INSTRUCTIONS

Pierce® IgM Fragmentation Kit

44887

0341.3

Number	Description
44887	**Pierce IgM Fragmentation Kit,** contains sufficient materials to prepare fragments from 1 ml of 1 mg/ml mouse or human IgM (kit may be used for other species)

Kit Contents:

Immobilized Pepsin, 2 × 2 ml columns

Immobilized Trypsin, 2 × 2 ml columns

2-mercaptoethylamine, 6 mg

Iodoacetamide, 16 mg

IgM Digestion Buffer, 400 ml, contains 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% NaN_3; pH 8.0

IgM F(ab´)$_2$ Digestion Buffer, 200 ml, contains 100 mM sodium acetate, 150 mM NaCl, 0.05% NaN_3; pH 4.5

C100 Concentrator, 7 each

C30 Concentrator, 5 each

Dextran Desalting Column, 5K MWCO, 2 × 5 ml

Storage: Upon receipt store the C100 and C30 Concentrators at room temperature. Store all other kit components at 4°C. Kit is shipped at ambient temperature.

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Introduction

The Thermo Scientific Pierce IgM Fragmentation Kit allows rapid, easy production of fragments from mouse and human IgM. The kit contains the necessary components for producing IgM fragments using trypsin, pepsin and 2-mercaptoethylamine (Figure 1). IgM is first prepared by performing a buffer exchange into an optimized digestion buffer. IgM can then be digested by the preferred method. The C100 and C30 Concentrators allow fragments to be separated and concentrated for downstream applications.

The trypsin and pepsin are supplied immobilized on an agarose support, eliminating the need to separate enzyme from IgM fragments. The IgM is digested as it passes through the pre-packed immobilized enzyme columns. Digestion can be easily regulated by digestion time or flow rate through the column. The enzyme remains bound to the support, ensuring there is no auto-digested enzyme to contaminate the IgM fragments. The immobilized enzymes also offer increased stability against heat

denaturation and autolysis and yield reproducible digests. (See the Additional Information Section for IgM nomenclature, as well as information concerning IgM structure, IgM fragmentation, digestion enzymes, reduction and alkylation.)
Note: Fragmentation of IgM from species other than human and mouse may require optimization (see Table 1 in the Additional Information Section).

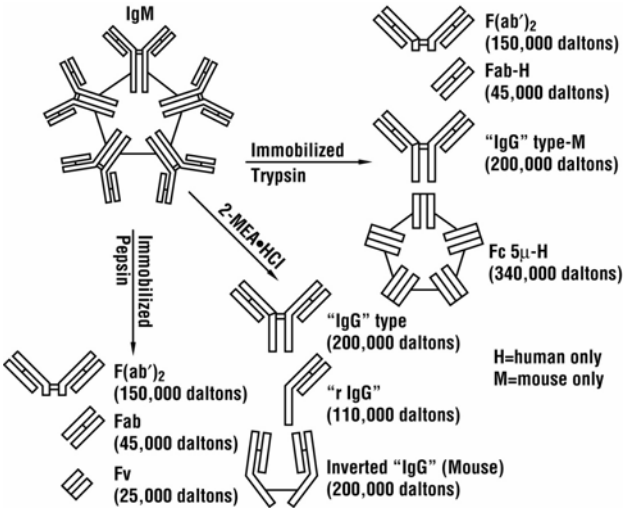


Figure 1. IgM fragments generated using trypsin, pepsin or 2-mercaptoethylamine.

Additional Materials Required

- Incubator, water bath or cooler capable of maintaining 4°C, 37°C and 63°C
- Centrifuge with a fixed-angle rotor: the C100 and C30 Concentrators have a maximum centrifugal force of 5,000 × *g* and can be used only with a fixed-angle rotor.
- Coomassie Plus (Bradford) Assay Kit (Product No. 23236) and a microplate reader or spectrophotometer

Procedure for IgM Buffer Exchange

Perform a buffer exchange of the IgM sample before proceeding to any of the digestion methods described in these instructions. Two methods of buffer exchange are available. The desalting column is most useful for concentrated samples. For samples with low IgM concentration, buffer exchange using the concentrator may be the preferred method.

Method 1: Desalting Column

1. Equilibrate the Dextran Desalting Column with three column volumes of IgM Digestion Buffer.
2. Apply 0.5-1.5 ml of IgM to the column and allow the sample to enter the gel bed.
3. Add 0.5 ml aliquots of IgM Digestion Buffer and collect each fraction in a separate tube. For a 0.5 ml sample, IgM will emerge in fractions 4-6. For a 1.5 ml sample, IgM will emerge in fractions 4-8.
4. Pool the protein-containing fractions and determine concentration. If IgM is not at 1 mg/ml, either adjust the concentration or alter digestion time.
5. Save 20-60 µl of sample IgM for protein recovery determination. Proceed to the preferred digestion method.

Method 2: C100 Concentrator

1. Apply ~1 mg of IgM in sample buffer to a C100 Concentrator. Centrifuge for 15-30 minutes (depending on the volume) at 5,000 × *g*. The maximum centrifugal force for the C100 Concentrator is 5,000 × *g*.
2. Add 0.5-1.0 ml of IgM Digestion Buffer to the retained IgM and centrifuge an additional 15-30 minutes at 5,000 × *g*.
3. Add the sample cup to the filter, invert and centrifuge 1-2 minutes at 5,000 × *g* to collect sample.
4. Adjust volume to 1 ml with IgM Digestion Buffer. Save 20-60 µl of sample IgM for protein recovery determination. Proceed to the desired digestion method.

Procedure for Hot Trypsin Digestion

Hot trypsin digestion (HTD) cleaves human IgM at Arg 214 and results in a loss of a few peptides and a major glycopeptide from Cμ2. Approximately 45% of Fc5μ and 50% Fab recovery can be obtained from HTD. Generally, increasing E/S ratio and temperature enhance digestion rate; however, IgM can be permanently denatured at 70°C. Digestion times and temperatures might require slight adjustments.

Fab also can be produced from tryptic digestion of mouse IgM; however, depending on the specific IgM, yields may be extremely low. Typically, Fc5μ and $F(ab')_2$ fragments are not generated and only ~2% of the Fab fragments are recovered (see the Additional Information section for nomenclature of IgM Fragments).

Notes:

- Increasing the NaCl concentration will decrease digestion rate.
- Heated IgM tends to unfold and precipitate when exposed to high temperatures for extended periods, therefore, heated IgM may migrate differently than unheated IgM on size exclusion columns or in gels.
- Trypsin columns may be used up to three times with the same IgM source.

A. Digestion

Note: Depending on IgM species, trypsin digestion followed by reduction and alkylation may produce similar fragments to reduction and alkylation alone.

1. Heat a water bath to 60°C. A temperature from 55 to 63°C is acceptable provided digestion time is adjusted.
2. Equilibrate the Immobilized Trypsin Column by adding at least 8 ml of IgM Digestion Buffer.
3. Pre-incubate separately the 1 ml of 1 mg/ml IgM prepared in IgM Digestion Buffer, 10-15 ml of IgM Digestion Buffer and the Immobilized Trypsin Column in a water bath at 60°C for 1-2 minutes.
4. Remove top and bottom caps from the Immobilized Trypsin column and add 1 ml of 1 mg/ml IgM. Allow sample to enter the gel. Add 200 µl of IgM Digestion Buffer to allow IgM to completely enter the gel and replace caps.
5. Incubate column at 60°C for 25-40 minutes or at 63°C for 15-25 minutes. To evaluate sample's susceptibility to heat denaturation or precipitation, incubate a sample of IgM (with no enzyme) along with the digest column.
6. Allow column to cool slightly.
7. Remove top and bottom caps from the column and apply 200 µl of IgM Digestion Buffer to remove some of the solution from the column tip. Transfer column to a clean test tube.
8. Apply 2 × 1 ml of IgM Digestion Buffer to the column and collect flow-through, which contains the majority (80-90%) of the digest. This tube will be used in the concentration step below. Transfer column to a clean test tube.
9. Apply 2 × 1 ml of IgM Digestion Buffer to recover any residual digest in the column.
10. Proceed with the concentration method below. Alternatively, separate Fc5μ and Fab using Immobilized MBP (Product No. 22212) or nickel immobilized to Iminodiacetic Acid (Product No. 20277).

B. Concentration and Separation of Fc5μ from Fab

1. Add 2 ml of digest from Step A8 to a C100 Concentrator. Centrifuge at 5,000 × *g* for 15-30 minutes.

   Note: Maximum centrifugal force for the C100 and C30 Concentrators is 5,000 × *g*.
2. Add the filtrate from the C100 Concentrator to a C30 Concentrator. Add the residual digest from Step A9 to the C100 Concentrator. Centrifuge at 5,000 × *g* for 15-30 minutes.
3. Apply filtrate from the C100 Concentrator to the C30 Concentrator. Wash by adding 2 ml of IgM Digestion Buffer to the C100 Concentrator. Centrifuge concentrator at 5,000 × *g* for 15-30 minutes.
4. Repeat washes 1-3 times to remove all Fab from the upper concentrator. When the C100 volume reaches 50-100 µl, add the sample cup, invert and centrifuge for 1-2 minutes at 5,000 × *g* to collect the Fc5μ.
5. Adjust the Fc5μ sample to desired volume with IgM Digestion Buffer for protein concentration determination. To simplify later calculations, adjust to a volume easily divisible by the original sample volume.

6. Repeat washes 1-3 times with IgM Digestion Buffer. When the C30 Concentrator volume reaches 50-100 µl, add sample cup, invert and centrifuge for 1-2 minutes at 5,000 × *g* to recover Fab. Adjust Fab to the desired concentration with IgM Digestion Buffer.

C. Protein Concentration Determination

Use the Coomassie Plus (Bradford) Assay Kit (Product No. 23236) for protein determination. The assay may be performed in test tubes or a microplate to conserve sample. Use a ratio of sample to Coomassie Reagent at 1:50. For example, in the test tube format combine 20 µl of sample with 1 ml of Coomassie Reagent, and in the microplate format combine 5 µl of sample with 250 µl of Coomassie Reagent.

1. Prepare standard curve at 0.25, 0.5 and 1.0 mg/ml.
2. Prepare the stock desalted IgM sample at 0.5 and 1.0 mg/ml.
3. Prepare the C100 and C30 retained samples in duplicate or triplicate.
4. Measure the absorbance of each sample at 595 nm. Calculate percent recovery.

D. Purity Determination Methods

Size and purity can be determined by gel electrophoresis or size exclusion chromatography. Note that IgM may react through hydrophobic/ionic interactions with column matrices. Superose® 6 or 12 Columns or Precise™ Gels 4-20% can be used for analyzing Fc5µ and IgM, and PW 300 (Waters) can be used for smaller fragments.

Procedure for Pepsin Digestion

Notes:

- Mouse and human IgM digestion with pepsin produces 150 kDa and 120 kDa fragments.
- The yield from mouse and human IgM is ~30% and ~20%, respectively.
- Incubating for 1.5 hours will contain undigested IgM; however, longer incubation may result in low recovery.
- Using a C30 Concentrator will remove peptides from the digest but will not eliminate IgM and Fab; however, for mouse, IgM contamination will be less than 4% and Fab contamination less than 10%. For human, Fab contamination will be 30% and IgM ~20%. To remove IgM, try using one of the following methods:
    - 300 K MWCO concentrator will retain IgM allowing smaller molecular weight components filter through
    - Protein A may bind some mouse and human IgM. Reports indicate the Cµ2 region must be intact and the Fc must be polyvalent for binding, and that IgM easily overloads Protein A columns.
    - Size-exclusion chromatography or slab gels
    - C1q agarose, C1q binds to the Fc portion of IgM via complement (will have IgG contamination and will not remove pH-denatured IgM)
    - Anti-Fc IgM agarose
    - Immobilized Mannan Binding Protein (Product No. 44897 and 22212) will not remove pH-denatured IgM
    - Nickel on Immobilized Iminodiacetic Acid (Product No. 20277) will work for some IgM
- Save 20-60 µl of sample IgM for protein recovery determination.
- Pepsin columns may be used up to three times with the same IgM source.

A. Pepsin Digestion of Mouse or Human IgM

1. Prepare IgM in the IgM Digestion Buffer using one of the methods described previously.
2. Set incubator or water bath to 37°C.
3. To equilibrate the Immobilized Pepsin, wash column with at least 3-4 column volumes of IgM $F(ab')_2$ Digestion Buffer.
4. Pre-incubate separately the 1 ml of 1 mg/ml IgM, 10-15 ml of IgM $F(ab')_2$ Digestion Buffer and Immobilized Pepsin Column at 37°C for 2-3 minutes.

5. Remove top and bottom caps from the Immobilized Pepsin column. Add the IgM sample and allow it to enter the gel. Add 200 µl of IgM F(ab´)$_2$ Digestion Buffer to allow the IgM to completely enter the gel; replace caps.
6. Incubate column at 37°C for 1.5 hours.
7. Allow column to cool slightly. Remove top and bottom caps, place column in a new test tube and add 200 µl of IgM F(ab´)$_2$ Digestion Buffer.
8. Transfer column to a new test tube. Apply 2 × 1 ml of IgM F(ab´)$_2$ Digestion Buffer to collect main fragment peak.
9. Transfer column to a new test tube. Apply 2 × 1 ml of IgM F(ab´)$_2$ Digestion Buffer to recover any residual digest.

B. Digest Concentration and Peptide Removal

1. Add 2 ml from Step A8 to a C30 Concentrator. Centrifuge at 5,000 × *g* for 15-30 minutes.

   Note: Maximum centrifugal force for the C100 and C30 Concentrators is 5,000 × *g*.
2. Add contents from the tube in Step A9 to the C30 Concentrator. Centrifuge at 5,000 × *g* for 15-30 minutes.
3. Wash 1-3 times with 2 ml of IgM Digestion Buffer. Centrifuge at 5,000 × *g* for 15-30 minutes.
4. When the C30 volume reaches 50-100 µl, add the sample cup, invert and centrifuge for 1-2 minutes at 5,000 × *g*.
5. Adjust digest to an appropriate volume with IgM Digestion Buffer for protein determination. To simplify calculations, adjust volume to a value easily divisible by the original sample volume.
6. Protein concentration can be determined using the Coomassie Plus (Bradford) Assay Kit as described in Section C of the hot trypsin protocol.

Procedure for IgM Reduction and Alkylation

Note: Depending on IgM species, trypsin digestion followed by reduction and alkylation may produce similar fragments to reduction and alkylation alone.

A. Reduction and Alkylation

1. Set incubator or water bath to 37°C.
2. Add 1 ml of IgM prepared in IgM Digestion Buffer to the 6 mg vial of 2-mercaptoethylamine and incubate at 37°C for 30 minutes.
3. Add the reduced IgM to the 16 mg of iodoacetamide.
4. Incubate reaction in the dark for 10 minutes at room temperature.

B. Concentration and Peptide Removal of Reduced IgM

1. Add 1 ml of a 1 mg/ml reduced and alkylated IgM to a C30 Concentrator and centrifuge at 5,000 × g for 15-30 minutes.
2. Note: Maximum centrifugal force for the C100 and C30 Concentrators is 5,000 × g.
3. Wash 1-3 times with 2 ml of IgM Digestion Buffer. Centrifuge at 5,000 × g for 15-30 minutes.
4. When the C30 volume reaches 50-100 µl, add the sample cup, invert and centrifuge for 1-2 minutes at 5,000 × g.
5. Adjust volume as desired with IgM Digestion Buffer for protein determination. To simplify calculations, adjust to a volume easily divisible by the original sample volume. IgM reduction and alkylation recoveries are approximately 70% IgG- and reduced IgG-type fragments.
6. Protein concentration can be determined using the Coomassie Plus - The Better Bradford™ Assay Kit as described in Section C of the hot trypsin protocol.

Additional Information

A. Nomenclature for IgM Fragments

L: 22 kDa peptide chain folded into two globular domains designated VL and CL

µ: 70 kDa peptide chain folded into five globular domains designated VH, Cµ1, Cµ2, Cµ3, Cµ4

Fab: proteolytic fragment consisting of L chain and Fd piece

Fd: piece of µ chain containing a VH and a Cµ1 domain

F(ab´)$_2$: dimeric disulfide linked proteolytic fragment consisting of two L chains and two Fd´ pieces

Fd´: µ chain piece containing VH, Cµ1 and Cµ2 domains and thus having the cysteine residue that forms the inter µ chain disulfide bond – few residues of the Cµ3 domain may also be present

Fab: a proteolytic fragment consisting of L chain and Fd piece

Fd: a piece of a chain containing VH and Cµ1 domains and most of the Cµ2 domain but not having the cysteine residue that forms the inter µ chain disulfide

(Fc)5: disulfide-linked pentameric fragment containing five Fc monomers (within this instruction book (Fc)5µ is used interchangeably with Fc5)

Fc monomers: a dimer of two Fc pieces

Fc: a piece of µ chain containing the Cµ3 and Cµ4 domains and a few residues of the Cµ2 domain including the cysteine residue that forms the inter µ chain disulfide bond

Fc: µ chain piece containing Cµ3 and Cµ4 domains and most or the entire Cµ2 domain

Fc´: µ chain piece containing the Cµ4 domain and most or the entire Cµ3 domain, but none of the Cµ2 domain

B. IgM Fragmentation from Various Species

Although the kit protocol has been optimized to produce fragments from mouse and human IgM, the kit components can be used to fragment IgM from other species (Table 1).

Table 1. Fragments of IgM produced from various species.

Enzyme Conditions	trypsin at 55°C E/S = 1:100	trypsin with urea at 25°C (preincubated with Tris) E/S = 1:50	pepsin at 37°C E/S = 1:100
Buffer Conditions	0.1 M Tris•HCl, 0.2 M NaCl, 0.01 M $CaCl_2$; pH 8.3	0.2 M Tris•HCl, 0.4 M NaCl 4-6 M urea; pH 8.0	0.1 M sodium acetate-acetic acid, 0.2 M NaCl; pH 4.6
Mouse	Fab, F(ab´)$_2$	Fab, F(ab´)$_2$, Fc5µ, Fc*5	Fab, F(ab´)$_2$
Human	Fab, Fc5µ	Fab, Fc5µ, Fc*5	Fab, F(ab´)$_2$
Pig	Fab, Fc5µ	Fab, Fc5µ, rapidly degraded	Fab, Fc5µ
Bovine	Fab, Fc5µ	Fab, Fc5µ, rapidly degraded	Fab, Fc5µ
Sheep	Fab	no information	low yields of Fab, Fc5µ
Rabbit	no information	no information	Fab, F(ab´)$_2$, Fc5µ, Fc*5

*Digested and therefore smaller versions of the fragments.

Note: This table was compiled from a review of the literature and serves as a guide for IgM fragmentation in certain conditions. In some cases, altering the stated conditions may produce different fragmentation patterns.

C. IgM General Information

IgM exists in two forms: a monomeric form present in the membrane and a secreted form, which exists as multiple monomers (~180 kDa) joined through disulfides and a J chain. The number of monomers present in the secreted form varies between species. Amino acids, disulfides and carbohydrates also vary with IgM species. There are significant differences in IgM fragments produced among species resulting from domain interactions, interchain disulfide bonds and oligosaccharides.

Human and mouse IgM are pentameric (700-1,100K). The J chain (15 kDa) is an acidic peptide rich in carbohydrate and cysteine, arginine and lysine residues. The J chain is involved in the polymerization of IgM. Each of the five IgM monomers

is composed of two identical light chains [kappa (κ) or lambda (λ)] and two identical heavy mu (μ) chains. The μ chain is composed of one variable region and four constant regions. There is less than 40% homology between μ chain of IgM and the gamma (γ) chains of IgG.[1] IgM does not have a hinge region, rather it contains the extra domain Cμ2. The Cμ3 domain corresponds to Cγ2 and Cμ4 corresponds to Cγ3. The Cμ2 domain of IgM correlates to the hinge region of IgG and has a large carbohydrate portion that may interfere sterically with proteolytic enzyme activity like papain.

The molar extinction coefficient of IgM is ~1.25 at A_{280} of a 0.1% solution. Solubility is dependent on solvent, pH and temperature. IgM often precipitates in low ionic solutions but will usually re-dissolve upon increasing salt concentration. Borate buffer may enhance precipitation from interactions with carbohydrate moieties. IgM has a tendency to associate with other proteins such as IgG and albumin.

Human and mouse IgM are structurally different. Human IgM disulfides are arranged in parallel, and mouse IgM disulfides are arranged in series (Figure 2). This disulfide bond arrangement affects the position of selective cleavage from an IgG-type arrangement to an inverted IgG-type arrangement. Homology between human and mouse IgM is 27% in Cμ1, 43% in Cμ2 with similar distance from disulfide to carbohydrate and 70% in Cμ4. There are no disulfides in Cμ3 of mouse IgM and a much stronger favor for κ light chains than human IgM. There is complete conservation of the general type and location of the carbohydrate moieties in the μ constant region between mouse and human μ chain.[2]

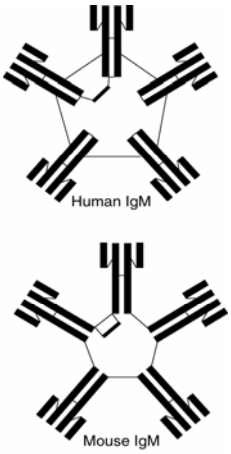


Figure 2. Comparison of J chain and monomer attachment of human and mouse IgM.

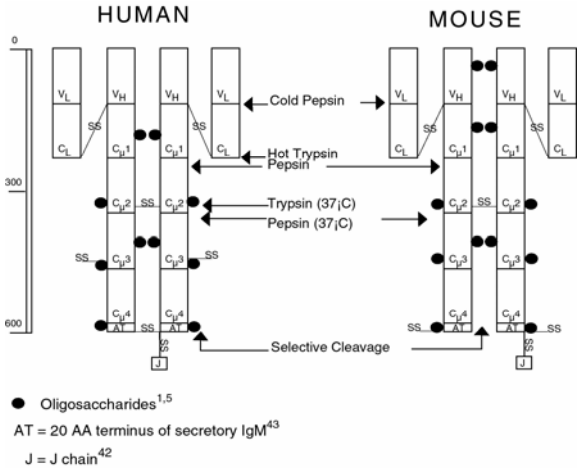


Figure 3. Comparison of human and mouse IgM fragmentation using various digestion methods.

D. IgM Fragmentation

The proteolytic fragmentation of IgM proceeds differently from IgG resulting from differences in structure (Figure 3). The μ chains are folded into multiple globular domains and IgM has an actual domain (Cμ2) in place of a hinge region. Cμ2 lacks the proline-rich sequence present in the hinge region of IgG and IgA that makes the hinge susceptible to cleavage. When IgM is cleaved with trypsin, pepsin or papain; Fabμ or $F(ab')_2\mu$ are usually generated.

Fv fragments (25 kDa) are the smallest fragments that still contain the antigen-binding site. Fv consists of both VH and VL domain held together by noncovalent interactions. Fv has the same binding properties as Fab or whole antibody. They also exhibit similar three-dimensional binding characteristics.[3]

$F(ab')_2$ fragments (150 kDa) have an increased binding valence over Fv and Fab. Because $F(ab')_2$ fragments are bivalent, they can cross-link antigens and form precipitates, unlike the univalent Fab or Fv. Fab fragments (45kDa) are monovalent and smaller than $F(ab')_2$, allowing for better penetration and faster clearance.

E. Digestion Enzymes

Pepsin (35.4 kDa) is a protease that is active only at acidic pH and is irreversibly denatured at neutral or alkaline pH. Pepsin normally cleaves IgM at the site between Cμ2 and Cμ3. It is possible to produce $F(ab')_2$, Fab and Fv fragments using pepsin with human and mouse IgM. There is often cleavage between domains to give Fc and other fragments. There can also be extensive degradation of the Fc portion with pepsin. Mouse IgM digested with pepsin produces heterogeneous fragments, which may imply the existence of multiple conformational states and/or differing degrees of unfolding.

Trypsin (23.8 kDa) is a serine protease with an optimal pH of 8 and normally cleaves at the C-terminal side of Arg or Lys. In general, increasing the enzyme-to-substrate ratio (E/S) and temperature increases digestion rate. Trypsin can generate Fab,

F(ab´)$_2$ or IgG-type fragments. Trypsin cleaves human IgM at Arg 214 using a 60°C digestion and Arg 239 using a 37°C digestion. No Fc is recovered from the 37°C. Hot tryptic digestion (HTD) of human IgM results in a loss of peptides and a major glycopeptide from the Cμ2.[4] Increasing the temperature significantly increases digestion rate, although precipitation and denaturation can occur. IgM is irreversibly denatured at 70°C in a Tris-$CaCl_2$ buffer with no NaCl.[5] Human IgM HTD digestion products are Fab (45 kDa) and Fc5μ (350-450 kDa). The cleavage of the Cμ2 domain at high temperatures suggests that heat causes the middle part of the IgM molecule to undergo a conformational change.[6] Fab and F(ab´)$_2$ can also be produced from mouse IgM; however, yields may be substantially lower.[6] Typically, Fc5μ and F(ab´)$_2$ fragments are not generated with tryptic digestion of mouse IgM and only ~2% Fab are recovered.

F. Reduction and Alkylation

Mild reduction of IgM is achieved using 2-mercaptoethylamine (2-MEA). Temperature, pH, salt concentration, digestion time and reductant-to-protein ratio can effect fragmentation. Reduction of IgM at 1 mg/ml with 2-MEA occurs at an optimal concentration of 50 mM. At 60°C, the antibody rapidly dissociates and is similar at 25-37°C, but at 4°C the reduction is decreased two-fold. Because disulfide arrangement of human IgM occurs in parallel, the ratio of IgG- and rIgG-type molecules will vary based on temperature and reduction time. The disulfide arrangement of mouse IgM occurs in series suggesting an inverted IgG and rIgG should be produced, again varying in ratio based on temperature and reduction time.

Trypsin digestion at 37°C without reduction or alkylation cleaves IgM, but the immunoglobulin is held intact by disulfide and protein interactions and migrates on nonreducing SDS-PAGE as an undigested IgM. Trypsin digestion and subsequent reduction and alkylation yields fragments similar to those produced with reduction and alkylation alone.

Related Products

44897	**IgM Purification Kit**
22212	**Immobilized Mannan Binding Protein**
44667	**Protein A IgG Purification Kit**
20277	**Immobilized Iminodiacetic Acid**
23236	**Coomassie Plus (Bradford) Assay Kit**

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