

Technical Data Sheet

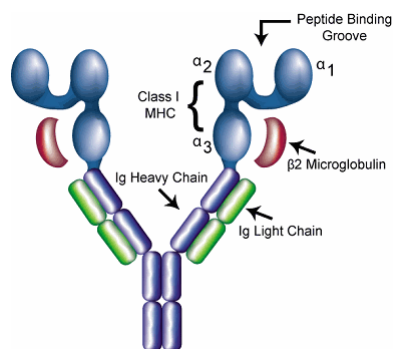
DimerX I: Recombinant Soluble Dimeric Mouse H-2D[b]:Ig**Product Information**

Material Number:	551323
Size:	0.25 mg
Concentration:	0.5 mg/ml
Isotype:	Mouse IgG1, λ
Storage Buffer:	Aqueous buffered solution containing $\leq 0.09\%$ sodium azide.

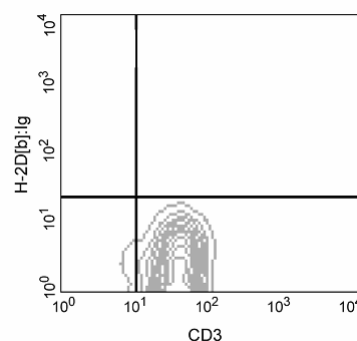
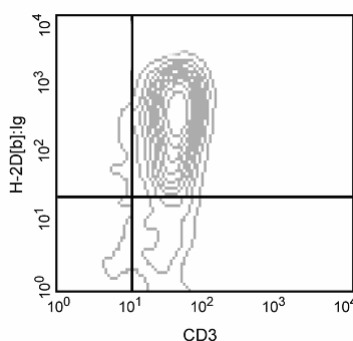
Description

The H-2D[b]:Ig fusion protein consists of three extracellular major histocompatibility complex (MHC) class I H-2D[b] domains that are fused to the VH regions of mouse IgG1 (see Figure 1). In order for the MHC class I to be functional, ie, capable of binding peptides, $\beta 2$ Microglobulin ($\beta 2M$) must be present. For this reason, BD™ DimerX consists of recombinant H-2D[b]:Ig fusion protein, supplemented with recombinant $\beta 2M$. Recombinant MHC molecules, like the DimerX fusion protein, are useful for studying T-cell function by immunofluorescent staining and flow cytometric analysis of antigen-specific T cells.

The MHC gene locus encodes a group of highly polymorphic, cell-surface proteins that play a broad role in the immune response to protein antigens. MHC molecules function by binding and presenting small antigenic protein fragments to antigen-specific receptors expressed by T cells (TCR). Human (*human leukocyte antigen/HLA*) and mouse (*histocompatibility 2/H-2*) MHC molecules are structurally and functionally related proteins that comprise two major classes. Class I MHC molecules consist of two separate polypeptide chains. The class I α chain is an MHC encoded, transmembrane polypeptide containing three extracellular domains: $\alpha 1$, $\alpha 2$, and $\alpha 3$. The second chain consists of a non-MHC encoded polypeptide called $\beta 2M$. Since $\beta 2M$ does not contain a transmembrane domain, it associates with the α chain through noncovalent interaction. Functionally, class I MHC molecules can bind peptides derived from intracellular antigens (eg, viral and some bacterial antigens) that are specifically recognized by CD8+ T cells. Class II MHC molecules consist of two different transmembrane proteins that can bind peptide fragments derived from extracellular proteins (eg, bacteria and fungi) and are specifically recognized by CD4+ T cells. TCR recognize both processed peptides bound to MHC, as well as regions of the MHC molecule itself. CD4 and CD8 accessory molecules strengthen formation of the TCR-MHC complex through their interaction with non-polymorphic regions of the MHC molecule.



Schematic representation of the MHC class I:Ig dimeric protein.



Flow cytometric analysis of a T-cell hybridoma using DimerX I H-2Db:Ig. Mouse DimerX I H-2Db:Ig was incubated with a 160-molar excess of a specific peptide MOG35-5 (MEVGWYRSPFSRVVHLYRNGK, Panel A) or irrelevant peptide Influenza A34 NP 366-374 (ASNENMETM, Panel B) at 37°C overnight. Peptide-loaded H-2Db:Ig was then used for the immunofluorescent staining of MOG TH10 hybridoma cells, along with FITCconjugated anti-mouse CD3e mAb 145-2C11 (Cat. no. 553061/553062) followed by PE-conjugated anti-mouse IgG1 mAb A85-1 (Cat. no. 550083). Flow cytometry was performed on a BD FACSCalibur™ flow cytometry system.

Preparation and Storage

Store undiluted at 4°C.

The H-2D[b] protein was expressed together with human $\beta 2M$ in the mouse plasmacytoma cell line, J558L (ATCC TIB-6). The H-2D[b] and $\beta 2M$ polypeptide chains are associated noncovalently as a consequence of their coexpression within J558L cells.

The H-2D[b]:Ig fusion protein was purified from tissue culture supernatant by affinity chromatography. The purity of the preparation was confirmed by SDS-PAGE.

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Application Notes

Application

Flow cytometry	Routinely Tested
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Recommended Assay Procedure:

This H-2D[b]:Ig fusion protein has been tested by immunofluorescent staining ($\leq 4 \mu\text{g}$ H-2D[b]:Ig/million cells) (see Figure 2) and flow cytometric analysis of antigen-specific T cells to assure specificity and reactivity. It is necessary to load the H-2D[b] portions of the dimeric protein with a relevant peptide of interest prior to immunofluorescent staining of T cells. H-2D[b]:Ig complexes are effectively loaded by incubation with excess relevant (specific) or irrelevant (control) peptides (see Protocol 1). Peptide-loaded H-2D[b]:Ig may be used for immunofluorescent staining (see Protocol 2). Since applications vary, each investigator must determine dilutions appropriate for individual use.

Protocol 1: Peptide Loading of H-2D[b]:Ig Dimeric Protein

Several peptide-loading protocols have been described. The method used at BD Biosciences Pharmingen involves passive loading of excess peptide in solution with H-2D[b]:Ig protein. We have found that passive loading works particularly well in the case of high-affinity peptides. For lower-affinity peptides, an increase in the molar ratio of peptide to H-2D[b]:Ig may improve loading, as determined by flow cytometric analysis. It is suggested that for each peptide, parameters such as the dose of H-2D[b]:Ig per million cells, molar ratio of peptide to H-2D[b]:Ig, and peptide loading time be determined empirically by the investigator. Parameters for peptide binding to H-2D[b] have been reported, including those peptides routinely used at BD Biosciences Pharmingen for evaluation of H-2D[b]:Ig. While this DimerX product contains $\beta 2$ Microglobulin, for investigators requiring excess recombinant Human $\beta 2$ Microglobulin, we recommend BD Biosciences Cat. no. 551089.

Peptide preparation and loading:

1. The molecular weight (MW) of a peptide of interest will need to be determined. A peptide's MW can be estimated by multiplying its number (n) of amino acids (AA) by 130 daltons (d) per amino acid:
MW of peptide (d) = n (AA) x 130 (d/AA)
2. A stock of peptide may be prepared at 20 mg/ml in DMSO. Dilute the peptide solution to 2 mg/ml in sterile DPBS, pH 7.2 for use in the H-2D[b]:Ig loading protocol.
3. Mix H-2D[b]:Ig protein with specific or control peptide at 40, 160, or 640 molar (M) excess.

The following calculation, using an 8 amino acid peptide (8mer) as an example, may be used:

Dp = Molecular Weight of peptide: eg, 8 amino acids x 130 = 1,040 daltons.

DDb = Molecular Weight of H-2Db:Ig = 250,000 daltons.

R = desired excess molar ratio, eg, 160.

Mp = micrograms (μg) peptide of interest.

Mdb = micrograms (μg) H-2D b:Ig in the reaction. A typical amount of peptide-loaded H-2Db:Ig to use for flow cytometry staining is 0.25 to 4 μg /million cells (test).

$$\text{Mp} = \text{Mdb} \times \text{R} \times \text{Dp} \div \text{DDb} = 4 \mu\text{g} \times 160 \times 1,040 \text{ d} \div 250,000 \text{ d} = 2.66 \mu\text{g}$$

Therefore, one would add 2.66 μg of peptide and 4 μg of H-2D[b] :Ig in solution for the optimal peptide loading of H-2D[b]:Ig.

Mix peptide and H-2D[b]:Ig together in PBS, pH 7.2, incubate at 37°C overnight. The peptide-loaded H-2D[b]:Ig can be stored at 4°C for up to 1 week.

Protocol 2: Immunofluorescent Staining Protocol

1. Prepare peptide-loaded H-2D[b] protein staining cocktail by mixing 0.25 -4 μg of peptide-loaded H-2D[b] protein/test with 0.25 -4 μg of PE-conjugated A85-1 mAb (anti-mouse IgG1, Cat. no. 550083)/test at a ratio of 1:1 or 1:2 of dimer:A85-1 mAb. Incubate the mixture for 60 minutes at RT, protect from exposure to light.
2. Add 0.25 - 4 μg of purified mouse IgG1 isotype control mAb A111-3 (Cat. no. 553485)/test to the staining cocktail (see Step 1 above). Incubate the staining cocktail for 30 minutes at RT, protect from exposure to light.
3. Resuspend mouse cells in FACS staining buffer [eg, DPBS, 1% FCS, 0.09% NaN3 or BD Pharmingen™ Stain Buffer (FBS), Cat. no. 554656], containing the appropriate amount of Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (Cat. no. 553141/553142), at a concentration of approximately 10^6 cells per 50 μl . Incubate 10 minutes at 4°C. Add $\sim 1 \times 10^6$ cells per staining tube (eg, 12 x 75 mm tube, BD Falcon™ Cat. no. 352008).
4. Add 50 μl FACS buffer containing the optimal per test amount of the staining cocktail to each sample, plus any other cell-surface marker-specific antibodies to be used. Incubate 60 minutes at 4°C.
5. Wash cells 2 \times with 2 ml FACS buffer, centrifuge for 5 minutes at 250 \times g, and discard supernatant. Resuspend cell pellet in approximately 0.5 ml staining buffer in a tube appropriate for the flow cytometer.

Protocol 3: Alternative: Immunofluorescent Staining Protocol

1. Resuspend mouse cells in FACS staining buffer [eg, DPBS, 1% FCS, 0.09% NaN3 or BD Pharmingen™ Stain Buffer (FBS), Cat. no. 554656], containing the appropriate amount of Mouse BD Fc Block™ purified anti-mouse CD16/CD32 mAb 2.4G2 (Cat.

- no. 553141/553142), at a concentration of approximately 10^6 cells per 50 μ l. Incubate 10 minutes at 4°C. Add $\sim 1 \times 10^6$ cells per staining tube (eg, 12 x 75 mm tube, BD Falcon™ Cat. no. 352008).
2. Add 0.25 to 4 μ g of peptide-loaded H-2D[b]:I γ protein to cell suspension. Incubate 60 minutes at 4°C.
 3. Wash cells $1 \times$ with 2 ml FACS buffer, centrifuge for 5 minutes at $250 \times g$, and aspirate supernatant.
 4. Resuspend cells in 100 μ l FACS buffer containing appropriately diluted fluorescent secondary reagent. We typically use PE-conjugated A85-1 mAb (anti-mouse IgG1, Cat. no. 550083). Incubate 30 - 60 minutes at 4°C.
 5. Wash cells $2 \times$ with 2 ml FACS buffer, centrifuge for 5 minutes at $250 \times g$, and discard supernatant. Resuspend cell pellet in approximately 0.5 ml staining buffer in a tube appropriate for the flow cytometer.

Product Notices

1. Please refer to www.bdbiosciences.com/pharming/en/protocols for technical protocols.
2. Since applications vary, each investigator should titrate the reagent to obtain optimal results.
3. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

References

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