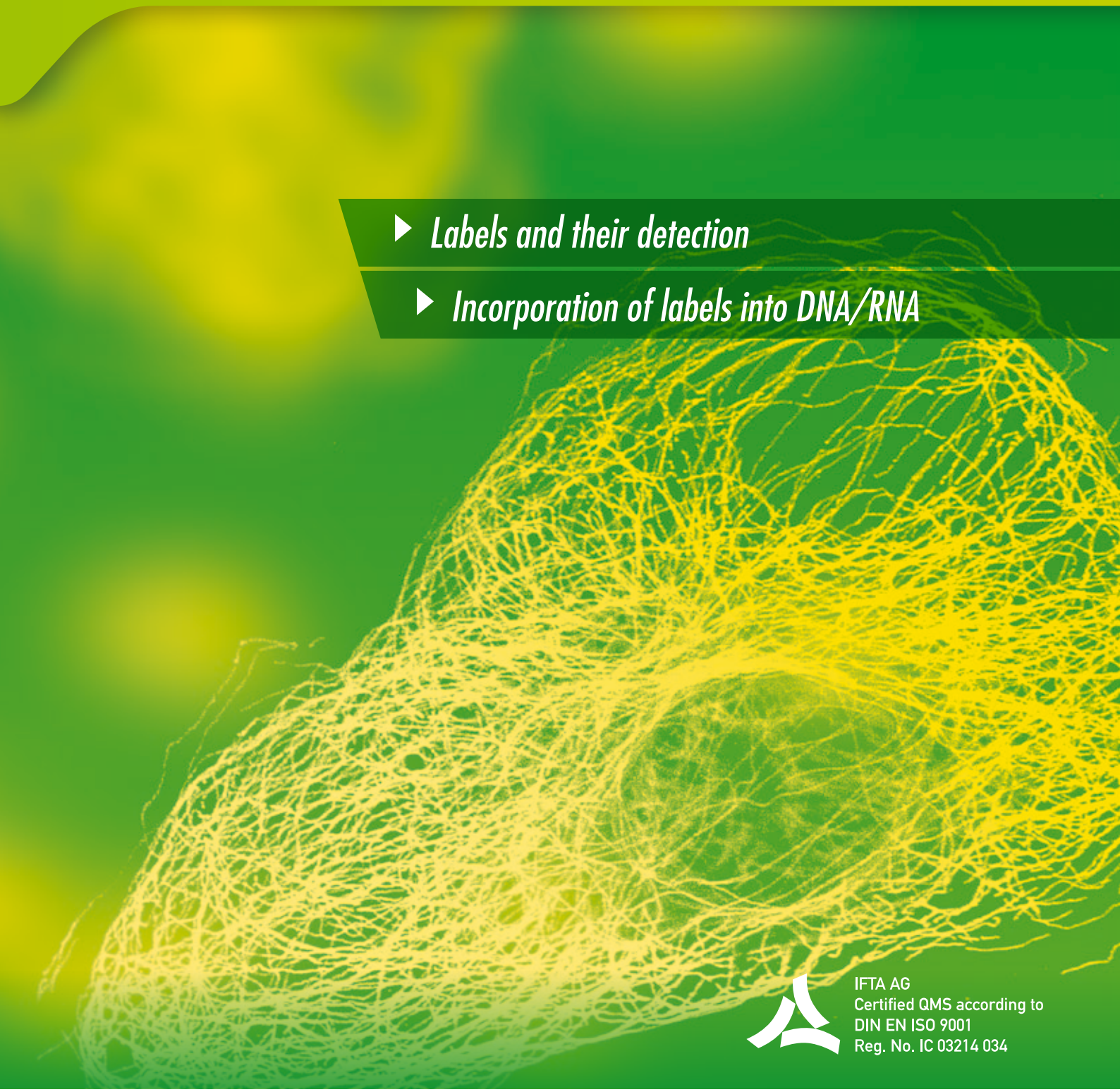
Non-radioactive Labeling of DNA and RNA



- Labels and their detection
- Incorporation of labels into DNA/RNA

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Jena Bioscience

Jena Bioscience GmbH was founded in 1998 by a team of scientists from the Max-Planck-Institute for Molecular Physiology in Dortmund. 25+ years of academic know how were condensed into the company in order to develop innovative reagents and technologies for the life science market.

Since the start up, the company has evolved into an established global reagent supplier with more than 6500 products on stock and > 4000 customers in 50+ countries. Jena Bioscience serves three major client groups:

- **Research laboratories at universities, industry, government, hospitals and medical schools**
- **Pharmaceutical industry in the process from lead discovery through to pre-clinical stages**
- **Laboratory & diagnostic reagent kit producers and re-sellers**

Our company premises are located in the city of Jena / Germany with a subsidiary in Teltow, in the vicinity of the German capital Berlin.

Jena Bioscience's products include nucleosides, nucleotides and their non-natural analogs, recombinant proteins & protein production systems, reagents for the crystallization of biological macromolecules and tailor-made solutions for molecular biology and biochemistry.

In our chemistry division, we have hundreds of natural and modified nucleotides available on stock. In addition, with our pre-made building blocks and in-house expertise we manufacture even the most exotic nucleotide analog from mg to kg scale.

In the field of recombinant protein production, Jena Bioscience has developed its proprietary LEXSY technology. LEXSY (*Leishmania* Expression System) is based on a S1-classified unicellular organism that combines easy handling with a full eukaryotic protein folding and modification machinery including mammalian-like glycosylation. LEXSY is primarily used for the expression of proteins that are expressed at low yields or are inactive in the established systems, and expression levels of up to 500 mg/L of culture were achieved.

For the crystallization of biological macromolecules – which is the bottle-neck in determining the 3D-structure of most proteins – we offer reagents and tools for crystal screening, crystal optimization and phasing that can reduce the time for obtaining a high resolution protein structure from several years to a few days.

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Imprint

Design and Layout by:
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Introduction

DNA and RNA fragments of variable length ("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 thus efficiently serve for detection of a defined DNA or RNA target sequence.

Typical applications of hybridization probes are **(fluorescence) *in situ* hybridization ((F)ISH), microarray-based gene expression profiling or electrophoretic mobility shift assays (EMSA)** that are routinely used in research or clinical diagnostics to

- detect and localize specific DNA sequences and their potential aberrations (mutations, deletions, duplications) on chromosomes ((F)ISH, Fig. 1A).
- identify and analyze protein-DNA/-RNA interactions (EMSA, Fig. 1B).
- simultaneously measure the relative abundance of multiple RNAs (e.g. microarray-based gene expression profiling).

Historically, mainly radioisotope labels were used for hybridization probes however, in recent years the incorporation of non-radioactive labels has gained increasing popularity. This is due to significant improvements of the available detection methods (speed, sensitivity, and versatility) that make the analytical performance of non-radioactive labels comparable to that of their radioactive counterparts. In addition, non-radioactive probes are clearly superior in many practical aspects such as **enhanced stability, convenient handling and greatly improved safety profiles.**

Therefore, and together with the corresponding detection systems and well-established incorporation methods, non-radioactive nucleic acid labeling probes have become a superior alternative over radioactive approaches.

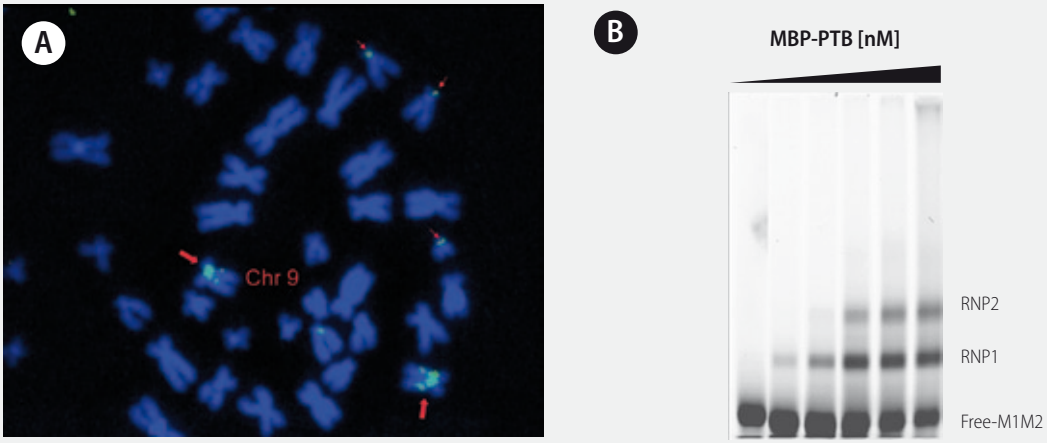

Figure 1

A: *Chromosome 9 was visualized by fluorescence in situ hybridization (FISH). A DNA probe (Bac268E1) was fluorescently labeled by nick translation using Jena Bioscience's Aminoallyl-dUTP-ATTO-488 (Cat.-No. PP-306S-488).*

B: *Polypyrimidine tract-binding protein (MBP-PTB) as a new component of the oskar mRNA/ ribonucleoprotein (M1M2/RNP1/2) complex was detected by an electrophoretic mobility shift assay (EMSA). The mRNA probe (M1M2) had been labeled by T7 RNA polymerase mediated in vitro transcription using Jena Bioscience's Aminoallyl-UTP-ATTO-680 (Cat.-No. NU-821-680) (adapted from [1]).*

Labels and their detection

The most commonly used labels for the generation of non-radioactively DNA or RNA hybridization probes are **fluorophores** and **haptens**, the latter meaning **Biotin** and **Digoxigenin**.

Fluorescent probes are detected directly after incorporation by fluorescence spectroscopy (Fig. 2A). Nowadays, a plethora of fluorescent dyes is available with optical properties that cover the whole UV-Vis spectrum and fit to common commercial light sources and filter systems. The dyes differ, however, greatly in terms of their incorporation efficiency, photostability, hydrophilicity, and size/bulkiness. Classical dyes such as rhodamine, fluorescein and cyanine derivatives have been the most widely used so far but recently an increasing number of new dyes (e.g. ATTO or Eterneon™ dyes) has become very popular.

In contrast to fluorophores, **Biotin** and **Digoxigenin** are indirect labels since their visualization requires a secondary reporter molecule (Fig. 2B). The detection of **Biotin**, also known as vitamin H, relies on its high affinity (K_D= 10^{-15} M) to Streptavidin, a 60 kDa tetrameric protein purified from the bacterium *Streptomyces avidinii* [2]. Biotinylated hybridization probes are therefore easily detected through Streptavidin carrying either a reporter enzyme or a fluorescent dye (Fig. 2B.1). Since however, endogenous biotin from biological samples often interferes with biotin detection (resulting in high backgrounds and false positives [3] [4]), the use of **Digoxigenin**, a steroid exclusively present in Digitalis plants, may sometimes be preferable [3] [5]. Visualization of incorporated Digoxigenin is achieved in a similar manner by Digoxigenin antibody conjugates with a reporter enzyme or a fluorescent dye (Fig. 2B.2).

The choice of label and method of detection is generally determined by the particular downstream application. While speed of detection and ease of quantification are major advantages of fluorescent labels, indirect methods with secondary reporter molecules allow signal amplification with resultant increased sensitivity.

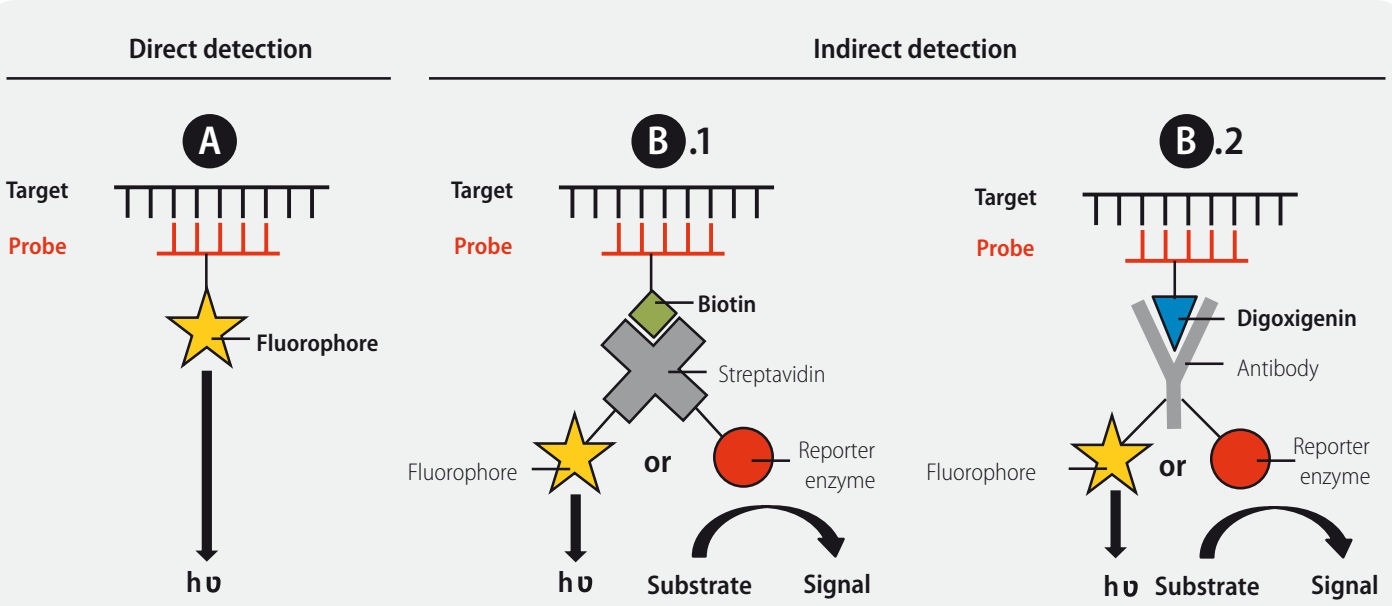

Figure 2

Fluorescently labeled probes can be detected directly after incorporation (A) whereas indirect detection via Biotin/Streptavidin (B.1) or Digoxigenin/Antibody (B.2) systems offers signal amplification and increased stability. Commonly used reporter enzymes include horseradish peroxidase (HRP) and alkaline phosphatase (AP) that generate signals through an enzymatic reaction with chemiluminescent or chromogenic substrates.

Incorporation of labels into DNA/RNA

The incorporation of fluorophores and haptens into DNA and RNA hybridization probes is achieved by enzymatic labeling techniques using modified nucleotides as substitutes for their natural counterparts (Fig. 3). In a **one-step procedure**, accordingly labeled nucleotides are incorporated for immediate detection (Fig. 3A). Alternatively, **two-step labeling** is achieved via i) incorporation of a reactive group followed by ii) coupling with the desired label (Fig. 3B).

To ensure optimal substrate properties of such modified nucleotides, their core structure is derived from natural analogs with the desired moiety attached via a linker to one of the available modifiable nucleotide positions (base, ribose, phosphate). Both, the position of linker attachment and the type of linker, are critical factors affecting substrate properties and resulting labeling efficiency [6].

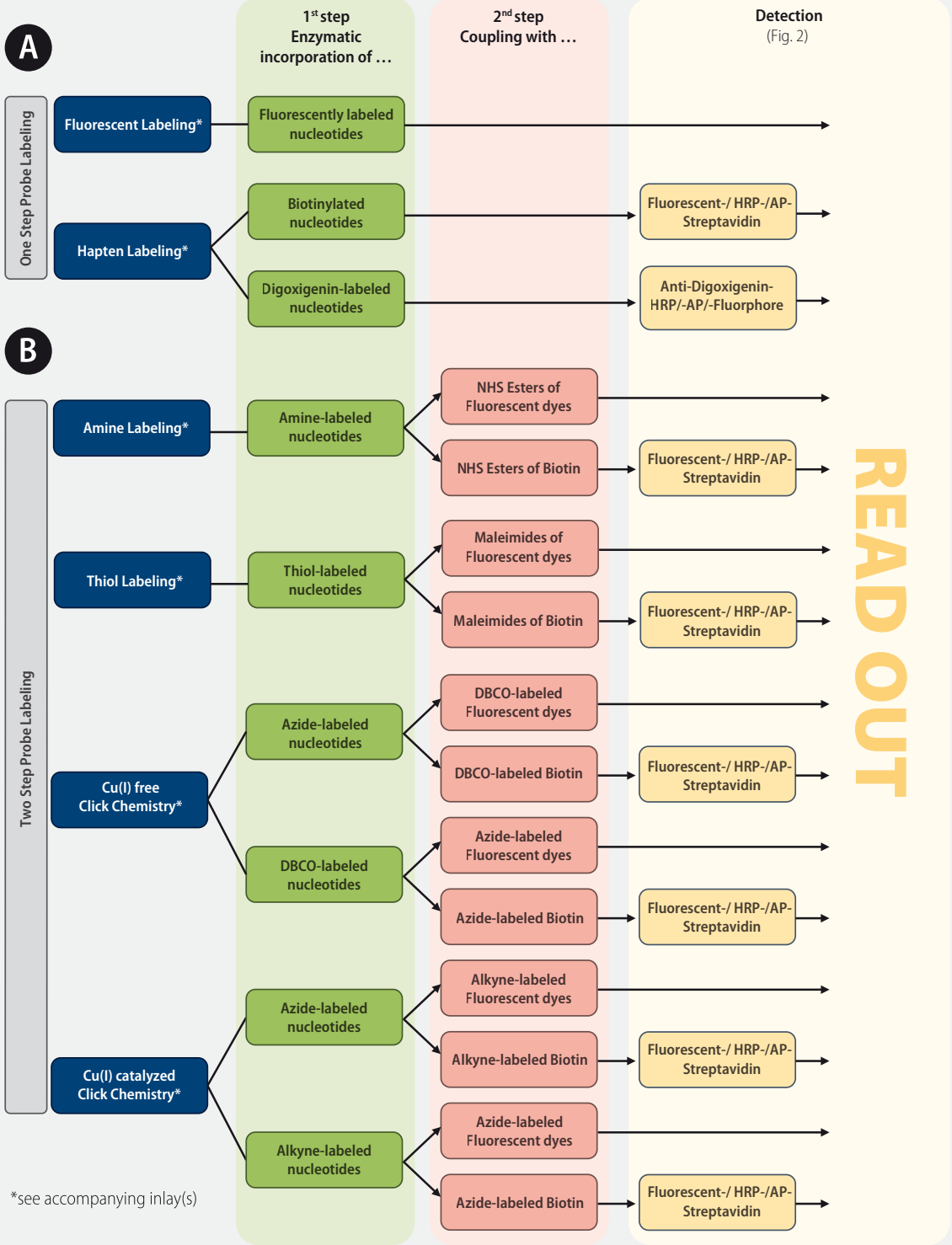

Figure 3

One-step probe labeling (A) is performed by incorporation of nucleotide analogs carrying a label (dye or hapten) while two-step probe labeling (B) requires in a first step introduction of a reactive group (amine, thiol, or "Clickable" moiety) followed by coupling with an appropriate label. NHS: N-hydroxy-succinimide, DBCO: Dibenzylcyclooctyne, HRP: horseradish peroxidase, AP: alkaline phosphatase, Cu: copper. Please note that this is a non-comprehensive illustration of existing labeling methods.

The labeling strategy depends on the size and type of nucleic acid to be labeled (DNA or RNA), the required sensitivity for the downstream application and the availability of appropriately labeled nucleotide analogs for enzymatic incorporation. For example, labeling with near-infrared dyes is, on the one hand, 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, on the other hand, often show insufficient labeling efficiencies due to sterical hindrance caused by the bulky fluorophore. This problem can be circumvented by the use of smaller labels such as haptens or reactive group-carrying nucleotides followed by coupling with a near-IR dye (Fig. 3)

Incorporation of modified nucleotides into DNA & RNA can be done random (i.e. incorporation of multiple nucleotides in a random fashion along the entire nucleic acid) or by selective end-labeling (incorporation of one or multiple nucleotides exclusively at the 3´- and/or 5´-end). Random labeling usually achieves higher labeling densities and thus higher sensitivities (Tab. 1).

Table 1: Standard enzymatic procedures are available for the preparation non-radioactively labeled probes.

Taq: *Thermus aquaticus*; Vent exo⁻: *Thermococcus litoralis* exo⁻ DNA polymerase; MMLV: *Moloney Murine Leukemia Virus*; AMV: *Avian Murine Virus*; exo⁻: lack of exonuclease activity.

Template	Method	Labeled Probe	Labeling site	Enzyme	References
DNA	**PCR**	**DNA**	**random**	Thermophilic polymerases e.g. Taq Polymerase or Vent exo⁻	[7], [8]
	Nick Translation	**DNA**	**random**	DNAse I/ DNA Polymerase I	[8], [9], [10]
	Primer Extension	**DNA**	**random**	Klenow Fragment 3'->5' exo⁻ Taq Polymerase	[8]
	3´-End Labeling	**DNA**	**3´-OH**	Terminal Deoxynucleotidyl Transferase (TdT)	[11], [12]
	5´-End Labeling	**DNA**	**5´-OH**	T4 Polynucleotide Kinase (T4 PNK)	[13]
	***In vitro* Transcription**	**cRNA**	**random**	SP6 RNA Polymerase T7 RNA Polymerase T3 RNA Polymerase	[14], [15]
RNA	**Reverse Transcription**	**cDNA**	**random**	MMLV Reverse Transcriptase AMV Reverse Transcriptase	[7], [14]
	3´-End Labeling	**RNA**	**3´-OH**	Terminal Deoxynucleotidyl Transferase (TdT)	[16], [17]
		RNA	**3´-OH**	Yeast Poly A Polymerase	[18]
		RNA	**3´-OH**	T4 RNA Ligase	[19]
	5´-End Labeling	**RNA**	**5´-OH**	T4 Polynucleotide Kinase (T4 PNK)	[13], [20]

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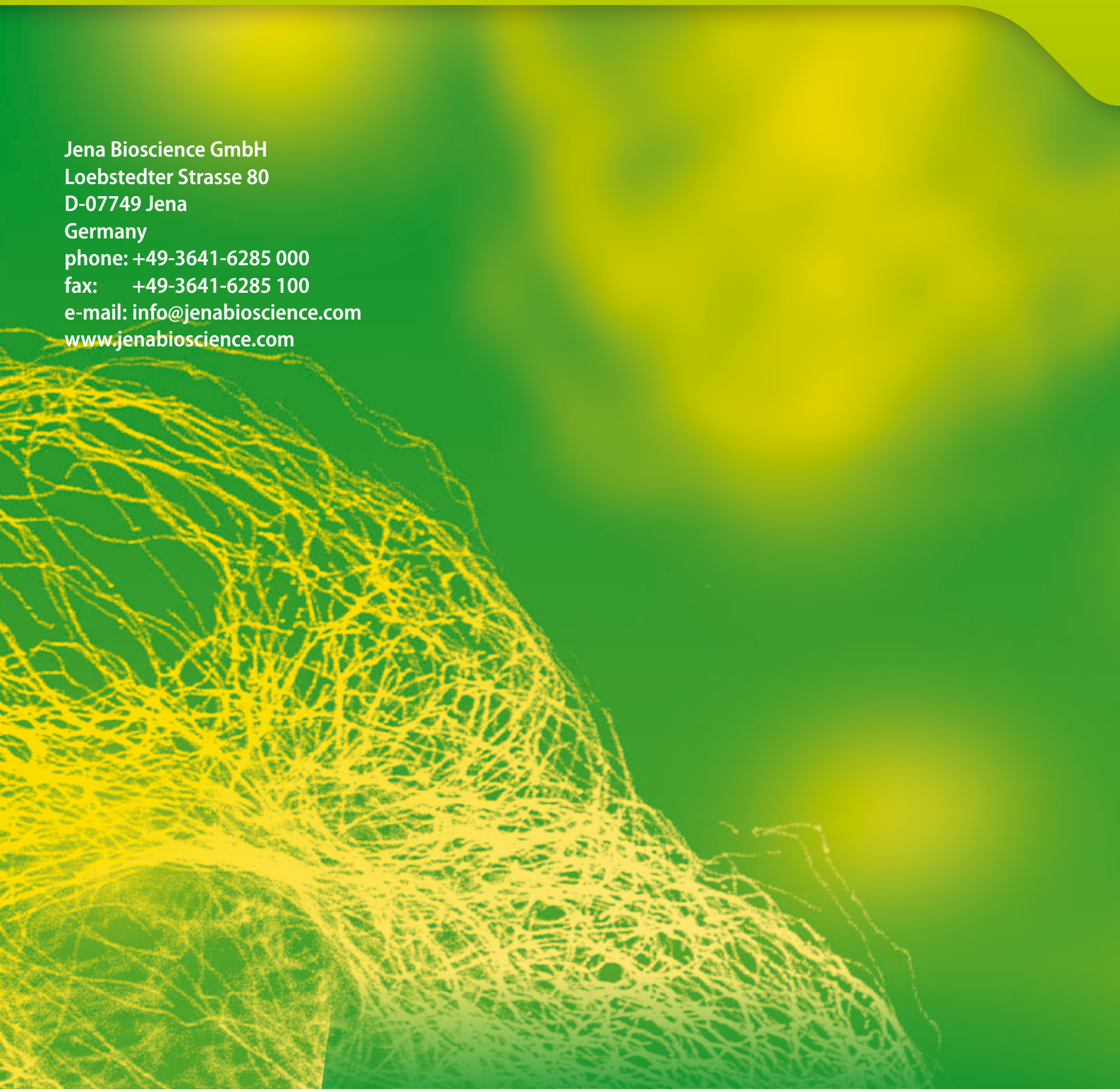

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