EF5 Compound, 50 MG (CAS 152721-37-4)

Cat. # EF5014-50MG

pack size: 50 mg

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.
NOT FOR HUMAN OR ANIMAL CONSUMPTION.

Store at -20ºC

Data Sheet

Background:

A number of imaging markers which target hypoxia exist and are widely used in research. EF5 (2-(2-Nitro-1*H*-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide) is a compound developed at the University of Pennsylvania by Dr. Cameron Koch and Dr. Sydney Evans. Upon injection into animal tissues, EF5 selectively binds to hypoxic cells and forms adducts. A fluorophore conjugated mouse monoclonal antibody, clone ELK3-51, binds to the EF5 adducts, enabling the detection and measurement of tissue hypoxia in animal and human tumors, normal tissues and cells.

Advantages of the EF5 hypoxia detection method:

- EF5 exists in only one form; Pimidozole, an alternative hypoxia marker, exists in two forms; one of which is charged and very hydrophilic, the other lipophilic. Pimidozole thus has a very complex biodistribution. In contrast, EF5 is lipophilic and uncharged and this allows very rapid and even tissue distribution.
- EF5 binding images can be calibrated to provide quantitative data on the pO2 values of each cell[1]. The fluorescent images obtained from EF5 binding can be calibrated according to camera settings and a "cube-binding" value which is obtained through a separate procedure. The intensity values of calibrated images are directly related to actual tissue pO2 values. As a result, these images provide information regarding not only where hypoxic areas may or may not be, but also data regarding the distribution and levels of hypoxia.

Storage & Handling

EF5 Compound, CAS 152721-37-4: 1 vial containing 50 mg. Store at -20°C.

Representative Data

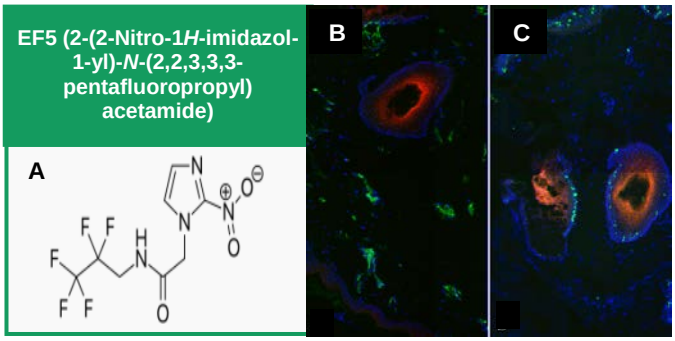

Figure 1. Chemical structure of EF5 (**A**). Human skin demonstrating the relationship between EF5 binding (red, **B**) and blood vessels (green; CD31, **B**) or proliferating cells (green; Ki67; **C**). Blue regions are viable nuclei (Hoechst 33342). Blood vessels (**B**) are not seen within hypoxic regions but are consistently in an inverse location.

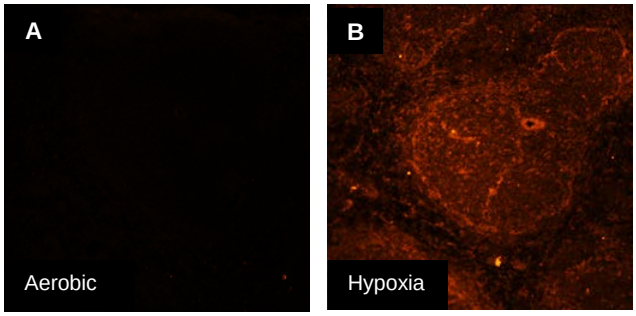

Figure 2. Fluorescence images of aerobic (**A**) and hypoxic (**B**) mouse spleen stained with 75 µg/mL of anti-EF5, clone ELK3-51 Cyanine 3 conjugated antibody.

References:

Koch CJ (2002) Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. *Methods in Enzymology* 352: 3-31.

EF5 is widely published. For a complete listing, please check on our website.

Rev.1.0/2018-01-18/EF5014-50MG/AN

Buffer Recipes

10 mM Injection-Ready EF5 (MW = 302 g/mol)
To prepare 10 mL of 10 mM EF5:

- Dissolve 30 mg EF5 in 0.24 mL of 100% ethanol. EF5 should dissolve readily. Vortex briefly to get EF5 into solution.
- Make a 5% glucose solution by adding 5 grams glucose (Sigma Cat. No. G7021-100G) to 100 mL sterile water. Sterile filter.
- Transfer the EF5 solution to a 15 mL polypropylene tube (don't use polystyrene) containing 9.76 mL of 5% glucose solution. Filter sterilize using Steriflip-GP with a 0.22 µm pore size membrane (Cat. No. SCGP00525).
- **Store at room temperature** and protect from light in amber glass vials or by covering with aluminum foil. Do not store injection ready EF5 in the fridge as it may form very long needle crystals over a period of weeks that may look a little like mold. If you see the crystals, warm briefly at 37°C; the EF5 will quickly redissolve and go back in solution.
- Reconstituted EF5 is stable for at least 1 year at room temperature.

CAUTION: It is very important to **NOT** add the 5% glucose solution directly to the 0.24 mL of 100% ethanol in the amber vial of EF5 drug supplied. Doing this will cause the EF5 drug to precipitate out in the amber vial and make it difficult to transfer into a 15 mL conical tube.

ELK Antibodies (available separately):
ELK antibodies are provided at 2 mg/mL. For optimal IHC tissue staining, ELK antibodies should be diluted to at least 75 µg/mL.

- **Regular Stain:** (ELK3-51 Cyanine 3 conjugate or ELK-51-Alexa Fluor 488 conjugate)
  - To make 400 µL at 75 µg/mL, add 15 µL of ELK3-51 Cy3 or Alexa Fluor 488 antibodies at 2 mg/mL into 385 µL of EF5 Antibody Dilution Buffer with Tween 20 (Cat. No. EF5013-10ML or see formulation below).
- **Competed Stain:** (Regular Stain + 0.5 mM EF5)
  - Competed stain is a negative control for non-specific binding of the ELK3-51 antibody. To conserve the antibody, only one slide of the competed stain is necessary per organ tissue. Below is the calculation for 1 slide at 100 - 150 µL volume.
  - Add 5 – 7.5 µL of 10 mM EF5 solution to 100 – 150 µL Regular Stain. Final concentration of EF5 in competed stain is 0.5 mM.

1X ttPBS (1X PBS with 0.3% Tween 20 and 2 mM sodium azide):
Store at 2-8°C. To prepare 4 liters, add:

- 12 mL of 100% Tween 20
- 0.5 g sodium azide
- Fill up to 4L volume with sterile filtered water.
- Stir until Tween 20 is completely in solution. pH to 7.2 – 7.5.

EF5 Antibody Dilution Buffer with Tween 20 (Cat. No. EF5013-10ML)
To prepare 100 mL, add the following:

- 100 mL of 1X ttPBS to a beaker with a stir bar.
- Slowly add in 1.5 g of lipid-free albumin (Cat. No. 126575-10GM) while stirring. Keep refrigerated.

Blocking Solution
Store at 2-8°C for up to 2-3 weeks
To prepare 8 mL, add the following. Scale up as necessary.

- 1.6 mL of a 10% non-fat dry milk (Blotting-Grade Blocker; Bio-Rad Cat. No. 1706404) made by dissolving 1 g of Blotting-Grade Blocker in 9 mL of 1X PBS.
- 0.4 mL of desired serum (mouse, rat, goat, etc.)
- 0.12 grams of lipid-free albumin (Cat. No. 126575-10GM)
- 6.0 mL 1X ttPBS

Competed ttPBS (1X ttPBS w/ 250 µM EF5)
Store at 2-8°C. To prepare 10 mL, add:

- 0.25 mL of 10 mM EF5 to 9.75 mL of 1X ttPBS.

5-bromo-2'deoxyuridine (BrdU; MW=307 g/mol)
BrdU is light sensitive. Wrap in tin foil.

- For in vivo applications, inject 5 mM BrdU solution with the amount (in mL) equal to 1/100th of the animal weight in grams. For a 25 gram mouse, this amounts to 0.25 mL of 5 mM BrdU.

Hoechst 33342 (MW= 561 g/mol)

- Make 5 mM stock solution by dissolving 2.8 mg in 1X PBS. Aliquot and store at -20°C.
- For tissue sections or cells in suspension, use at 20 µM final concentration.

Peroxidase Reagents
For peroxidase-based staining, it is essential to have final rinses and all reagents mixed in azide-free solutions. These include PBS and ttPBS.

- Make a 3% hydrogen peroxide solution (e.g. 4 mL 30% stock + 36 mL 1X PBS)
- Stain (10 µL of 3% hydrogen peroxide + 40 µL of AEC concentrate in DMF + 950 µL of acetate buffer pH 5.5).
- If crystals are forming in this solution over a typical period of uses (up to 1 hour at RT), increase the volume of acetate buffer.

Protocols

In Vivo Assay
For small animal studies, be sure to include a no EF5 injection control where EF5 has not been injected and a competed stain antibody control.

1. EF5 Dosage

- Make a tail-vein injection using 10 mM drug with the amount (in mL) equal to 1/100 of the animal weight, in grams. For a 25 gram mouse, this amounts to 0.25 mL of 10 mM EF5.
- Freeze tissues in OCT (Tissue-Tek Cat. No. 4583). Store frozen OCT samples at -80°C freezer until ready to section.

2. Obtaining Sections

- Using a cryostat, slice 10 µm sections and place on poly-lysine coated slides. Obtain at least 5 good (minimal folds and rips), preferably sequential sections for simple staining procedure.
- Number slides to keep track of relative distances and depth in tissues.

3. Fix Slides

All rinses are done in staining dishes. Between all rinses and changes of solutions, slides are to be blotted on a paper towel to eliminate carry-over between solutions. Below are various ways to fix slides. Store fixed slides at -80°C if not used immediately.

A. 4% Paraformaldehyde (PFA):
   - Leave slides in 4% PFA for 60 ± 5 min.
   - 2 x 10 min rinses in 1X PBS w/ azide.
   - Blot away moisture and block (see below)

B. Acetone Fixation:
   - Dry slides for at least 30 min.
   - Incubate 5 min in ice cold acetone. Let dry for 30 min.

C. Ethanol Fixation:
   - Fix sections in ice-cold 70% ethanol for 30 minutes.
   - Transfer tissue sections into ice-cold 85% ethanol for 10 minutes and then 5 minutes in ice-cold 100% ethanol.
   - To prevent condensation, transfer to 100% ethanol at RT for approximately 1 minute and then allow to air dry.

4. Blocking

- Blot away moisture around section(s).
- Mark section(s) with a PAP pen (hydrophobic pen).
- Add Blocking Solution.
- Place slides in a staining tray containing a small amount of water covered with a lid.
- Store at 2-8°C overnight.

5. IHC Staining Procedures

NOTE: All rinses are done in staining dishes. Between all rinses and changes of solutions, slides are to be blotted on a paper towel to eliminate carry-over between solutions. All staining procedures and rinses are done in subdued light using cold reagents. Unless otherwise noted slides are refrigerated or kept on ice between steps.

General EF5 Staining

A. 5 slides are recommended

- 2 for EF5 regular stains (RS) with conjugated ELK3-51 antibody.
- 2 for EF5 competed stains (CS) with conjugated ELK3-51 antibody + EF5 drug (Refer to Buffers Recipes Section).
- 1 for no stain (NS) which is left in blocking solution.

NOTE: For IHC, 75 µg/mL working concentration of conjugated antibody is recommended. Use from ~100 – 150 µL of conjugated antibody for a typical section.

B. For regular stain (RS) and competed stain (CS) only:

- Remove blocking buffer from slides by tipping and blotting on a paper towel.
- Dip in 1X ttPBS to rinse
- Blot away moisture with Kimwipes
- Add 100 – 150 µL of the Regular Stain (RS) and Competed Stain (CS) to the appropriate slides and let sit for 4-6 hours in a tin-foil covered staining tray with a lid. Keep refrigerated.

C. General EF5 Staining Rinses

NOTE: the parallel steps are to be completed simultaneously (All rinses should be in opaque or covered staining dishes as slides are now light sensitive).

Regular EF5 Stain (RS)	Competed EF5 Stain	No Stain
Tip to remove stain	Tip to remove stain	Leave in blocking solution
Dip in 1X ttPBS	Dip in 1X ttPBS	Leave in blocking solution
45 minutes 1X ttPBS rinse (use 200-300 µL per rinse)	Add 200-300 µL of 1X ttPBS with 250 µM EF5 onto the tissue slide. Let sit for 2 minutes and then tip to remove. Repeat this rinse for a total of 5 times. Leave the 5th rinse on the slide and allow to sit for 45 minutes in a tin foil covered staining tray.	Leave in blocking solution
45 minutes 1X ttPBS rinse (use 200-300 µL per rinse)	Repeat above step for this rinse	Leave in blocking solution
45 minutes 1X PBS rinse	45 minutes 1X PBS rinse	45 minutes 1X PBS rinse
Add a drop of ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Cat. No. P36935) and cover with a no. 1 cover glass. Alternatively, slide can be stored in an opaque dish containing 1% PFA for a maximum of 2 weeks.	Add a drop of ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher Cat. No. P36935) and cover with a no. 1 cover glass. Alternatively, slide can be stored in an opaque dish containing 1% PFA for a maximum of 2 weeks.	Add a drop of ProLong™ Gold Antifade Mountant with DAPI and (Thermo Fisher Cat. No. P36935) cover with a no. 1 cover glass. Alternatively, slide can be stored in an opaque dish containing 1% PFA for a maximum of 2 weeks.

D. Co-staining with EF5

Note: These co-stained slides are considered qualitative rather than quantitative as the EF5 staining may be affected by the co-stain.

1. Endogenous Hoechst (Administered by blood permeation through vessels before tissues is removed from the animal and stains for vessels. The tissue is then light sensitive so exposure to light must be kept at a minimum for the remainder of the procedure.
   - Section and fix as usual
   - Photograph immediately after fixation
   - Be very careful of photo-bleaching the section by exposing it to too much light while imaging.
   - After imaging, follow the general EF5 staining procedure to co-stain for EF5.

2. CD-31/PECAM (blood vessels), KI-67 (proliferation) and MN75 (CA9)
   - Section, fix and rinse as usual
   - Make blocking reagent appropriate for specific stain and tissue type and block for 30-60 minutes at room temperature.
   - Dip in 1X ttPBS, blot and add primary antibody at 1:100 dilution of antibody to carrier and refrigerate overnight.
   - Tip off antibody, dip in 1X ttPBS
   - 2 x 15 minute rinses in 1X ttPBS at room temperature.
   - 1 x 15 minute rinse in 1X PBS at room temperature.
   - Blot away moisture and add secondary antibody (Cy5 usually) at 1:100 dilution of antibody in carrier for 45-60 minutes at room temperature. From this point on, the slides are light sensitive.
   - 2 x 15 minute rinses in 1X ttPBS at room temperature.
   - 1 x 15 minute rinse in 1X PBS at room temperature
   - Re-fix in 4% PFA for 20-30 minutes at room temperature.
   - 2 x 10 min rinse in 1X ttPBS at room temperature.
   - 1 x 10 minute rinse in 1X PBS at room temperature.
   - Block with mouse block for 1 hour at room temperature.
   - Stain with CY3 conjugated ELK-351 overnight in the refrigerator
   - Rinse as usual (2 x 45 minutes in 1X ttPBS, 1 x 45 minutes in 1X PBS, store in 1% PFA).

3. BrdU and Iurd (Cell proliferation)
   - BrdU or Iurd are given in vivo at 0.1g/kg (300 μM) or in vitro tissue is treated with 10 μM concentration.
   - Place slides in cold (4°C) 70% ethanol.
   - Pour off ethanol and rinse in 1X ttPBS 3 x 15 minutes.
   - Add 50 μL pepsin (5.5 mg crude Sigma 7000 pepsin in 2.75 mL 0.5N HCL/1X PBS dilution of 1:10) (0.2 mg/mL in 0.5N HCL with 10% 10X PBS) to each slide and incubate for 20 minutes at 37°C (warm room).
   - Tip off pepsin and add 150 μL of Borax neutralizing buffer. Do this for 3 exchanges.
   - Chill slides on ice in trays for 1-2 minutes.
   - 2 x 10 min rinses in 1X ttPBS.
   - Add appropriate block and incubate for 30 min at 4°C.
   - Dip in 1X ttPBS.
   - Add primary antibody (1:50 concentration) and leave for 2 hours at 4°C.
   - 2 x 10 min rinse in 1X ttPBS.
   - 1 x 30 min rinse in 1X ttPBS.
   - Add secondary antibody (1:50 – usually FITC) for 30 min at 4°C.
   - 2 x 10 min rinse in 1X ttPBS.
   - 1 x 30 min rinse in 1X ttPBS.
   - Check staining
   - Re-fix in 4% PFA to stabilize
   - Counterstain nuclei with 20 μM Hoechst 33342
   - Block with mouse block for 1 hour and stain for EF5 overnight in the refrigerator.
   - Rinse and store as usual.

FAQ

1. **Can I fix tissue in formalin and use paraffin embedded sections with the EF5 antibody system?**
   Ans. Yes, but the antibody detection system is only validated for frozen tissue samples. Use no more than 24 hr in fresh formalin, with standard antigen retrieval methods. Expect more background and less signal (so reduced contrast).

2. **Do I really need to run the competed antibody controls?**
   Ans. Yes. The competed stain antibody control is the best method available to determine what a 'no EF5' drug control would look like. Some tissues are consistent in this property; but others are quite variable.

3. **I am getting staining, but the background seems to be high. Is there a problem?**
   Ans. This question brings together all aspects of development of a calibrated fluorescent scale, including the use of calibration dye and use of 'no-stain' and competed-stain' controls. In other words, you have to know exactly where you are on the scale to determine whether or not you really have a high background. However, there are a few specific reasons for a true problem with background staining. The main one is the failure to keep the sections moist at all times, especially during photography. Another common source of background is infrared light leaking through the optics. Digital cameras are especially sensitive to IR, but it can also efficiently fog regular film.

4. **How long can my stained slides be kept before loss of signal?**
   Ans. If stored in 1-4% paraformaldehyde, in the dark at 4°C, the signal is stable for at least a month.

5. **There are crystals in the EF5 solution. Is it still usable?**
   Ans. EF5 crystals look like long needles. They will re-dissolve if the solution is heated to 70°C and stirred or sonicated. Any other shape of particulate may be a contaminant (mold, yeast, etc.)

6. **Do I need to inject the EF5 intravenously? Or can I do an i.p. injection?**
   Ans. There is nothing inherently wrong with IP injections. However, you can better assure the drug biodistribution after an IV injection.

7. **I am using nude mice with a human xenograft. Why do you recommend I give the animal an i.v. injection followed by an i.p. injection?**
   Ans. Human cells bind EF5 at a 2-3 fold slower rate than their rodent counterparts. This procedure provides roughly similar overall binding rates for both cell types, but obviously violates the "IV only" injection rule.

8. **Can I do multiple staining with EF5?**
   Ans. Yes, procedures are available for CD31, Ki67, apoptosis, Hoechst, HIF1 alpha, etc. However, treatments which gain access to the DNA and RNA (in situ hybridization; halogenated pyrimidines for cells in 'S' phase) are too harsh to provide normal EF5 antigen binding (both loss of signal and higher non-specific binding).