INSTRUCTIONS

Pierce® Fab Preparation Kit

44985

2088.2

Number	Description
44985	**Pierce Fab Preparation Kit,** contains sufficient reagents to generate and purify Fab fragments from ten 0.5 ml samples containing 0.25-4 mg IgG

Kit Contents:

Immobilized Papain, 1.25 ml settled resin, contains 250 µg (7 BAEE units) of papain per milliliter of settled resin; support is 6% crosslinked beaded agarose supplied as a 50% slurry in 50% glycerol, 0.1 M sodium acetate, pH 4.4; contains 0.05% sodium azide as a preservative

Cysteine•HCl•H_2O, 1 g, MW 175.63

Fab Digestion Buffer, 120 ml, pH 10.0

NAb™ Protein A Spin Column, 1 ml, 1 each; binding capacity: ≥ 34 mg of human IgG per column

BupH™ Phosphate Buffered Saline, 2 packs, makes 1 L of 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2

IgG Elution Buffer, 120 ml, pH 2.8, contains primary amine

Spin Columns, 10 each, 0.8 ml spin columns with top caps and bottom plugs

Microcentrifuge Tubes, 30 each, 2 ml spin column collection tubes

Zeba™ Desalt Spin Columns, 2 ml, 10 each, for 200-700 µl samples

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

Introduction

The Thermo Scientific Pierce Fab Preparation Kit enables efficient Fab generation from IgG. This kit uses papain, a nonspecific thiol-endopeptidase, immobilized on agarose resin. Immobilized enzyme is advantageous because digestion can be immediately stopped by simply removing the IgG solution from the resin, resulting in a digest that is enzyme-free. Digestion by papain produces 50 kDa Fab and Fc fragments (Figure 1).

This complete kit makes Fab generation and purification simple, fast and effective. The kit includes spin columns for easy manipulation of the enzyme resin. The prepacked immobilized Protein A binds the Fc fragments and undigested IgG, allowing the Fab fragments to pass through the spin column for maximum recovery. The Zeba Desalt Spin Columns contain an exclusive high-performance resin that offers exceptional desalting, ensuring that the IgG sample is in the optimal buffer for efficient digestion.

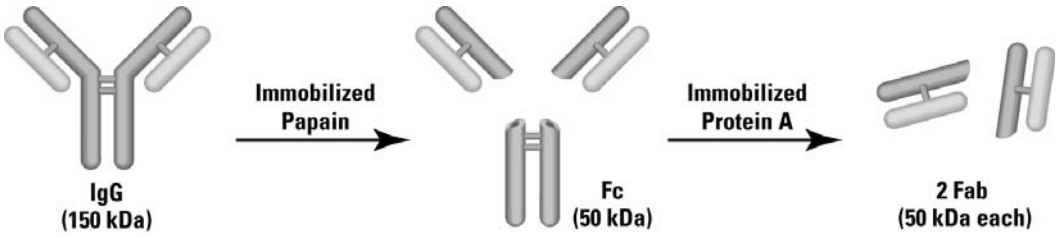

Figure 1. Schematic for preparing and purifying Fab using Immobilized Papain and Protein A.

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Important Product Information

- These instructions are optimized for rabbit, human and mouse IgG (250 µg-4 mg per sample). Fragmentation of IgG from other species might require optimization. For purification, the IgG species must be able to bind to Protein A (see Appendix Section C for Tech Tip #34). For mouse IgG_1, use the Pierce IgG_1 Fab and $F(ab')_2$ Preparation Kit (Product No. 44980).
- The kit components and protocol are for 0.5 ml samples containing 0.25-4 mg IgG. For 25-250 µg samples use the Pierce Fab Micro Preparation Kit (Product No. 44685).
- Proper sample preparation is essential for successful fragment generation using this kit. If the IgG sample contains a carrier protein such as BSA, use the Pierce Antibody Clean-up Kit (Product No. 44600) to remove it before performing the buffer exchange (Section B).

Additional Materials Required

- Incubator capable of maintaining 37°C
- Microcentrifuge capable of 5,000 × *g*
- Variable speed centrifuge
- 15 ml conical collection tubes
- End-over-end mixer or tabletop rocker

Material Preparation

Digestion Buffer	Dissolve 35 mg cysteine•HCl in 10 ml of the supplied Fab Digestion Buffer (pH 10). After adding the cysteine•HCl the pH should be ~7.0. **Note:** Cysteine readily oxidizes to cystine; therefore, prepare this buffer on the same day of use.
Phosphate-buffered Saline (PBS)	Dissolve contents of a package in 500 ml of ultrapure water. For long-term storage, add 0.05% sodium azide and store at 4°C.

Procedure for Fab Generation and Purification

A. Immobilized Papain Equilibration

1. Gently swirl the Immobilized Papain vial to obtain an even suspension. Seat the spin-column frit with an inverted 200 µl pipette tip.
2. Using a wide-bore or cut pipette tip, place 0.25 ml of the 50% slurry (i.e., 0.125 ml of settled resin) into a 0.8 ml spin column. Cap column and place into a microcentrifuge tube. Centrifuge column at 5,000 × *g* for 1 minute and discard buffer.
3. Wash resin with 0.5 ml Digestion Buffer. Centrifuge column at 5,000 × *g* for 1 minute and discard buffer. Cap bottom of spin column with supplied rubber cap.

B. IgG Sample Preparation

1. Twist off the bottom closure of a Zeba Desalt Spin Column and loosen cap. Place column in a 15 ml collection tube.
2. Centrifuge column at 1,000 × *g* for 2 minutes 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.
3. Add 1 ml of Digestion Buffer to column. Centrifuge at 1,000 × *g* for 2 minutes to remove buffer. Repeat this step three additional times, discarding buffer from the collection tube.
4. Place column in a new collection tube, remove cap and slowly apply 0.5 ml sample to the center of the compacted resin bed.

5. Replace cap and centrifuge at 1,000 × *g* for 2 minutes to collect the sample. Discard the column after use.
6. If IgG sample is 0.5-8 mg/ml (i.e., 250 µg to 4 mg), no further preparation is necessary. If sample volume is less than 0.5 ml, add Digestion Buffer to a final volume of 0.5 ml.

C. Generation of Fragments

1. Add 0.5 ml of the prepared IgG sample to the spin column tube containing the equilibrated Immobilized Papain. Place top cap and bottom plug on spin column.
2. Incubate the digestion reaction for the appropriate time (see Appendix A) with an end-over-end mixer or a tabletop rocker at 37°C. Maintain constant mixing of resin during incubation.
3. Remove bottom cap and place spin column into a microcentrifuge tube. Centrifuge column at 5,000 × *g* for 1 minute to separate digest from the Immobilized Papain.
4. Wash resin with 0.5 ml PBS. Place spin column into a microcentrifuge tube. Centrifuge column at 5,000 × *g* for 1 minute.
5. Add the wash fraction to the digested antibody from Step 3. Total sample volume should be 1.0 ml. Discard the used Immobilized Papain.

   Note: To assess digestion completion, evaluate the digest and wash fraction via SDS-PAGE. The separated digest and wash fraction contains cysteine. Boiling samples in non-reducing SDS-PAGE loading buffer will reduce the sample. To avoid reducing the 50 kDa Fab fragment on SDS-PAGE, do not boil the samples. See representative gel in Appendix B.

D. Fab Purification

1. Equilibrate the NAb Protein A Column, PBS and IgG Elution Buffer to room temperature. Set centrifuge to 1,000 × *g*.
2. Loosen top cap on spin column and snap off bottom closure. Place column in a 15 ml collection tube and centrifuge for 1 minute to remove storage solution (contains 0.02% sodium azide). Discard the flow-through.
3. Equilibrate column by adding 2 ml of PBS, centrifuge for 1 minute and discard the flow-through. Repeat this step once.
4. Cap bottom of column with the included rubber cap. Apply sample to column and tightly cap top. Resuspend the resin and sample by inversion. Incubate at room temperature with end-over-end mixing for 10 minutes.
5. Loosen top cap and remove bottom cap. Place column in a new 15 ml collection tube and centrifuge for 1 minute. Save the flow-through as this fraction contains Fab fragments.
6. For optimal recovery, wash column with 1 ml of PBS. Centrifuge for 1 minute and collect the flow-through. Repeat and combine wash fractions with the Fab fraction from Step 5.
7. Apply 1 ml of IgG Elution Buffer to the Protein A spin column and centrifuge for 1 minute. Repeat this step two times to obtain three fractions, which will contain undigested IgG and Fc fragments. To save the undigested IgG or Fc fragments, add 100 µl of a neutralization buffer (e.g., 1 M phosphate or 1 M Tris at pH 8-9) to each of the elution fractions.
8. Measure protein concentration by absorbance at 280 nm. Use an estimated extinction coefficient of 1.4. Assuming complete IgG digestion, Fab yields may vary from 50 to 65%, depending on the amount of starting antibody and the protein assays used. Protein concentration may also be measured using the Reducing Agent Compatible BCA Protein Assay (Product No. 23252); however, the sample must contain less than 2.5 mM cysteine. The undiluted digest and Protein A fraction contains approximately 5 mM cysteine.

E. Regeneration of the Immobilized Protein A Column

1. Add 3 ml of IgG Elution Buffer and centrifuge for 1 minute. Repeat and discard flow-through.
2. Add 3 ml of PBS to the column, centrifuge for 1 minute and discard the flow-through. Repeat three times.
3. For storage, add 3 ml 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.

Troubleshooting

Problem	Possible Cause	Solution
Low amounts of Fab (50 kDa) produced as visualized by non-reducing SDS-PAGE	The IgG sample was not properly prepared	Dialyze or buffer-exchange IgG into the Digestion Buffer
	Cysteine in the Digestion Buffer oxidized to cystine	Prepare Digestion Buffer with cysteine on the same day of usage
	Sample loading buffer contains reducing reagent	Use SDS loading buffer that does not contain β-mercaptoethanol, DTT or TCEP
	Digested material contains cysteine	Desalt before SDS-PAGE
	Resin not equilibrated in Digestion Buffer	Wash resin with 0.5 ml of Digestion Buffer before adding IgG sample
	Sample contains protein other than IgG (e.g., BSA), which can increase digestion time	Purify the antibody sample with the Pierce Antibody Clean-up Kit
Fab has low immunoreactivity	Sample was digested for too long	Reduce digestion time and do not exceed 20 hours or try using the Pierce $F(ab')_2$ Preparation Kit (Product No. 44988)
A portion of undigested IgG or Fc does not bind to Protein A	Sample is goat IgG	Try an alternative purification method such as ion-exchange chromatography
	Sample is mouse IgG_1	Dilute mouse IgG_1 sample in Pierce Protein A Binding Buffer (Product No. 21001) before adding to the Protein A column

Appendix

A. Recommended Digestion Times

This kit is for digesting ten 0.5 ml samples of rabbit, human or mouse IgG at 0.5-8 mg/ml. Digestion effectiveness will vary depending on antibody preparation and source (rate and completeness of digestion: mouse> rabbit > human). Digestion times listed in Table 1 result in > 90% digestion for mouse and rabbit IgG and > 80% digestion for human IgG. Data was generated using serum purified by Protein A or G affinity chromatography. No significant increase in digestion is obtained for more than 10 hours. Extended digestion times > 20 hours can degrade Fc, which might not bind to Protein A.

Table 1. Recommended digestion times for various species and concentrations of IgG.

Species	IgG (mg/ml)	Digestion Time (hours)
Rabbit	8	8-9
	4	6-7
	1.5	4-5
	0.5	3-4
Human	8	5-6
	4	5-6
	1.5	3-4
	0.5	2-3
Mouse	8	4-5
	4	3-4
	1.5	2-3
	0.5	2-3

B. Protein Gel Interpretation

The Fab and Fc analyzed by non-reducing and non-boiled SDS-PAGE typically migrate with an apparent molecular weight of 45-50 kDa, depending on the antibody species. In reducing SDS-PAGE, Fab fragments migrate near 25 kDa, and Fc fragments migrate at 28-30 kDa (Figure 2). The presence of the Fc at 28-30 kDa confirms digestion of IgG. Boiling the IgG digest before gel loading will result in a reduced sample, because of the cysteine present. Also, an additional band might be present in reduced SDS-PAGE, which is likely the undigested IgG heavy chain (50 kDa).

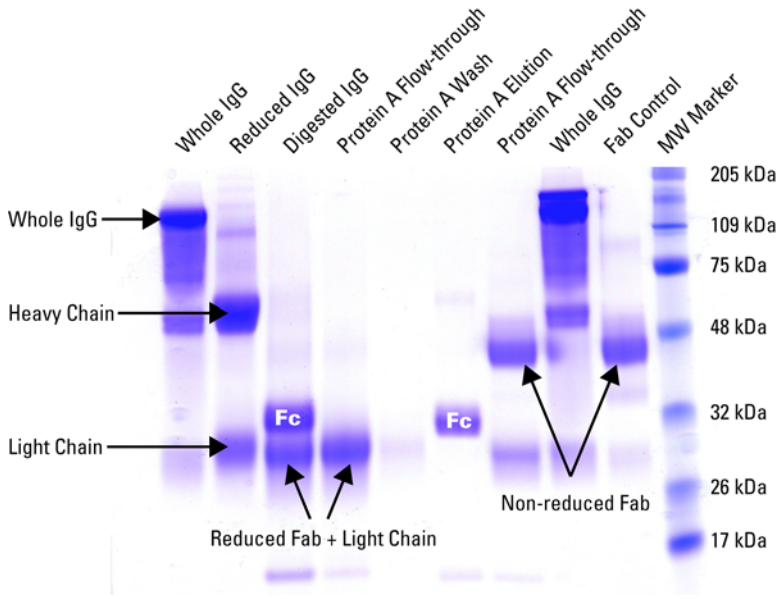

Figure 2. Typical SDS-PAGE (10% Bis-Tris) results of reduced and non-reduced Fab and Fc fragments from rabbit IgG.

C. Additional Information from the Web Site (*www.thermo.com/pierce*)

- Tech Tip #34: Binding characteristics of Protein A, G, A/G and L for immunoglobulins
- Tech Tip #43: Protein stability and storage
- Tech Tip #40: Convert between times gravity (× *g*) and centrifuge rotor speed (RPM)
- Tech Tip #6: Extinction coefficients guide
- Tech Tip #62: Ion exchange chromatography

Related Products

89868	**Pierce Centrifuge Columns, 0.8 ml,** 50 units
89956	**NAb Protein A Spin Columns, 1 ml,** 5 × 1 ml pre-packed columns for centrifuge or gravity-flow
44685	**Pierce Fab Micro Preparation Kit**
44988	**Pierce F(ab´)$_2$ Preparation Kit,** uses Immobilized Pepsin to prepare F(ab´)$_2$ fragments from IgG
44688	**Pierce F(ab´)$_2$ Micro Preparation Kit**
44980	**Pierce IgG$_1$ Fab and F(ab´)$_2$ Preparation Kit**, uses Immobilized Ficin, optimized for mouse IgG$_1$
44680	**Pierce IgG$_1$ Fab and F(ab´)$_2$ Micro Preparation Kit**
23225	**Pierce BCA Protein Assay Kit,** sufficient to perform 500 standard tube assays
23252	**Pierce Microplate BCA Protein Assay Kit – Reducing Reagent Compatible,** sufficient reagents for 1,000 microplate assays
25200-25244	**Precise™ Protein Gels** (see catalog or web site for a complete listing)

References

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Product References

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