INSTRUCTIONS

Pierce™ F(ab´)$_2$ Preparation Kit

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44988

Number	Description
44988	**Pierce F(ab´)$_2$ Preparation Kit,** contains sufficient reagents to generate and purify F(ab´)$_2$ fragments from up to 10 0.5mL samples containing 0.25-4mg of IgG

Kit Contents:

Immobilized Pepsin, 1.25mL settled resin, contains 2-3mg (> 6,000 units) of pepsin 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; 0.05% sodium azide

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 columns with 10 caps and 11 bottom plugs

Microcentrifuge Tubes, 30 each, 2.0mL collection tubes

Digestion Buffer, 120mL, 20mM sodium acetate, pH 4.4; 0.05% sodium azide

Zeba™ Spin Desalting Columns, 2mL, 10 each, for 200-700µL samples

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

Introduction

The Thermo Scientific™ Pierce™ 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 Thermo Scientific™ NAb™ Protein A Plus Spin 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.

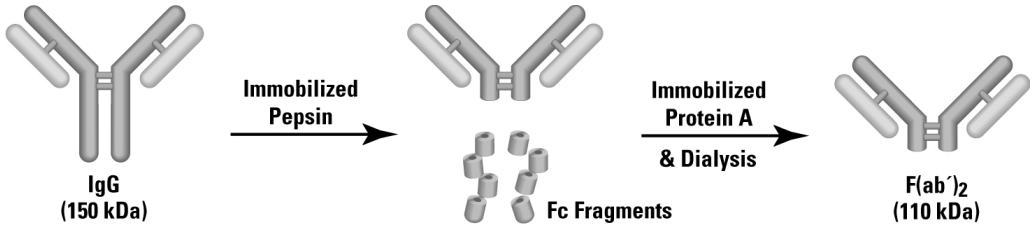

Figure 1. Schematic for preparing F(ab´)$_2$ using Immobilized Pepsin and Protein A.

Important Product Information

- 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).
- 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. For best results with 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 0.5mL samples containing 250µg-4mg of IgG per sample. For 25-250µg samples use the Pierce $F(ab')_2$ Micro Preparation Kit (Product No. 44688).

Additional Materials Required

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

Material Preparation

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 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. Twist off the bottom tab from a 0.8mL spin column and place into a 2.0mL 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 5,000 × *g* for 1 minute and discard buffer.
3. Wash resin with 0.5mL of Digestion Buffer. Centrifuge column at 5,000 × *g* for 1 minute and discard buffer. Cap bottom of spin column with included rubber cap.

B. IgG Sample Preparation

1. Twist off the bottom closure of a Zeba Spin Desalting Column and loosen cap (SAVE bottom closure for later use). Place column in a 15mL 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 1mL 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 0.5mL of 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 0.5-8mg/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. Fragment Generation

1. Add 0.5mL of the prepared IgG sample to the spin column containing the equilibrated Immobilized Pepsin (Section A). Reseal the column by inverting the original snap-off closure, and with a slight twisting motion, press it firmly to the bottom tip of the column, then seal the top cap of the column.
2. Incubate digestion reaction for the appropriate time (see the Appendix) with an end-over-end mixer or a tabletop rocker at 37°C. Maintain constant mixing of resin during incubation.
3. Remove bottom closure and place column into a 2.0mL microcentrifuge tube. Centrifuge column at 5,000 × *g* for 1 minute to separate digest from the Immobilized Pepsin.
4. Wash resin with 0.5mL of PBS. Place column into a tube and centrifuge at 5,000 × *g* for 1 minute. Repeat this step once.
5. Add both wash fractions to the digested antibody. Total sample volume should be 1.5mL. 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 NAb Protein A Plus Column, PBS and IgG Elution Buffer to room temperature. Set centrifuge to 1,000 × *g*.
2. Loosen the top cap on the NAb Protein A Plus Spin Column and snap off the bottom closure (SAVE closure for later uses). Place column in a 15mL collection tube and centrifuge for 1 minute to remove storage solution (contains 0.02% sodium azide). Discard the flowthrough.
3. Equilibrate column by adding 2mL of PBS. Centrifuge for 1 minute and discard the flowthrough. Repeat this step once.
4. Reseal bottom of the column by inverting the original snap-off closure, and with a slight twisting motion, press it firmly to the bottom tip of the column.
5. Apply 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 10 minutes.
6. Loosen top cap and remove bottom cap. Place column in a new 15mL collection tube and centrifuge for 1 minute. Save the flowthrough as this fraction contains $F(ab')_2$ and Fc fragments that are too small to bind to Protein A.
7. For optimal recovery, wash column with 1mLof PBS. Centrifuge for 1 minute and collect flowthrough. Repeat and combine wash fractions with the $F(ab')_2$ fraction from Step 5.
8. Measure protein concentration using the Thermo Scientific™ 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.
9. 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 NAb Protein A Plus Spin Column

1. 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. To save the undigested IgG, add 100µL of a neutralization buffer (e.g., 1M phosphate or 1M Tris at pH 8-9) to each of the elution fractions.
2. Add 3mL of IgG Elution Buffer to the column and centrifuge for 1 minute. Discard flowthrough and repeat.
3. Add 3mL of PBS to the column and centrifuge for 1 minute.
4. For storage, add 3mL of 0.02% sodium azide in PBS to column. Reseal the column by inverting the original snap-off closure, and with a slight twisting motion, press it firmly to the bottom tip of the column, then seal the top cap of the column. 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 $F(ab')_2$ 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 that 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 was goat or mouse IgG_1.	Reduce IgG concentration and increase digestion time to 8 hours.
	Some mouse IgG_1 were resistant to pepsin cleavage[1].	Use the Pierce IgG_1 Fab and $F(ab')_2$ Preparation Kit (Product No. 44980 or 44680).
	Sample contained protein other than IgG (e.g., BSA), which can increase digestion time.	Remove BSA with the Pierce Antibody Clean-up Kit (Product No. 44600).
$F(ab')_2$ 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 Pierce IgG_1 Fab and $F(ab')_2$ Preparation Kit.
Low $F(ab')_2$ recovery	Incomplete washing of the pepsin resin.	Two 500µL washes of PBS are required for maximum recovery.
A portion of undigested IgG does not bind to Protein A	Sample was 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 Pierce Protein A Binding Buffer (Product No. 21001) before adding to the Protein A Column.

Appendix

Recommended Digestion Times

This kit is for digesting 0.5mL of IgG at 0.5-8mg/mL from rabbit, human or mouse. Digestion effectiveness will vary depending on antibody preparation and source (rate and completeness of digestion: rabbit > human > mouse ≥ goat). The times listed in Table 1 result in > 90% digestion of IgG. Data was generated using serum purified by immobilized Protein A or G affinity chromatography. Digestion over 8 hours is not recommended.

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

Species	IgG (mg/mL)	Digestion Time (hours)
Rabbit	8	2
	3.5	1-2
	1.5	0.5
	0.5	0.5
Human	5.0	6-7
	2.5	3-4
	1.0	2-3
	0.5	1-2
Mouse	5.0	6-7
	2.5	2-3
	1.0	0.5-1
	0.5	0.5-1

Related Thermo Scientific Products

90009	**Pierce Strong Cation Exchange Spin Column, Maxi,** 8 spin columns and 16 collection tubes
90011	**Pierce Strong Anion Exchange Spin Column, Maxi,** 8 spin columns and 16 collection tubes
89868	**Pierce Centrifuge Columns, 0.8mL,** 50 units
89956	**NAb Protein A Plus Spin Columns, 1mL**
44688	**Pierce F(ab´)$_2$ Micro Preparation Kit**
44985	**Pierce Fab Preparation Kit,** uses Immobilized Papain to prepare Fab fragments from IgG
44685	**Pierce Fab 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
44600	**Pierce Antibody Clean-up Kit**
XP04200BOX	**Novex™ Tris-Glycine protein gels** (see thermofisher.com/proteingels for a complete listing)

General References

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

Ellerson, J.R., *et al.* (1972). A fragment corresponding to the CH2 region of immunoglobulin G (IgG) with complement fixing activity. *FEBS Letters* **24(3):**318-22.

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

Kulkarni, 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 Immunotherapy* **19:**211-4.

Lamoyi, E. (1986). Preparation of F(ab´)$_2$ Fragments from mouse IgG of various subclasses. *Meth Enzymol* **121:**652-663.

Parham, P., *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.

Rousseaux, J., *et al.* (1980). The differential enzyme sensitivity of rat immunoglobulin G subclasses to papain an pepsin. *Mol Immunol* **17:**469-82.

Rousseaux, J., *et al.* (1983). Optimal condition for the preparation of Fab and F(ab´)$_2$ fragments from monoclonal IgG of different rat IgG subclasses. *J Immunol Meth* **64:**141-6.

Wilson, K.M., *et al.* (1991). Rapid whole blood assay for HIV-1 seropositivity using a Fab-peptide conjugate. *J Immunol Meth* **138:**111-9.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition. The information in this guide is subject to change without notice.

Revision history: Pub. No. MAN0017019 B

Revision	Date	Description
B	31 July 2024	Correcting spin column usage.
A	17 October 2015	New document for Pierce™ F(ab')2 Preparation Kit.