INSTRUCTIONS

Pierce™ Fab Preparation Kit

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44985

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

Kit Contents:

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

Cysteine•HCl•H_2O, 1g, MW 175.63

Fab Digestion Buffer, 120mL, pH 10.0

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

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

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

Spin Columns, 10 each, 0.8mL spin columns with 10 top caps and 11 bottom plugs

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

Zeba™ Spin Desalting Columns, 2mL, 10 each, for 200-700µL 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 50kDa 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 Thermo Scientific™ NAb™ Protein A Plus Spin Column binds the Fc fragments and undigested IgG, allowing the Fab fragments to pass through the spin column for maximum recovery. The Thermo Scientific™ Zeba™ Spin Desalting Columns contain an exclusive high-performance resin that offers exceptional desalting, ensuring that the IgG sample is in the optimal buffer for efficient digestion.

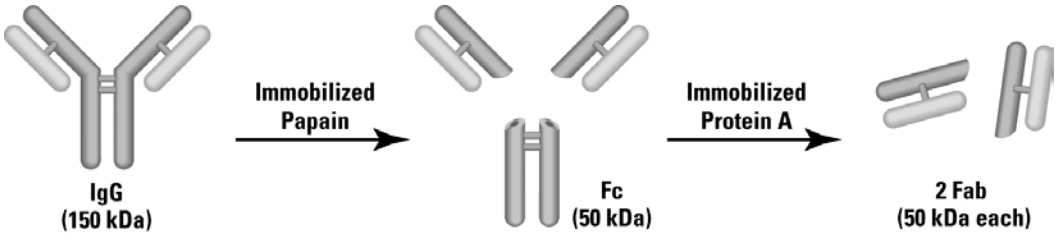

Figure 1. Schematic for preparing and purifying Fab using Immobilized Papain and Protein A.

Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford, IL 61105 USA
(815) 968-0747
(815) 968-7316 fax
thermofisher.com

Important Product Information

- These instructions are optimized for rabbit, human and mouse IgG (250µg-4mg 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 Tech Tip #34 on thermofisher.com). For mouse IgG_1, use the Thermo Scientific Pierce IgG_1 Fab and $F(ab')_2$ Preparation Kit (Product No. 44980).
- The kit components and protocol are for 0.5mL samples containing 0.25-4mg IgG. For 25-250µg samples use the Thermo Scientific 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 Thermo Scientific 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 5000 × *g*
- Variable speed centrifuge
- 15mL conical collection tubes
- End-over-end mixer or tabletop rocker

Material Preparation

Digestion Buffer	Dissolve 35mg cysteine•HCl in 10mL 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 500mL 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 200µL pipette tip.
2. Twist off the bottom tab from a 0.8mL spin column and place into a 2mL microcentrifuge tube. Using a wide-bore or cut pipette tip, place 0.25mL of the 50% slurry (i.e., 0.125mL of settled resin) into the 0.8mL spin column. Centrifuge the column at 5000 × *g* for 1 minute and discard buffer.
3. Wash resin with 0.5mL of Digestion Buffer. Centrifuge column at 5000 × *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 Spin Desalting Column and loosen cap. Place column in a 15mL collection tube.
2. Centrifuge column at 1000 × *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 1mL of Digestion Buffer to column. Centrifuge at 1000 × *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 0.5mL sample to the center of the compacted resin bed.
5. Replace cap and centrifuge at 1000 × *g* for 2 minutes to collect the sample. Discard the column after use.
6. If IgG sample is 0.5-8mg/mL (i.e., 250µg to 4mg), no further preparation is necessary. If sample volume is less than 0.5mL, add Digestion Buffer to a final volume of 0.5mL.

C. Generation of Fragments

1. Add 0.5mL 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 5000 × *g* for 1 minute to separate digest from the Immobilized Papain.
4. Wash resin with 0.5mL PBS. Place spin column into a microcentrifuge tube. Centrifuge column at 5000 × *g* for 1 minute.
5. Add the wash fraction to the digested antibody from Step 3. Total sample volume should be 1.0mL. 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 50kDa 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 Plus Spin Column, PBS and IgG Elution Buffer to room temperature. Set centrifuge to 1000 × *g*.
2. Loosen top cap on spin column and snap off bottom closure. Place column in a 15mL 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 2mL 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 15mL collection tube and centrifuge for 1 minute. Save the flow-through as this fraction contains Fab fragments.
6. For optimal recovery, wash column with 1mL 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 1mL of IgG Elution Buffer to the NAb Protein A Plus 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 100µL of a neutralization buffer (e.g., 1M phosphate or 1M Tris at pH 8-9) to each of the elution fractions.
8. Measure protein concentration by absorbance at 280nm. 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 2.5mM cysteine. The undiluted digest and Protein A fraction contains approximately 5mM cysteine.

E. Regeneration of the Immobilized Protein A Column

1. Add 3mL of IgG Elution Buffer and centrifuge for 1 minute. Repeat and discard flow-through.
2. Add 3mL of PBS to the column, centrifuge for 1 minute and discard the flow-through. Repeat three times.
3. For storage, add 3mL 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 (50kDa) 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 0.5mL 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 Thermo Scientific Pierce Protein A Binding Buffer (Product No. 21001) before adding to the NAb Protein A Plus Spin Column

Appendix

A. Recommended Digestion Times

This kit is for digesting ten 0.5mL samples of rabbit, human or mouse IgG at 0.5-8mg/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 45-50kDa, depending on the antibody species. In reducing SDS-PAGE, Fab fragments migrate near 25kDa, and Fc fragments migrate at 28-30kDa (Figure 2). The presence of the Fc at 28-30kDa 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 (50kDa).

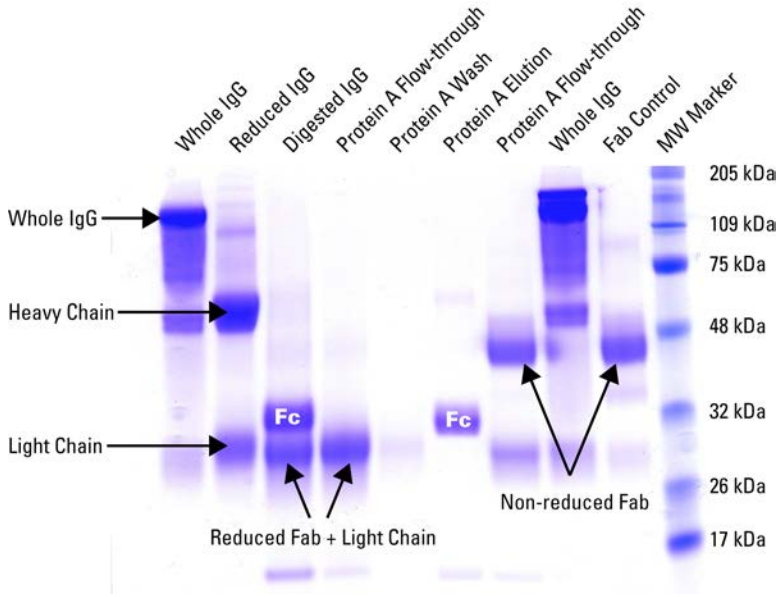

Figure 2. Typical SDS-PAGE (10% Bis-Tris) results of reduced and non-reduced Fab and Fc fragments from rabbit IgG.

C. Additional Information on Our Website (*theromofisher.com*)

- Tech Tip #34: Binding characteristics of Protein A, G, A/G and L
- 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, 0.8mL,** 50 units
89956	**NAb Protein A Plus Spin Columns, 1mL**
44685	**Pierce Fab Micro Preparation Kit**
44988	**Pierce F(ab´)$_2$ Preparation Kit**
44688	**Pierce F(ab´)$_2$ Micro Preparation Kit**
44980	**Pierce IgG$_1$ Fab and F(ab´)$_2$ Preparation Kit**
44680	**Pierce IgG$_1$ Fab and F(ab´)$_2$ Micro Preparation Kit**
23225	**Pierce BCA Protein Assay Kit**
23252	**Pierce Microplate BCA Protein Assay Kit – Reducing Reagent Compatible**
XP04200BOX	**Novex™ Tris-Glycine protein gels** (see thermofisher.com/proteingels for a complete listing)

General References

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Raychaudhuri, G., *et al.* (1985). Human IgG_1 and its Fc fragment bind with different affinities to the Fc receptors on the human U937, HL-60 and ML-1 cell lines. *Mol Immunol* **22(99):**1009-19.

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Rousseaux, J., *et al.* (1983). Optimal conditions for the preparation of Fab and F(ab') fragments from monoclonal IgG of different rat IgG subclasses. *J Immunol Meth* **64:**141-6.

Product References

Chang, P., *et al.* (2004). Poly (ADP-ribose) is required for spindle assembly and structure. *Nature* **432:**645-9.

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Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | thermofisher.com