**Introduction**

The ImmunoPure® IgG1 Fab and F(ab')2 Preparation Kit utilizes Immobilized Ficin to prepare Fab and F(ab')2 fragments from mouse IgG1. Ficin activity at pH 6 is slightly greater than the activity at pH 7 or 8. Ficin will generate F(ab')2 fragments in the presence of 1 mM cysteine; and Fab fragments will be generated in the presence of 10 mM cysteine.

Mouse IgG1 has shown significant resistance to pepsin digestion. The pepsin cleavage site on human IgG contains a Leu 234 that is conserved in most species except mouse IgG1. The lack of this residue and others in mouse IgG1 gamma chain may contribute to the restricted hinge region and resulting resistance to pepsin cleavage.1 Also, pepsin digestion takes place at low pH, which can destroy or damage sensitive antibodies. Ficin is a thiol protease that catalyzes a broad range of reactions involving interconversion of carboxylic acids, esters or amides.2 Ficin is purified from fig latex and has a molecular weight of 25,000 and an extinction coefficient of 21 at 280 nm for a 1% solution. Mild reducing agents such as cysteine, sulfide, sulfite and cyanide are activators. EDTA, N-bromosuccinimide, acridine dyes and cysteine are enhancers of ficin. Inhibitors include sulfhydryl blocking agents such as N-ethyl maleimide and iodoacetamide, heavy metal ions, mercuric chloride and carbonyls.

A digestion study of five IgG1 monoclonal antibodies from mouse with pepsin, bromelain, ficin and elastase was performed to determine how to best generate high yields of stable, active F(ab')2 fragments. 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 that of intact IgG.1

**Additional Materials Required**

- 0.02% Sodium azide storage solution for the Immobilized Protein A
- 0.1 M Citric acid, pH 3.0, regeneration buffer for the Immobilized Protein A
- Microconcentrators with 10,000, 30,000 and/or 100,000 molecular weight cut-off
Example Protocol for Digestion of Mouse IgG1

**Note:** Digestion time in the standard protocol has been optimized for 0.5-1 mg/ml of mouse IgG1. Higher concentrations of IgG1 (≤ 10 mg/ml) may be digested with appropriate incubation times determined empirically.

### Material Preparation

**IgG1 Solution for Digestion**

Add 0.5 ml of IgG1 solution to 0.5 ml of ImmunoPure® IgG1 Mild Elution Buffer or buffer exchange IgG1 into ImmunoPure® IgG1 Mild Elution Buffer.

**Note:** If IgG1 was purified using ImmunoPure® Mouse IgG1 Mild Elution Buffer Kit, it is ready for digestion.

**Stock Digestion Buffer**

For the preparation of F(ab')2 fragments, add 4 mg of cysteine•HCl to 2 ml of ImmunoPure® IgG1 Digestion Buffer (10X).

For the preparation of Fab fragments, add 40 mg of cysteine•HCl to 2 ml of ImmunoPure® IgG1 Digestion Buffer (10X).

**Working Digestion Buffer**

Mix 1.5 ml of appropriate Stock Digestion Buffer with 15 ml of ImmunoPure® IgG1 Mild Elution Buffer.

### A. Sample Addition

1. Equilibrate a 2 ml column of Immobilized Ficin with 15 ml of Working Digestion Buffer.
2. Add 100 µl of Stock Digestion Buffer to 1 ml of the prepared IgG1 solution.
3. Apply sample to Immobilized Ficin column (50-100 µl of sample can be set aside for determination of protein recovery).
4. Allow sample to enter gel bed. Apply 200 µl of Working Digestion Buffer.
5. Replace the bottom cap and the top cap sequentially. Digest at 37°C for appropriate incubation time. Fab fragments are generated in 3-5 hours. Preparation of F(ab')2 fragments usually requires 15-20 hours of digestion. These times are optimized for 0.5-1 mg/ml of mouse IgG1.

**Note:** Higher IgG1 concentrations will require longer digestion times (determined empirically) for comparable yields.

### B. Collection of Digest from Immobilized Ficin Column

1. Collect a 4 ml fraction of digest by adding 4 ml of ImmunoPure® Binding Buffer to column.
2. Wash the Immobilized Ficin with 4-6 ml of Binding Buffer.
3. Add 4 ml of Immobilized Ficin Storage Buffer. This regenerates the column for use with the same monoclonal antibody. Allow buffer to flow through the column. Add 1 ml of storage buffer and replace top and bottom caps.
4. Store the column at 4°C. The column may be reused with the same monoclonal antibody.

**Note:** The columns can be reused up to five times with up to 85% retention of activity.

### C. Separation of Fab or F(ab')2 Fragments from Nondigested IgG and Fc Fragments

1. Allow the Immobilized Protein A and ImmunoPure® Binding Buffer to equilibrate to room temperature.
2. Open a Protein A Column (caution: remove top cap first, which avoids air bubbles being drawn into the gel) and decant the storage solution.

**Note:** Storage solution contains 0.02% sodium azide and should be disposed of according to local regulations.

3. Equilibrate a 2.5 ml Immobilized Protein A Column with 12 ml of ImmunoPure® Binding Buffer.
4. Apply the 4 ml of digested sample from Step B1. Collect four 1 ml fractions containing Fab or F(ab')2 fragments.
5. Wash column with 2 ml of Binding Buffer. Collect 1 ml fractions. These fractions may also contain Fab or F(\(\text{ab}'\))\(_2\) fragments. Measure the absorbance at 280 nm, and pool those fractions with peak absorbance measurements.

6. Wash Immobilized Protein A with 4 x 2 ml of Binding Buffer.

7. Elute Fc fragments and nondigested IgG1 with 8 x 1 ml of ImmunoPure\textsuperscript{®} IgG1 Mild Elution Buffer.

8. Regenerate Immobilized Protein A with 8 ml of 0.1 M citric acid, pH 3.0, or 0.1 M glycine, pH 2.8. For storage, wash column with 10 ml of water containing 0.02% sodium azide. When about 3 ml of solution remains above the gel bed, place bottom cap over the column tip. Replace top cap. Columns may be regenerated a maximum of 10 times without significant loss of binding capacity.

Note: Bound and nonbound fractions can be concentrated using appropriate microconcentrators. F(\(\text{ab}'\))\(_2\) and Fab fragments can be concentrated using a microconcentrator with either a 10,000 or 30,000 molecular weight cut-off. To concentrate the nondigested IgG and separate them from Fc, use a microconcentrator with a 100,000 molecular weight cut-off. Approximate molecular weights of the fragments are F(\(\text{ab}'\))\(_2\)-105,000; Fab-50,000 and Fc-50,000. The molecular weight of intact IgG is approximately 150,000.

Note: Protein recovery can be determined by measuring absorbance values at 280 nm or by Coomassie\textsuperscript{®} Protein Assay Reagent Kit (Product No. 23200). Fragment purity can be determined by gel electrophoresis.

Cited References


General References


