

A Method for High Quality Digoxigenin-Labeled RNA Probes for *In Situ* Hybridization

1. Introduction

Digoxigenin (DIG)-labeled RNA antisense probes are widely used for *in situ* hybridization due to their high sensitivity and specificity. DIG-labeled RNA probes are also stable for more than a year, making them ideal for long-term studies with high consistency and low technical variation.

DIG-labeled RNA antisense probes are easily produced using PCR and *in vitro* transcription, avoiding tedious cloning steps. In this note, we describe a method for optimizing probe sensitivity and specificity for greater hybridization consistency. For quality control, hybridization probes should be tested using dot blot to analyze the sensitivity and northern blot for specificity, prior to *in situ* hybridization. This method allows rare transcripts with late cycle threshold (Ct) values in real-time qRT-PCR to be detected using northern blots, indicating high sensitivity when using DIG-labeled RNA probes.

Here we are using DIG-labeled probes to investigate the localization of human hepatocyte growth factor (hHGF), a cell surface cytokine binding the c-MET-receptor, implicated in the tyrosine kinase signaling cascade causing tumor genesis. Her2 was also examined which binds and stabilizes numerous EGF-receptor-ligand-interactions in downstream signaling pathways. The overexpression of both genes has been demonstrated in variety of cancers. In addition, the housekeeping genes, ALAS and MRPL19, were used as reference genes.

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Not for use in diagnostic procedures.

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2. Materials and Methods

RNA isolation from cell cultures

Total RNA was isolated from cell cultures (RKO, U87 and A549) using the High Pure RNA Isolation Kit (Roche). Cells were washed with cold PBS and lysed by adding lysis buffer to the culture bottle. The content of one bottle was processed on one purification spin column per sample. Resulting total RNA was quantified using the NanoDrop Instrument. RNA quality was verified using the Bioanalyzer 2100 (Agilent). RNA-Integrity-Number (RIN) values were obtained using capillary electrophoresis. RIN values of 7 to 9 indicate high quality RNA suitable for qPCR and northern blot hybridization.

cDNA synthesis

cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

Real-time qPCR

cDNAs were tested using the reference (housekeeping) gene, ALAS1 to analyze relative expression. qPCR assays for ALAS1, MRPL19, hHGF and Her2 were designed using the Universal ProbeLibrary (UPL). ProbeFinder Software was used to identify PCR primers and matching UPL probes. The LightCycler® 480 Instrument was used for all PCR assays.

Generating DIG labeled RNA probes from PCR templates by *in vitro* transcription

For each gene of interest antisense and sense RNA probes were prepared. These probes were designed to be intron spanning with lengths between 400 and 1900 bases. The following reference gene sequences were used:

ALAS1: RefSeq NM_000688 selected probe length: 448 bases
hHGF: RefSeq NM_000601.4 selected probe length: 508 nucleotides
Her2: RefSeq NM_004448 selected probe length: 1895 nucleotides

For initiation of *in vitro* transcription, T7 promoter sequences were added to the respective PCR primers. For antisense RNA probes, the T7 RNA polymerase promoter sequence 5'-taatacgaactcactataggaga3' was added to the reverse primer, and for sense probes to the forward primer; 50 pg of plasmid clones containing the respective cDNAs were used as PCR templates. Amplicons were generated using the Expand High Fidelity^{PLUS} PCR System, dNTPack (Roche). Amplicons were verified for size and purity using a 1.2 % agarose gel (Lonza). *In vitro* transcription for producing DIG-labeled RNA probes was performed using the DIG Northern Starter Kit (Roche). Due to DIG incorporation, DIG-labeled RNA probes show a shift in molecular weight. DIG probe sensitivity was tested in a dot blot assay using the DIG Northern Starter Kit (Roche).

Technical Tip 1:

Retroviral reverse transcriptases commonly used for cDNA synthesis exhibit a higher error rate than other DNA polymerases. Low accuracy can lead to base exchanges and frame shifts which are propagated by PCR. High fidelity (proofreading) PCR enzymes have been available for many years. Roche Applied Science Transcriptor High Fidelity cDNA Synthesis Kit provides high accuracy reverse transcriptase to synthesize high yield, full-length cDNA, using a blend of recombinant reverse transcriptase and proofreading enzyme. This synergy enables a sevenfold higher fidelity compared to other commonly used reverse transcriptases. In combination with a proofreading DNA polymerase with higher accuracy, the overall fidelity for RT-PCR can be significantly increased.

Technical Tip 2:

Template concentration affects labeling reactions

When excessive template amounts containing primary extension products are used for labeling reactions, longer than desired and unspecific hybridization probes may be created, disturbing accurate hybridization signals. Therefore it is crucial to use only 10 – 50 pg of plasmid DNA as PCR template for probe labeling, whether in direct labeling of a DNA probe or for the generation of a DNA template for subsequent labeling by *in vitro* transcription.

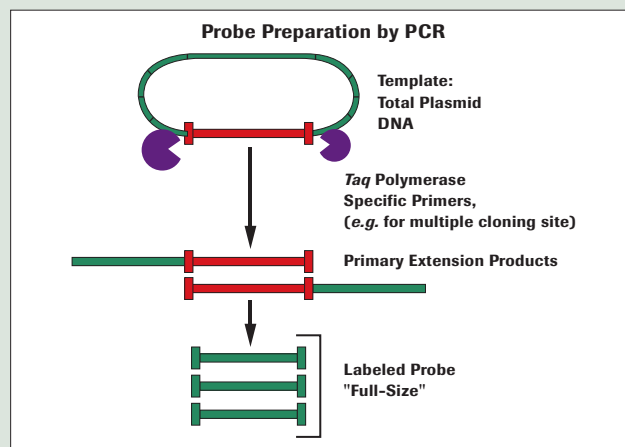


Figure 1: Scheme of PCR amplification of plasmid.

Flanking forward and reverse primers limit elongation of PCR product. This accounts for every PCR cycle except the first. Here, so-called "primary extension products" are generated, because the polymerase can run past the priming sites. These long products may contain vector sequences or in case of complex genomic DNA undesired repetitive element sequences or even unrelated products from secondary priming sites. Linear co-amplification of these products can eventually cause undesired signals in the hybridization reaction. This also applies to the generation of PCR templates. A straightforward method for generating templates for *in vitro* transcription labeling of RNA using DIG without cloning, is to prepare a PCR template using specially designed primers, including the sequence of the appropriate RNA polymerase promoter. In general, T7 and T3 RNA polymerase promoters are most widely used. The SP6 RNA polymerase promoter was the first promoter used for *in vitro* transcription. However, the SP6 polymerase sometimes transcribes less reliably. We designed individual primer sets using the longer T7 promoter sequence which is 4 bases longer compared to the sequence provided in the DIG Northern Starter Kit (Roche). For comparison data, see also Cancer Research Application Note 10. For simplicity, we used only the T7 promoter; see also Technical Tip, Dec 2012: RNA Labeling using *In Vitro* Transcription.

2. Materials and Methods

Northern blot assay and hybridization

Formamide/formaldehyde denaturation of target RNAs was performed. MOPS/formaldehyde gels were run according to the manufacturer's protocol for the DIG Northern Starter Kit (Roche). RNA transfer to positively charged nylon membranes (Roche) was done overnight using capillary transfer with $20 \times$ SSC (Roche) in sterile and RNase-free solutions and equipment. RNA was fixed to the membrane using UV crosslinking (HL-2000 HybriLinker 2000, UVP Ltd.). After crosslinking, membranes were rinsed briefly with RNase-free water and air dried.

Overnight hybridization was performed in DIG Easy Hyb Buffer (Roche) with 100 ng of denatured RNA probe/ml of hybridization solution at $+68^\circ\text{C}$ in roller bottles under constant rotation in a hybridization oven (HL-2000 HybriLinker 2000, UVP Ltd.). Stringency washes and immunological detection were performed following the manufacturer's protocol for the DIG Northern Starter Kit (Roche).

RNA *in situ* hybridization

Formalin fixed and paraffin embedded (FFPE) tissues were cut into $3 \mu\text{m}$ sections using a Microm HM 355S rotation microtome (Thermo Scientific). *In situ* hybridization was performed on sections using a Discovery XT Automated Slide Stainer (Ventana Medical Systems). The RiboMap Kit was used for tissue fixation, pretreatment and post-fixation. For antigen retrieval, CCI buffer and Protease 3 were used. The DIG-labeled HGF probe was hybridized in RiboHybe diluent at $+66^\circ\text{C}$ for 6 hours. Nonspecifically bound probes were washed off using high stringency conditions at $+70^\circ\text{C}$. DIG-labeled probes were detected using AP-coupled anti-digoxigenin Fab fragments (Roche Applied Science) at 1:100 in alkaline phosphatase buffer using the DIG Wash and Block Buffer Set (Roche), and visualized using the BlueMap Kit. Red Stain II was used for counterstaining. Negative controls were done using the DIG-labeled sense strand in RiboHybe Buffer, in place of the DIG-labeled antisense probe. All reagents used were obtained from Ventana Medical Systems, unless specified otherwise.

3. Results

Total RNA isolation from RKO, U87 and A549 cells

Total RNA was isolated from 1×10^7 RKO cells, and 2×10^6 U87 cells and 2×10^6 A549 cells from Kryostocks, divided into 5 tubes each. RNA quantity was measured using a Nanodrop Photometer. RNA yields were $59.5 \mu\text{g}$ for RKO cells, $72.6 \mu\text{g}$ for U87 cells, and $121.4 \mu\text{g}$ for A549 cells. RNA quality was analyzed using a Bioanalyzer 2100 (Agilent), producing RIN values between 9.5 and 10 (see Figures 2).

Technical Tip 3:

RNA quality is important for reliable qRT-PCR, even more important when gene expression is monitored using northern blots. RNA quality was assessed using the Bioanalyzer 2100 (Agilent). RNA-Integrity-Number (RIN) values using capillary electrophoresis between 9.5 and 10 were obtained here, indicating RNA suitable for northern and *in situ* hybridization, and qPCR. RNase free conditions were used, with buffers prepared using DMPC-treated water and equipment treated with RNase Zap (Ambion). When isolating RNA from FFPE material, the RNA quantity and quality can be assessed as described above. Because of the fixation and embedding of the sample material, the RNAs are highly degraded. RIN values found for these RNAs made them unsuitable for northern blot analysis, but can still be used for qRT-PCR.

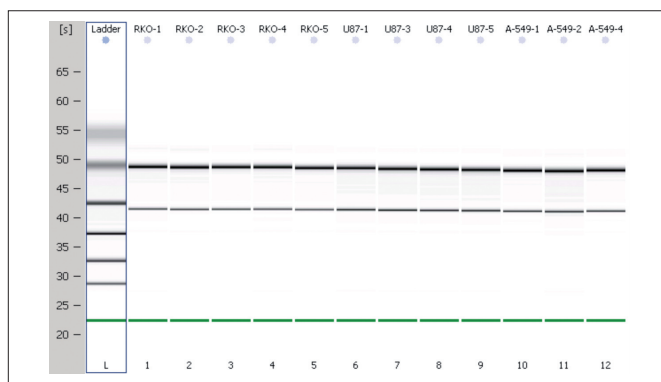


Figure 2a: Isolated total cellular RNAs reproducibly show high quality RNA in each of 5 tubes per cell type.

	Sample Name	Status	Result Label
1	RKO-1	✓	RIN: 10
2	RKO-2	✓	RIN: 10
3	RKO-3	✓	RIN: 10
4	RKO-4	✓	RIN: 10
5	RKO-5	✓	RIN: 10
6	U87-1	✓	RIN: 9.80
7	U87-3	✓	RIN: 9.80
8	U87-4	✓	RIN: 9.70
9	U87-5	✓	RIN: 9.80
10	A-549-1	✓	RIN: 10
11	A-549-2	✓	RIN: 9.50
12	A-549-4	✓	RIN: 10

Figure 2b: RIN values are reproducibly between 9.5 and 10, indicating excellent quality.

3. Results

RT-qPCR using RNA from cell lines

cDNAs were synthesized using hexamer-primers, and diluted to 10 ng/μl; 20 or 50 ng cDNA were used for each PCR. RT minus controls used water and 5 ng human genomic DNA. Positive controls were prepared using appropriate plasmid DNAs.

HGF and Her2 verification assay

PCR products were diluted 1:10, and 3 μl were mixed with 3 μl 2× loading buffer, and loaded on a 1.2 % agarose gel (Lonza), using a 100 bp to 4 kb molecular weight marker (Lonza); see Figure 3. PCR assays were also verified using the LightCycler® 480 Real-Time PCR Instrument (data not shown).

Resulting PCR products showed the correct band size. The HGF and Her2 assays also showed the appropriate results in positive and negative control reactions (data not shown).

MRPL19, HGF and Her2 m RNA gene expression

As shown in Table 1, using 20 ng cDNA, LightCycler® 480 Real-Time PCR produced the following crossing point (Cp) values (see Table 1).

As expected, the reference (housekeeping) genes ALAS1 and MRPL19 were expressed similarly in all cell lines. Cell line A549 was found to be negative for HGF showing a Cp of 40. This cell line was used as a negative control for the HGF northern blot hybridizations. Her2 was expressed at levels in U87 cells which were tenfold less than that found for RKO and A549 cells, both showing Cps of 31.

DIG-labeled RNA probes for Her2, ALAS1 and hHGF

DNA templates for *in vitro* transcription have the T7 promoter; 50 pg plasmid was used for PCR with the Expand High Fidelity^{PLUS} PCR System, dNTPack (Roche). Probe lengths were as follows: Her2 –1895 bp; ALAS1 – 448 bp, and hHGF – 508 bp; (see Figure 4).

PCR products were run on agarose gels to verify size. RNA probes were diluted 1:10 with TE buffer; 3 μl of each dilution were combined with 3 μl 2× Loading Buffer for a 1.2 % agarose gels (Lonza) with the 100 bp to 4 kb molecular weight marker (Lonza).

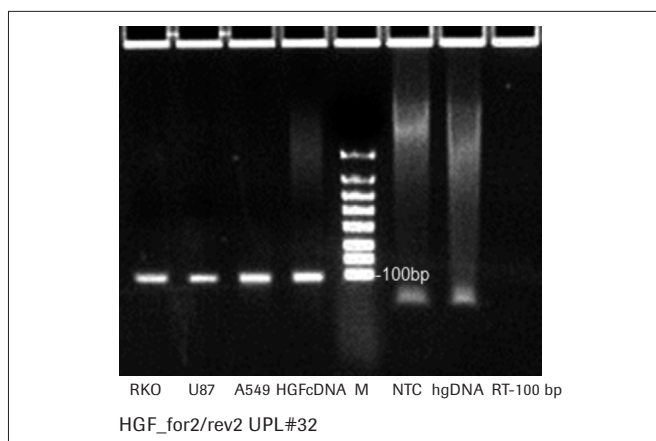


Figure 3: Gel image of PCR products generated using the HGF (81 bp) RT-PCR assay.

Template (20 ng)	ALAS1 CP	MRPL19 CP	HGF CP	Her2 CP
RKO	28.8	28.8	31.2	31.3
U87	29.7	29.6	32.6	35.0
A549	29.6	28.7	40.0	31.3

Table 1: Crossing point (Cp) values s generated for the different genes for each sample type.

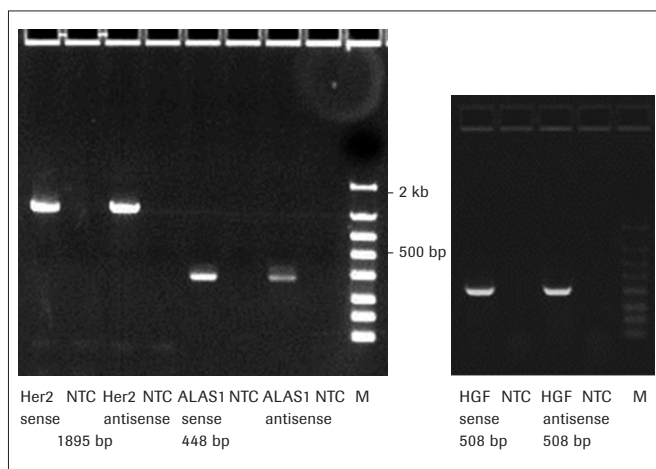


Figure 4: PCR products show correct size and no secondary products; see text for details.

3. Results

DIG labeling using *in vitro* transcription

Four microliters of each PCR product were used for *in vitro* transcription. DIG-labeled and unlabeled antisense and sense RNA probes were prepared for ALAS1, Her2 and HGF. *In vitro* transcribed RNAs were verified for size and quantity using agarose gels (see Figure 5).

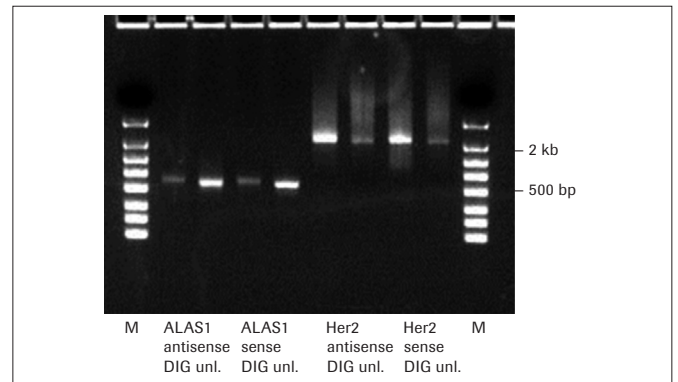


Figure 5: DIG-labeled RNA probes loaded with labeled and unlabeled transcripts on a non-denaturing agarose gel.

Sensitivity verified by dot blot assay

Dilution series of DIG labeled probes were applied to positively charged nylon membrane. After crosslinking, immunological detection using anti-DIG-AP-antibody and CDP-Star was done, according to the DIG Northern Starter Kit (see Figure 6). Probes could detect 1 pg. For rare transcript ISH, sensitivity of 0.1 pg is desirable. For ISH, it is important that antisense and sense RNA probes show the same detection sensitivity.

The sensitivity of all DIG-labeled RNA probes was found to be sufficient for northern blot and *in situ* hybridization. Dot blot assay showed the following concentrations could be detected: ALAS: 0.1 pg/ μ l; Her2: 0.03 pg/ μ l, and HGF: 0.03 pg/ μ l).

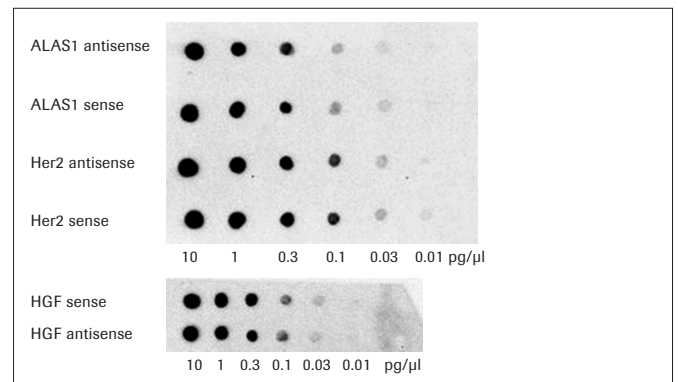


Figure 6: Dot blot analysis of DIG-labeled RNA probes; see text for details.

Northern blot analysis of DIG-labeled Her2 probe

One and ten micrograms of total RNA and DIG-labeled RNA molecular weight marker III (Roche) were separated on a denaturing MOPS/formaldehyde gel, transferred to positively charged nylon membrane, and hybridized using the Her2 antisense RNA probe (100 ng/ml) in DIG Easy Hyb Buffer (see Figure 7).

Technical Tip 4:

Load 1 μ g and 10 μ g DIG labeled RNA probes for northern blot analysis to identify both faint and robust signals. The weak expression of Her2 in U87 cells is readily detected when using 1 μ g RNA and a 3 min exposure. In contrast, signals using 10 μ g RNA are so strong that even brief exposures result in too strong a signal and high background.

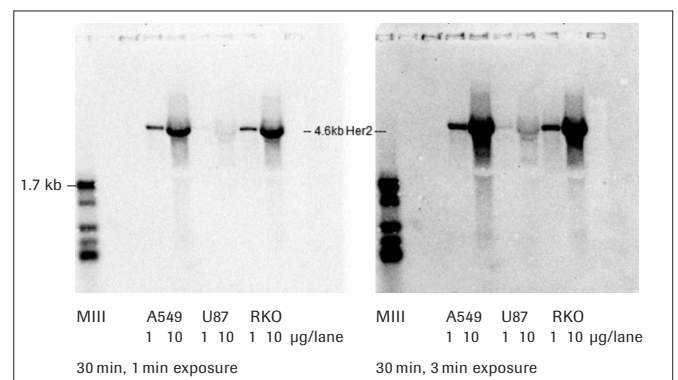


Figure 7: Northern blot analysis of total RNA from cell lines A549, U87 and RKO using the DIG-labeled Her2 antisense RNA probe. Her2 mRNA was detected in a band with the expected size of 4.6 kb. As expected from RT-PCR, U87 show only a faint band. Images show 1 min and 3 min exposure time results.

3. Results

Northern blot analysis of A549, U87 and RKO cells using DIG-labeled HGF RNA probe

The DIG-labeled HGF probe detected two HGF splice variants of 5.9 and 2.9 kb in RKO cells using 100 ng total RNA (see Figure 8). Results in U87 cells showed a weaker signal, detecting predominantly the 5.9 kb transcript. In A549 cells, no HGF was detected, consistent with our RT-PCR results. These findings underscore the importance of testing RNA probes using filter hybridization prior to ISH. These real-time PCR results indicate that having qRT-PCR data available for the sample materials you are using, prior to northern blot analysis, is beneficial.

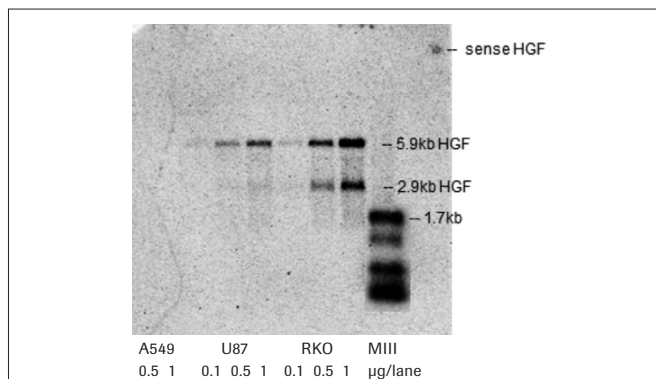


Figure 8: Northern blot hybridization with the DIG-labeled HGF RNA probe. This probe detects two splice variants. Even in U87 which expresses approximately tenfold less HGF mRNA as seen from the qRT-PCRs, the respective bands can be detected in as little as 100 ng of total RNA. Incubation with CDP-Star for 4 hrs, followed by an 8 min exposure on film.

In situ hybridization using DIG-labeled HGF and Her2 RNA probes

FFPE tissue samples were deparaffinized, and used for *in situ* hybridization. HGF and Her2 transcripts were visualized on tissue sections as a blue nitro blue tetrazolium reaction product. Sense strand hybridization produced only low background. Red Stain II counterstaining (Ventana Medical Systems) shows useable morphology for cellular localization.

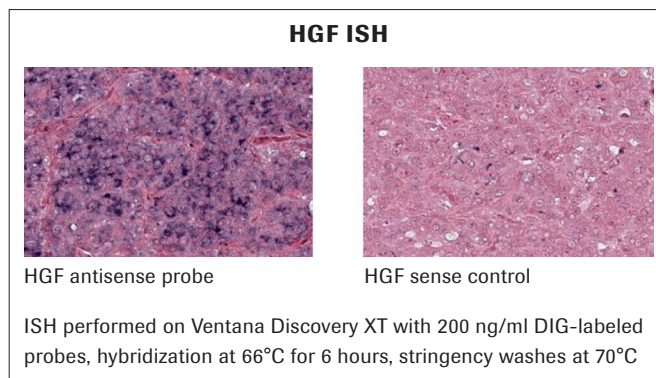


Figure 9: mRNA ISH staining of HGF transcripts in FFPE section.

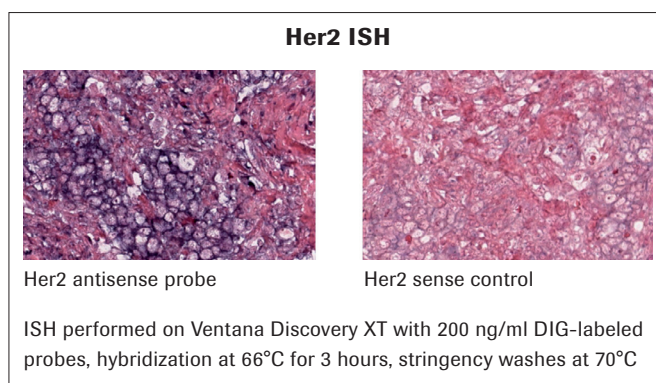


Figure 10: mRNA ISH staining of Her2 transcripts in an FFPE section.

4. Conclusion

In situ hybridization can be challenging. Here, we present a straightforward procedure using DIG-labeled RNA probes for *in situ* hybridization in a standardized workflow. The key steps are:

- Design intron-spanning RNA probes using reliable sequences.
- Generate templates, containing either T3 or T7 RNA polymerase promoters for *in vitro* transcription, using PCR.
- Prepare labeled sense and antisense RNA probes using *in vitro* transcription.
- Test RNA Probe detection sensitivity using a dot blot assay, with sense and antisense probe having identical sensitivity
- Perform qRT-PCR to determine cellular and/or tissue transcript abundance
- Perform northern blot analysis to verify *in situ* hybridization
- Perform *in situ* hybridization and DIG detection

The DIG System produces high sensitivity in a reliable and reproducible manner. As shown here, rare transcripts are detectable in as little as 100 ng total RNA. Synthesizing DIG labeled RNA probes from PCR templates with T7 or T3 RNA polymerase promoter sequences is rapid and robust.

4. Conclusion

Ordering Information

Product	Catalog Number	Pack sizes
High Pure RNA Isolation Kit	11 828 665 001	1 Kit for up to 50 reactions
High Pure FFPE RNA Micro Kit#*	04 823 125 001	1 Kit for up to 50 reactions
Expand High Fidelity ^{Plus} PCR System, dNTPack	04 743 725 001	125 units
Transcriptor High Fidelity cDNA Synthesis Kit	05 091 284 001	Kit for 100 reactions
Sp6/T7 Transcription Kit	10 999 644 001	1 Kit (2 × 20 transcription reactions)
RNA molecular weight marker I, digoxigenin-labeled (0.3 – 6.9 kb)	11 526 529 910	4 µg (200 µl)
RNA molecular weight marker III, digoxigenin-labeled (0.3 – 1.5 kb)	11 373 099 910	2 µg (200 µl)
DIG Northern Starter Kit	12 039 672 910	1 kit (10 labeling reactions and detection of 10 blots of 10 × 10 cm ²)
DIG Wash and Block Buffer Set	11 585 762 001	for at least 30 blots à 100 cm ²
DIG Easy Hyb	11 603 558 001	500 ml
Nylon Membranes, positively charged	11 209 299 001	20 sheets with 10 × 15 cm
Anti-Digoxigenin-AP, Fab fragments	11 093 274 910	150 units (200 µl)
CDP-Star, ready-to-use	12 041 677 001	2 × 50 ml
Actin RNA Probe, DIG-labeled	11 498 045 910	2 µg
HGF: Universal ProbeLibrary Probe#32	04 687 655 001	2 vials with 125 µl (10 µM)
Her2: Universal ProbeLibrary Probe#85	04 689 097 001	2 vials with 125 µl (10 µM)
ALAS1: Universal ProbeLibrary Probe RTR ID#102108		
MRPL19: Universal ProbeLibrary Probe#144	04 694 309 001	2 vials with 125 µl (10 µM)
RealTime ready Catalog Assay Design #102108 ALAS1, human	05 532 957 001	300 reactions
Buffers in a Box, Premixed SSC Buffer, 20X	11 666 681 001	4 l
Agarose MP	11 388 991 001	500 g
LightCycler [®] 480 Instrument II	05 015 278 001	1 instrument (96-well version)
LightCycler [®] 480 Probes Master	04 887 301 001	10 × 500 reactions, 20 µl volume
LightCycler [®] 480 Multiwell Plate 96, white	04 729 692 001	5 × 10 plates with sealing foils

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