FIDIS™ HUMAN CYTOKINE DEATH RECEPTOR THREE-PLEX

REF **MX HC0306** *For Research Use Only*

English

INTRODUCTION

Advances in the field of cell biology have defined a complex and interdependent set of extracellular and intracellular signaling molecules that control normal cell function. Perturbations in signaling pathways may be important indicators, and possibly the root cause, of many diseases. Therefore, there is growing interest among clinicians as well as drug discovery groups in monitoring multiple components of signaling pathways simultaneously. Solid phase multiplex protein assays have become the primary tools of choice in these studies as they maximize efficiency by permitting several analytes to be profiled within individual samples.

BMD's Multiplex Bead Immunoassays are solid phase protein assays which use spectrally encoded antibody-conjugated beads as the solid support. The spectral beads are suitable for use in singleplex assays or may be mixed for multiplex assays according to the researcher's needs. Each assay is carefully designed and tested to assure that sensitivity, range and correlation are maximized. The assay is performed in a 96 well plate format and analyzed with a FIDIS™ instrument which monitors the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. Standard curves generated with this assay system extend over several orders of magnitudes of concentrations, while the sensitivity and quantitation of the assays are comparable to ELISAs (Enzyme Linked-Immuno-Sorbent Assays). Assay standards are calibrated to NIBSC reference preparations, when available, to assure accurate and reliable results.

This kit provides a series of combined reagents for the simultaneous measurement of three different human death receptors, including TNF-RI, TNF-RII, and DR5.

The 3-Plex assay is designed for the *in vitro* quantitative determination of the designated three analytes. The 3-Plex may also be combined with other extracellular bead reagents, allowing higher multiplexing of the assay. Samples may include serum, plasma, or tissue culture supernatant.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.

PRINCIPLE OF THE METHOD

BMD's Multiplex Bead Immunoassays are solid phase sandwich immunoassays, which are designed to be analyzed with a FIDIS™ instrument. The spectral properties of 100 distinct bead regions can be monitored with the FIDIS™ instrument, a capability that affords this assay system the potential for measuring up to 100 different analytes in a single sample. Beads of defined spectral properties conjugated to analyte specific capture antibodies and samples (including standards of known analyte concentration, control specimens, and unknowns) are pipetted into the wells of a filter bottom microplate and incubated for 2 hours. During this first incubation, analytes bind to the capture antibodies on the beads. After washing the beads, analyte-specific biotinylated detector antibodies are added and incubated with the beads for 1 hour. During this second incubation, the analyte-specific biotinylated detector antibodies recognize their epitopes and bind to the appropriate immobilized analytes. After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (Streptavidin-RPE), is added and incubated for 30 minutes. During this final incubation, the Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich. After washing to remove unbound Streptavidin-RPE, the beads are analyzed with the FIDIS™ instrument. By monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin (RPE) fluorescence, the concentration of one or more analytes can be determined.

REAGENTS PROVIDED

Note: *Store all reagents at 2 – 8°C.*

- 1 vial of Hu Cytokine Death Receptor Three-Plex Bead Concentrate (10x). Contains 7.5 mM sodium azide; 0.25mL per vial.
- 1 vial of Hu Cytokine Death Receptor Three-Plex Biotinylated Antibody Concentrate (10x). Contains 15 mM sodium azide; 1mL per vial.
- 2 vials of Hu Cytokine Death Receptor Standard.
- 1 bottle of Wash Solution Concentrate (20x); 15 mL per bottle.
- 1 bottle of Assay Diluent. Contains 15 mM sodium azide; 15mL per bottle.
- 1 bottle of Incubation Buffer. Contains 15 mM sodium azide; 12mL per bottle.
- 1 bottle of Biotin Diluent. Contains 3.3 mM thymol; 12mL per bottle.
- 1 vial of Streptavidin-RPE Concentrate (10x). Contains 15 mM sodium azide; 1mL per vial.
- 1 bottle of Streptavidin-RPE Diluent. Contains 3.3 mM thymol; 12 mL per bottle.
- 1 plate of Filter plate, 96 wells per plate (Extra plates can be purchased from Millipore/Fischer Cat. # MSBVN-1250.)

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

- FIDIS™ system. Please contact Bmd for instrument and software placement services.
- Filtration manifold for bead washing (e.g., Millipore, Cat. # MAVM 096 0R; Qiagen, Cat. # 9014579).
- Data analysis and graphing software program.
- Sonicating water bath (e.g., Cole Parmer, Cat. # 08849-00).
- Orbital shaker (e.g., Fischer, Cat. # 14-271-9).
- Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable.)
- Distilled or deionized water.
- Glass or plastic tubes.
- Beakers and graduated cylinders in various sizes.
- Aluminum foil.
- Extra filter plate covers.
- Adhesive plate covers.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. This 3-Plex assay may be combined with other Extracellular Antibody Bead Kits for higher level multiplexing. See the **INFORMATION SHEET and APPENDIX I** for further information. To ensure that several Antibody Bead Kits are compatible in a multiplexed assay, verify that the bead region for each analyte (stated on the **INFORMATION SHEETS** in the Antibody Bead Kits used) is unique.
2. Do not freeze any component of this kit. When not in use, kit components should be stored at 2 to 8°C. All reagents should be brought to room temperature before use.
3. **The fluorescent beads are light-sensitive.** Protect the beads from light to avoid photobleaching of the embedded dye. Aluminum foil should be used to cover test tubes used in the assay. Filter plates containing beads should be shielded with an aluminum foil-wrapped plate cover. The amber vial does not provide full protection, and should therefore be kept covered in the box when not in use.
4. Do not expose beads to organic solvents.
5. Do not use reagents after kit expiration date.
6. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
7. Do not invert the filter plates during the assay. The filter plates are designed to be used in conjunction with a vacuum manifold (DO NOT EXCEED 5 psi) and emptied from the bottom.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This method ensures equal incubation times for all wells.
9. Avoid touching the filter plate membrane with pipette tips to prevent tearing.
10. Do not place filter plates on absorbent paper towels during loading or incubations, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.

PREPARATION OF WASH SOLUTION

Upon storage at 2 to 8°C, a precipitate may form in the 20x *Wash Solution Concentrate*. If this occurs, warm the 20x *Wash Solution Concentrate* to 37°C and vortex until the precipitate is dissolved.

The *Wash Solution Concentrate* is provided as a 20x concentrate. To prepare the Working Wash Solution for use with a 96 well plate, transfer the entire contents of bottle to a 500 mL container and add 285 mL of deionized water. If not using an entire 96 well plate, smaller volumes of 1x Working Wash Solution can be made by mixing 1 part of 20x concentrate with 19 parts deionized water.

The Working Wash Solution is stable for up to two weeks when stored at 2 to 8°C.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect assay outcome. All washing must be performed with the Wash Solution provided. All phases of the assay, including incubation steps, washing steps, and loading the beads into the FIDIS™ instrument, are performed in the filter bottom plate provided. Unused wells may be left dry during the assay and used at a later time.

1. To wash the beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum. The vacuum setting should be adjusted so 3 seconds are required to empty 0.2 mL solution from the wells. (DO NOT EXCEED 5 psi.) Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface.
2. If solution remains in the wells during vacuum aspiration **DO NOT DETACH THE BOTTOM OF THE 96 WELL FILTER PLATE.** In some cases, minor clogs in the filter plate may be dislodged by carefully pressing the bottom of the plate under the clogged well with the pointed end of a 15 mL plastic conical tube.
3. Following the final aspiration step, gently blot the bottom of the filter plate on clean paper towels to remove residual liquid.

PREPARATION OF SAMPLE

1. Serum, plasma, and tissue culture medium samples have been evaluated with BMD's Multiplex Bead Assays. When possible, avoid the use of hemolyzed or lipemic sera. Suitable sample types are defined on the **INFORMATION SHEET** included in this kit.
2. Samples should be collected in pyrogen/endotoxin-free polypropylene tubes.
3. Samples should be analyzed shortly after collection or frozen. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
4. All samples need to be clarified by centrifugation (14,000 x g for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates.
5. Samples with concentrations that exceed the standard curve should be diluted and reanalyzed. Serum or plasma samples should be diluted in *Assay Diluent*. Tissue culture supernatants should be diluted in the corresponding tissue culture medium.

REAGENT PREPARATION

This 3-Plex kit may also be used in conjunction with other bead reagents allowing for a higher level of multiplexing (please see **APPENDIX I** for preparation).
Prior to starting the assay, prepare standard and antibody conjugated beads in accordance with instructions below, then proceed to **ASSAY PROCEDURE**, **Step 1**.

- **Preparation of Standard**

Reconstitute the protein standard within one hour of performing the assay. All standards are calibrated to NIBSC preparations, when available. Additional standards are available from BMD. The standard included in this kit is provided as a premixed set of related markers. The concentrations of the protein components of the standard are indicated on the **INFORMATION SHEET** included in this kit. Standard dilutions may be performed in glass or plastic tubes.

When using serum or plasma samples, reconstitute the standard with *Assay Diluent* provided.

If using other sample types (e.g., tissue culture supernatant), reconstitute the standard with a mixture composed of 50% *Assay Diluent* and 50% of the matrix which most closely matches the sample type (50%/50% mixture).

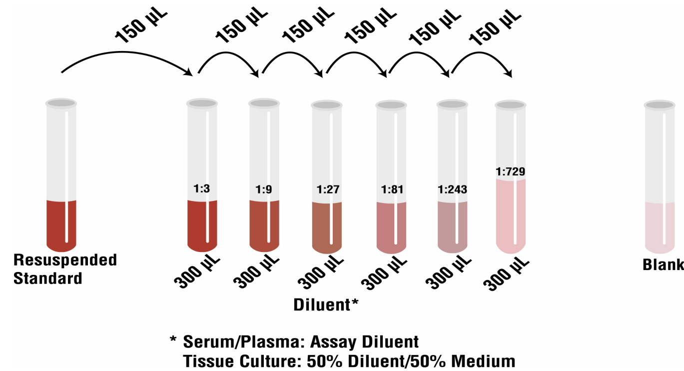
For example: When the sample type is RPMI medium containing 5% FBS, the standards should be reconstituted in a mixture composed of 50% *Assay Diluent* and 50% RPMI containing 5% FBS.

Protein standards may be analyzed alone, or may be combined with other protein standards for higher levels of multiplexing. **DO NOT COMBINE MORE THAN 4 VIALS.**

One vial of standards	Reconstitute the standard vial in the suggested reconstitution volume, usually 1 mL, of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes.
Two vials of standards	Reconstitute each vial with 0.5 mL of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes, mix gently. Combine equal volumes from each vial.
Three vials of standards	Reconstitute each vial with 0.333 mL of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes, mix gently. Combine equal volumes from each vial.
Four vials of standards	Reconstitute each vial with 0.250 mL of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes, mix gently. Combine equal volumes from each vial.

- **Prepare the Standard Curve**

The standard curve is made by serially diluting the reconstituted standard in *Assay Diluent* (serum and plasma samples) or 50% *Assay Diluent*/50% culture medium. See diagram below.



Discard all remaining reconstituted and diluted standards after completing assay. Return the *Assay Diluent* to the refrigerator.

- **Preparation of antibody conjugated beads**

Determine the number of wells required for the assay. Standard curves and samples may be run singly or in replicates, as desired.

The beads are provided as a 10x concentrate. Immediately before dispensing, vortex the 10x bead concentrate for 30 seconds, then sonicate for 30 seconds. To make a 1x capture bead stock, mix 1 part 10x bead concentrate with 9 parts Working Wash Solution. Each well requires 25 µL of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Capture Beads	Vol. Working Wash Solution	Total Vol.
16	0.05 mL	0.45 mL	0.5 mL
24	0.07 mL	0.63 mL	0.7 mL
32	0.09 mL	0.81 mL	0.9 mL
40	0.11 mL	0.99 mL	1.1 mL
48	0.13 mL	1.17 mL	1.3 mL
56	0.15 mL	1.35 mL	1.5 mL
64	0.17 mL	1.53 mL	1.7 mL
72	0.19 mL	1.71 mL	1.9 mL
80	0.21 mL	1.89 mL	2.1 mL
88	0.23 mL	2.07 mL	2.3 mL
96	0.25 mL	2.25 mL	2.5 mL

NOTE: We included 4 extra volumes in our calculations to assure enough reagent when pipetting.

Assay Procedure

1. Pre-wet the wells designated for the assay. Pipette 0.2 mL of Working Wash Solution into designated wells. Wait 15 to 30 seconds then aspirate the Working Wash Solution from the wells using the vacuum manifold. Wells not used during the assay should be kept dry for future use. An adhesive plate cover may be used to seal the unused wells.
2. Vortex the diluted bead solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.
3. Pipette 25 µL of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then remove the Working Wash Solution from the wells by aspiration with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
5. Pipette 50 µL *Incubation Buffer* into each well.

6. To the wells designated for the standard curve, pipette 100 µL of appropriate standard dilution.
7. To the wells designated for the sample, pipette 50 µL *Assay Diluent* followed by 50 µL sample.
8. Incubate the plate for 2 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500-600 rpm).
9. Ten to fifteen minutes prior to the end of this incubation, prepare the biotinylated detector antibody in accordance with instructions below, then proceed to **ASSAY PROCEDURE**, **Step 10**.

- **Preparation of Detector Antibody**

The biotinylated detector antibody is provided as a 10x concentrate. To make a 1x stock, dilute 1 part *Biotinylated Detector Antibody Concentrate* with 9 parts *Biotin Diluent*. Each well requires 100 µL of the diluted biotinylated detector antibody. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Biotinylated Detector Antibody	Vol. Biotin Diluent	Total Vol.
16	0.200 mL	1.8 mL	2.00 mL
24	0.280 mL	2.52 mL	2.80 mL
32	0.360 mL	3.24 mL	3.60 mL
40	0.440 mL	3.96 mL	4.40 mL
48	0.520 mL	4.68 mL	5.20 mL
56	0.600 mL	5.4 mL	6.00 mL
64	0.680 mL	6.12 mL	6.80 mL
72	0.760 mL	6.84 mL	7.60 mL
80	0.840 mL	7.56 mL	8.40 mL
88	0.920 mL	8.28 mL	9.20 mL
96	1.00 mL	9.00 mL	10.00 mL

NOTE: We included 4 extra volumes in our calculations to assure enough reagent when pipetting.

10. After the 2 hour capture bead incubation, remove the liquid from the wells by aspiration with the vacuum manifold. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
11. Add 100µL of prepared 1x Biotinylated Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).
12. Ten to fifteen minutes prior to the end of the detector incubation step, prepare the Streptavidin-RPE in accordance with instructions below, then proceed to **ASSAY PROCEDURE**, **Step 13**.

- **Preparation of Streptavidin-RPE**

The Streptavidin-RPE is provided as a 10x concentrate. To prepare a 1x stock, dilute 1 part *Streptavidin-RPE Concentrate* with 9 parts *Streptavidin-RPE Diluent*. Prepare fresh and store in a foil wrapped tube and use within 15 minutes of preparation. Each well requires 100 µL of the diluted Streptavidin-RPE. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Streptavidin-RPE	Vol. Streptavidin RPE Diluent	Total Vol.
16	0.200 mL	1.8 mL	2.00 mL
24	0.280 mL	2.52 mL	2.80 mL
32	0.360 mL	3.24 mL	3.60 mL
40	0.440 mL	3.96 mL	4.40 mL
48	0.520 mL	4.68 mL	5.20 mL
56	0.600 mL	5.40 mL	6.00 mL
64	0.680 mL	6.12 mL	6.80 mL
72	0.760 mL	6.84 mL	7.60 mL
80	0.840 mL	7.56 mL	8.40 mL
88	0.920 mL	8.28 mL	9.20 mL
96	1.00 mL	9.00 mL	10.00 mL

NOTE: We included 4 extra volumes in our calculations to assure enough reagent when pipetting.

13. Remove the liquid from the wells by aspiration with the vacuum manifold. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
14. Add 100 µL of prepared 1x *Streptavidin-RPE* to each well and incubate the plate for 30 minutes at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500-600 rpm).
15. Prepare the FIDIS™ instrument during this incubation step. Please see **APPENDIX II.**
16. Remove the liquid from the wells by aspiration with the vacuum manifold (DO NOT EXCEED 5 psi). Wash the beads by adding 0.2 mL Working Wash Solution to the wells; allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step two additional times for a total of 3 washes.
17. Add 100 µL of Working Wash Solution to each well. Shake the plates on an orbital shaker (500-600 rpm) for 2-3 minutes to resuspend the beads. If the plates cannot be read on the day of the assay, they may be covered and stored in a dark location overnight at 2 to 8°C for reading the following day without significant loss of fluorescent intensity. Aspirate Working Wash Solution from stored plates and add 100 µL fresh Working Wash Solution. Place the plate on an orbital shaker 2-3 minutes prior to analysis.
18. Uncover the plate; insert the plate into the XY platform of the FIDIS™ instrument, and analyze the samples.
19. Determine the concentration of samples from the standard curve using curve fitting software. The four parameter algorithm usually provides the best fit. **IMPORTANT:** In addition to dilutions performed on the sample prior to running the assay, the sample concentration calculated from the standard curve must be multiplied by an additional factor of 2 to correct for the 1:2 dilution in **Step 7**.

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the highest standard point; the dose-response is non-linear in this region and inaccurate. Dilute samples that are greater than the highest standard with *Assay Diluent* or appropriate matrix diluent; reanalyze these samples and multiply results by the appropriate dilution factor. The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum/plasma and tissue culture medium samples have not been thoroughly investigated. The rate of degradation of analytes in various matrices may not have been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies.

This kit is for research use only.

Not for human therapeutic or diagnostic use.

PERFORMANCE CHARACTERISTICS

Please refer to analyte specific **INFORMATION SHEET** for performance claims.

APPENDIX I:

MULTIPLEXING ASSAYS

Before mixing plexes it is important to check that each analyte is represented by a unique bead region. Up to 10 bead concentrates (singleplexes or pre-mixed multiplexes) can be combined to increase the number of analytes being monitored.

- **Capture bead preparation**

  Calculation:

  [Total Volume] - [(Vol. 10x beads) x (# of plexes)] = Vol. Working Wash Solution

  Total Volume = (0.025 mL x (# of wells + 4))
  Volume 10x bead concentrate = (Total volume)/10

  Example Calculation: Combining 5 plexes using 48 wells

  [(0.025 mL x (48 + 4))] - [(0.13 mL) x (5)] = 0.65 mL

If desired, premixed beads can be stored at 2 - 8°C until the expiration date printed on the kit box.

- **Biotinylated Antibody preparation**

  Calculation:
  [Total Vol. needed] – [(Vol. 1x *Biotintylated Detector Antibody*) x (# of *Biotinylated Antibody Concentrates*)] = Biotin Diluent

  Total Volume = (0.1 mL x (# of wells + 4))
  Volume 10x *Biotintylated Detector Antibody* = (Total volume)/10

  Example Calculation: Combining 5 plexes using 48 wells
  (0.1 mL x (48 + 4)) – [(0.520 mL) x (5)] = 2.6 mL

If desired, premixed *Biotinylated Detector Antibody* can be stored at 2 - 8°C until the expiration date printed on the kit box.

APPENDIX II:

INSTRUMENT SETUP

- **Helpful Bmd guides for FIDIS™ cytokine users**

- Creating a Cytokine method*, April 2006*

- How to run Cytokine*, April 2006*

REFERENCES

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Pre-wet plate
Add 25µL 1x Antibody Coated Beads
200µL Wash Solution

Wash 1 x 200µL

Sample type:

Standard	Serum/Plasma/Tissue Culture Supernatant
Add 50µL Incubation Buffer	Add 50µL Incubation Buffer
Add 100µL Standard	Add 50µL Assay Diluent
	Add 50µL Sample

Shake for 2 hours at RT in the dark
Wash 2 x 200µL

Add 100µL 1 x Biotinylated Detector Antibody

Shake for 1 hour at RT in the dark
Wash 2 x 200µL

Add 100µL 1 x SAV-RPE

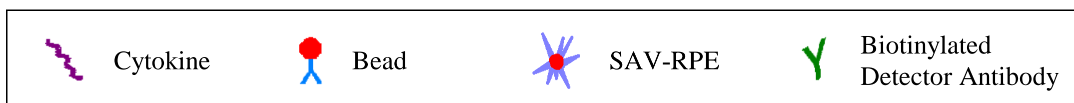
Shake for 30 min. at RT in the dark
Wash 3 x 200µL

Add 100µL Wash Buffer

Shake for 2-3 min

Read in FIDIS™ system

Total time: 3.5 hours



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