F(ab´)$_2$ Micro Preparation Kit

BIOK2023

F(ab´)2 Micro Preparation Kit, contains sufficient reagents to generate and purify F(ab´)2 fragments from up to ten 0.125mL samples containing 25-250µg of IgG

Kit Contents:

Immobilized Pepsin	0.5mL*1
Microcentrifuge Tubes	1.5mL*10
Protein A Column	0.2mL*2
Phosphate Buffered Saline	1 pack for 1L
IgG Elution Buffer pH 2.8	30mL*1
Microcentrifuge Tubes	2.0mL*30
F(ab´)2 Digestion Buffer pH 4.4	30mL*1
Desalting Columns	0.5mL*10

Storage: Upon receipt store kit at 4-8°C. Kit is shipped at ambient temperature

The F(ab´)2 Preparation Kit enables efficient generation of F(ab´)2 from IgG. This kit uses Immobilized Pepsin, a nonspecific endopeptidase that is active only at acid pH and irreversibly denatured at neutral or alkaline pH. Pepsin digestion typically produces a F(ab´)2 fragment (~110kDa by SDS-PAGE under non-reducing conditions) and numerous small peptides of the Fc portion (Figure 1). The resulting F(ab´)2 fragment is composed of a pair of Fab´ units connected by two disulfide bonds. The Fc fragment is extensively degraded and can be separated from F(ab´)2 by dialysis, gel filtration or ion exchange chromatography.

This kit contains the necessary components for F(ab´)2 generation and subsequent purification. Immobilized Pepsin is advantageous because the digestion can be immediately stopped by simply removing the resin from the antibody digest solution. The included Spin Columns allow easy manipulation of the resin and maximum F(ab´)2 recovery. The prepacked, immobilized Protein A Column binds the large Fc fragments and undigested IgG, allowing the F(ab´)2 fragments to pass through the column for efficient purification. This complete kit makes F(ab´)2 generation and purification simple, fast and effective.

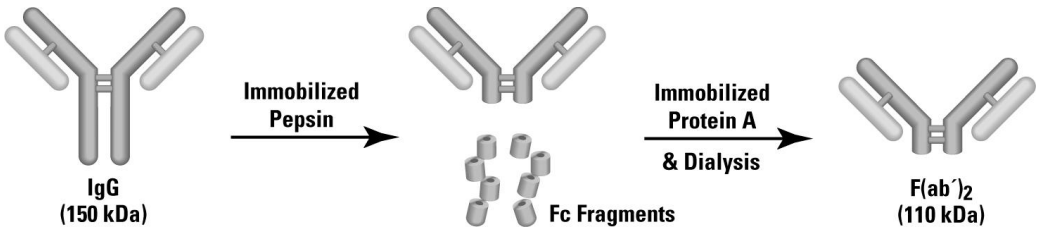

Figure 1. Schematic for preparing F(ab´)$_2$ using Immobilized Pepsin and Protein A

Important Product Information

- The kit components and protocol is for 125µL samples containing 25-250µg of IgG per sample.
- Proper sample preparation is essential for successful fragment generation using this kit. If the IgG sample

contains a carrier protein such as BSA, removing it before performing the buffer exchange.

- For best results, use rabbit, human or mouse IgG. Fragmentation of IgG from other species may require optimization. For purification, the IgG species must be able to bind to Protein A.
- Digestion effectiveness will vary depending on antibody preparation and source (rate and completeness of digestion: rabbit > human > mouse ≥ goat). Digestion times in the protocol result in > 90% digestion of IgG using serum purified by Protein A or G affinity chromatography. Digestion over 3 hours is not recommended.

Additional Materials Required

- Incubator capable of maintaining 37°C
- Microcentrifuge capable of 5000 × *g*
- Variable speed centrifuge
- 1.5mL microcentrifuge tubes
- End-over-end mixer or tabletop rocker

Material Preparation

Phosphate-buffered Saline (PBS): Dissolve contents of a package in 1L of ultrapure water. For long-term storage, add 0.05% sodium azide and store at 4°C.

Procedure for Generating and Purifying F(ab´)2 Fragments

A. Immobilized Pepsin Equilibration

1. Gently swirl the Immobilized Pepsin vial to obtain an even suspension. Seat the spin column frit with an inverted 200µL pipette tip.
2. Tplace 65µL of the 50% slurry (i.e., 32.5µL of settled resin) into the 1.5mL Microcentrifuge Tubes at 5000 × *g* for 1 minute and discard buffer.
3. Wash resin with 130µL of Digestion Buffer. Centrifuge column at 5000 × *g* for 1 minute and discard buffer.

B. IgG Sample Preparation

1. Desalting Column, Centrifuge column at 1500 × *g* for 1 minute to remove storage solution. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps.

   Note: Resin will appear compacted after centrifugation.
2. Add 300µL of Digestion Buffer to column. Centrifuge at 1500 × *g* for 1 minute to remove buffer. Repeat this step three additional times, discarding buffer from the collection tube.
3. Place column in a new Microcentrifuge Tubes, remove cap and slowly apply 125µL of sample to the center of the compacted resin bed.
4. Centrifuge at 1500 × *g* for 2 minutes to collect the sample. Discard the column after use.
5. If IgG sample is 0.2-2mg/mL (i.e., 25-250µg), no further preparation is necessary. If sample volume is less than

125µL, add Digestion Buffer to a final volume of 125µL.

C. Fragment Generation

1. Add 125µL of the prepared IgG sample to the Microcentrifuge Tubes containing the equilibrated Immobilized Pepsin (Section A). Briefly vortex to mix.
2. Incubate digestion reaction for 2 hours for rabbit or human IgG or 3 hours for mouse IgG with an end-over-end mixer or tabletop rocker at 37°C. Maintain constant mixing of resin during incubation.
3. Centrifuge at 5000 × *g* for 1 minute to separate digest from the Immobilized Pepsin.
4. Wash resin with 130µL of PBS. centrifuge at 5000 × *g* for 1 minute. Repeat this step once.
5. Add both wash fractions to the digested antibody. Total sample volume should be 385µL. Discard the Immobilized Pepsin.

Note: For best results, evaluate the digest and wash fraction via SDS-PAGE to assess digestion completion. Protein A purification is only required to remove undigested IgG. $F(ab')_2$ and degraded Fc do not bind to Protein A. The resulting $F(ab')_2$ in non-reducing SDS-PAGE derived from human and mouse IgG will migrate with an apparent molecular weight of ~110kDa. Rabbit $F(ab')_2$ will migrate with a lower apparent molecular weight of ~88kDa.

D. F(ab´)2 Purification

1. Equilibrate the Protein A Plus Spin Column, PBS and IgG Elution Buffer to room temperature. Set centrifuge to 1000 × *g*.
2. Place column in a collection tube and centrifuge for 1 minute to remove storage solution (contains 0.02% sodium azide). Discard the flow-through.
3. To equilibrate column, add 400µL of PBS and briefly mix. Centrifuge for 1 minute and discard the flow-through. Repeat this step once.
4. Apply 25-500µL sample to column and cap the top tightly. Resuspend the resin and sample by inversion. Incubate at room temperature with end-over-end mixing for 2 minutes.
5. Place column in a new collection tube and centrifuge for 1 minute. Save the flow-through as this fraction contains $F(ab')_2$ and Fc fragments that are too small to bind to Protein A.
6. For optimal recovery, wash column with 200µL of PBS. Centrifuge for 1 minute and collect flow-through. Repeat and combine wash fractions with the $F(ab')_2$ fraction from Step 5.
7. Measure protein concentration using the BCA Protein Assay or by measuring the absorbance at 280nm. Use an estimated extinction coefficient of 1.4. Assuming complete IgG digestion, $F(ab')_2$ yields may vary from 50 to 70%, depending on the amount of starting antibody and the protein assays used.
8. If desired, perform dialysis (50K MWCO), gel filtration or ion-exchange chromatography to remove the Fc fragments that are too small to bind to Protein A.

E. Regeneration of the Immobilized Protein A Plus Spin Column

1. Apply 400µL of IgG Elution Buffer to the Protein A Plus Spin Column. Centrifuge for 1 minute. Repeat this step two times to obtain three fractions, which will contain undigested IgG. To save the undigested IgG, add

40µL of a neutralization buffer (e.g., 1M phosphate or 1M Tris at pH 8-9) to each elution fraction.

2. Add 400µL of IgG Elution Buffer to the column and centrifuge for 1 minute. Discard flow-through and repeat.
3. Add 400µL of PBS to the column and centrifuge for 1 minute. Discard flow-through and repeat two times.
4. For storage, add 400µL of 0.02% sodium azide in PBS to column. Replace top and bottom caps. Store column upright at 4°C. Columns can be regenerated at least 10 times without significant loss of binding capacity.

F. Troubleshootin

Problem	**Possible Cause**	**Solution**
Low amounts of $F(ab')_2$ produced as determined by non-reducing SDS-PAGE	IgG sample was not in Digestion Buffer	Dialyze or buffer exchange IgG into Digestion Buffer, or decrease the Digestion Buffer pH to 3-4.3 **Note:** Decreasing the pH might increase the $F(ab')_2$ amount produced but can reduce its immunoreactivity]
	Sample loading buffer contains reducing reagent	Use SDS loading buffer that does not contain β-mercaptoethanol, DTT or TCEP
	Resin was not equilibrated in Digestion Buffer before adding IgG	Wash resin with 0.5mL of Digestion Buffer before adding IgG sample
	Sample is goat or mouse IgG_1	Reduce IgG concentration and increase digestion time to 8 hours
	Some mouse IgG_1 are resistant to pepsin cleavage[1]	Use the IgG_1 Fab and $F(ab')_2$ Preparation kit
	Sample contains protein other than IgG (e.g., BSA), which can increase digestion time	Remove BSA with the Antibody Clean-up Kit
$F(ab')_2$ has low immunoreactivity	Sample digested for too long	Reduce digestion time; do not exceed 8 hours
	The low pH of Digestion Buffer decreased $F(ab')_2$ activity	Use the IgG_1 Fab and $F(ab')_2$ Preparation Kit
Low $F(ab')_2$ recovery	Incomplete washing of the pepsin resin	Two 130µL washes of PBS are required for maximum recovery
A portion of undigested IgG does not bind to Protein A	Sample is goat or mouse IgG_1	Goat IgG binds weakly to Protein A, so try an alternative purification method such as ion-exchange
		Dilute sample in Protein A Binding Buffer before adding to the Protein A Plus Spin Column