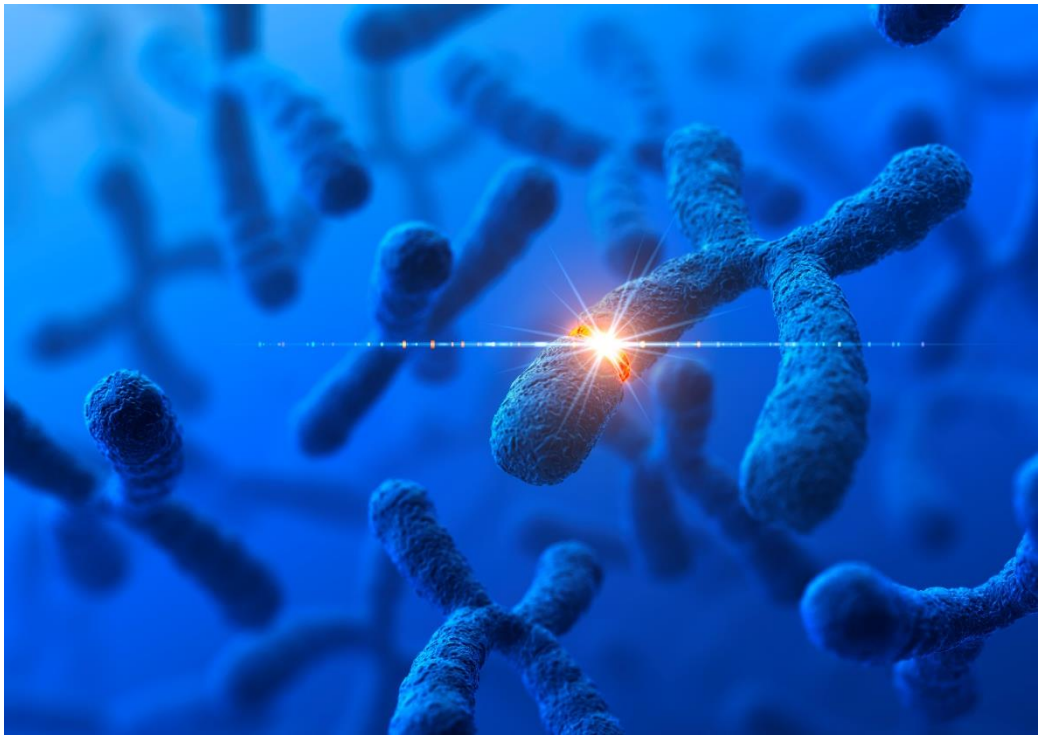
User Manual

ClickTech DNA FISH Kit



Version 1.1

Ordering information

(for detailed kit content see **Table 1**)

ClickTech DNA FISH Kits:

Product Number	Used fluorescent dye	Excitation (nm)	Emission (nm)	Spectral range
BCK-DNA-FISH-488	6-FAM Azide (FITC alternative)	496	516	**green**
BCK-DNA-FISH-555	5-TAMRA-PEG3-Azide (Cy3 alternative)	546	579	**yellow**
BCK-DNA-FISH-594	5/6-Sulforhodamine 101-PEG3-Azide (Texas Red alternative)	584	603	**orange**
BCK-DNA-FISH-647	Eterneon-Red 645 Azide (Cy5 Azide alternative)	643	662	**red**

For References, FAQs and ordering please see online or contact us:

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ClickTech DNA FISH Kit

The ClickTech DNA FISH Kits contain chemicals to perform 20 PCR reactions (50 µL each) and 40 independent labeling reactions (FISH experiments).

Introduction and product description:

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique that enables the detection and localization of specific DNA or RNA sequences within cells/tissues. DNA FISH is a very important tool not only for basic research, but also for clinical applications such as cytogenetic screening for diagnosis and prognosis of cancer (Oliveira and French 2005) and genetic syndromes (Volpi and Bridger 2008a), including prenatal screening. The principle of DNA FISH is to hybridize small single stranded DNA or RNA molecules (probes) to complementary sequences on chromosome spreads or in nuclei of whole cells (target), which are immobilized on glass slides. The probes are labeled directly or indirectly using fluorescent or radioactive nucleotides or through fluorescent antibodies (Volpi and Bridger 2008b). After labeling, FISH probes and targets are thermally denatured and hybridized.

The standard FISH technique, however, often suffers from limitations, namely poor signal to background noise ratio due to unspecific binding of probes to only partially complementary sequences or other cellular and tissue structures. In addition, while even routine *in situ* detection highly benefits from densely labelled, sensitive probes, the labelling rate of FISH probes generated by traditional methods is typically low.

baseclick's ClickTech DNA FISH Kit overcomes these limitations and provides all reagents required for the generation of highly labelled FISH probes *via* an easy-to-handle method based on click chemistry (see Workflow page 6).

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Please read the material safety data sheets (MSDS) provided for each product/component.

Literature Citation: When describing a procedure for publication using this product, please refer to it as baseclick *ClickTech DNA FISH Kit*.

1. Materials Provided with the Kit and Storage Conditions

Table 1: Contents of the kits and storage conditions

Color code	Amount	Component	Storage
blue	25 µL	dNTP Mix (10 mM)	– 20 °C
white	25 µL	EdUTP (5 mM)	– 20 °C
purple	35 µL	baseclick Ethynyl Polymerase (2 U/µl)	– 20 °C
orange	300 µL	PCR Buffer (5x)	– 20 °C
yellow	150 µL	10x Activator[2]	– 20 °C
red	50 µL	• 6-FAM Azide[(1)] • 5-TAMRA-PEG3-Azide[(1)] • 5/6-Sulforhodamine101-PEG3-Azide[(1)] • Eterneon Red Azide[(1)]	– 20 °C, dark
green	40x	Reactor 25	-20 - +20 °C

[(1)] The solution can be stored at – 20 °C in the dark for several months. (Note: The azide functionality is very stable and does not hydrolyse in the presence of water.)

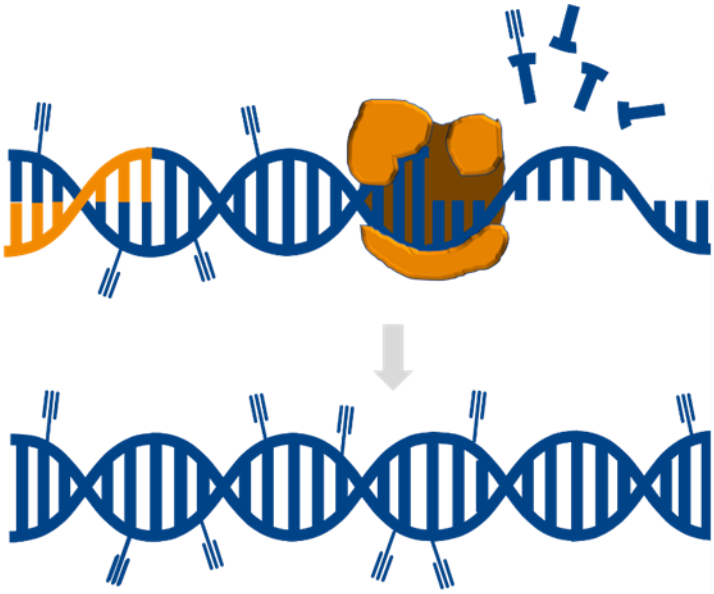
2. Required Material and Equipment not included in this Kit

- Primers (specific primers can be ordered as a custom synthesis at baseclick, more information at www.baseclick.eu) and DNA template
- Reaction tubes (size depends on the volume of reaction cocktail needed)
- DNA purification kit / columns (e.g. QIAquick PCR purification kit from Qiagen)
- PCR grade water
- Thermocycler
- Microcentrifuge
- Thermomixer or water bath
- Fluorescence microscope
- Slides and coverslips for sample preparation, rubber cement to seal the coverslips to the slide during hybridization and at final coverslip mounting before imaging (alternatively, clear nail polish may be used for the latter procedure)
- Ethanol and DAPI or other suitable fluorescent DNA dye for chromosome/nuclear counterstaining.
- Formamide (deionized)
- 20x saline sodium citrate buffer (SSC)
- Nonidet P-40 (NP-40)
- Dextran sulphate

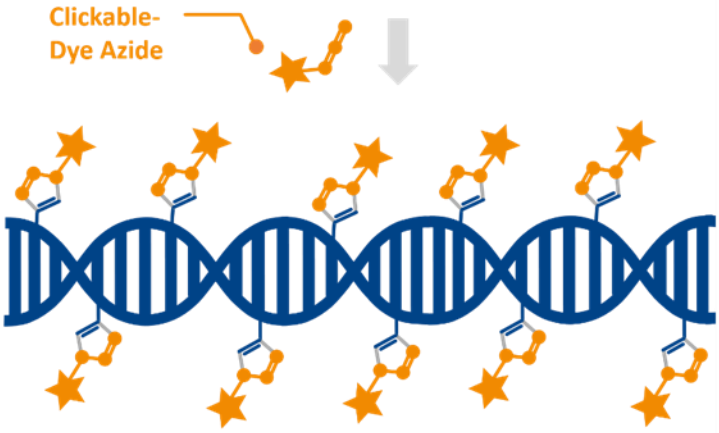
3. Workflow

1. Enzymatic synthesis -PCR
Native (dNTPs) and alkyne-modified (EdUTP) nucleoside triphosphates are used by our special baseclick-Ethynyl Polymerase....

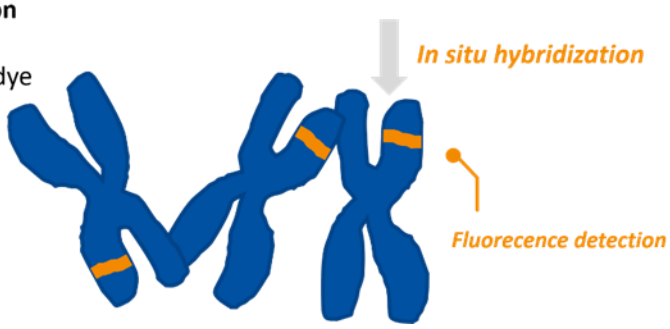
...to produce a clickable PCR product (DNA FISH probe).



2. Click reaction
By using the click reagents and a clickable dye of your choice you obtain DNA fluorescent labeled DNA FISH probes



3. Hybridization and Immobilization
Preparation of the slide containing chromosome and incubation with dye labelled DNA FISH probes



4. General Considerations

- This manual contains protocols and reagents for the synthesis alkyne-modified DNA FISH probes *via* polymerase chain reaction (PCR) and subsequent labelling with dye-azides by click chemistry.

- Excess of reagents should be removed by spin column purification of the DNA FISH probes. We recommend using a PCR purification kit (e.g. QIAquick PCR Purification Kit, Qiagen) to clean up both the PCR product and the click reaction. Please, consider fragment cut-off size when using spin column purification.

- In section 7. a general protocol for DNA FISH on slide preparations is also provided. This protocol is meant as general guidance; more tailored and detailed procedures may need to be followed and/or optimized according to specific experimental designs. For detailed, standard protocols we refer to the scientific literature (e.g., Bayani and Squire, 2004, Fluorescent in situ hybridization, in Current Protocols in Cell Biology, 23:22-4).

5. PCR conditions

The optimal conditions for PCR vary depending on DNA template and primers. Especially cycle times and temperatures have to be optimized for every primer/template pair.

20 PCR reactions (50 µL each) can be performed with the material provided in this kit.

The standard setup for PCR is given below:

Table 2: Setup for a 50 µL standard PCR reaction:

Color code	Reagent	Amount	Final concentration
not included	PCR grade water	Up to 50 µL final volume	n.a.
orange	5x PCR Buffer	10 µL	1x
not included	Forward Primer (10 µM)	2.5 µL	0.5 µM
not included	Backward Primer (10 µM)	2.5 µL	0.5 µM
not included	Template DNA	1 pg - 10 ng	n.a.
white	EdUTP	1 µL	0.1 mM
blue	dNTP Mix	1 µL	0.2 mM (each dNTP)
purple	baseclick Ethynyl Polymerase	1.5 µL (3 Units)	n.a.

Table 3: PCR program:

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1
Denaturation	98 °C	5 - 10 s	25 - 35
Annealing	X °C	10 - 30 s	
Extension	72 °C	15 - 30 s/kb	
Final extension	72 °C	5 - 10 min	1
Hold	4 °C	Hold	Hold

Assess the specificity and yield of PCR products by agarose gel electrophoresis. It is highly recommended to purify PCR products using a PCR purification kit (e.g. QIAquick PCR Purification Kit, Qiagen) to eliminate excess of unincorporated EdUTP.

6. Click Protocol for DNA probe Labelling

Table 4: Setup for a standard click reaction.

Color code	Component	Amount
green	Reactor 25 (Solid Catalyst)	n.a.
yellow	10x Activator^2	2.5 µL
not included	DNA probe solution (100 ng/µL)	20 µL
red	Dye azide (10 mM in DMSO)	1 µL
not included	PCR grade H_2O	1.5 µL

- Centrifuge the **green vial** with the solid catalyst shortly to collect all catalyst at the bottom of the vial.
- Pipette 2.5 µL of the 10x Activator^2 (**yellow vial**) into one of the **green vials** with the catalyst. (Be aware that the catalyst is solid and will not dissolve during the click reaction!)
- Pipette 20 µL of the DNA probe solution (PCR Product) in the same **green vial** with the solid catalyst and the Activator^2.
- Add 1 µL of the dye azide solution (**red vial**, 10 mM in DMSO) into the **green vial** with solid catalyst, Activator^2 and DNA probe solution.
- Vortex shortly, spin down and place the vial in a thermomixer. Incubate the reaction for 30 min at 45 °C and 600 rpm. You can also use a water bath, but apply intermittent mixing during the incubation.
- Recover the supernatant for further purification. It is important to remove the remaining Dye-Azide after the click reaction. We recommend to use a PCR purification kit (e.g. QIAquick PCR Purification Kit, Qiagen) for clean-up of the click reaction. Elute the labelled probe with 5 mM Tris-HCl, pH 8, so that its concentration will be at least 15 ng/µL.

Table 5: Standard dye azides contained in the different kit variants.

Product number	Dye	Excitation/ Absorption (nm)	Emission (nm)
BCFA-001	6-FAM Azide	496	516
BCFA-037	5-TAMRA-PEG3-Azide	546	579
BCFA-044	5/6-Sulforhodamine 101-PEG3-Azide	584	603
BCFA-201	Eterneon-Red 645 Azide (Cy5 Azide alternative)	643	662

7. General Protocol for sample preparation and hybridization

In this section a general protocol for DNA FISH on slide preparations is also provided. This protocol is meant as general guidance; more tailored and detailed procedures may need to be followed and/or optimized according to specific experimental designs. For detailed, standard protocols we refer to the scientific literature (e.g., Bayani and Squire, 2004, Fluorescent in situ hybridization, in Current Protocols in Cell Biology, 23:22-4).

1. Use standard protocols for the preparation of slide samples. (e.g., Bayani and Squire, 2004, Fluorescent in situ hybridization, in Current Protocols in Cell Biology, 23:22-4).

2. For each slide where the specimen covers a surface no larger than 22x22 mm pipette 30 ng of purified labelled probe in a volume of 2 µL into an empty PCR-vial and mix with 8 µL of the following Hybridization Buffer: 62,5% (v/v) formamide, 2.5x SSC, 12.5% (w/v) dextran sulphate.

   Note: For more slides or specimens with a larger surface, we recommend preparing a larger volume of the probe solution in Hybridization Buffer to avoid formation of air bubbles and patches of specimen running dry during denaturation and hybridization.

3. Denature the labelled probe for 5 min at 75 °C (e.g. in a thermocycler and immediately quick-chill on ice to avoid re-annealing of the probe.)

   Note: Protect from light!

4. Add the probe solution to each slide and cover with a coverslip. Subsequently, seal the coverslip with a thin, but seamless lining of rubber cement.

5. Heat the slides to 80 °C for 2 min. Alternatively, pre-denature your slide sample as part of its preparation procedure (see, e.g., Bayani and Squire, 2004, Fluorescent in situ hybridization, in Current Protocols in Cell Biology, 23:22-4).

6. Incubate the slides overnight at 37 °C in a humid chamber to avoid dehydration.

7. Carefully remove the rubber cement lining before taking off the coverslip (a short wash with 2x SSC in 50% formamide helps remove the coverslip).

8. Using suitable reservoirs (e.g., Coplin jars) wash the slides three times 5 min with each of the following washing solutions at 45 °C: i) 2x SSC in 50% formamide; ii) 1x SSC; iii) 4x SSC, 0.1% Tween 20.

   Note: more or less stringent washing may be required depending on GC content and length of the probe.

9. Dehydrate in ethanol (70%, 90%, 100%; each step 2 minutes).
   Note: Protect from light!

10. Let the slides air-dry in the dark (recommended time: 30 minutes) and counterstain with a suitable DNA dye (e.g., DAPI). Mount a coverslip over the hybridized specimen using a suitable mounting medium containing an anti-fading agent. Seal the coverslip to the slide with rubber cement or clear nail polish. Proceed to fluorescence detection.

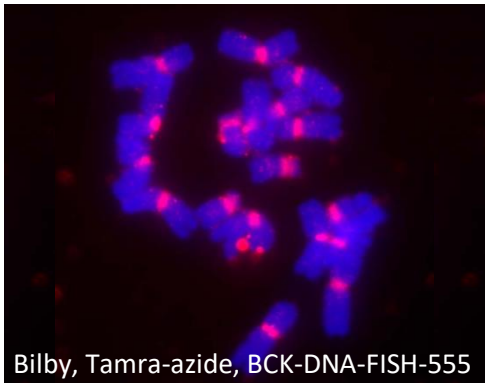


Figure 1. Mitotic chromosomes of rye (*Secale cereale*) after hybridization (image in the middle) click with the centromere-specific repeat *Bilby* (in red).

8. Troubleshooting

a. If no labelled probe could be recovered, the labelling rate might have been too high and labelled DNA was lost during spin column purification (dyed spin column). In this case use a lower concentration of EdUTP in the PCR reaction.

b. Some standard commercial buffer components can decrease the click reaction efficiency or even block the reaction process. For example, TE buffer contains EDTA, which can chelate Cu^{II} ions and decrease the reaction rate. Thiol groups from reducing agents like β-mercaptoethanol or dithiothreitol (DTT) can stop the click reaction.

c. PCR fragments can have folded structures, which hinder accessibility of the functional groups that are needed for the click reaction. By adding some DMSO (5 - 10% (v/v) final) these folded structures are destabilized and improved click yields may be obtained.

d. Some dye-azide compounds show low solubility in the final reaction mixture, when used at higher concentrations and amounts, especially when high labeling rates are desired. This can impair reaction progress and decrease the yield. Try to use additional or alternative co-solvents like methanol or tetrahydrofuran if possible.

e. Some PCR target sequences with supercoiled and self-hybridizing structures might also cause problems. A prolonged denaturation step could help as well as the reduction of MgCl2 content. Varying the MgCl2 content leads to less specific primer binding but can help avoid unspecific fragments.