INSTRUCTIONS

Pierce™ Mouse IgG_1 Fab and $F(ab')_2$ Preparation Kit

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44980

Number	Description
44980	**Pierce Mouse IgG_1 Fab and $F(ab')_2$ Preparation Kit,** contains sufficient reagents to generate and purify Fab and $F(ab')_2$ fragments from ten 0.5mL samples containing 0.25-4mg IgG
	Kit Contents:
	Immobilized Ficin, 2.5mL settled resin, support is 6% crosslinked beaded agarose supplied as 33% slurry
	Cysteine•HCl•H_2O, 1g, MW 175.63
	Mouse IgG_1 Digestion Buffer, 120mL, pH 6.0
	NAb™ Protein A Plus Spin Column, 1mL, 1 each, binding capacity: ≥ 34mg of human IgG per column
	Protein A Binding Buffer, 120mL
	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, 2.0mL 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™ Mouse IgG_1 Fab and $F(ab')_2$ Preparation Kit uses Immobilized Ficin to prepare fragments from mouse IgG_1. Ficin generates $F(ab')_2$ fragments exclusively in the presence of 1-4mM cysteine; Fab fragments are generated in the presence of 25mM cysteine (Figure 1). Fragment generation from other IgG species and isotypes might be possible by modifying the cysteine concentration and other digestion parameters.

Pepsin is commonly used for generating $F(ab')_2$ fragments because the pepsin cleavage site on human IgG contains a Leu 234, which is conserved in most species; however, mouse IgG_1 lacks this residue and others, which possibly contributes to the restricted hinge region and resistance to pepsin cleaveage. Also, the low pH required for pepsin digestion can destroy or damage antibodies. For comparison, mouse IgG_1 monoclonal antibodies were digested with ficin, pepsin, bromelain and elastase. Ficin digestion produced high yields of $F(ab')_2$ fragments with the highest residual antigen-binding activity and immunoreactivity. Affinity constants of ficin-generated $F(ab')_2$ fragments were near those of intact antibody.

This kit contains the necessary components for Fab or $F(ab')_2$ generation of mouse IgG_1 and subsequent purification. Immobilized Ficin enables immediate cessation of the digestion by simply removing the resin from the antibody digest solution. The included Spin Columns allow easy manipulation of the resin and maximum Fab and $F(ab')_2$ recoveries. The prepacked, immobilized Thermo Scientific™ NAb™ Protein A Plus Spin Column and optimized binding buffer binds the intact Fc fragments and undigested IgG, allowing for efficient Fab or $F(ab')_2$ fragments purification. The optimized cysteine concentration produces Fab or $F(ab')_2$ with maximum purity. This complete kit makes Fab and $F(ab')_2$ generation and purification simple, fast and effective.

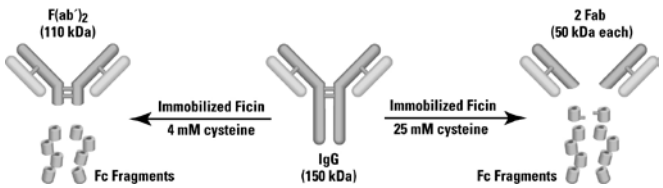

Figure 1. Using ficin with different concentrations of cysteine produces either Fab or $F(ab')_2$ fragments.

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Important Product Information

- These instructions are optimized for mouse IgG_1. Fragmentation of other mouse IgG isotypes or IgG from other species might require optimization.
- The kit components and protocol are configured for 0.5mL samples containing 0.25-4mg of IgG. For 25-250µg samples, use the Pierce Mouse IgG_1 Fab and $F(ab')_2$ Micro Preparation Kit (Product No. 44680).
- 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).
- Protein A Binding Buffer will precipitate in SDS-PAGE Loading Buffer. Dilute sample 1:5 or desalt or dialyze before loading onto a gel.

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
- 0.02% Sodium azide storage solution (in PBS or TBS) for the Immobilized Protein A

Material Preparation

Digestion Buffer	**Fab generation:** Dissolve 43.9mg cysteine•HCl in 10mL of the supplied Mouse IgG_1 Digestion Buffer. After adding the cysteine•HCl the pH should be ~5.6. **$F(ab')_2$ generation:** Dissolve 7mg cysteine•HCl in 10mL of the supplied Mouse IgG_1 Digestion Buffer. After adding the cysteine•HCl the pH should be ~5.9. **Note:** Cysteine readily oxidizes to cystine; therefore, prepare this buffer on the same day of use.

Procedure for Fab or $F(ab')_2$ Generation and Purification

A. Immobilized Ficin Equilibration

1. Gently swirl the Immobilized Ficin vial to obtain an even suspension. Seat the spin-column frit with an inverted 200µL pipette tip.
2. Twist off the bottom tab of a 0.8mL spin column and place into a 2mL microcentrifuge tube. Using a wide-bore or cut pipette tip, place 0.750mL of the 33% slurry (i.e., 0.25mL of settled resin) into the 0.8mL spin column. Centrifuge the column at 5000 × *g* for 1 minute and discard the flow-through.
3. Wash resin with 0.5mL of Digestion Buffer. Centrifuge column at 5000 × *g* for 1 minute and discard the flow-through. Cap bottom of spin column with the 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.

5. Replace cap and centrifuge at 1000 × *g* for 2 minutes to collect the sample. Discard 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 for 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 Ficin. Place top cap and bottom plug on spin column.
2. Incubate digestion reaction 3-5 hours to generate Fab fragments or 24-30 hours to generate F(ab´)$_2$ fragments with 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 2.0mL microcentrifuge tube. Centrifuge column at 5000 × *g* for 1 minute to separate digest from the Immobilized Ficin.
4. Wash resin with 0.5mL Protein A Binding Buffer. Place spin column into a 2mL microcentrifuge tube. Centrifuge column at 5000 × *g* for 1 minute. Repeat this step for a total of three washes.
5. Add the wash fractions to the digested antibody from Step 3. Total sample volume should be 2.0mL. Discard used Immobilized Ficin.

   Note: To assess digestion completion, evaluate the digest and wash fraction via SDS-PAGE. Dilute digest 1:5 before adding to SDS-PAGE loading buffer. Because of the presence of cysteine, boiling samples in non-reducing SDS-PAGE loading buffer will reduce the sample. To avoid reducing the 50kDa Fab fragment or 110kDa F(ab´)$_2$ fragment do not boil the samples. For best interpretation, desalt or dialyze samples before electrophoresis. See representative gels in the Additional Information Section.

D. Fab and F(ab´)$_2$ Purification

1. Equilibrate the NAb Protein A Plus Spin Column, Protein A Binding Buffer and 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 flow-through.
3. Equilibrate column by adding 2mL of Protein A Binding Buffer. Centrifuge for 1 minute and discard the flow-through. Repeat this step once.
4. Cap bottom of column with the included rubber cap. Add the digested antibody sample (Step C.5) to column and tightly cap top. Suspend 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. The flow-through contains Fab or F(ab´)$_2$ fragments.
6. For optimal recovery, wash column with 1mL of Protein A Binding Buffer. Centrifuge for 1 minute and collect flow-through. Repeat and combine wash fractions with the Fab and F(ab´)$_2$ fraction (Step D.5).
7. Apply 1mL of Elution Buffer to the NAb Protein A Plus Spin Column. 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 fragments, add 100µL of a neutralization buffer (e.g., 1M phosphate or 1M Tris at pH 8-9) to each elution fraction.
8. Estimate protein concentration by measuring the absorbance at 280nm. Use an estimated extinction coefficient of 1.4. Alternatively, measure the concentration using the Thermo Scientific Reducing Agent Compatible BCA Protein Assay (Product No. 23252); however, the sample must contain less than 2.5mM cysteine. The combined digest and Protein A fraction might contain up to 2mM cysteine. The Protein A Binding Buffer might also interfere with colorimetric protein assays. For best results, desalt or dialyze the sample and use the Reducing Agent Compatible BCA Protein Assay.

E. Regeneration of the Immobilized Protein A Column

1. Add 3mL of Elution Buffer and centrifuge for 1 minute. Repeat and discard flow-through.
2. Add 3mL of a suitable storage buffer (PBS or TBS with 0.02% azide) to column and centrifuge for 1 minute. Discard flow-through. Repeat three times.
3. Replace top and bottom caps. Store column upright at 4°C. Columns may be regenerated at least 10 times without significant loss of binding capacity.

Troubleshooting

Problem	Possible Cause	Solution
Low amounts of Fab (50kDa) or F(ab´)$_2$ (110kDa) produced as visualized by non-reducing SDS-PAGE	IgG sample was not prepared properly	Buffer exchange IgG into Digestion Buffer
	Sample loading buffer contains reducing reagent	Use SDS loading buffer that does not contain β-mercaptoethanol, DTT or TCEP
	Digested material contains cysteine	Desalt digest before SDS-PAGE
	Sample contains protein other than IgG (e.g., BSA)	Remove BSA with the Pierce Antibody Clean-up Kit
	Some mouse IgG$_1$ clones may generate alternate fragments of different molecular weight[2]	Use the Pierce Fab Preparation Kit (Product No. 44985) or Pierce F(ab´)$_2$ Preparation Kit (Product No. 44988) and dilute mouse IgG$_1$ samples with Protein A Binding Buffer (Product No. 21001) for purification
Fab or F(ab´)$_2$ has low immunoreactivity	Sample digested for too long	Reduce digestion time; do not exceed 8 hours for Fab or 40 hours for F(ab´)$_2$ or try using the Pierce F(ab´)$_2$ or Fab Preparation Kit
Protein A flow-through contains Fab and F(ab´)$_2$	Extended digestion times for F(ab´)$_2$ production might result in the formation of Fab	Use recommended cysteine concentrations and digestion times

Additional Information

A. Gel Interpretation

Fab analyzed by non-reducing and non-boiled SDS-PAGE will migrate with an apparent molecular weight of 45-50kDa, depending on the IgG$_1$ clone, while intact Fc may also migrate at the same molecular weight. F(ab´)$_2$ migrates with an apparent molecular weight of 110 kDa. In reducing SDS-PAGE, Fab, F(ab´)$_2$ and IgG light chain will migrate near 25kDa, Fc fragments migrate at 25-30kDa, and IgG heavy chain at migrate 50kDa (Figure 2). The IgG digest purified by Protein A will be free of Fc fragments and contain Fab or F(ab´)$_2$. Undigested IgG, Fc or Fc-containing fragments will be in the elution fraction (Figure 3).

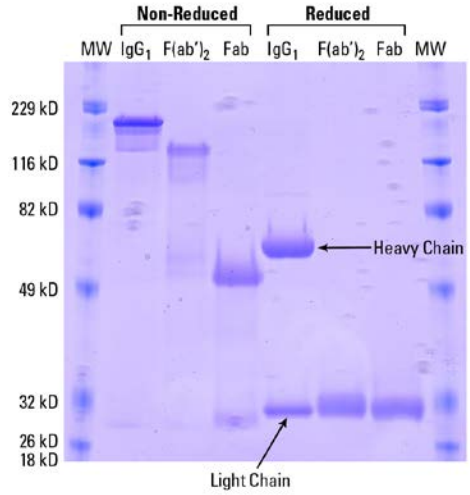

Figure 2. Mouse IgG$_1$ and IgG$_1$ fragments analyzed by non-reducing and reducing SDS-PAGE (10%). Each well was loaded with 4µg of protein. Thermo Scientific™ Imperial™ Protein Stain (Product No. 24615) was used for detection.

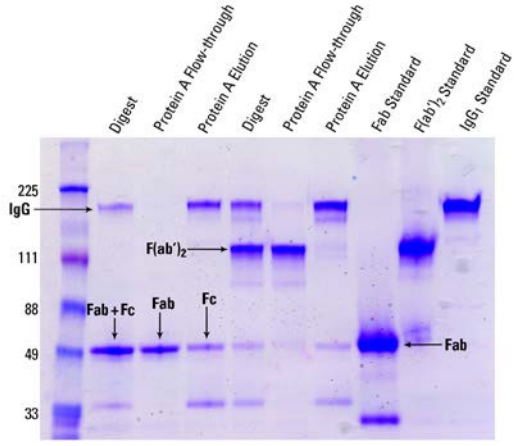

Figure 3. Analysis of various experimental fractions by non-reducing SDS-PAGE (8-16%). Each well was loaded with 2µg of protein. Imperial Protein Stain was used for detection.

B. Additional Information from Our Website (*thermofisher.com*)

- Tech Tip #34: Binding characteristics of Protein A, G, A/G and L
- Tech Tip #43: Protein stability and storage
- 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,** 5/pkg
44985	**Pierce Fab Preparation Kit**
44685	**Pierce Fab Micro Preparation Kit**
44988	**Pierce F(ab´)$_2$ Preparation Kit**
44688	**Pierce F(ab´)$_2$ Micro Preparation Kit**
23252	**Pierce Microplate BCA Protein Assay Kit – Reducing Reagent Compatible**
XP08160BOX	**Novex™ 8-16% Tris-Glycine protein gels** (see thermofisher.com/proteingels for a complete listing)
NW04120BOX	**Bolt™ 4-12% Bis-Tris Plus protein gels** (see thermofisher.com/proteingels for a complete listing)

Cited References

1. Parham, P. (1983). On the fragmentation of monoclonal IgG$_1$, IgG$_{2a}$, and IgG$_{2b}$ from BALB/c mice. *J Immunol* **131:**2895-2902.

General References

Beale, D. (1987). Molecular fragmentations. Some applications in immunology. *Dev Comp Immunol* **11:**287-96.

Boguslawski, S.J., *et al.* (1989). Improved procedure for preparation of F(ab´)$_2$ fragments of mouse IgGs by papain digestion. *J Immunol Meth* **120:**51-56.

Kerbel, R.S. and Elliot, B.E. (1983). Detection of Fc receptors. *Method Enzymol* **93**:113-147.

Kulkami, P.N., *et al.* (1985). Conjugation of methotrexate to IgG antibodies and their F(ab´)$_2$ fragments and the effect of conjugated methotrexate on tumor growth *in vivo*. *Cancer Immunol Immunother***19:**211-14.

Milenic, D.E., *et al.* (1989). Comparison of methods for the generation of immunoreactive fragments of a monoclonal antibody (B72.3) reactive with human carcinomas. *J Immunol Meth* **120**:71-83.

Nisonoff, A., *et al.* (1960). Properties of the major component of a peptic digest of rabbit antibody. *Science* **132:**1770.

Parham, P.L., *et al.* (1982). Monoclonal antibodies: Purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J Immunol Meth* **53:**133-73.

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(9)**:1009-19.

Wilson, K.M., *et al.* (1991). A new enzymatic method to obtain high-yield F(ab´)$_2$ suitable for clinical use from mouse IgG$_1$. *Mol Immunol* **28:**69-77.

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