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Building Blocks of Life

Labeling of *in situ* hybridization DNA probes

- Fluorescent dUTP & dCTP
- Hapten-modified dUTP & dCTP
- CLICKable dUTP & dCTP
- Amine-modified dUTP & dCTP

IFTA AG
Certified QMS and EMS according to
DIN EN ISO 9001 and DIN EN ISO 14001
Reg.-No.: ICV03597 034 and ICV03597 534

ACCCACGAAAGGGAA ATAAGC AACO TTCAGGGAAGAA CTAUAACTGCCAC ACCCACGAAAGGGAA ATAAGC AACO TTCAGGGAAGAA CT
TTCAGGGAAGAA CTAUAACTGCCAC **ACCCACGAAAGGGAA ATAAGC AACO TTCAGGGAAGAA CTAUAACTGCCAC** ACCCACGAAAGG
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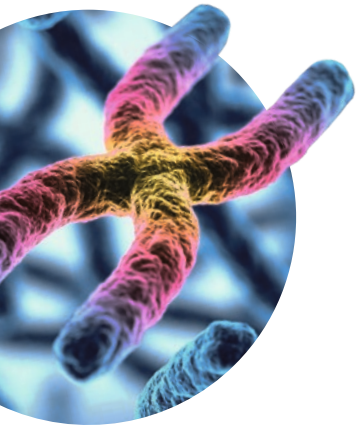
Probes & Epigenetics



Introduction

DNA fragments of variable length ("DNA probes") are able to form non-covalent, highly specific duplexes with a complementary nucleic acid strand (termed hybridization). When a label is attached to such a hybridization probe it can efficiently serve for the detection of a defined DNA target sequence.

Labeled DNA probes are routinely used in research and clinical diagnostics for *in situ* hybridization experiments where specific chromosomal DNA sequences as well as their potential aberrations (mutations, deletions and/or duplications) are detected and localized within fixed tissues and cells.

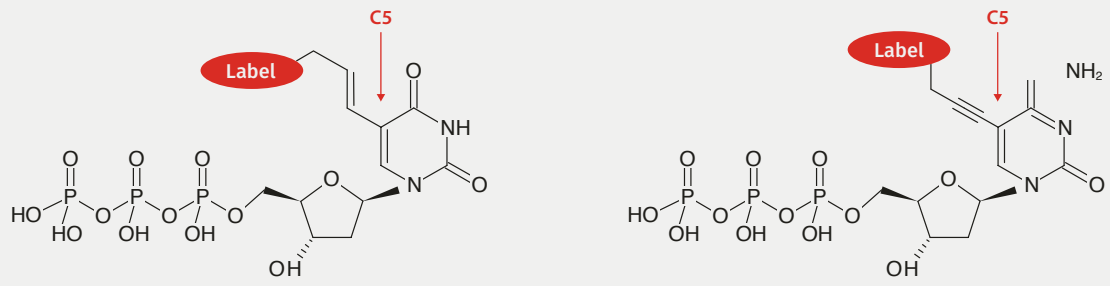
Visualization of DNA *in situ* is either performed by a fluorescence read-out (Fluorescence *in situ* hybridization (FISH)) or by chromogenic detection (Chromogenic *in situ* hybridization (CISH)) that relies on an enzymatic reaction causing precipitation of colored substrates.

Labeling of DNA probes for *in situ* hybridization is most often performed by enzymatic incorporation of labeled dNTPs using *Taq* DNA polymerase (= Polymerase Chain Reaction (PCR)) or a mixture of DNase I/ DNA polymerase I (=Nick Translation) (Tab. 1) [1,2].

Table 1 DNA probes for *in situ* hybridization can be enzymatically labeled via Nick Translation or PCR.

	Nick Translation	PCR
Principle	Introduction of random single-strand breaks ("nicks") into dsDNA by DNAse I that are subsequently filled by DNA Polymerase I using a labeled dNTP as substitute for its natural counterpart.	Amplification of a specific DNA fragment (PCR with specific primer or multiple DNA loci (PCR with degenerated oligonucleotide primer (DOP-PCR) using Taq polymerase for incorporation of a labeled dNTP substituting its natural counterpart.
Template	pmol amounts of linearized dsDNA	fmol amounts of linearized dsDNA
Size of labeled fragment	100–500 bp dsDNA	up to 1500 bp
DNA amplification	no	yes

The most prominent labeled dNTP is dUTP labeled at the C5 position (Fig. 1) which is incorporated as partial substitute for its natural counterpart dTTP. Depending on the template sequence (e.g GC-rich) however, the usage of labeled dCTP or a combination of both might be favorable for achieving the desired labeling.



5-Aminoallyl-dUTP **5-Propargylamino-dCTP**

Figure 1 *dUTP and dCTP labeled at the C5 position via aliphatic aminoallyl or propargylamino linker, respectively, are compatible with enzymatic incorporation, show minimized influence on Watson-Crick base pairing and thus yield good hybridization results. Label: fluorescent dye, hapten (e.g. Biotin, Digoxigenin, DNP) or reactive group such as amine or CLICKable moiety.*

Probe labeling can be performed in a one-step or two step procedure (Fig. 2). The labeling strategy depends on the required sensitivity for the downstream application and the suitability of an appropriately labeled dUTP or dCTP for an enzymatic incorporation method.

For example, labeling with near-infrared dyes is of great importance when working with biological samples since near IR-fluorescence is not disturbed by biological autofluorescence (haemoglobins or cytochromes). Nucleotides labeled with near IR-dyes however, often show insufficient labeling efficiencies caused by the bulky fluorophore. Furthermore, dUTP or dCTP labeled with hydrophobic fluorescent dyes often show poor PCR incorporation efficiencies compared to hydrophilic dyes.

These obstacles can be circumvented by a) use of smaller labels such as haptens (Fig. 2A) or b) use of dUTPs/dCTPs carrying a reactive group that is coupled with the desired fluorescent dye in a second step (Fig. 2B).

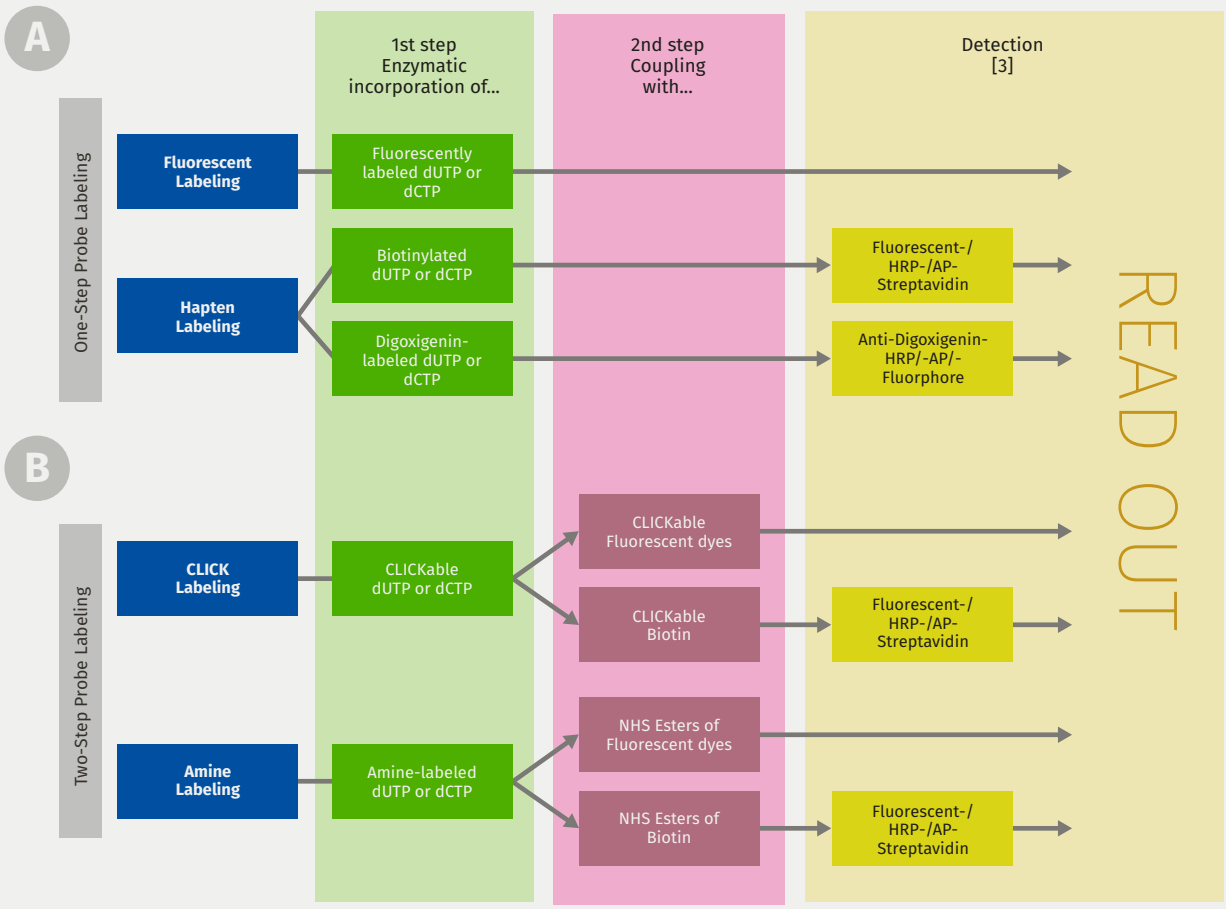


Figure 2 *One-step probe labeling (A) is performed by incorporation of dUTP or dCTP carrying a label (dye or hapten) while two-step probe labeling (B) requires first introduction of a reactive group (amine or "Clickable" moiety) followed by coupling with an appropriate label. HRP: horse radish peroxidase, AP: alkaline phosphatase, NHS: N-hydroxy-succinimide.*

Recommended Reading:

[1] Morrison *et al.* (2003) Labeling Fluorescence *In situ* Hybridization Probes for Genomic Targets. Methods in Molecular Biology **204**: 21.

[2] Wiegant *et al.* (1997) Probe Labeling and Fluorescence *In situ* Hybridization. In: Current Protocols in Cytometry 8.3.1, John Wiley & Sons Inc.

Fluorescent dUTP & dCTP

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Fluorescent dUTP and dCTP can be used for enzymatic labeling of DNA probes in a one-step procedure (Fig. 2). The labeled DNA probe is directly visualized by fluorescence microscopy[3] or spectroscopy. Combinatorial labeling, e.g. labeling of probes with different or a unique set of fluorophores, allows simultaneous visualization of multiple targets (multiplexing)[4–7].

The choice of fluorophore(s) depends on the available excitation source, filter sets, application (single-colour or multi-colour imaging) (Tab. 2) and desired enzymatic labeling technique (Nick Translation or PCR) (Tab. 3).

Both photostability and hydrophilicity of a fluorophore are critical parameters that should be taken into account during decision making. For example, fluorophores with improved photostability may generally increase sensitivity thus improving detection limits of target sequences. The hydrophilicity of a fluorophore however, directly affects the substrate properties of correspondingly labeled dUTP & dCTP.

dUTP & dCTP labeled with hydrophilic (e.g. Cy3) or moderately hydrophobic dyes (e.g. Texas Red) are ideally suited for both PCR and Nick Translation labeling. In contrast, hydrophobic fluorophores (e.g. ATTO647N) cause an early termination of DNA amplification, most likely due to dye-dye or dye-enzyme interactions, and are thus recommended for Nick Translation only.

Table 2 Spectral properties of selected fluorescent dyes. ++: good, +: moderate, -: poor.

Please note: The choice of fluorophore depends on the final application and is a compromise between hydrophilicity and photostability that determine enzymatic incorporation efficiency and detection sensitivity, respectively.

Emission colour	Dye	Exc_{max} [nm]*	Em_{max} [nm]*	ε_{max} [L × mmol^{-1} cm^{-1}]**	Substitute for	Hydrophilicity	Photostability
Blue	AMCA	350	450	19.0		+	+
	DEAC	426	480	57.0		–	+
	ATTO425	436	484	45.0	DEAC	+	++
Green	Rhodamine	505	530	85.0		–	+
	Fluorescein	492	517	83.0		++	–
	ATTO488	501	523	90.0	Fluorescein, Rhodamine, Alexa Fluor 488, Cy2	++	++ (>fluorescein, rhodamine, Cy2)
	AF488	494	515	73.0	Fluorescein, Rhodamine, ATTO488, Cy2	++	++ (>fluorescein, rhodamine, Cy2)
Yellow-Green	ATTO532	532	553	115.0	Alexa Fluor 532	++	++
Yellow	Cy3	550	570	150.0		++	+
	ATTO550	554	576	120.0	Cy3	–	++ (> Cy3)
Orange	Texas Red	588	609	80.0	Cy3.5	+	+
	AF594	590	617	92.0	Texas Red	++	++
Red	J647	637	670	94.0	Cy5, ATTO647N, Alexa Fluor 647	++	++ (> Cy5)
	ATTO647N	644	669	150.0	Cy5, Alexa Fluor 647	–	++ (> Cy5)
	Cy5	649	670	250.0		++	–

* dUTP-dye conjugate, ** free acid

Table 3 Enzymatically incorporable fluorescent dUTP and dCTP. n/a: not applicable

		Recommended dTTP or dCTP substitution by modified dNTP [%]*	
Nucleotide	**Cat. No.**	**PCR (Taq Pol)**	**Nick Translation (DNAse I , DNA Pol I)**
		dUTP	
AMCA-6-dUTP	NU-803-AMCA	20–30 %	30–50 %
DEAC-dUTP	NU-803-DEAC	n/a	30–50 %
dUTP-ATTO425	NU-803-425	20–30 %	30–50 %
Rhodamine-12-dUTP	NU-803-RHOX	n/a	30–50 %
Fluorescein-12-dUTP	NU-803-FAMX	30–50 %	30–50 %
dUTP-XX-ATTO488	NU-803-XX-488	30–50 %	30–50 %
dUTP-XX-ATTO532	NU-803-XX-532	30–50 %	30–50 %
dUTP-Cy3	NU-803-Cy3	30–50 %	30–50 %
dUTP-ATTO550	NU-803-550	n/a	30–50 %
dUTP-Texas Red	NU-803-TXR	20–30 %	30–50 %
dUTP-J647	NU-803-J647	30–50 %	30–50 %
dUTP-Cy5	NU-803-Cy5	30–50 %	30–50 %
dUTP-ATTO647N	NU-803-647N	n/a	30–50 %
		dCTP	
Fluorescein-12-dCTP	NU-809-FAMX	30–50 %	30–50 %
Rhodamine-12-dCTP	NU-809-RHOX	n/a	30–50 %
dCTP-Cy3	NU-809-Cy3	30–50 %	30–50 %
dCTP-ATTO550	NU-809-550	n/a	30–50 %
dCTP-Texas Red	NU-809-TXR	20–30 %	30–50 %
dCTP-J647	NU-809-J647	30–50 %	30–50 %
dCTP-Cy5	NU-809-Cy5	30–50 %	30–50 %
dCTP-ATTO647N	NU-809-647N	n/a	30–50 %

***Please note:**

Optimal final concentration of the dye-labeled dNTP may vary depending on the application and assay conditions. For optimal product yields and optimal incorporation rates an individual optimization of the dye-labeled dUTP/ dTTP or dye-labeled dCTP/dCTP ratio is recommended.

dTTP is substituted by modified dUTP, dCTP is substituted by modified dCTP

Recommended reading:

[3] Jena Bioscience GmbH (a): Non-radioactive Labeling of DNA and RNA, http://www.jenabioscience.com/images/741d0cd7d0/bro_FluorescentProbes_WEB.pdf, 23.04.2015.

[4] Speicher *et al.* (1996) Karyotyping human chromosomes by combinatorial Multi-Fluor FISH. *Nat. Genetics* **12**:368.

[5] Fauth *et al.* 2001) Classifying by colors: FISH-based genome analysis. *Cytogen. Cell Genet.* **93**:1.

[6] Bayani *et al.* (2004) Multi-color FISH techniques. Curr. Protoc. Cell. Biol. **22**: Unit 22.5.

[7] Anderson *et al.* (2004) Multiplex fluorescence *in situ* hybridization (M-FISH). *Methods Mol. Biol.* **659**:83.

Website
fluorescent PCR Labeling Kits at ↗

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fluorescent Nick Translation Labeling Kits at ↗

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Hapten-modified dUTP & dCTP

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Hapten-modified dUTP & dCTP are suitable for enzymatic labeling of DNA probes in a one-step procedure (Fig. 2) using reporter molecules for subsequent detection[8].

Such reporter molecules are either coupled with a fluorophore for visualization by fluorescence microscopy (Fluorescence *in situ* hybridization (FISH)) or with a reporter enzyme that generates signals through an enzymatic reaction with chromogenic substrates (Chromogenic *in situ* hybridization (CISH)) with subsequent detection by bright-field microscopy[8]. This signal amplification usually results in higher sensitivity of indirect detection methods compared to direct fluorescent labeling.

The most commonly used haptens are Biotin, Digoxigenin and Dinitrophenol (DNP). Accordingly labeled dUTP & dCTP are excellent enzymatic substrates (Tab. 4)[9-12] whose incorporation and detection efficiency by secondary reporter molecules is strongly influenced by linker length.

In general short linkers result in high probe yields but may prevent sufficient accessibility of label on the probe surface while long linkers result in increased label accessibility towards detection reagents but result in lower probe yields. An optimal balance between incorporation rate and detection efficiency is achieved by an 11- atom or 16-atom linker[12].

For most applications Biotin is the preferred label however, in some cases endogenous Biotin from biological samples may interfere with detection, resulting in high backgrounds or even false positives. In such cases, the use of Digoxigenin, a steroid exclusively present in Digitalis plants, is recommended.

Table 4 Enzymatically incorporable Hapten-modified dUTP and dCTP. n/a: not applicable

		Recommended dTTP or dCTP substitution by modified dNTP [%]*	
Nucleotide	**Cat. No.**	**PCR (Taq Pol)**	**Nick Translation (DNAse I , DNA Pol I)**
dUTP			
Biotin-11-dUTP	NU-803-BIOX	30–50 %	30–50 %
Biotin-16-dUTP	NU-803-BIO16	30–50 %	30–50 %
Digoxigenin-11-dUTP	NU-803-DIGX	30–50 %	30–50 %
DNP-dUTP	NU-803-DNP	n/a	30–50 %
dCTP			
Biotin-11-dCTP	NU-809-BIOX	30–50 %	30–50 %
Biotin-16-dCTP	NU-809-BIO16	30–50 %	30–50 %

***Please note:**
Optimal final concentration of the Hapten-labeled dNTP may vary depending on the application and assay conditions. For optimal product yields and optimal incorporation rates an individual optimization of the Hapten-labeled dUTP/ dTTP or Hapten-labeled dCTP/dCTP ratio is recommended.
dTTP is substituted by modified dUTP, dCTP is substituted by modified dCTP

Recommended reading:

[8] Jena Bioscience GmbH (a): Non-radioactive Labeling of DNA and RNA, http://www.jenabioscience.com/images/741d0cd7d0/bro_FluorescentProbes_WEB.pdf, 23.04.2015
[9] Anderson *et al.* (2005) Incorporation of reporter-labeled nucleotides by DNA polymerases. *Biotechniques* **38**:257.
[10] Jackson *et al.* (1991) Detection of Shiga Toxin-Producing Shigella dysenteriae Type 1 and Escherichia coli by Using Polymerase Chain Reaction with Incorporation of Digoxigenin-11-dUTP. *J Clin Microbiol.* **29(9)**:1910.
[11] Dauwerse *et al.* (1999) Two-colour FISH detection of the inv(16) in interphase nuclei of patients with acute myeloid leukemia. *Br J Haematol* **106**:111.
[12] Jena Bioscience GmbH (b): Biotin and Digoxigenin Labeled Nucleotides, http://www.jenabioscience.com/images/741d0cd7d0/Biotin_DIG_Nuleotides.pdf, 23.04.2015.

Website
Biotin and Digoxigenin PCR Labeling Kits at ↗

Website
Biotin and Digoxigenin Nick Translation Labeling Kits at ↗

CLICKable dUTP & dCTP

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CLICKable dUTP & dCTP (Tab. 5) can be used for both hapten and fluorescent DNA labeling by a two-step procedure (Fig. 3).

The small-sized CLICK reactive moieties (DBCO or Azide) possess excellent substrate properties resulting in incorporation efficiencies of CLICKable dUTP & dCTP analogous to their Amine-modified counterpart (Tab. 5).

CLICK labeling however, offers a superior labeling procedure compared to strongly pH-dependent Amine-NHS-Ester-based reactions that are often hampered by competing NHS ester hydrolysis.

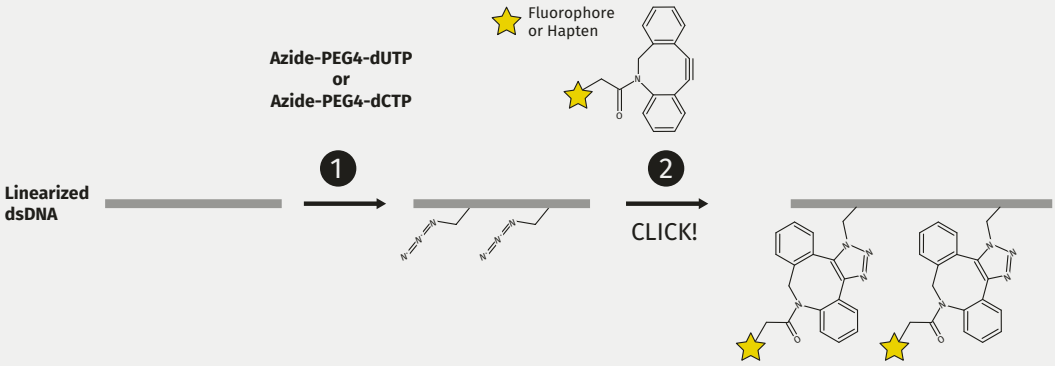

Figure 3 *CLICK DNA Labeling is performed by a two-step procedure. 1) Enzymatic Azide-functionalization of dsDNA by PCR or Nick Translation. 2) Copper-free CLICK-labeling via a DBCO-functionalized detection molecule. DBCO-PEG4-dUTP and –dCTP are similarly incorporated but labeled with Azide-functionalized detection molecules.*

Table 5 Enzymatically incorporable CLICKable dUTP & dCTP.

		Recommended dTTP or dCTP substitution by modified dNTP [%]*	
Nucleotide	**Cat. No.**	**PCR (Taq Pol)**	**Nick Translation (DNAse I , DNA Pol I)**
	dUTP		
Azide-PEG4-dUTP	NU-1705	30–50 %	30–50 %
DBCO-PEG4-dUTP	CLK-T09	30–50 %	30–50 %
	dCTP		
Azide-PEG4-dCTP	CLK- 061	30–50 %	30–50 %
DBCO-PEG4-dCTP	CLK- 060	30–50 %	30–50 %

***Please note:**
Optimal final concentration of the CLICKable dNTP may vary depending on the application and assay conditions. For optimal product yields and optimal incorporation rates an individual optimization of the CLICKable dUTP/ dTTP ratio is recommended.
dTTP is substituted by modified dUTP, dCTP is substituted by modified dCTP

Recommended reading:
[13] Jena Bioscience GmbH: CLICK Chemistry Background Information, http://www.jenabioscience.com/images/741d0cd7d0/20140306_Click_Chemistry_Background_information_pdf_creator.pdf, 06.01.2016.

Website
Azide and DBCO detection molecules at ↗

Website
CLICK PCR Labeling Kits at ↗

Website
CLICK Nick Translation Labeling Kits at ↗

Amine-modified dUTP & dCTP

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Amine-modified dUTP & dCTP are historically used for both hapten and fluorescent DNA labeling by a two-step procedure (Fig. 4).

Step 1 consists of the enzymatic incorporation of the Amine-modified dNTP (Tab. 6) which is subsequently labeled with an Amine-reactive (e.g. NHS ester) fluorescent dye or hapten[14–15].

Due to the small size of the amino group, higher incorporation efficiencies may be achieved compared to enzymatic labeling with fluorescent dUTPs and dCTPs – especially when carrying bulky or hydrophobic fluorescent dyes.

Table 6 Enzymatically incorporable Amine-modified dUTP and dCTP.

		Recommended dTTP or dCTP substitution by modified dNTP [%]*	
Nucleotide	**Cat. No.**	**PCR (Taq Pol)**	**Nick Translation (DNAse I , DNA Pol I)**
	dUTP		
Aminoallyl-dUTP	NU-803	30–50 %	30–50 %
	dCTP		
Propargylamino-dCTP	NU-809	30–50 %	30–50 %

***Please note:**
Optimal final concentration of the Amine-labeled dNTP may vary depending on the application and assay conditions. For optimal product yields and optimal incorporation rates an individual optimization of the Amine-labeled dUTP/ dTTP or Amine-labeled dCTP/dCTP ratio is recommended.
dTTP is substituted by modified dUTP, dCTP is substituted by modified dCTP

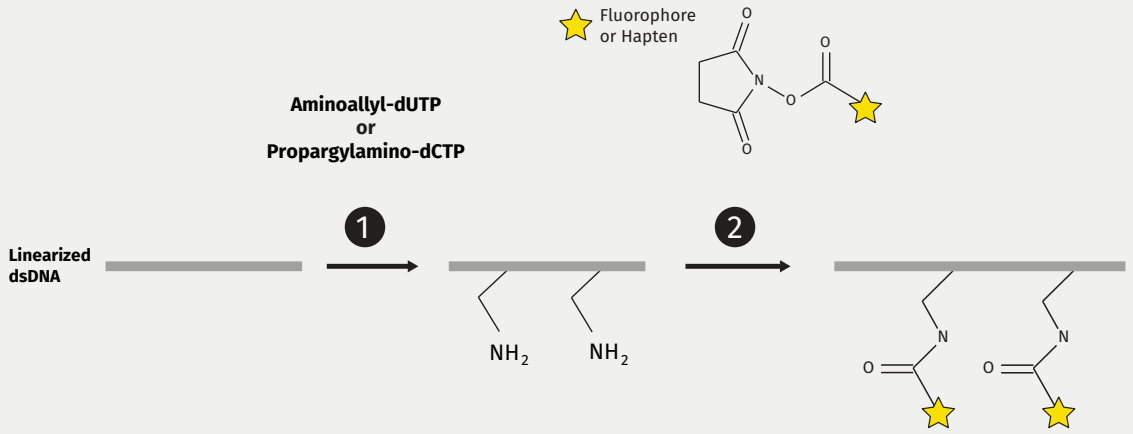

Figure 4 *Amine DNA Labeling is performed by a two-step procedure. 1) Enzymatic Amine-functionalization of dsDNA by PCR or Nick Translation. 2) Amine Labeling via a N-Hydroxysuccinimid (NHS)-functionalized detection molecule.*

Recommended reading:
[14] Dirsch *et al.* (2007) Probe production for *in situ* hybridization by PCR and subsequent covalent labeling with fluorescent dyes. *Appl. Immunohistochem. Mol. Morphol.* **3**:332.
[15] Cox *et al.* (2004) Fluorescent DNA hybridization probe preparation using amine modification and reactive dye coupling. *BioTechniques* **36**:114.

Website
NHS Ester detection molecules at ↗

Quality control

Jena Bioscience is a primary nucleotide manufacturer offering bulk amounts as well as custom formulations, packaging & labeling.

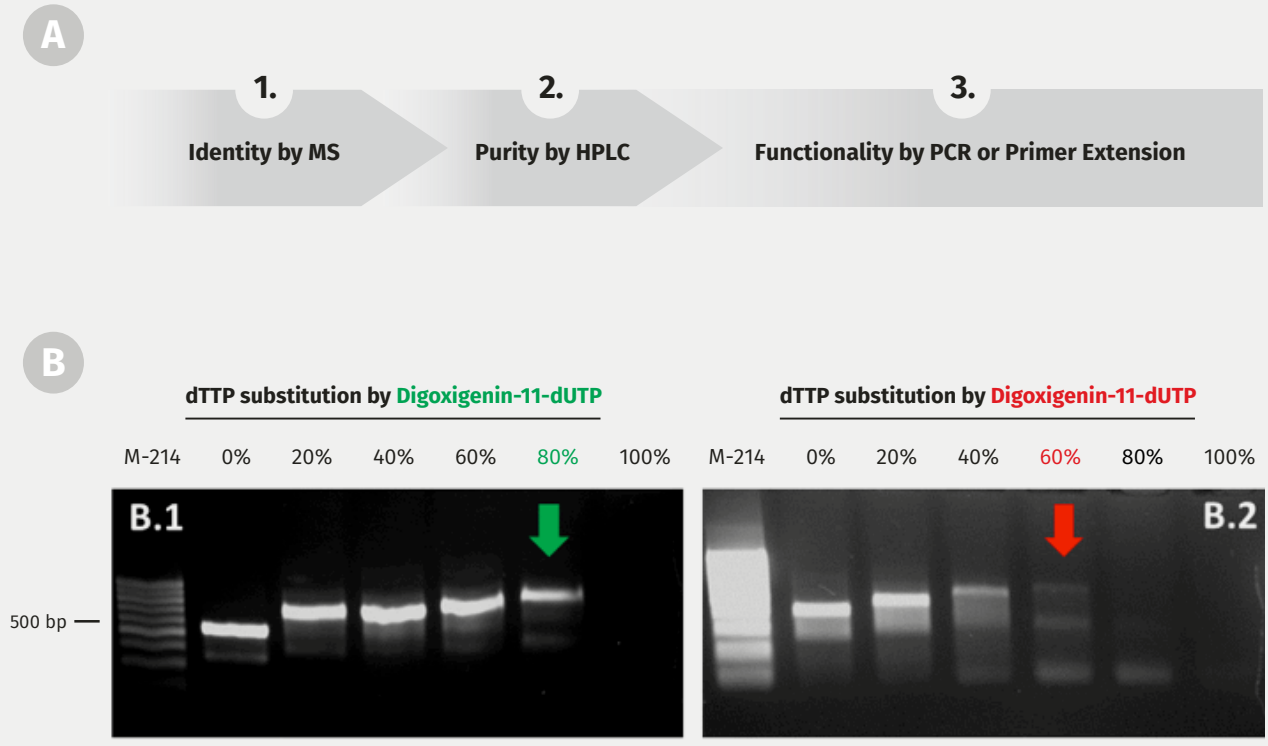

Figure 5 *Quality control of selected labeled nucleotides. A) Three-step quality control procedure B) Functional Quality Control Example (Step 3): Each lot of Digoxigenin-11-dUTP is – besides a number of other tests – analyzed in a PCR reaction with increasing substitution of dTTP by Digoxigenin-11-dUTP (0–100%). The band shift indicates Digoxigenin-11-dUTP incorporation. Lots yielding a visible PCR product at 80 % substitution of dTTP by Digoxigenin-11-dUTP are accepted (B.1). B.2: Non-Conforming Digoxigenin-11-dUTP lot (source: other supplier).*

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Jena Bioscience has established a quality management system according to DIN EN ISO 9001 and an environmental management system according to DIN EN ISO 14001. We commit ourselves to international standards and continuously improve our quality and environmental management system and our inhouse processes to meet our high quality standards.

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