ProteinSEQ™ Protein A Quantification Kit

Workflow for Standard (non-FAST) PCR plates

Catalog Number 4469343

Pub. No. MAN0013526 **Rev.** A.0

Contents	Cap color	Storage conditions
Repligen Protein A Standard (1 mg/mL)	Blue	−20°C
Protein A 5′ Probe	Gray	
Protein A 3′ Probe	Yellow	
2X Protein A Sample Prep Reagent	White	
ProteinSEQ™ Ligation and Assay Mix	Green	
ProteinSEQ™ Ligase	Orange	
Fast Master Mix, 2X	White	4°C
Wash Buffer	White	
Protein A Capture Beads	White	
ProteinSEQ™ Elution Buffer	White	
ProteinSEQ™ Diluent	White	

Note: For safety and biohazard guidelines, refer to the "Safety" appendix in the *ProteinSEQ™ Protein A Quantification Kit User Guide* (Pub. no. MAN0013524). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Important procedural guidelines

- The MagMAX™ Express-96 PCR Well Magnetic Head is fragile. Handle with care.
- Use serially diluted standards when performing spiking studies. See *ProteinSEQ™ Protein A Quantification Kit User Guide* (Pub. no. MAN0013524) for spiking guidelines.

Pre-dilute the Protein A standard

Dispense the following to four labeled 1.5-mL microfuge tubes. After you add the Protein A standard to each tube, vortex the tube for 2 seconds, then briefly centrifuge before making the next transfer.

Table 1 Repligen Protein A standard

Dilution Tubes	ProteinSEQ™ Diluent	Repligen Protein A standard	Final volume
D1	480 µL	20 µL (1 mg/mL)	500 µL
D2	900 µL	100 µL from D1	1000 µL
D3	900 µL	100 µL from D2	1000 µL
D4	900 µL	100 µL from D3	1000 µL

Table 2 MabSelect SuRe™ Protein A Standard

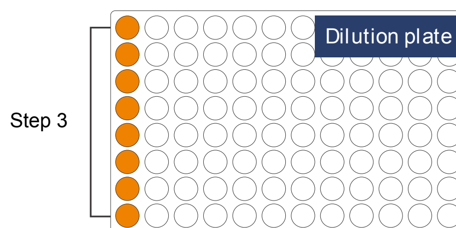
Dilution Tubes	ProteinSEQ™ Diluent	MabSelect SuRe™ Protein A Standard	Final volume
D1	490 µL	10 µL (2 mg/mL)	500 µL
D2	900 µL	100 µL from D1	1000 µL
D3	900 µL	100 µL from D2	1000 µL
D4	900 µL	100 µL from D3	1000 µL

Note: The final Protein A concentration of D4 is 40 ng/mL.

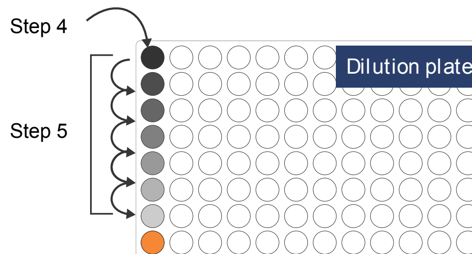
Prepare serial dilutions of the diluted Protein A standard

Prepare a dilution series using D4 from the previous step.

1. Label a MagMAX™ Express-96 Standard Plate "Dilution".
2. Aliquot approximately 2 mL of ProteinSEQ™ Diluent into a 25-mL reagent reservoir.
3. Dispense 200 µL ProteinSEQ™ Diluent to wells A1 through H1 of the labeled plate. Use a multi-channel pipette.



4. Add 50 µL diluted Protein A Standard (40 ng/mL; D4 from "Pre-dilute the Protein A standard" on page 1) to the A1 plate well, then pipet up and down 5 times to mix.



5. Serially dilute the standard from A1 to G1 by transferring 50 µL. Use a new pipette tip for each transfer. After each transfer, pipet up and down 5 times to mix.

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Prepare samples

Adjust sample pH and/or concentration

For best results, adjust the sample pH, IgG concentration, and buffer concentration according to the table:

Sample type	Recommendation
pH < 6	Adjust the pH to > 6 with 0.5M Sodium Phosphate buffer pH 7
pH > 8	Adjust the pH to <8 with 0.5M Sodium Phosphate buffer pH 7
IgG > 25 mg/mL	Dilute to < 25 mg/mL with ProteinSEQ™ Diluent
Carboxylate buffer (Citrate)	Dilute to < 50 mM with ProteinSEQ™ Diluent

IMPORTANT! Do not dilute samples with 2X Protein A Sample Prep Reagent; the final concentration of the 2X Protein A Sample Prep Reagent must be 1X. If necessary to dilute samples, use the ProteinSEQ™ Diluent.

Treat samples with 2X Protein A Sample Prep Reagent

1. In a 1.5-mL screw cap or safe-lock tube, add 150 µL of sample to 150 µL of 2X Protein A Sample Prep Reagent.
2. Vortex for 5–10 seconds, then quick spin to collect the contents at the bottom of the tube.
3. Place the sample tubes in a heat block, cover the tubes with aluminum foil, then incubate the tubes at 100°C for 10 minutes.

CAUTION! Adding water to heat blocks may create an electrical hazard. Refer to the manufacturer's guidelines for use of water in heat blocks.

4. Remove the tubes from the heat block and cool at room temperature for 5 minutes.
5. Vortex the tubes on a bench-top vortexer for 3 seconds at maximum speed.
6. Centrifuge the tubes for 5 minutes at 16,000 × g (~14,000 rpm).
7. Transfer the entire volume of supernatant to an empty well in the Dilution plate that contains the serially diluted standards .

Prepare plates for the MagMAX™ Express-96 run

Label plates

1. Label 8 plates:

No. of plates	Plate type		Cat. no.	Label(s)
6	MagMAX™ Express-96 Skirted Low Profile Plates		4472783	Capture Probes Wash 1 Wash 2 Wash 3 Wash 4
1	MicroAmp™ Optical 96-Well Reaction Plate with Barcode (0.2 mL)		4306737	qPCR
1	MagMAX™ Express-96 Standard Plate (200 µL)		4388475	Comb

2. Insert the plate labelled "qPCR" into a Standard PCR Plate Adapter (request from your local sales or service representative).
3. Place a MagMAX™ Express PCR Head Tip Comb (Cat. no. 4472784) in the plate labelled "Comb".

Prepare wash plates

Dispense 100 µL Wash Buffer into each well of the 4 wash plates with a multi-channel pipette.

Prepare qPCR plate

Dispense 25 µL of ProteinSEQ™ Elution Buffer into each well of the qPCR plate.

Prepare probes plate

1. Add the assay probe reagents to a 15-mL tube in the order shown in the table. Scale the volumes as needed for the number of reactions. Vortex and keep the 15-mL tube on ice.

Reagent	Cap color	Volume[1]		
		1 rxn	48 rxn	96 rxn
ProteinSEQ™ Diluent	Clear	56.3 µL	2700 µL	5400 µL
Protein A 5′ Probe	Grey	3.1 µL	150 µL	300 µL
Protein A 3′ Probe	Yellow	3.1 µL	150 µL	300 µL
Total		62.5 µL	3000 µL	6000 µL

[1] Includes 25% overage.

2. Invert the assay probe mix tube several times to mix, transfer to a reagent reservoir, then dispense 50 µL assay probe mix into each well of the Probes plate with a multi-channel pipette.

Prepare the capture plate

1. In a 15-mL conical tube, mix the Protein A Capture Beads and the ProteinSEQ™ Diluent using the volumes from the table:

Reagent	Cap color	Volume[1]		
		1 rxn	48 rxn	96 rxn
ProteinSEQ™ Diluent	Clear	21.3 µL	1020 µL	2040 µL
Protein A Capture Beads	Clear	3.8 µL	180 µL	360 µL
Total		25.1 µL	1200 µL	2400 µL

[1] Includes 25% overage.

2. Immediately dispense 20 µL of diluted Protein A Capture Beads into each well of the Capture plate. Use a multi-channel pipette.
3. Transfer 50 µL of each standard and sample from the Dilution plate to the capture plate in triplicate. Use a multi-channel pipette.

   The final volume in the capture plate is 70 µL per well.

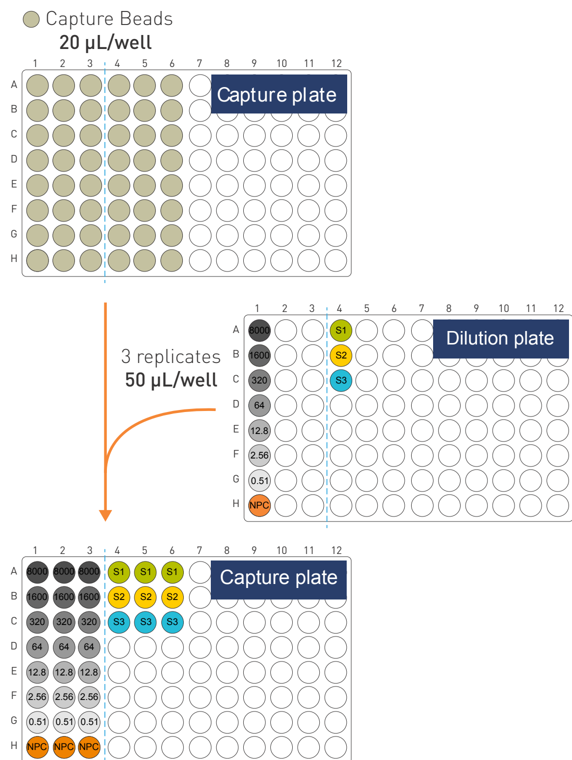

Figure 1 Transfer standards and samples to the capture plate

Run plates in the MagMAX™ Express-96 Magnetic Particle Processor

1. Turn on the MagMAX™ Express-96 instrument and select the **Protein A** program from the screen.
2. Press **START** to initiate plate loading. Follow the prompts on the display screen to load each plate onto the MagMAX™ Express-96 turntable, starting with "Comb" (see Figure 2). Slide each plate into the plate hold-down (if present).

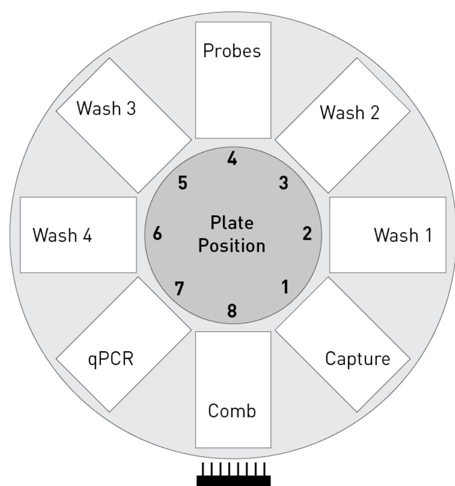

Figure 2 Plate positions in the MagMAX™ Express-96 turntable

3. Load the last (Capture) plate, then press **START** to begin the run.

   The run requires ~2 hours. When the run is complete, the screen displays "Proceed to qPCR".
4. When the MagMAX™ Express-96 program is complete, carefully remove the qPCR plate. Discard the Capture, Wash, and Probes plates.

Proceed immediately to "Run qPCR reaction" on page 3.

Run qPCR reaction

1. Prepare the Ligation/qPCR mix in a 15-mL tube, then briefly vortex to mix.

   Table 3 Reagent volumes for Standard (**non-FAST**) PCR plates

Reagent	Cap color	Volumes[1]		
		1 rxn	48 rxn	96 rxn
Fast Master Mix, 2X	Clear	32.5 µL	1560 µL	3120 µL
ProteinSEQ™ Ligation and Assay Mix	Green	3.25 µL	156 µL	312 µL
ProteinSEQ™ Ligase	Orange	0.26 µL	12.5 µL	25 µL
Total		36.01 µL	1728.5 µL	3457 µL

   [1] Includes 30% overage; volumes for 48 and 96 reactions are rounded to nearest tenth.
2. Transfer 25 µL of the Ligation/qPCR mix to each bead-containing well of the qPCR plate that was prepared on the MagMAX™ Express-96 instrument.
3. Seal the qPCR plate with an optical film, centrifuge for 3 seconds at 500 rpm, then load the plate on a 7500 Real-Time PCR System (or equivalent).
4. Set up the run using AccuSEQ™ system software (or equivalent, for example, SDS 1.4 software) as follows:
   -

Stage	Temperature	Time
Hold	37°C	10 minutes
Hold	95°C	20 seconds
40 cycles	95°C	3 seconds
	60°C	30 seconds

   - Set the Protein A standards and sample wells to a volume of **50 µL** with detection dye set to **FAM** and the quencher set to **none**.
5. Start the run.
6. After the run completes, dispose of the qPCR plate. Do not remove the optical film from the qPCR plate; removing the film introduces amplicon contamination into the local environment.

Perform data analysis

Perform data analysis with AccuSEQ™ software v2.0 or later

1. In the AccuSEQ™ software, select autobaseline **on** and set the threshold manually to **0.2**.
2. Use the AccuSEQ™ software to fit standards to a curve using a non-linear method and obtain interpolated values for the unknowns.
   - 4PL is commonly used for symmetric curves with asymptotes for both the lower and upper Protein A concentrations.
   - 5PL is commonly used if the curve is asymmetric or if either the lower or upper asymptote is not present.
   - Apply 1/Y or 1/Y^2 weighting according to your criteria.
3. Export the data to a Microsoft™ Excel™ spreadsheet for custom statistical analysis.

4. Evaluate the dynamic range using %CV and the quality of the curve fit.
   - R^2 is appropriate for judging linear fits but it is not an appropriate metric for evaluating the quality of a non-linear fit.
   - Common acceptance criteria for non-linear curve fits are back-calculation values of 80–120% throughout the curve and 75–125% at the LLOQ.
   - Common acceptance criteria for precision are %CV ≤20% throughout the curve and≤25% at the LLOQ.
5. Obtain the final concentration for each sample by correcting for sample dilution and spike concentrations, if used.

Perform data analysis without AccuSEQ™ software

1. Select autobaseline **on** and set the C_t threshold manually to **0.2**. Determine the C_t values.
2. Export the raw data from the qPCR software to a Microsoft™ Excel™ spreadsheet, then export from Microsoft™ Excel™ to your fitting program of choice. Transform the values to logarithmic values.

   Note: If you use GraphPad™, the Protein A Master Template (a Microsoft™ Excel™ template available from your local sales or service representative) facilitates this process.
3. Fit standards to a curve using a non-linear method and obtain interpolated values for the unknowns.
   - 4PL is commonly used for symmetric curves with asymptotes for both the lower and upper Protein A concentrations.
   - 5PL is commonly used if the curve is asymmetric or if either the lower or upper asymptote is not present.
   - Apply 1/Y or 1/Y^2 weighting according to your criteria.
4. Transform concentration values from logarithmic to linear values.
5. Evaluate the dynamic range using %CV and the quality of the curve fit.
   - R^2 is appropriate for judging linear fits but it is not an appropriate metric for evaluating the quality of a non-linear fit.
   - Common acceptance criteria for non-linear curve fits are back-calculation values of 80–120% throughout the curve and 75–125% at the LLOQ.
   - Common acceptance criteria for precision are %CV ≤20% throughout the curve and ≤25% at the LLOQ.
6. Obtain the final concentration for each sample by correcting for sample dilution and spike concentrations, if used.

Limited product warranty

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