading	15 h	28 h
Probe Unloading	45 h	45 h

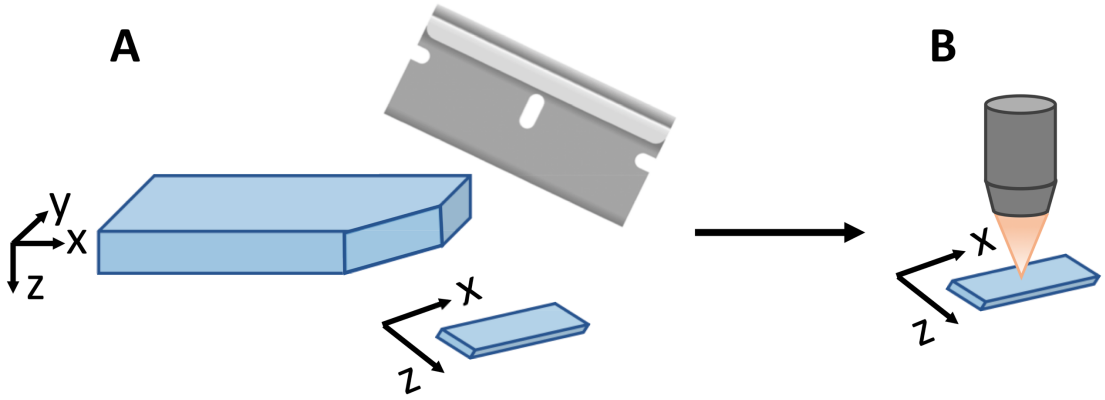

Figure S6: Workflow for imaging gel-sliver. (**A**) A sizing gel was cut using a razor to produce a gel-sliver (dimensions of gel sliver: 10mm x ~0.5mm x 1mm in x-y-z). (**B**) The gel sliver was imaged such that the gel x-z face was parallel with the microscope plane-of-focus.

Note S1: Design considerations for diffusive probing system

In designing the diffusive probing control, we considered systems for (i) uniform probe delivery to the sizing gel and (ii) consumption of the antibody probe mass as equivalent to that consumed in the electrotransfer probing system. To satisfy this latter criterion when diffusive probing is accomplished by submerging a sizing gel in a probe solution, requires prohibitively large consumption of antibody probe material. For example, sufficient probe-solution volume to submerge a sizing gel requires an antibody probe concentration that is low compared to the probe concentration maintained in the loading gel (using single well of 12 well plate). The in-gel probe concentration at equilibrium is further reduced compared to the in-solution probe concentration, as size exclusion partitioning strongly impedes diffusive probe entry into the sizing gel. Thus, to satisfy the latter criterion, an alternative method for interfacing probe solution to the sizing gel without reducing the in-solution probe concentration was devised by pipetting a smaller volume of probe solution directly onto the sizing gel (the reduced solution volume preserves in-solution antibody probe concentration). The primary challenge in this system design is that fluid tends to pool around gel edges, resulting in non-uniform probe delivery to the sizing gel surface that is in contact with the probe solution, as we have previously demonstrated.[1] Consequently, the loading gel – sizing gel – loading gel diffusive probing system was ultimately chosen as it meets the needs of our diffusive probing control system by (i) preventing probe solution from pooling to gel edges and (ii) not diluting probe in large solution volumes.

1 A. Geldert, H. Huang and A. E. Herr, *Sci. Rep.*, 2020, **10**, 1–12.