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PerKit™ F(ab')2 Preparation Kit (CM51408x1 and CM51408x3) User Reference Guide

Contents

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Important Notes & Contact Information

READ BEFORE USING ANY RESOURCES PROVIDED HEREIN

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Sample data are provided for illustration and example purposes only and represent a small dataset used to verify kit performance in the CellMosaic laboratory. Information about the chemicals and reagents used in the kit are provided as necessary.

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Kit Components

This kit provides materials to fragment 0.25-3 mg of mouse IgG_1 to produce F(ab')2 with the options to choose from one (CM51408x1) or three (CM51408x3) reactions. Scale of each reaction: 0.25-3 mg (protein content). Upon receipt, please remove the plastic bag containing Reagent A and B and store in a freezer below -20°C. Store the rest of the items and box in a refrigerator at 2-8°C.

Name	Part #	Quantity (CM51408)	Quantity (CM51408x3)	Storage condition
Reagent A (green label)	CM12105	1 unit	3 units	-20°C, dry
Reagent B (red label)	CM53213	1 unit	3 units	-20°C
Reagent C (Blue)	CM12005	40 µL	40 µL	-20°C
Protein A spin column	CM71520.1	1 column	3 columns	2-8°C
Digestion Buffer (chocolate label)	CM02019	4 mL	12 mL	2-8°C
Storage Buffer (1 x PBS buffer) (grey label)	CM02013	5 mL	20 mL	2-8°C
Centrifugal Filter Devices	CM03CD050A	3	9	2-8°C
Collection Tubes	CM03CT0	6	18	2-8°C
1.5 mL Centrifuge Tubes	CM03CT2	3	9	2-8°C
2.0 mL Eppendorf Tubes	CM03CT12	2	6	2-8°C
Mouse IgG_1	N/A	NOT PROVIDED (User Supplied Material, 0.25-3 mg per reaction)		

Safety Information

Warning: some of the chemicals used can be potentially hazardous and can cause injury or illness. Please read and understand the Safety Data Sheets (SDS) available at CellMosaic.com before you store, handle, or use any of the materials.

Fragmentation Chemistry

The kit is designed to fragment any mouse IgG_1 antibody with Ficin. The procedure we use involves cleaving the IgG_1 molecules below the disulfide groups in the hinge region using Ficin to create a bivalent fragment and degraded smaller peptide fragments, and then purify to obtain F(ab')2. F(ab')2 can be further reduced to produce Fab' fragment. If you need Fab', contact CellMosaic for a custom synthesis. Key features of this kit:

- Easy preparation: 1 day preparation and <4 h hands-on time
- All reagents and supplies included for preparation and purification
- 30-60% recovery and 80-95% of pure F(ab')2 by SEC (size-exclusion chromatography)

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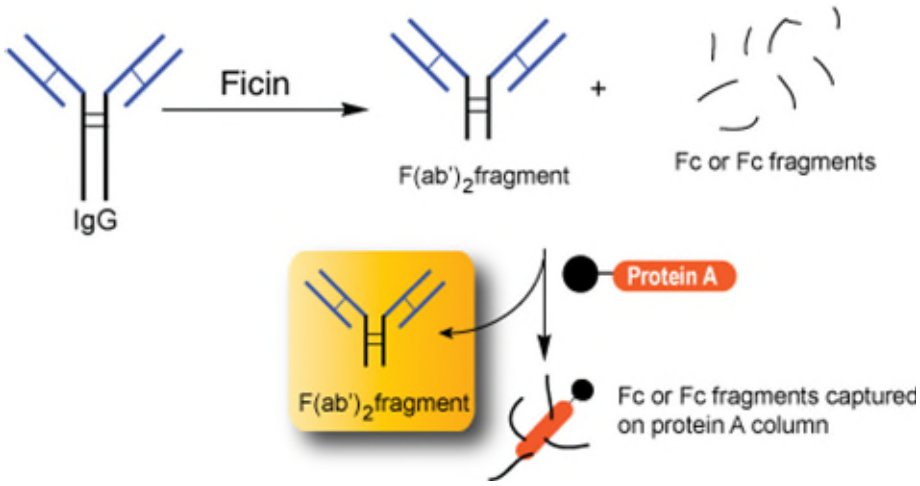
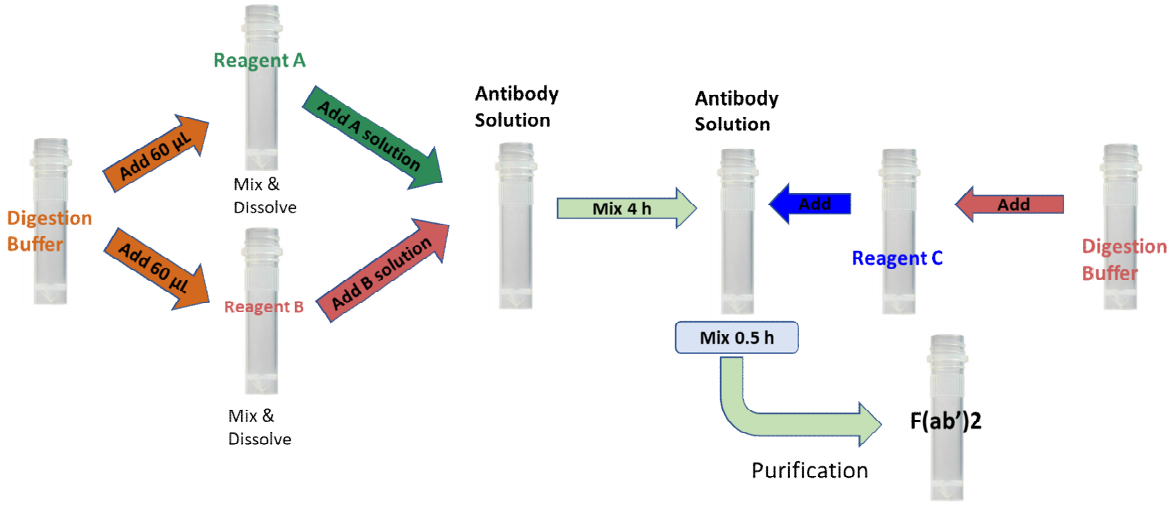


Requirement for Mouse IgG_1:
1. Preferably > 90% pure by gel electrophoresis
2. Preferably monoclonal antibody
3. Total amount: 0.25-3 mg (protein content)

Support

CellMosaic provides additional support services to customers who need help analyzing the F(ab')2 product by HPLC.

Protocol



Scheme 1. Schematic diagram of the work flow for F(ab')2 preparation

Table 1. Calculation of the total volume of Reagent Solution and Buffers

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Amt of IgG	3 mg	2 mg	1 mg	0.5 mg	0.25 mg	Other IgG Amt (mg)
IgG Solution (µL)	480	320	160	80	40	**= 160 x Amt (IgG)**
Reagent A Solution (µL)	60	40	20	10	5	**= 20 x Amt (IgG)**
Reagent B Solution (µL)	60	40	20	10	5	**= 20 x Amt (IgG)**
Reagent C Solution (µL)	12	8	4	2	1	**= 4 x Amt (IgG)**

1. Lab Instrumentation Needed

- Vortex mixer, centrifuge (preferably refrigerated)
- Pipettes and tips
- Timer
- Incubator or shaker set at 37 °C

2. Preparation of Antibody Samples (IgG_1) for Conjugation

Total amount of antibody used for the conjugation is 0.25-3 mg (protein content measured by UV) per reaction.

B1. Insert the **Filter Device** into one of the provided collection tube (microcentrifuge tube with the cap attached). Perform the step based on the following conditions.

- ✓ If your antibody is supplied as a lyophilized solid, dissolve the antibody in 500 µL of **deionized water** and then transfer the entire contents to the **Filter Device**.
- ✓ If your antibody is supplied in < 500 µL buffer, transfer your antibody sample to the **Filter Device** directly. Add **Digestion Buffer** (chocolate label) to make up the total volume to 500 µL and cap it.
- ✓ If the volume of your antibody sample is between 500 and 1000 µL, divide the volume into two **Centrifugal Filter Devices** and add the antibody sample to the filter device. Add **Digestion Buffer** to make up the total volume to 500 µL in each device and cap them.
- ✓ If the volume of your antibody sample is >1000 µL, add up to 500 µL of sample to the two **Filter Devices** and cap them. Repeat Step **B1-B4** until all of the antibody sample goes into the **Filter Device**. Move on to Step **B5**. Add **Digestion Buffer** to make up the total volume to 500 µL in each device for the last refill.

B2. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

B3. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to **< 100 µL** (Spin time depends on many factors. The typical spin time for a 500 µL sample is approximately 8 to 20 minutes. The typical volume is ~40 µL after spinning for 8 minutes on an Eppendorf 5417R at 4°C.)

B4. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

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B5. Insert the **Filter Device** back to the collection tube. Add 400-450 µL of **Digestion Buffer** to make up the total volume to 500 µL. Then place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x g to concentrate to **< 100 µL**. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

B6. Repeat **Step B5** two more times. If the total amount of IgG is less than 0.5 mg, for the last repeat, spin the **Filter Device** at 14,000 x g to concentrate to **< 20 µL**.

B7. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube (use the pipetman to estimate the approximate volume of the concentrated sample).

B8. Add appropriate amount of **Digestion Buffer** to the **Filter Device** to rinse (check the total volume in Step B10). Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B7.**

B9. Repeat **Step B8** once.

B10. Add **Digestion Buffer** to the 1. 5 mL micro-centrifuge tube from **Step B9** to make up the total volume shown below and cap it (**160 µL per mg of IgG**).

Total volume for antibody:

Amt of IgG	**3 mg**	**2 mg**	**1 mg**	**0.5 mg**	**0.25 mg**	**IgG Amt (mg)**
IgG Solution (µL)	480	320	160	80	40	**= 160 x Amt (IgG)**

B11. Vortex the combined antibody sample for 30 seconds and then centrifuge to ensure no liquid is in the cap.

3. Ficin Fragmentation

C1. Spin the centrifuge tubes containing **Reagent B** (red label) before opening it.

C2. Add **60 µL** of **Digestion Buffer** (chocolate label) to the **Reagent B** tube. Vortex for 30 seconds to 1 minute to dissolve the reagent and then centrifuge to ensure no liquid is in the cap (**Digestion Buffer** will be also used in **Step C7**).

Tip for solubility check: Check the bottom of the micro-centrifuge tube to see if the solution is clear of any solid residue.

C3. Transfer **Reagent B Solution** to the antibody solution from **Step B11** based on the following table (**20 µL Reagent B solution per mg of IgG**).

Total volume for antibody:

Amt of IgG	**3 mg**	**2 mg**	**1 mg**	**0.5 mg**	**0.25 mg**	**IgG Amt (mg)**
Reagent B Solution (µL)	60	40	20	10	5	**= 20 x Amt (IgG)**

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C4. Spin the centrifuge tubes containing **Reagent A** (green label) before opening it.

C5. Add **60 µL** of **Digestion Buffer** to the **Reagent A** tube. Vortex for 30 seconds to 1 minute to dissolve the reagent and then centrifuge to ensure no liquid is in the cap.

C6. Transfer **Reagent A Solution** from **Step C5** to the antibody solution from **Step B11** based on the following table (**20 µL Reagent A solution per mg of IgG**).

Total volume for antibody:

Amt of IgG	3 mg	2 mg	1 mg	0.5 mg	0.25 mg	IgG Amt (mg)
Reagent A Solution (µL)	60	40	20	10	5	**= 20 x Amt (IgG)**

C7. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Incubate with mixing at 37 °C for 4 h.

Tip for mixing: You can use a nutator, a shaker, vortex, or an incubator shaker for mixing. If you are using end to end nutating, make sure your centrifuge is capped properly.

C8. Spin the centrifuge tubes containing **Reagent C solution** (blue label) before opening it. Pipette **Reagent C Solution** to the antibody solution from **C7** (after4 h incubation) based on the following table (**4 µL Reagent A solution per mg of IgG**).

Total volume for antibody:

Amt of IgG	3 mg	2 mg	1 mg	0.5 mg	0.25 mg	IgG Amt (mg)
Reagent C Solution (µL)	12	8	4	2	1	**= 4 x Amt (IgG)**

C9. Vortex the solution for 30 seconds, and then centrifuge to ensure no liquid is in the cap. Incubate with mixing at RT for 30 minutes.

4. Purification

D1. Insert the **protein A spin column** into an Eppendorf tube. Remove the bottom and top cap, centrifuge at 1000 x g for 1 minutes and discard the flow-through. Washing the top and bottom caps with deionized water and set aside for reuse.

D2. Add 0.75 mL of **Digestion buffer** onto the column, centrifuge at 1000 x g for 1 minutes and discard the flow-through.

D3. Repeat **Step D2** two more times.

D4. Cap bottom of column. Apply sample from **Step C9** to column. If the amount of your IgG is over 2.5 mg, go to next step. If the amount of your IgG is less than 2.5 mg, washing the sample centrifuge tube with **Digestion Buffer** and add **Digestion Buffer** to make up the total volume to 500 µL.

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Total volume for Digestion Buffer:

Amt of IgG	3 mg	2 mg	1 mg	0.5 mg	0.25 mg	IgG Amt (mg)
Buffer A (µL)	0	92	296	398	449	**= 500 – 204 x Amt (IgG)**

D5. Cap the top of the column. Make sure the caps are tight. You can also use parafilm to wrap around the top and bottom caps. Resuspend the resin and sample by inversion. Nutate the spin column at RT with end-over-end mixing for 30 minutes.

D6. Invert the column and slowly open the bottom of the cap. Insert the spin column into a clean Eppendorf tube. Centrifuge at 1000 x g for 1 minutes. Collect the flow-through and label the Eppendorf tube as **Step D6 F(ab')2**

D7. Insert the spin column into a new Eppendorf tube. Add 250 µL of **PBS buffer** to the column. Centrifuge at 1000 x g for 1 minutes. Collect the flow-through and label the Eppendorf tube as **Step D7 F(ab')2**.

D8. Insert the **Filter Device** into one of the provided collection tube (microcentrifuge tube with the cap attached). Transfer the F(ab')2 solution from **Step D6** into the **Filter Device** directly (up to 500 µL total) and cap it. Place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

D9. Spin the **Filter Device** at 14,000 x g for 8 minutes (preferably cooled to 4°C) to concentrate to < 100 µL.

D10. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

D11. Transfer the remainder of the F(ab')2 solution from **Step D6** if any and **Step D7** into the **Filter Device** directly. Add **PBS buffer** to make up the total volume to 500 µL and cap it. aligning the cap strap toward the center of the rotor; counterbalance with a similar device.

D12. Spin the device at 14,000 x g to concentrate to **< 100 µL**.

D13. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

D14. Add 400-450 µL of **PBS Buffer** to make up the total volume to 500 µL. Then place the capped **Filter Device** into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device. Spin the device at 14,000 x g to concentrate to **< 100 µL**. Remove the assembled device from the centrifuge and separate the **Filter Device** from the collection tube. Transfer the filtrate from the collection tube to a clean centrifuge tube (not provided). **Save the filtrate until the experiments are done.**

D15. Repeat **Step D14** two more times.

D16. Transfer the concentrated sample from the **Filter Device** to a 1.5 mL micro-centrifuge tube.

D17. Add 20-100 µL of **PBS buffer** to the **Filter Device** to rinse. Stir it gently with a pipet tip, then transfer the entire contents to the 1.5 mL micro-centrifuge tube from **Step B7.**

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D18. Repeat **Step D17** once. The amount of **PBS buffer** added will depend on how concentrate you want your F(ab')2 Sample to be.

D19. Vortex the combined **F(ab')2 sample** from **Step D18** for 30 seconds and then centrifuge to ensure no liquid is in the cap.

F(ab')2 is Ready for Your Experiment

- **Specification for your product:** A typical batch contains over 80% of purified F(ab')2 and free of ficin enzyme, Fc, and any other smaller fragments. The recovery is 30-60% recovery. You can determine the concentration by UV/Vis spectrophotometer (see other considerations).

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Other Considerations

1. Concentration Determination

To determine the concentration, dilute your conjugate from **Step D19** with 1x PBS buffer. Measure the UV absorbance of the F(ab')2 at 280 nm (A280) using a UV spectrometer and calculate the concentration based on the following formula:

$$Concentration\ (\mu M) of\ the\ dilute\ sample = \frac{(A280) * 100}{L * 11}$$

$$Concentration\ (mg/mL) of\ the\ dilute\ sample = \frac{(A280)}{L * 1.4}$$

Where **L** is the UV cell path length (cm). If you are using a 1 cm UV cell, you can dilute the conjugate 4 times to obtain a good reading.

Where **n** is the average molar ratio of DM1 per antibody. Use 4.0 if you do not have the experimental value of your conjugates.

For a typical F(ab')2 with MW of 110,000, use an estimate molar extinction coefficient of 154,000 $M^{-1}cm^{-1}$

2. Recommended Storage Conditions

Recommend storage at -20°C for long term.

3. Submit Samples for HPLC Analysis

If you are submitting samples to CellMosaic for SEC analysis, please follow these instructions:

1) Dilute your F(ab')2 sample to 1 mg/mL in PBS buffer, then transfer 50 µL of the diluted solution to a 500 µL microcentrifuge tube. Label the vial properly.
2) Ship your samples with a cold pack for overnight delivery.

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Appendix: Typical Kit Performance Data (LC analysis, CellMosaic)

Figure 1: SEC analysis of a mouse IgG1, crude digestion mixture of the IgG1, and the purified F(ab')2 (UV at 220 nm)