

Caution: For Laboratory Use. A research chemical for research purposes only.

## HCA ImagAmp™

### Material Provided

**Kit content:** Each HCA ImagAmp kit contains the following 2 components necessary for signal amplification: Fluorophore Amplification Reagent and 1X Plus Amplification Diluent.

**Kit format:**

	Format*	Catalog #	Kit Components
<b>HCA ImagAmp 488 Kit</b> (Fluorescein)	One 384-well microplate	NEL771001KT	Fluorescein Amplification Reagent (2 vials, green caps) 1X Plus Amplification Diluent, 1 X 15 mL
	Five 384-well microplates	NEL771B001KT	Fluorescein Amplification Reagent (10 vials, green caps) 1X Plus Amplification Diluent, 5 X 15 mL
<b>HCA ImagAmp 546 Kit</b> (Cyanine 3)	One 384-well microplate	NEL774001KT	Cyanine 3 Amplification Reagent (2 vials, red caps) 1X Plus Amplification Diluent, 1 X 15 mL
	Five 384-well microplates	NEL774B001KT	Cyanine 3 Amplification Reagent (10 vials, red caps) 1X Plus Amplification Diluent, 5 X 15 mL
<b>HCA ImagAmp 647 Kit</b> (Cyanine 5)	One 384-well microplate	NEL775001KT	Cyanine 5 Amplification Reagent (2 vials, purple caps) 1X Plus Amplification Diluent, 1 X 15 mL
	Five 384-well microplates	NEL775B001KT	Cyanine 5 Amplification Reagent (10 vials, purple caps) 1X Plus Amplification Diluent, 5 X 15 mL

\*The format of the kit is based on 30 µL per well of Fluorophore Amplification Reagent Working Solution.

### Product Information

**Storage:** Store kits in the dark at 4°C.

**Stability:** The components in the kits are stable for a minimum of 3 months after the delivery date, if stored under proper storage conditions.

**Application:** The HCA ImagAmp kit is used for sensitive detection of markers in High Content Analysis. The reagents in this kit have been optimized for use with adherent cells in 384-well microplates. Cells must be fixed before applying HCA ImagAmp reagents.

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

## Safety Note

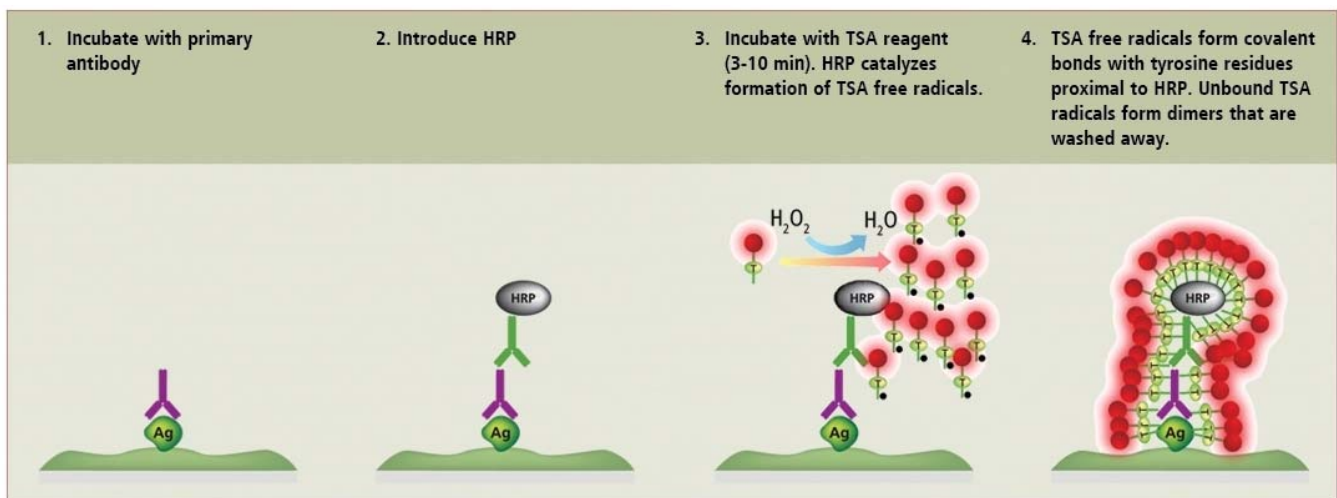
All reagents are classified as nonhazardous. However, it is strongly recommended to wear disposable gloves and safety glasses while working. Thorough washing of hands after handling is also recommended.

## Quality Control

We certify that QC results of these reagents meet our quality release criteria.

## What is HCA ImagAmp?

HCA ImagAmp is based on Tyramide Signal Amplification (TSA™), a technology from PerkinElmer that generally provides 10 to 100-fold of improved sensitivity while allowing reduced consumption of primary antibodies. TSA uses horseradish peroxidase (HRP) to catalyze covalent labeling directly adjacent to the immobilized enzyme. The labeling reaction is quick (less than 10 minutes) and deposited fluorophores can then be detected immediately by standard fluorescence visualization techniques, with significant enhancement of the signal.



## Reagents and Materials

### Critical Reagents Required but not Supplied

- 384-CellCarrier™ microplates (PerkinElmer cat. no. 6007550)
- Cell fixative
- Counterstain (e.g. nuclear dye)
- HRP-labeled reagent, for example:
  - HRP-labeled secondary antibody:
    - Anti-rabbit IgG (goat) HRP (PerkinElmer cat. no. NEF812001EA)
    - Anti-mouse IgG (goat) HRP (PerkinElmer cat. no. NEF822001EA)
- Blocking Reagent (PerkinElmer cat. no. FP1012)
- DMSO (molecular biology or HPLC grade)
- Buffer components and detergents such as PBS, Triton-X100, Tween®-20
- TopSeal™-A NEW Adhesive Sealing Film (PerkinElmer cat. no. 6050195)

## Solutions to prepare

The following buffers and reagents are required for cell fixation, permeabilization and staining as well as fluorescence amplification:

**Fixation Solution:** 3.7% Formaldehyde in 1X PBS pH 7.4

**Permeabilization Solution:** 0.1% Triton X-100 in 1X PBS pH 7.4

**PBS Wash Buffer:** 0.1% Tween<sup>®</sup>-20 in 1X PBS pH 7.4

### **PBS Blocking Buffer: 0.5% Blocking Reagent in 1X PBS pH 7.4**

Add Blocking Reagent slowly in small increments to 1X PBS pH 7.4 while stirring. Heat gradually to 50°C with continuous stirring to completely dissolve the Blocking Reagent (this may take up to several hours). Aliquot and store at -20°C for long term use. Discard any unused PBS Blocking Buffer that has been stored for greater than 24 hours at room temperature. The Blocking Reagent has been found to be optimal for use with HCA ImagAmp kits. The user should validate the use of alternative blocking reagents.

### **Fluorophore Amplification Reagent Stock Solution:**

The Fluorophore Amplification Reagents (Fluorescein, Cyanine 3 or Cyanine 5) are supplied as a solid. Each vial must be reconstituted with 150 µL DMSO to make the Fluorophore Amplification Reagent Stock Solution. Fluorophore Amplification Reagent Stock Solution is stable for at least 3 months when stored at 4°C protected from light. (Note: DMSO freezes at 4°C; therefore, thaw stock solution before each use).

## Recommendations

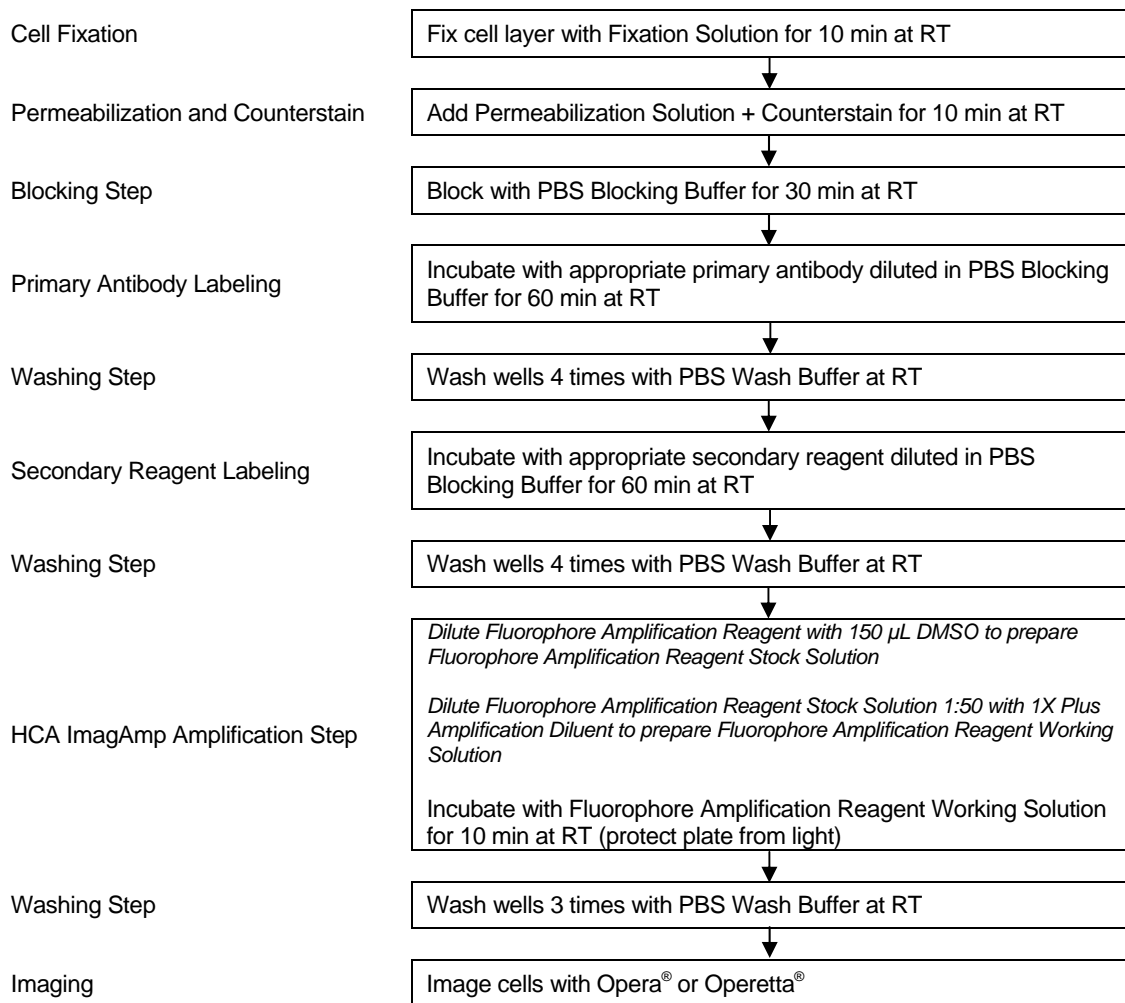
- Remove as much of the incubation solutions as possible before the addition of the next solution to prevent reagent dilution.
- Prevent liquid in wells from completely drying out.
- Always run control wells in each experiment. These should include amplified negative control (i.e. no primary antibody or replace with a non-specific antibody). In addition to proving validity of results, control wells are often beneficial in determining the cause of non-specific background.
- If present, endogenous peroxidases will cause non-specific signal. Endogenous peroxidases may be quenched by a preliminary incubation with 1-3% H<sub>2</sub>O<sub>2</sub> for 30 min.
- Plate must be protected from light during and after incubation with Fluorophore Amplification Reagent Working Solution.
- HCA ImagAmp will provide a more sensitive staining than commonly used fluorescent dyes, but the presence of excess HRP will favor formation of dimers over the labeling reaction, resulting in decreased signal and increased background. Therefore, it is critical to determine the appropriate dilution of primary and secondary antibodies to obtain optimal staining.
- Optimization of the primary and secondary antibody dilutions is highly recommended. Use of the same primary antibody dilution as in an unamplified detection method typically results in excess background with low signal. Suggested optimization steps are described below (primary antibody followed by a secondary HRP-labeled conjugate). All dilutions of primary antibody should be tested with all dilutions of HRP-labeled conjugate in one experiment:
  - Titration of primary antibody dilution:
    - A: Supplier's recommendations
    - B: 5-fold dilution from A
    - C: 5-fold dilution from B
    - D: 5-fold dilution from C
    - Negative control (primary antibody omitted)
  - Titration of HRP-labeled conjugate
    - A: Supplier's recommendations
    - B: 2-fold dilution from A
    - C: 2-fold dilution from B

*N.B. For the secondary HRP-antibodies listed in the Reagents and Materials section, the suggested dilution is 1:1000.*

## Protocol

The protocol below describes a standard labeling process working with a primary antibody followed by a secondary HRP-labeled conjugate. The protocol is based on a working volume of 30  $\mu\text{L}$  per well of Fluorophore Amplification Reagent Working Solution in a 384-well microplate. Optimization of each step can be done as required.

### Overview Protocol for Cell Labeling



### Step by Step Protocol for Cell Labeling

The protocol is based on a working volume of 30  $\mu\text{L}$  per well of Fluorophore Amplification Reagent Working Solution in a 384-well microplate. If working in 96-well microplates, all volumes should be a minimum of 50  $\mu\text{L}$ .

#### Cell Fixation

- Aspirate and discard cell culture medium
- Add 50  $\mu\text{L}$  Fixation Solution per well
- Incubate for 10 minutes at RT
- Aspirate and discard

*N.B. If the protocol needs to be paused for any reason, it can be done at that stage by replacing Fixation Solution with 1X PBS pH 7.4, and store the plate at 4°C.*

### Cell Permeabilization and Counterstaining

- Add 50 µL Permeabilization Solution + counterstain at appropriate dilution per well (ex: Hoechst® 33342 diluted at 2 µg/mL for nuclei staining)
- Incubate for 10 minutes at RT
- Aspirate and discard

*N.B. Certain types of cells are known to have a high level of endogenous peroxidase activity and might benefit from a treatment with 1-3% H<sub>2</sub>O<sub>2</sub> to inactivate endogenous peroxidase activity before proceeding to the next step.*

### Blocking Step

- Add 50 µL PBS Blocking Buffer per well
- Incubate for 30 minutes at RT
- Aspirate and discard

### Primary Antibody Labeling

*N.B. Primary antibody must be diluted to appropriate dilution in PBS Blocking Buffer*

- Add 30 µL primary antibody diluted in PBS Blocking Buffer per well
- Incubate for 1h at RT
- Wash wells 4 times with 50 µL PBS Wash Buffer per well, at RT
- Aspirate and discard each wash

### Secondary Reagent Labeling

*N.B. Secondary labeling reagent must be diluted to appropriate dilution in PBS Blocking Buffer*

- Add 30 µL HRP-labeled reagent diluted in PBS Blocking Buffer per well
- Incubate for 1h at RT
- Wash wells 4 times with 50 µL PBS Wash Buffer per well, at RT
- Aspirate and discard each wash

### HCA ImagAmp Amplification Step

*N.B. Reconstitute each vial of Fluorophore Amplification Reagent supplied in the kit with 150 µL DMSO to prepare Fluorophore Amplification Reagent Stock Solution.*

*Before each amplification with HCA ImagAmp, dilute 1:50 the required amount of Fluorophore Amplification Reagent Stock Solution in 1X Plus Amplification Diluent supplied in the kit, to make the Fluorophore Amplification Reagent Working Solution. Discard any unused portion of Amplification Reagent Working Solution afterwards.*

- Add 30 µL Fluorophore Amplification Reagent Working Solution per well
- Incubate for 10 minutes at RT; protect the plate from light
- Aspirate and discard
- Wash wells 3 times with 50 µL PBS Wash Buffer per well, at RT
- Aspirate and discard each wash. Add 50 µL of 1X PBS pH 7.4 after removing the last wash solution. Seal the wells with a TopSeal-A and protect the plate from light
- Image cells or store plate at 4°C (protected from light) for subsequent imaging

## Imaging

Excitation and emission wavelengths for Fluorescein, Cyanine 3 and Cyanine 5 fluorophores:

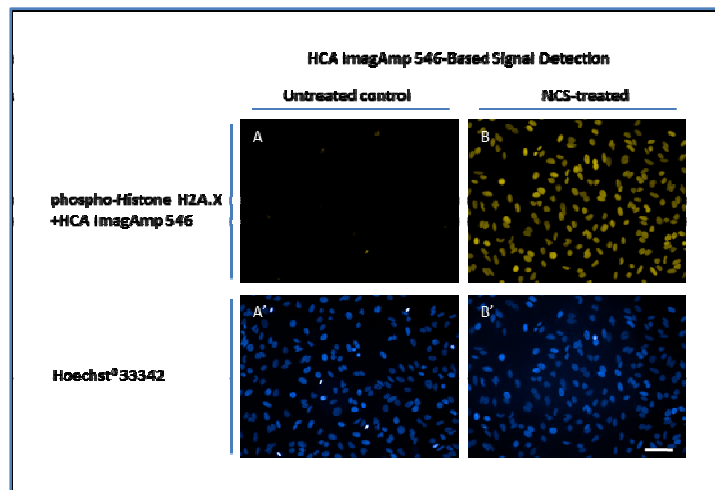
Kit	Fluorophore	Excitation	Emission
HCA ImagAmp 488	Fluorescein	494 nm	517 nm
HCA ImagAmp 546	Cyanine 3	550 nm	570 nm
HCA ImagAmp 647	Cyanine 5	648 nm	667 nm

### Suggested procedure for imaging with the Operetta imaging system

- For imaging, set appropriate filter sets for each channel (e.g. Hoechst<sup>®</sup> 33342 and Fluorescein)
- Start with one well where strong staining of target is expected
- Acquire image in counterstain channel first, then determine appropriate exposure time and focal plane (it is recommended to refer to the histogram of the image to achieve a good intensity distribution and to avoid over- or underexposure)
- Acquire image in target channel, optimize as above
- Take images in several wells to verify that settings are optimal, modify as needed

*N.B. The recommendations above are based on images captured on a PerkinElmer Operetta imaging system. If the user images the plate on another system, these recommendations can serve as guidelines, but need to be revised according to the instrument supplier's recommendations.*

## Typical Data



Staining with a marker for DNA damage using the HCA ImagAmp 546 Kit. HeLa cells were plated in a 384-CellCarrier microplate, treated for 1 hr with 1  $\mu$ g/ml Neocarzinostatin (NCS) and stained according to the protocol described above with the following reagents: Primary antibody: mouse anti-phospho-Histone H2A.X (Ser139), secondary antibody: goat anti-mouse HRP, and HCA ImagAmp 546. Nuclei were counterstained with Hoechst<sup>®</sup> 33342.

A) Control (untreated cells), anti-phospho-Histone H2A.X. A') Hoechst<sup>®</sup> 33342.

B) Cells treated with 1  $\mu$ g/ml NCS, anti-phospho-Histone H2A.X. B') Hoechst<sup>®</sup> 33342.

Images were taken on an Operetta imaging system with a 20x objective and 40 msec exposure time for each channel. Bar: 100  $\mu$ m.

TSA reagents and related methods are covered by U.S. Patents 5,863,748, 6,372,937, 6,593,100, and 6,617,125, related patent applications, and international equivalents thereof.

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