NIR-FLIVO® Free Dye Control Assays

Catalog #9113 & #9115

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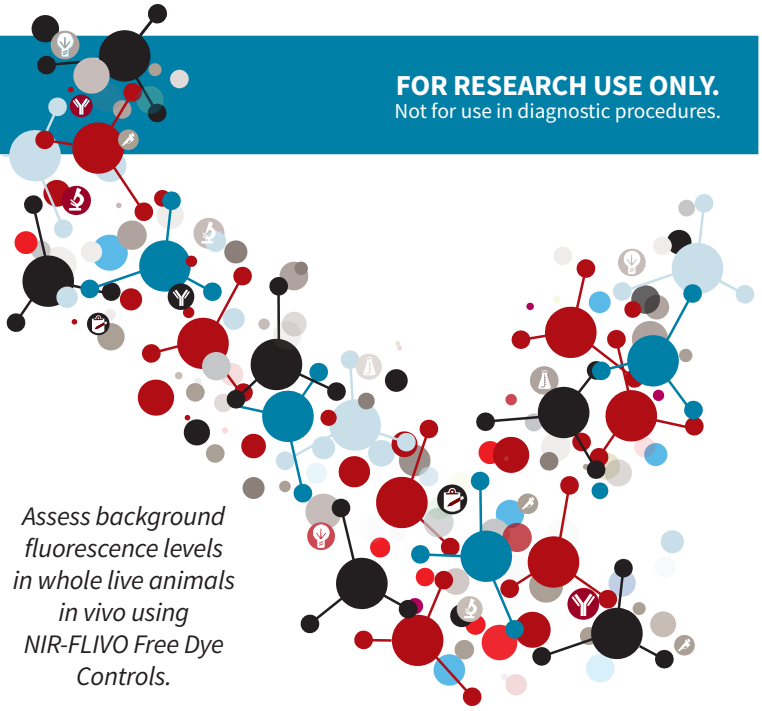
Not for use in diagnostic procedures.



Assess background fluorescence levels in whole live animals in vivo using NIR-FLIVO Free Dye Controls.

1. INTRODUCTION

ICT's near-infrared (NIR)-FLIVO® Tracers are used in conjunction with NIR-FLIVO Free Dye Control Assays. The NIR-FLIVO Free Dye Control Assay uses the NIR fluorescent dye molecule. When injected, both the Free Dye Control and the Tracer reagents will generate a fluorescent signal, but only the Tracer should bind to active caspases and remain inside an apoptotic cell. The fluorescent signal generated from the Free Dye Control reveals where the fluorescent reagent may have become trapped inside the cell, tissues, or body of the animal without specifically binding with an active caspase. In the context of the overall experiment, this base level of fluorescence is considered background noise compared with the signal generated in animals that were injected with the NIR-FLIVO Tracer.

FLIVO (FLuorescence in vIVO) is a powerful method for assessing caspase activity *in vivo*. Like our FLICA® probes[1,2], but optimized for whole live animal imaging, NIR-FLIVO Tracers (sold separately) are non-cytotoxic fluorescent inhibitors of caspases. NIR-FLIVO poly caspase tracers contain the preferred binding sequence for most caspases, Val-Ala-Asp (VAD). This preferred poly caspase tripeptide binding sequence is labeled at the amino terminus end with *Dylight® 690 or 747 NIR label and linked at the carboxyl end to a fluoromethyl ketone (FMK) reactive entity.

Apoptosis is an evolutionarily conserved process of programmed cell suicide. It is centered on a cascade of proteolytic enzymes called caspases that are triggered in response to pro-apoptotic signals. Like most other proteases, caspases are synthesized as pro-form precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity[3]. Active caspase enzymes consist of two large (~20 kD) and two small (~10 kD) subunits that non-covalently associate to form a two heterodimer, tetrameric active caspase[4-6]. Once activated, caspases cleave protein substrates leading to the eventual disassembly of the cell. Caspases have been identified in organisms ranging from *C. elegans* to humans. Mammalian caspases play distinct roles in both apoptosis and inflammation.

NIR-FLIVO Tracer Assays provide a simple yet accurate method to detect caspase activity *in vivo*. To label cells containing elevated levels of active caspases, inject the FLIVO Tracer intravenously and let it circulate. Because the Tracer is cell-permeant, it readily diffuses in and out of all cells it encounters as it circulates throughout the body. If there are active caspase enzymes inside a cell, the Tracer will form an irreversible covalent bond with a reactive cysteine on the large subunit of the caspase heterodimer, thereby inhibiting further enzymatic activity. The bound NIR-FLIVO Tracer will remain inside the cell if the cell membrane is intact. Any unbound FLIVO is removed from the circulation of the animal in about an hour. The remaining NIR fluorescent signal in the tissue is a direct measure of caspase activity that occurred at the time the reagent was injected. Apoptotic cells will retain a higher concentration of FLIVO Tracer and fluoresce brighter than non-apoptotic cells. There is no interference from pro-caspases or inactive forms of the enzyme. If the treatment is causing cell death via apoptosis, apoptotic cells will have an elevated level of caspase activity relative to non-apoptotic or negative control cells and fluoresce near-infrared with FLIVO.

An initial experiment may be necessary to determine when and how much NIR-FLIVO to inject based on the size of the animal, tissue type, experimental conditions, rate of apoptosis, and method of analysis. Generally, the longer FLIVO circulates, the lower the non-specific background signal; however, some apoptotic cells may be lost over time. After 60 minutes, most of the unbound NIR-FLIVO Tracer or Free Dye Control will have cleared the bloodstream. The bound NIR-FLIVO Tracer will remain inside an apoptotic cell and generate a positive signal if the cell membrane is still intact.

Once the animals have been injected with NIR-FLIVO, they are ready for analysis and no further staining is necessary. Because NIR-FLIVO Tracers are a direct stain, it eliminates any false positives that may arise from manipulation of the tissue. This gives a true representation of the induction of apoptosis *in vivo* as a result of the experimental condition. Live animals may be analyzed in a whole animal imager, and /or tissues may be prepared and further analyzed by histological methods. Tissues labeled with NIR-FLIVO can be counter-stained with other reagents such as DAPI and fixed or frozen for future analysis. The fluorescence intensity can be quantified by excising the tissue and analyzing cells with a flow cytometer. NIR-FLIVO 690 Free Dye optimally excites at 690 nm and has a peak emission at 709 nm. NIR-FLIVO 747 Free Dye optimally excites at 747 nm and has a peak emission at 776 nm.

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2. KIT CONTENTS

Catalog #9113 contains:

- 2 vials of NIR-FLIVO 690 Free Dye Control reagent (*Dylight® 690 Free Dye), 10 Tests per vial, 47.7 µg per vial, #6307
- 1 bottle of 10X Injection Buffer, 5 mL, #6220

Catalog #9115 contains:

- 2 vials of NIR-FLIVO 747 Free Dye Control reagent (*Dylight® 747 Free Dye), 10 Tests per vial, 53.6 µg per vial, #6309
- 1 bottle of 10X Injection Buffer, 5 mL, #6220

3. STORAGE

Store the unopened kit and each unopened component at -20°C until the expiration date. Once reconstituted with DMSO, use NIR-FLIVO Free Dye immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

4. SAFETY DATA SHEETS (SDS)

SDS are available at online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute NIR-FLIVO Free Dye
- DiH_20, 45 mL to dilute 10X Injection Buffer
- 0.2 µm syringe filter to sterilize Injection Buffer
- Injection materials such as a syringe and needle
- Experimental and control animals ready to be assessed
- Optional: tools to dissect, extract, and examine labeled tissues
- NIR-FLIVO® 690 /747 Tracer Assays (kits #9112 and #9114, respectively)

6. DETECTION EQUIPMENT

Detection equipment such as instrumentation designed for non-invasive live whole animal imaging, alternatively, excised tissues can be analyzed by fluorescence microscope or flow cytometer.

- NIR-FLIVO 690 Free Dye optimally excites at 690 nm and has a peak emission at 709 nm.
- NIR-FLIVO 747 Free Dye optimally excites at 747 nm and has a peak emission at 776 nm.

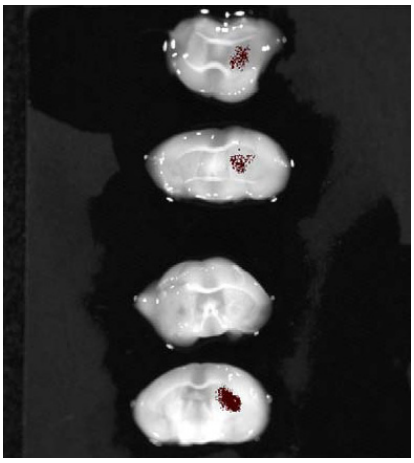
7. EXPERIMENTAL PREPARATION

NIR-FLIVO Tracers are used in conjunction with NIR-FLIVO Free Dye Control Assays. When injected, both the Free Dye Control and the Tracer reagents will generate a fluorescent signal, but only the Tracer should bind to active caspases and remain inside an apoptotic cell. The fluorescent signal generated from the Free Dye Control reveals where the fluorescent reagent may have become trapped inside the cell, tissues, or body of the animal without specifically binding with an active caspase. In the context of the overall experiment, this base level of fluorescence is considered background noise compared with the signal generated in animals that were injected with the NIR-FLIVO Tracer.

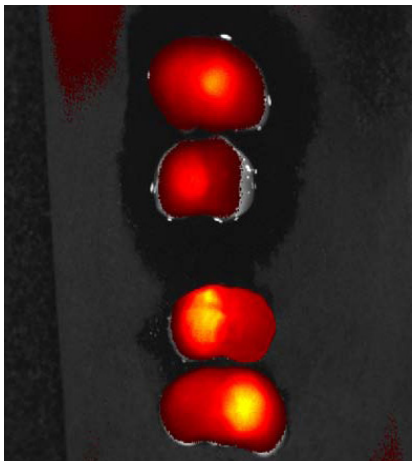
Plan your experiment so that NIR-FLIVO Tracer and Free Dye can be reconstituted, diluted and then injected at the time when caspase

FIGURE 1. DETECT APOPTOSIS IN MURINE BRAIN ABSCESSES USING NIR-FLIVO 690 TRACER

Brain abscesses were induced in mice following the intracerebral inoculation of live *Staphylococcus aureus*. Animals received intravenous injections of NIR-FLIVO 690 (*DyLight® 690-VAD-FMK, kit# 9112) or NIR-FLIVO 690 Free Dye Control (kit #9113) at 17 hours post-infection, whereupon signals were acquired 1 hour later from brain tissues *ex vivo* using an IVIS® Spectrum™ (Caliper Life Sciences). Strong caspase activity was associated with brain abscesses following administration of the NIR-FLIVO 690 tracer (right image), whereas minimal signal was detected in animals injected with the NIR-FLIVO 690 Free Dye Control (left image).



FREE DYE CONTROL



NIR-FLIVO 690 TRACER

activity is expected to be occurring in the animal. It may be necessary to set up an initial experiment to determine when and how much NIR-FLIVO to inject as the resulting positive fluorescent signal is a direct measure of caspase activity that occurred at the time of injection. The amount of NIR-FLIVO may need to be adjusted to accommodate the experimental model and research conditions being investigated.

1. Expose the test animals to your experimental conditions to assess caspase activity in the target tissue(s).
2. Prepare the animals for intravenous NIR-FLIVO injection.

8. PREPARATION OF 1X INJECTION BUFFER

ICT's Injection Buffer is an isotonic solution used for diluting and injecting NIR-FLIVO Free Dye.

1. 10X Injection Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Injection Buffer 1:10 in diH_20. For example, add 1 mL 10X Injection Buffer to 9 mL diH_20 for a total of 10 mL.
3. Sterilize the 1X Injection Buffer by filtering through a 0.2 µm syringe filter or equivalent.
    - 1X Injection Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

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9. PREPARATION OF NIR-FLIVO FREE DYE CONTROL

NIR-FLIVO Free Dyes are supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. Protect from light and use gloves and eye protection when handling. Each vial of NIR-FLIVO 690 Free Dye contains 47.7 µg. Each vial of NIR-FLIVO 747 Free Dye contains 53.6 µg. This amount is typically enough to inject 10 mice or 2 young rats at approximately 300 nanomoles/Kg.

1. Reconstitute each vial of NIR-FLIVO Free Dye with 50 µL DMSO to form the 10X stock solution. The stock solution may have a blue-green color. Once reconstituted, it may be stored at ≤-20°C for 6 months protected from light and thawed no more than twice during that time. Wear gloves and eye protection.
2. Immediately prior to injection into the animal, further dilute the 10X stock NIR-FLIVO Free Dye 1:10 by adding 450 µL sterile 1X Injection Buffer to each vial to form 500 µL of the 1X NIR-FLIVO Free Dye solution. Inject 1X NIR-FLIVO Free Dye within 1 hour of dilution into aqueous buffer; protect from light during handling.

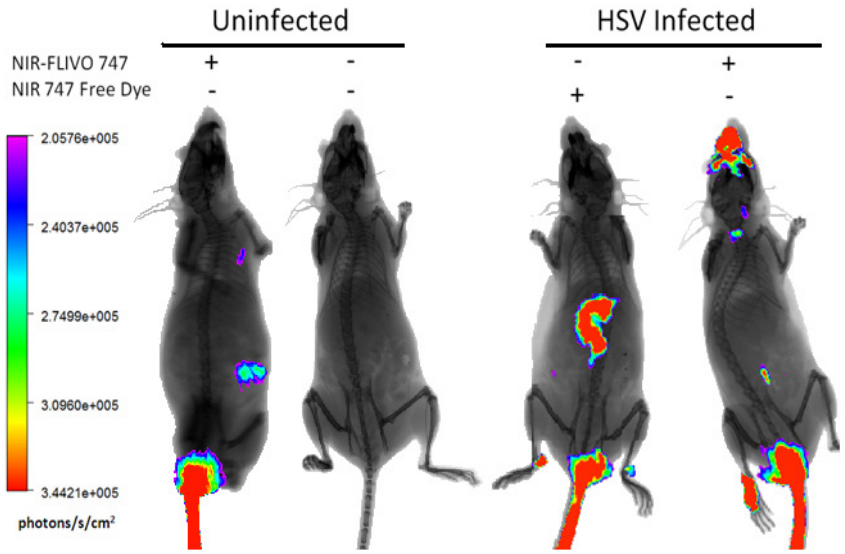
10. INTRAVENOUS INJECTION

The recommended dose of NIR-FLIVO Free Dye is 300 nanomoles/Kg per injection, but the necessary amount will depend on the extent of apoptosis in the animal. Each vial of reagent will test 10 tumor-bearing mice or 2 tumor-bearing young rats at approximately 300 nanomoles/Kg. Animal models that do not involve tumors may require less reagent. An initial experiment may be necessary to determine how much of the NIR-FLIVO Free Dye to inject.

1. Inject 50 µL 1X FLIVO into the tail vein or other large vein. Intraperitoneal or intravitreal injection is not advised. The exact IV

FIGURE 2. NON-INVASIVE IMAGING OF APOPTOSIS RESULTING FROM HSV-1 VIRAL INFECTION

Adult wild-type Balb/c mice were either inoculated with HSV-1 virus, which is known to induce apoptosis in the brain, or given a sham treatment. Seven days after viral inoculation, the mice were injected intravenously with either the NIR-FLIVO 747 Tracer (DyLight® 747-VAD-FMK, kit #9114) to detect caspase activity, the NIR-FLIVO 747 Free Dye (DyLight® 747, kit #9115) to detect where the dye reagent is getting trapped within tissues, or no reagent. The animals were then imaged with a Carestream In Vivo FX PRO imager seven hours after reagent injection. Strong caspase activity was located in the brain of the animal treated with HSV-1 and injected with NIR-FLIVO 747 Tracer (NIR-FLIVO 747 +). Minimal signal was detected in the liver region of the HSV-treated animal injected with the free dye control (HSV-treated; NIR 747 Free Dye +) and of the uninfected mouse injected with NIR-FLIVO 747 Tracer. The liver is the route of clearance for FLIVO Tracers. All mice show fluorescence signal in the tail where reagent is likely to pool after IV injection.



location and amount of NIR-FLIVO Free Dye injected may vary depending on the size of the animal, the target tissue, and the experimental conditions.

2. Optional: If a larger injection volume is preferred, dilute NIR-FLIVO Free Dye with a greater amount of 1X Injection Buffer and inject 1/10th of the final volume into the animal.
3. Allow NIR-FLIVO Free Dye to circulate within the animal for at least 30 minutes. After 60 minutes, most of the Free Dye will have cleared from the bloodstream. Generally, the longer the reagent circulates, the lower the background signal.

11. CIRCULATION

When determining the circulation time, it is important to consider both the reagent clearance rate as well as the rate of cellular apoptosis in the model system. NIR-FLIVO Tracers will clear from the circulating bloodstream within an hour and clear the liver in 3-4 hours. In general, non-specific background signal will decrease with increased circulation time, but caspase-positive cells may be lost over time via natural degradation. The NIR-FLIVO Tracer will be retained inside an apoptotic cell if the cell membrane is intact; it will generate the specific signal. The NIR-FLIVO Free Dye Control should not remain in the body; any remaining signal is background fluorescence. The Free Dye Control should circulate for the same length of time as the Tracer. An initial experiment may be necessary to determine the optimal circulation time, which will vary by the method of analysis and the tissue of interest:

- Analysis by *in vivo* imaging: Allow the NIR-FLIVO to circulate at least 4 hours before imaging. Depending on the rate of cellular apoptosis in the animals receiving the NIR-FLIVO Tracer, the animals should be able to be reinjected with another dose of the Tracer or Control and reanalyzed within 1-4 days if desired.
- Analysis of non-hepatic histological samples *ex vivo:* Allow the reagent to circulate *in vivo* at least 30-60 minutes before preparing samples. Protect samples from light.
- Analysis of hepatic histological samples *ex vivo:* Allow the reagent to circulate *in vivo* at least 4 hours before preparing samples. Protect samples from light.

12. PREPARATION OF ANIMALS FOR LIVE IMAGING

Anesthetize animals according to the experimental protocol and place in optical imaging machine. Consult instrument manufacturer for assistance with equipment set up and data collection.

13. PREPARATION OF HISTOLOGY SAMPLES

Prepare the animal tissues according to your desired protocol. We have listed several methods here:

- Excise the tissue, freeze, and make thin tissue sections for histology.
- Perfuse with formalin or a non-methanol, non-ethanol fixative or embedding agent.
- Extract the tissue and prepare cells for flow cytometry.

14. REFERENCES

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ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

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