INSTRUCTIONS

Pierce Mouse IgG₁ Fab and F(ab’)₂ Preparation Kit

44980  Pierce Mouse IgG₁ Fab and F(ab’)₂ Preparation Kit, contains sufficient reagents to generate and purify Fab and F(ab’)₂ fragments from ten 0.5 ml samples containing 0.25-4 mg IgG

Kit Contents:

- Immobilized Ficin, 2.5 ml settled resin, support is 6% crosslinked beaded agarose supplied as 33% slurry
- Cysteine•HCl•H₂O, 1 g, MW 175.63
- Mouse IgG₁ Digestion Buffer, 120 ml, pH 6.0
- NAb™ Protein A Spin Column, 1 ml, 1 each, binding capacity: ≥ 34 mg of human IgG per column
- Protein A Binding Buffer, 120 ml
- 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, 2.0 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 Mouse IgG₁ Fab and F(ab’)₂ Preparation Kit uses Immobilized Ficin to prepare fragments from mouse IgG₁. Ficin generates F(ab’)₂ fragments exclusively in the presence of 1-4 mM cysteine; Fab fragments are generated in the presence of 25 mM 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 IgG₁ lacks this residue and others, which possibly contributes to the restricted hinge region and resistance to pepsin cleavage. Also, the low pH required for pepsin digestion can destroy or damage antibodies. For comparison, mouse IgG₁ 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 IgG₁ 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 Protein A 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.
**Important Product Information**

- These instructions are optimized for mouse IgG1. Fragmentation of other mouse IgG isotypes or IgG from other species might require optimization.

- The kit components and protocol are configured for 0.5 ml samples containing 0.25-4 mg of IgG. For 25-250 μg samples, use the Pierce Mouse IgG1 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 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
- 0.02% Sodium azide storage solution (in PBS or TBS) for the Immobilized Protein A

**Material Preparation**

**Digestion Buffer**

**Fab generation:** Dissolve 43.9 mg cysteine•HCl in 10 ml of the supplied Mouse IgG1 Digestion Buffer. After adding the cysteine•HCl the pH should be ~5.6.

**F(ab´)2 generation:** Dissolve 7 mg cysteine•HCl in 10 ml of the supplied Mouse IgG1 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 200 μl pipette tip.

2. Using a wide-bore or cut pipette tip, place 0.750 ml of the 33% slurry (i.e., 0.25 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 the flow-through.

3. Wash resin with 0.5 ml of Digestion Buffer. Centrifuge column at 5,000 × 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 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.

5. Replace cap and centrifuge at 1,000 × g for 2 minutes to collect the sample. Discard 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 for 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 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 2.0 ml microcentrifuge tube. Centrifuge column at 5,000 × g for 1 minute to separate digest from the Immobilized Ficin.

4. Wash resin with 0.5 ml Protein A Binding Buffer. Place spin column into a 2 ml microcentrifuge tube. Centrifuge column at 5,000 × 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 2.0 ml. 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 50 kDa Fab fragment or 110 kDa 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 Column, Protein A Binding Buffer and 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 flow-through.

3. Equilibrate column by adding 2 ml 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 15 ml collection tube and centrifuge for 1 minute. The flow-through contains Fab or F(ab′)₂ fragments.

6. For optimal recovery, wash column with 1 ml 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 1 ml of Elution Buffer to the Protein A 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 100 µl of a neutralization buffer (e.g., 1 M phosphate or 1 M Tris at pH 8-9) to each elution fraction.

8. Estimate protein concentration by measuring the absorbance at 280 nm. 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 2.5 mM cysteine. The combined digest and Protein A fraction might contain up to 2 mM 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 3 ml of Elution Buffer and centrifuge for 1 minute. Repeat and discard flow-through.

2. Add 3 ml 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

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<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution</th>
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<td>IgG sample was not prepared properly</td>
<td></td>
<td>Buffer exchange IgG into Digestion Buffer</td>
</tr>
<tr>
<td>Sample loading buffer contains reducing reagent</td>
<td></td>
<td>Use SDS loading buffer that does not contain β-mercaptoethanol, DTT or TCEP</td>
</tr>
<tr>
<td>Digested material contains cysteine</td>
<td></td>
<td>Desalt digest before SDS-PAGE</td>
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<tr>
<td>Sample contains protein other than IgG (e.g., BSA)</td>
<td></td>
<td>Remove BSA with the Pierce Antibody Clean-up Kit</td>
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<tr>
<td>Some mouse IgG(_1) clones may generate alternate fragments of different molecular weight(^2)</td>
<td></td>
<td>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</td>
</tr>
<tr>
<td>Fab or F(ab’(_2)) has low immunoreactivity</td>
<td>Sample digested for too long</td>
<td>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</td>
</tr>
<tr>
<td>Protein A flow-through contains Fab and F(ab’(_2))</td>
<td>Extended digestion times for F(ab’(_2)) production might result in the formation of Fab</td>
<td>Use recommended cysteine concentrations and digestion times</td>
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### Additional Information

#### A. Gel Interpretation

Fab analyzed by non-reducing and non-boiled SDS-PAGE will migrate with an apparent molecular weight of 45-50 kDa, 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 25 kDa, Fc fragments migrate at 25-30 kDa, and IgG heavy chain at migrate 50 kDa (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 4 μg 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 2 μg of protein. Imperial Protein Stain was used for detection.
B. 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 (x g) and centrifuge rotor speed (RPM)
- Tech Tip #6: Extinction coefficients guide
- Tech Tip #62: Ion exchange chromatography

Related Thermo Scientific Products

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<tr>
<th>Code</th>
<th>Description</th>
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<tr>
<td>89868</td>
<td>Pierce Centrifuge Columns, 0.8 ml, 50 units</td>
</tr>
<tr>
<td>89956</td>
<td>NAab Protein A Spin Columns, 1 ml, 5 x 1 ml pre-packed columns for centrifuge or gravity-flow</td>
</tr>
<tr>
<td>44985</td>
<td>Pierce Fab Preparation Kit</td>
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<tr>
<td>44685</td>
<td>Pierce Fab Micro Preparation Kit</td>
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<tr>
<td>44988</td>
<td>Pierce F(ab')₂ Preparation Kit, uses Immobilized Pepsin to prepare F(ab')₂ fragments from IgG</td>
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<tr>
<td>44688</td>
<td>Pierce F(ab')₂ Micro Preparation Kit</td>
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<tr>
<td>44680</td>
<td>Pierce Mouse IgG₁, Fab and F(ab')₂ Micro Preparation Kit</td>
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<tr>
<td>23252</td>
<td>Pierce Microplate BCA Protein Assay Kit – Reducing Reagent Compatible</td>
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<tr>
<td>25200-25244</td>
<td>Precise™ Protein Gels (see catalog or web site for a complete listing)</td>
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Cited References

References

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