Milenia GenLine

HybriDetect 2T

Universal lateral flow dipstick for simultaneous detection of **two different analytes** (proteins, genomic amplificates) labelled with FITC/FAM and biotin or digoxigenin

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Milenia GenLine **HybriDetect 2T** REF **MGHD2 1** Σ **100**

IFU / REF MGHD2 1 / REV F / 2025-07-29

Note: Significant changes are indicated by dotted lines in the margin.
A change history can be found at the end of the manual.

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Table of Contents

Explanation of Symbols

Symbol	Explanation	Symbol	Explanation
	*Manufacturer*		Temperature limit
	Use-by date		Consult *instructions for use* or consult electronic *instructions for use*
	*Batch Code*		Contains biological material of animal origin
	*Catalogue number*		Contains sufficient for <n> tests
	Unique Device Identification		

Warnings and Precautions

- Store all reagents at 2-8 °C in their original containers.
- Before use, bring all reagents to room temperature (18–28 °C).
- The expiration date of all components must be observed. Do not use components past the expiration date!
- Protect Dispsticks from humidity. Containers must always be closed.
- Touch and label only the foil-covered areas of the dipsticks.
- Do not interchange components from different lots.
- The disposal of waste materials must be carried out according to current local regulations.
- The assay buffer contains an anti-microbial reagent; therefore avoid contact with skin and/or mucous membranes.
- Animal materials which are used in the reagent are free from infection pathogens and are derived from animals kept in Germany
- For professional users

Materials Supplied, Storage and Stability

Materials Supplied

REF	Contents		Number of Tests
MGHD2 1	2 x 50 2 x 10 ml	HybriDetect 2T **Dipsticks** (MGDS2A) HybriDetect 2T **Assay Buffer** (MGCBB)	100

Storage and Stability

Components	Description	Preparation	Storage	Shelf Life
HybriDetect 2T **Dipsticks** (MGDS2A)	Membrane coated with biotin-ligand, polyclonal anti-digoxigenin antibody (goat) and polyclonal anti-goat antibody (rabbit) Gold coated with polyclonal anti-FAM/FITC antibody (goat)	Ready to use	2-8 °C Container must always be closed (protected from moisture)!	Until expiry date
HybriDetect 2T **Assay Buffer** (MGCBB)	Citrate-Phosphate buffered saline	Ready to use	2-8 °C	Until expiry date

Materials Required but not Supplied

- Pipets
- Pipet tips (containing protective filters for PCR)
- Reaction tubes or 96-well microtiter plate

Intended Use

HybriDetect 2T is a universal lateral flow dipstick for simultaneous detection of two different analytes (proteins, genomic amplificates) labelled with FITC, biotin and digoxigenin. It is a development platform. The test is for research use only, not for diagnostic purposes.

Method

Milenia HybriDetect 2T is a ready-to-use, universal test strip (dipstick), which is based on the lateral flow technology using gold particles. The dipstick is designed to develop qualitative or semi-quantitative rapid test systems for simultaneous detection of two different analytes such as proteins, antibodies, or gene amplifications. The user needs to develop two analyte-specific solutions.

Solution A: Contains a first detector (e.g. antibody, antigen, specific probe) labeled with FITC or FAM and a second one (e.g. antibody, primer) labeled with biotin.

Solution B: Contains a first detector (e.g. antibody, antigen, specific probe) labeled with FITC or FAM, too and a second one (e.g. antibody, primer) labeled with digoxigenin (see "Common Testprinciple").

The sample to be determined is mixed with the developed analyte-specific solutions and then applied to the sample application area.

The analyte A-complex labeled with FITC/FAM and biotin binds first to the gold-labeled FITC/FAM-specific antibodies in the sample application area of the dipstick. The analyte B-complex (labeled with FITC/FAM and digoxigenin) also binds to the FITC/FAM labeled gold nanoparticles in this region. Capillary forces cause the gold complexes A and B to diffuse across the analytical membrane. Only the analyte captured gold particles will bind when they overflow the immobilized biotin-ligand molecules at the respective test band (test band A- analyt A, test band B- analyt B) and generate a red-blue band over the time. Not-captured gold particles flow over the control band and will be fixed there by species-specific antibodies. With increasing incubation time, the formation of an intensely colored control band appears.

Common Test Principle

The test strip consists of three major zones: the SAP, the nitrocellulose membrane, and the wicking pad (Figure 1).

The **SAP** (= sample application pad) is the zone where the sample is applied to the test strip. Additionally, it contains the gold nanoparticles which are responsible for the visualization of the test and control line. The gold nanoparticles on the SAP have a diameter of 40 nm and are coupled with polyclonal goat anti-FITC/FAM antibodies.

The nitrocellulose **membrane** contains the two test lines (T1 and T2) and the control line (C). T1 consists of immobilized streptavidin, T2 consists of immobilized polyclonal goat anti-digoxigenin (anti-DIG) antibodies and the control line consists of immobilized polyclonal rabbit anti-goat antibodies.

The **wicking pad** is an absorbent pad which moves the liquid to the top of the test strip due to capillary forces.

Additionally, a transparent **cover foil** is added on top of the test strip to protect the membrane from physical damage.

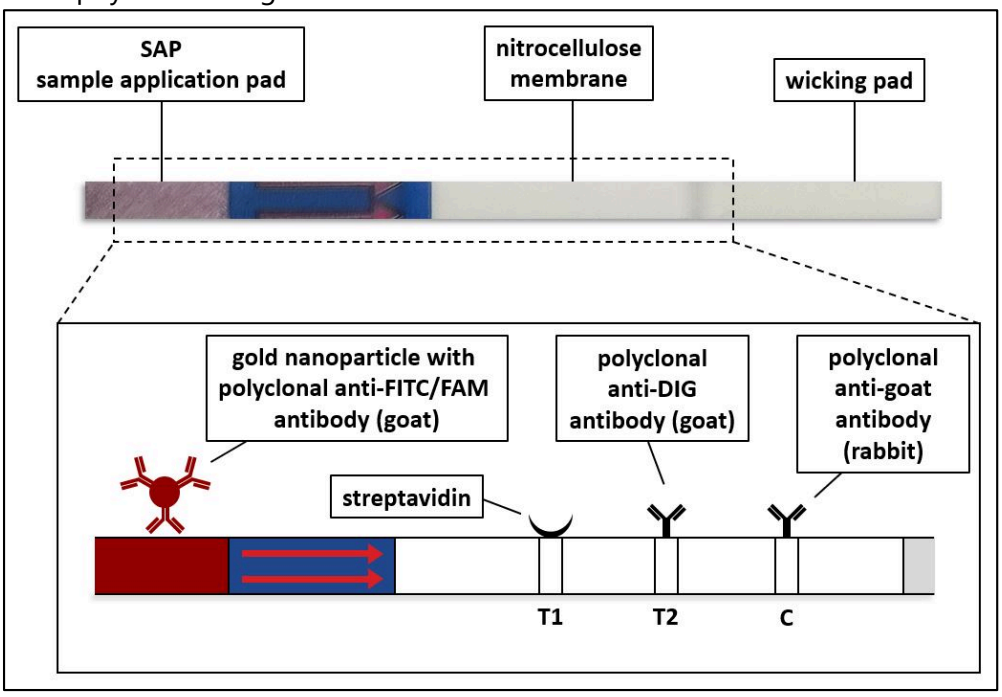

Figure 1: Design of the Milenia GenLine HybriDetect 2T test strip (MGDS2A) (simplified)

In absence of a dual labelled analyte (i.e., the sample is negative) the test line will stay invisible (Figure 2). Unlabelled analytes cannot bind to the streptavidin or the anti-digoxigening antibody and will rush through the test lines. The gold nanoparticles coupled with goat antibodies are caught by the anti-goat antibodies immobilized on the control line, which lead to a colouration of the control line.

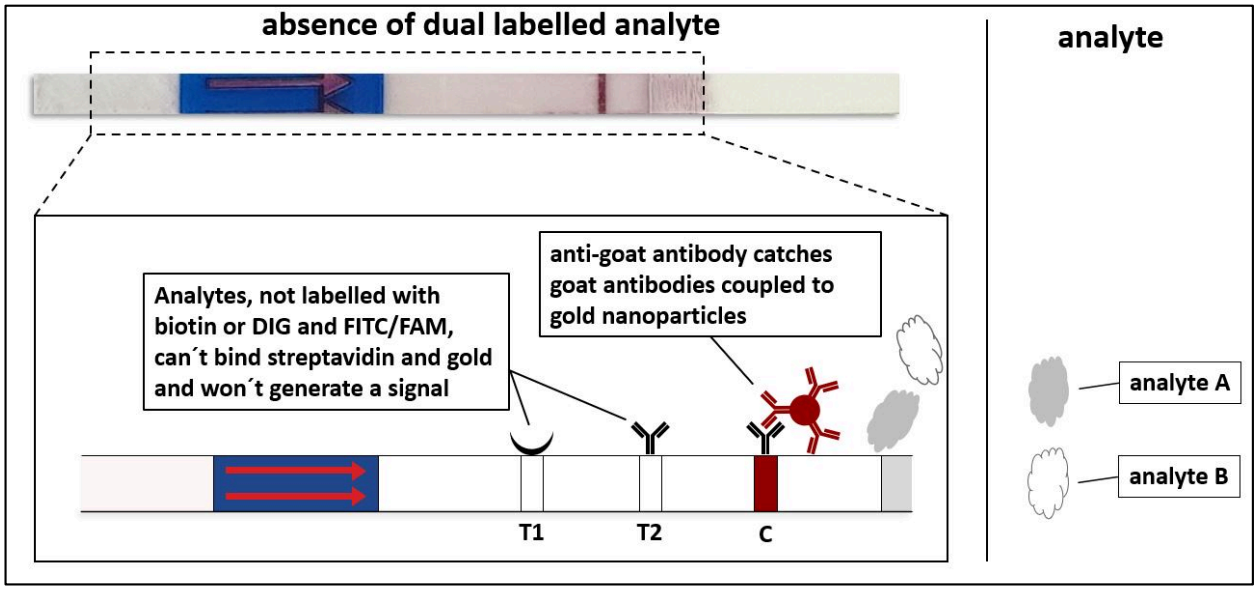

Figure 2: Result of a negative sample applied to the Milenia GenLine HybriDetect 2T test strip (MGDS2A)

In the presence of a dual labelled analyte (i.e., the sample is positive) the test line will turn red (Figure 3). Biotin bound to the analyte is captured by the streptavidin immobilized on test line 1. Digoxigenin bound to the analyte is captured by the anti-digoxigenin antibody immobilized on test line 2. The FITC/FAM of the analyte interacts with the anti-FITC/FAM antibodies of the gold nanoparticles. Holding them back leads to an accumulation of the gold that colours the test lines red. Additionally, the gold nanoparticles which were not immobilized by the test line are caught by the anti-rabbit antibodies immobilized on the control line. This leads to a colouration of the control line.

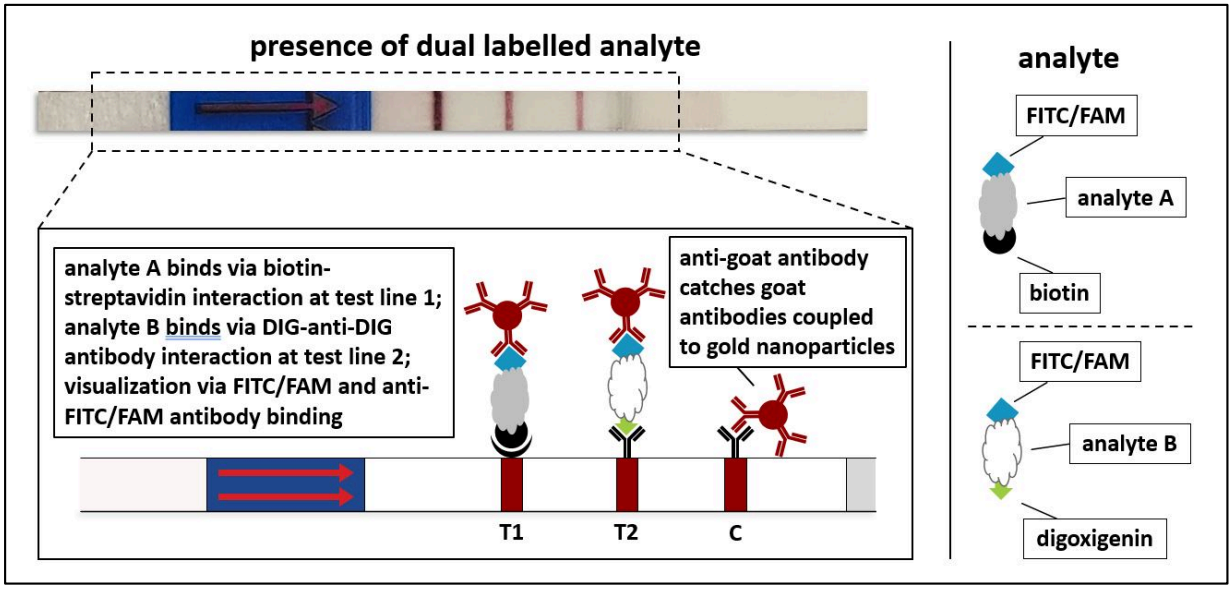

Figure 3: Result of a positive sample applied to the Milenia GenLine HybriDetect 2T test strip (MGDS2A)

Sample Application

The sample application has an impact on the evaluation result. There are two different ways to apply the sample to the Milenia GenLine HybriDetect 2T test strip (MGDS2A).

1. The sample can be applied directly to the SAP (Sample Application Pad). Typical sample volumes are 2-10 µl. Subsequently the test strip is dipped into the chase buffer (MGCBB) with the SAP-site first and is incubated for several minutes in an upright position. Typical buffer volumes are 80-100 µl.
2. The sample can be mixed with the chase buffer (MGCBB). Subsequently the test strip is dipped into the sample-chase buffer-mix with the SAP-site first and is incubated for several minutes in an upright position. Use about 100 µL fluid (sample material and analyte-specific solution/chase buffer) for the assay procedure.

There is no general recommendation for one sample application method. The best method is to be chosen by your own personal preference. The provided assay buffer (MGCBB) may be used as a basic buffer and works well for most applications. In some cases, it can be helpful to optimize this buffer for your specific application.

The minimum incubation time of Milenia GenLine HybriDetect test strip (MGDS2A) is 2 minutes. We recommend an incubation time of 5 minutes for good results. The incubation time should not exceed 15 minutes.

The evaluation (by eye, picture, etc.) should be done immediately after the run is finished. During the drying process of the membrane (after the run finished) some gold particles can agglomerate at the test lines and form a slight false-positive band after several minutes. Once dried, the result becomes invalid.

Note: Volumes, analyte-specific solution, chase buffer and incubation time can be adjusted to your needs and are part of the individual test development.

Interpretation of Results

There are two possibilities to evaluate Milenia GenLine HybriDetect dipstick results: a qualitative evaluation and a semi-quantitative evaluation.

a) Qualitative Evaluation

With our Milenia GenLine HybriDetect test strip no additional instrument is required for evaluation. The evaluation can be done by the naked eye due to the presence and absence of the test and control line (see Figure 4 and Table 1).

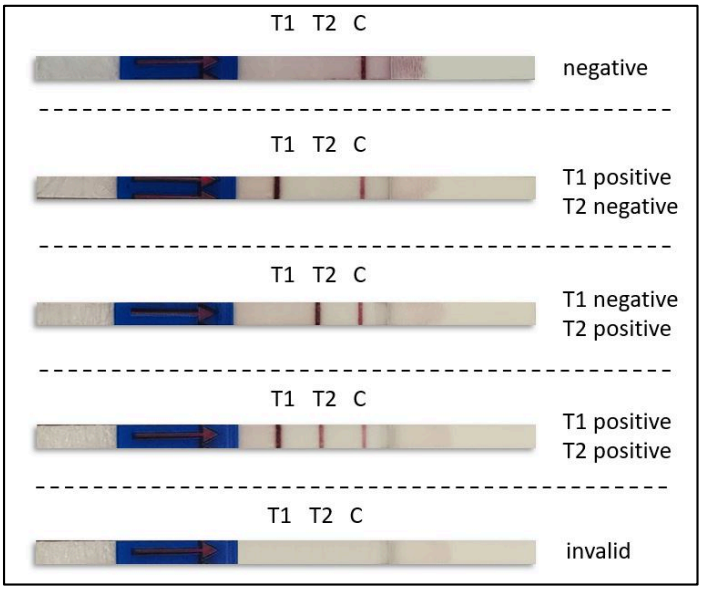

Figure 4: Visual evaluation of the Milenia GenLine HybriDetect 2T test strip (MGDS2A). C indicates the position of the control line. T1 and T2 are indicating the position of the first and second test line.

In any case, the control band must be visible! It is a control function and cannot be used to assess the quality of the result of the test band. If the control band is not visible after the incubation period, the result is invalid! The test must be repeated with a new dipstick!

Table 1: Correct interpretation of the Milenia GenLine HybriDetect 2T test strip (MGDS2A).
T1 = test band 1; T2 = test band 2; C = control line.

T1	T2	C	Interpretation
positive	positive	positive	• Control band is clearly visible, test run is valid • **Amplicon A and B is detected (positive)**
positive	negative	positive	• Control band is clearly visible, test run is valid • **Amplicon A is detected (positive)** • **Amplicon B is not detected (negative)**
negative	positive	positive	• Control band is clearly visible, test run is valid • **Amplicon A is not detected (negative)** • **Amplicon B is detected (positive)**
negative	negative	positive	• Control band is clearly visible, test run is valid • **Amplicon A is not detected (negative)** • **Amplicon B is not detected (negative)**
negative	negative	negative	• Control band is not visible, **test run is invalid** • No reliable information regarding amplicon detection • Repeat the test with a new test strip

b) Semi-quantitative Evaluation

The intensity of the test line correlates positively with the amount of dual-labelled analyte in a certain range. This allows for a semi-quantitative evaluation if only one test line is visible. The easiest way for a semi-quantitative evaluation is via evaluation cards (Figure 5).

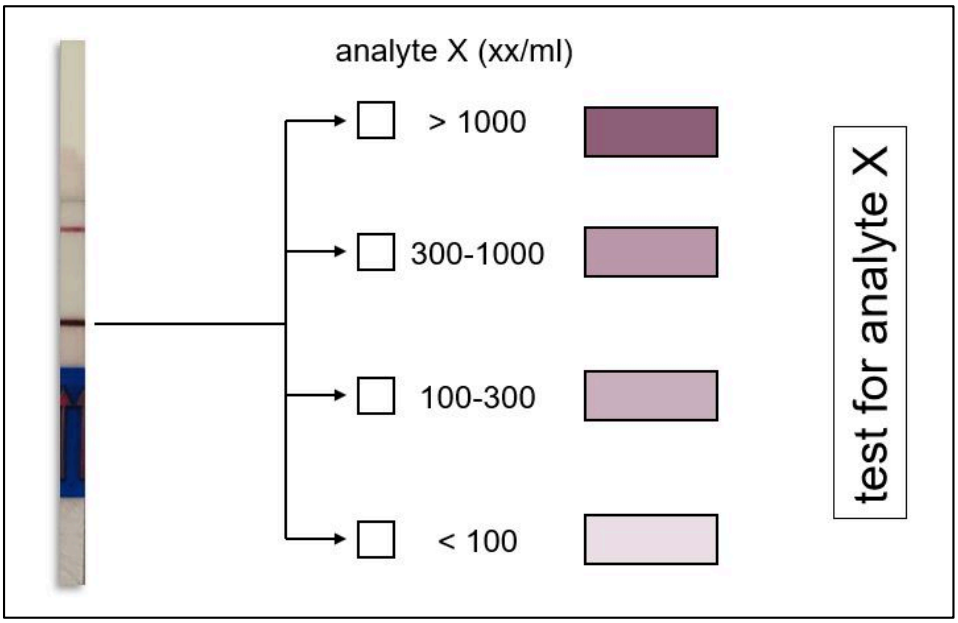

Figure 5: Example for a semi-quantitative evaluation of the Milenia GenLine HybriDetect 2T test strip (MGDS2A) via evaluation card for the analyte X

Note: The presence of test line 1 weakens the signal intensity of test line 2. Therefore, a semi-quantitative evaluation for both analyts at the same time is not possible. Semi-quantitative evaluation is only possible for one analyte.

Other common ways are the use of evaluation apps and/or extra devices such as our **Lateral Flow Reader myilab research** (REF: MYILABR 1).

The myilab research is a universal dipstick reader that can be used to quantify all our HybriDetect products. The HybriDetect Cassette (MGHC 1) or the myilab research adapter can be inserted directly into the reader (Figure 6 A). The HybriDetect (MGHD 1) and HybriDetect 2T (MGHD2 1) test strips can also be measured directly without any intermediate steps thanks to the adapter (Figure 6 B).
For more information regarding the semi-quantitative evaluation with our reader take a closer look at our blog article or contact us.

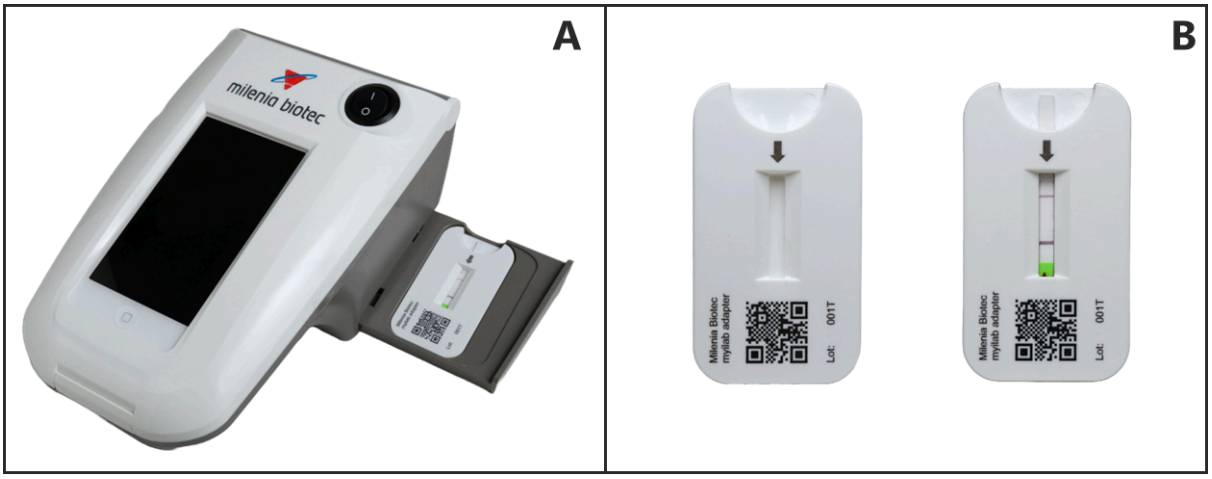

Figure 6: The myilab research. (A) The console of myilab research with an open drawer for the insertion of the Milenia GenLine HybriDetect Cassette (REF: MGHC 1) or the myilab research adapter. (B) The myilab research adapter for the evaluation of the Milenia GenLine HybriDetect (REF: MGHD 1) and Milenia GenLine HybriDetect 2T (REF: MGHD 1) test strip.

Assay Development Guide

The HybriDetect is an evaluation platform. The development of a solution containing two different labelled detectors for the analyte is up to the customer.

The Milenia GenLine HybriDetect strip can be used for the detection of **proteins** and **genomic amplicons**. In general, the aim for each assay is to generate an analyte labelled with biotin and/or digoxigenin and FITC/FAM. For the detection of proteins and antibodies this can be achieved due to four labelled protein specific antibodies. Detection of RNA/DNA can be accomplished by using an amplification reaction like PCR (polymerase chain reaction), RPA (recombinase polymerase amplification), LAMP (loop-mediated isothermal amplification) or CRISPR/Cas reaction. The combination of each of those methods with HybriDetect is described in more detail in the following sections.

a) Assay Development Guide: Protein and Antibody Detection

There are two options for the detection of proteins or antibodies. The first option is the usage of two different protein specific monoclonal antibodies. The antibodies target different locations of the protein to prevent competition for the same binding location. For the first target visualized on test line T1 one antibody must be labelled with biotin, the other one with FITC/FAM. For the second target visualized on test line T2 one antibody must be labelled with digoxigenin, the other one with FITC/FAM. Protein labelling kits are available from different companies.

The second option is the usage of one protein specific polyclonal antibody. The polyclonal antibody gets separated into two fractions. For the first target visualized on test line T1 one fraction is getting labelled with biotin, the other one is labelled with FITC/FAM. For the second target visualized on test line T2 one fraction gets labelled with digoxigenin, the other one with FITC/FAM. The labelled antibodies are mixed with the target protein. After a short incubation period the sample can be applied to the test strip. The test procedure is shown below (Figure 7).

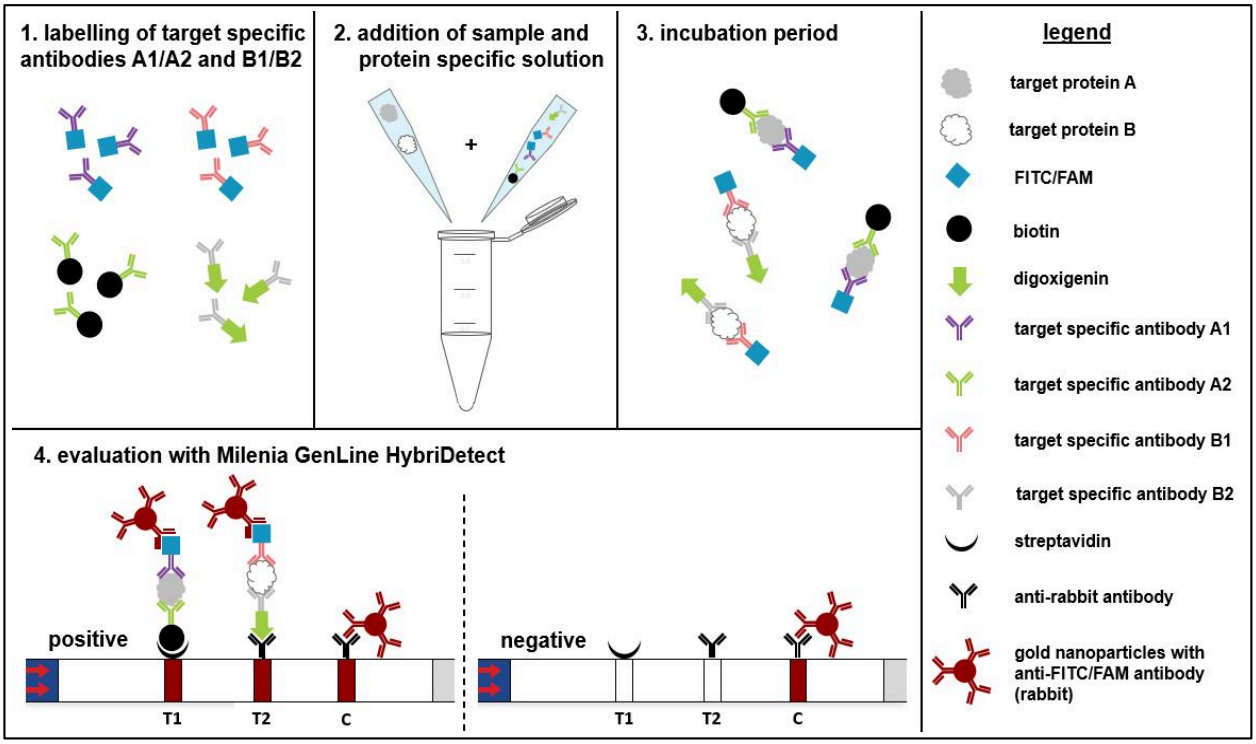

Figure 7: Test procedure for proteins and antibodies with Milenia HybriDetect 2T strip

b) Assay Development Guide: PCR

The polymerase chain reaction (PCR, RT-PCR) is the easiest way to detect DNA/RNA. The HybriDetect can visualize dual labelled oligo sequences. These sequences must be labelled with either biotin and FAM/FITC or digoxigenin and FAM/FITC.

Primer design

For the first target visualized on test line T1 the label incorporation is achieved during the PCR due to one FITC/FAM primer and another biotin labelled primer. For the second target visualized on test line T2 the label incorporation is achieved due to one FITC/FAM primer and another digoxigenin labelled primer. For primer design stick to common PCR and primer design rules:

- Length: The recommended primer length for PCR is 18-30 bp.
- Melting temperature: The primer annealing depends on the melting temperature T_m. The melting temperature should usually be between 65-75 °C and the T_m difference of the primers should not be higher than 5 °C. The melting temperature can be adjusted by the GC-content of the primer and the primer length.
- GC-content: The GC-content should be between 40-60 %.
- Sequence: Avoid repeating sequences as well as the repeat of mononucleotides like "AAAA" or "ATATATAT". The 3´ end should be a C or G to enhance binding.
- Label incorporation: The label (FITC/FAM or biotin) must be incorporated at the 5´end of the primer to allow elongation by the polymerase.

Dimer formation of the labelled primers should be avoided. If the sequences of the primers match partially, they will hybridize and form dimers. Those dimers which have both a FITC/FAM and biotin label would be shown as positive by the HybriDetect strip. See chapter Assay development guide: Check for primer dimers for more information on how to prevent dimerization and how to test your designed primers in lab.

For more information regarding the combination of PCR and HybriDetect check out the article Polymerase Chain Reaction & Lateral Flow on our website.

Do you want to know how other scientists have combined PCR and HybriDetect? Then have a look at our online literature database.

c) Assay Development Guide: RPA

The Recombinase Polymerase Amplification (RPA; RT-RPA) is one of the most used amplification strategies that is combined with the Milenia HybriDetect platform. RPA is an isothermal amplification method (37-42 °C) that can be performed without additional devices, allowing specific detection of DNA at the point-of-care if combined with our universal lateral flow strip. The patent holder of RPA and therefore only supplier of RPA reagents is TwistDx, which is part of Abbott since 2018 (status: April 2023).
RPA amplicons can be visualized due to FITC/FAM and biotin or digoxigenin label. The label incorporation is achieved during the RPA reaction due to two labelled primer, two labelled probes and non-labelled primers. Generally, RPA allows extremely rapid and robust analysis. But RPA is not known to be the perfect technique for multiplexing. Nevertheless, it is possible to develop multiplex RPA assays that are perfectly compatible with the multiplex Lateral Flow Device. Please take a look on on website to learn more about Multiplex Applications & Lateral Flow.

Primer design

Even if PCR primers can work as well, the design of special RPA primers will lead to higher sensitivities and shorter reaction times. The following list is a short guideline on how to design your RPA primers:

- Length: RPA primers are longer than PCR primers. The recommended primer length is 30-35 bp, sometimes 45 bp.
- Melting temperature: For RPA the melting temperature is irrelevant. Primer annealing is driven by the recombinase-primer-complex which is independent of the melting temperature of the primer.
- Label incorporation: The label (FITC/FAM or biotin) must be incorporated at the 5´end of the primer to allow elongation by the polymerase.
- GC-content: The GC content should be between 30-70 %.
- Sequence: Avoid repeating sequences as well as the repeat of mononucleotides like “AAAAA”.

Note: With increasing primer length, the risk of secondary structures will rise, decreasing the sensitivity of the assay. Primers that are too short will reduce the reaction speed and lower the sensitivity as well.

Nfo probe design

RPA is a highly processive amplification method. In order to avoid the detection of non-specific RPA-products via lateral flow, it is highly recommended to add an additional specificity-generating step to the RPA, the Endonuclease IV (nfo). TwistDx has so far offered the TwistAmp® nfo kit for this purpose. On the basis of this kit no longer being available since 2022, it should be recreated itself by adding endonuclease IV to the TwistAmp® Basic-Kit.

For the use of Endonuclease IV, a special nfo probe is needed (Figure 8). This probe consists of a tetrahydrofuran (THF), an internal abasic nucleotide which replaces a nucleotide. Additionally, it consists of a C3-spacer at the 3´end and a label (FITC/FAM or biotin) at the 5´end. The THF is the binding site for Endonuclease IV which will cleave the nfo probe if the probe is fully hybridized to their complementary target sequence.

The C3-spacer acts as a blocking group so elongation due to polymerase activity is not possible if the probe is not hybridized to the target sequence. This way the formation of dual labelled false amplicons is prevented. The following list is a brief guide on how to design your nfo probe:

- The probe length should be between 46 and 52 bp
- There should be a minimum of 30 bp between the THF and the 5´end
- There should be a minimum of 15 bp between the THF and the 3´end
- The THF replaces a nucleotide and is not an additional nucleotide
- The C3- spacer is located at the 3´end
- The label (FITC/FAM or biotin) is incorporated at the 5´end

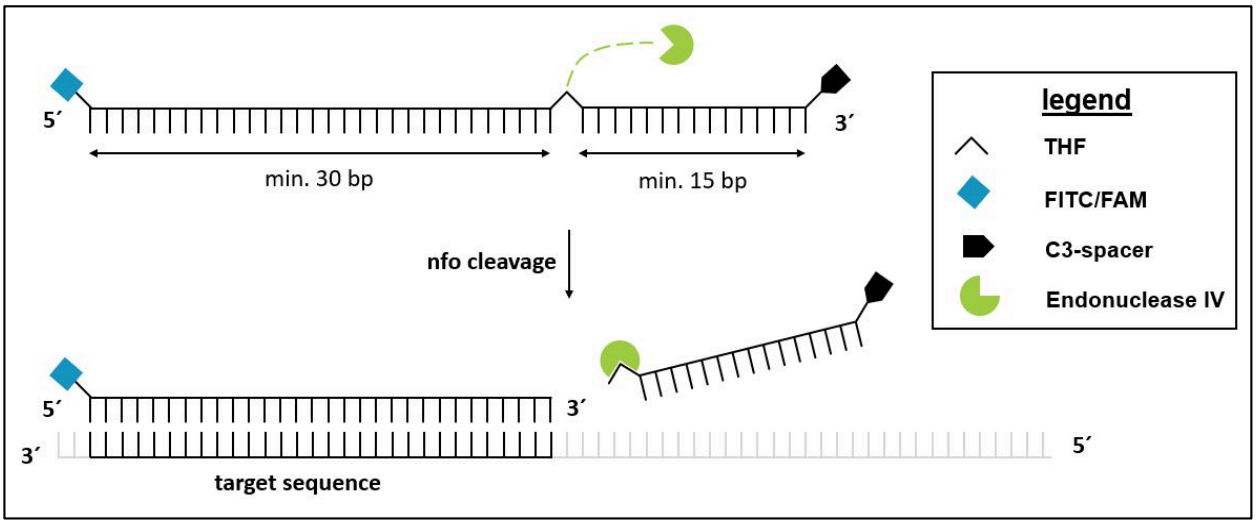

Figure 8: Structure of a nfo probe for the combination of RPA and lateral flow. As label FITC/FAM is shown. It can also be labelled with biotin instead of FITC/FAM. Endonuclease IV (nfo) cleaves nfo probe after completely binding to the target sequence.

For more detailed information regarding RPA primer and nfo probe design, target selection and reaction conditions we highly recommend the RPA Assay Design Manual from TwistDx.

Note: Please make sure to avoid dimer formation of the labelled primer and probe. If the sequences of the primers match partially, they will hybridize and form dimers. Those dimers would have both a FITC/FAM and biotin or digoxigenin label which would be shown as positive by the HybriDetect strip. See chapter Assay development guide: Check for primer dimers for more information on how to prevent dimerization and how to test your designed primers in lab.

For more information regarding the combination of RPA and HybriDetect check out the article Recombinase Polymerase Amplification & Lateral Flow on our website.

Do you want to know how other scientists have combined RPA and HybriDetect? Then have a look at our online literature database.

d) Assay Development Guide: LAMP

The Loop mediated isothermal Amplification (LAMP) is another molecular method that can be combined with the Milenia HybriDetect strips and is a patent-free alternative to RPA (status: April 2023). LAMP is an isothermal amplification method (60-72 °C) that can be performed with a low-cost heating device, allowing a cheap and specific detection of DNA at the point-of-care if combined with the HybriDetect lateral flow strip. LAMP amplicons can be visualized due to FITC/FAM and biotin or digoxigenin label. The label incorporation is achieved during the LAMP reaction due to two labelled primers and at least four non-labelled primers for one target.

Primer design

In contrast to PCR and RPA, the LAMP reaction is more complex due to the presence of more primers. The following abstract describes the primer design recommendation for the generation of a biotin FITC/FAM fragment which can be visualized on the T1 line. For generation of a digoxigenin and FITC/FAM fragment the recommendations are equivalent.
Four primers are necessary to initiate the LAMP reaction (Figure 9). This includes two loop-forming, modular designed inner primers (FIP and BIP) and two outer primers (F3 and B3), which are necessary for strand displacement of the characteristic FIP- and BIP-related elongation product. Additional loop primers (LF and LB) will improve the amplification efficiency. Due to the complexity, it is not recommended to design LAMP-Primers by hand. Rather, it makes sense to use available online tools to find an appropriate primer set and use this as a basis for further optimization. Eiken's PrimerExplorer (V5) and the NEB LAMP – Primer Design Tool are useful tools when it comes to LAMP primer design.

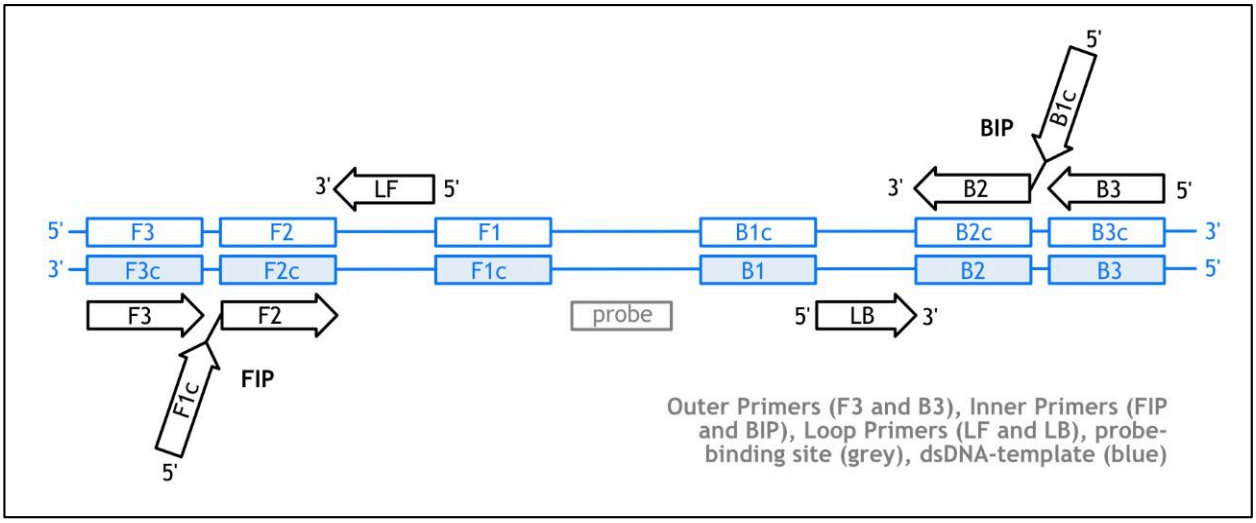

Figure 9: Primers (and probe) needed for LAMP reaction

There are different options for FITC/FAM and biotin incorporation when it comes to LAMP. In general, it is possible to label all, the FIP, BIP, LF and LB primer. The easiest option is labelling of the FIP and BIP primer. This combination shows a high sensitivity but it´s more likely to form unspecific dual labelled amplicons which will be shown as false positive by the lateral flow strip. Therefore, testing different combinations can be helpful.
Another option is the use of a labelled probe which is added to the LAMP product. Using a labelled probe can significantly enhance the overall specificity, but an additional work step makes the workflow more time consuming. Table 2 will give a brief overview of existing labelling strategies used by scientists.

Table 2: Different primer labeling strategies for the combination of LAMP and Milenia GenLine HybriDetect

Labelled Component	Label Location	Post LAMP Hybridization	Specificity	Probability Cross Primer Dimers	Ref
FIP + BIP	FIP: 5' Biotin BIP: 5' FITC/FAM	no	++	+++	(1-3)
FIP + LF/BIP + BF	FIP/BIP: 5' Biotin LF/BF: 5' FITC/FAM	no	++	++	(4,5)
LF + BF	LF: 5' Biotin BF: 5' FITC/FAM	no	++	+	(6)
dNTPs + LF or BF	dNTPs: Biotin-11-dUTP LF/BF: 5' FITC/FAM	no	++	-	(7)
dNTPs only	dNTP-1: Biotin-11-dUTP dNTP-2: FITC-aha-dUTP	no	+	-	(8)
FIP or BIP + probe	FIP/BIP: 5' Biotin probe: 5' FITC/FAM	yes	+++	++	(9-12)
LB + probe (in LAMP)	LB: 5' Biotin probe: 5' FITC/FAM, 3' inversed dT	no	+++	+	(13)
FIP + probe (in LAMP)	FIP: 5' Biotin probe: 5' FITC/FAM , 3' Spacer C3	no	+++	++	(14)

For more information regarding the combination of LAMP and HybriDetect check out the articles Loop mediated isothermal Amplification & Lateral Flow on our website and our summary about LAMP and lateral flow.

To learn more about LAMP primer design and assay optimization we highly recommend Lucigen's webinar video.

Do you want to know how other scientists have combined LAMP and HybriDetect? Then have a look at our online literature database.

e) Assay Development Guide: CRISPR/Cas

The usage of Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated nuclease (CRISPR/Cas) systems has increased dramatically since the first publication describing this technique. In general, there are two different mechanisms of CRISPR/Cas based methods: label separation and the label incorporation.
While label separation is not combinable with the Milenia HybriDetect 2T strip label incorperation allows you multiplexing.

The **label incorporation** method focuses on the Cas9 protein. For this method the Cas9 protein is labelled with FITC/FAM. The amplicon is labelled with biotin or digoxigenin during a previous amplification method like PCR, RPA or LAMP. The complex of a chimeric guide RNA and Cas9 enables the specific "recognition" of the amplicon. This leads to a "molecular sandwich" which has both a FITC/FAM label on the one side and a biotin or digoxigenin label on the other. This amplicon-CRISPR-Cas9 complex is detectable via Lateral Flow using the Milenia HybriDetect 2T (Figure 10).

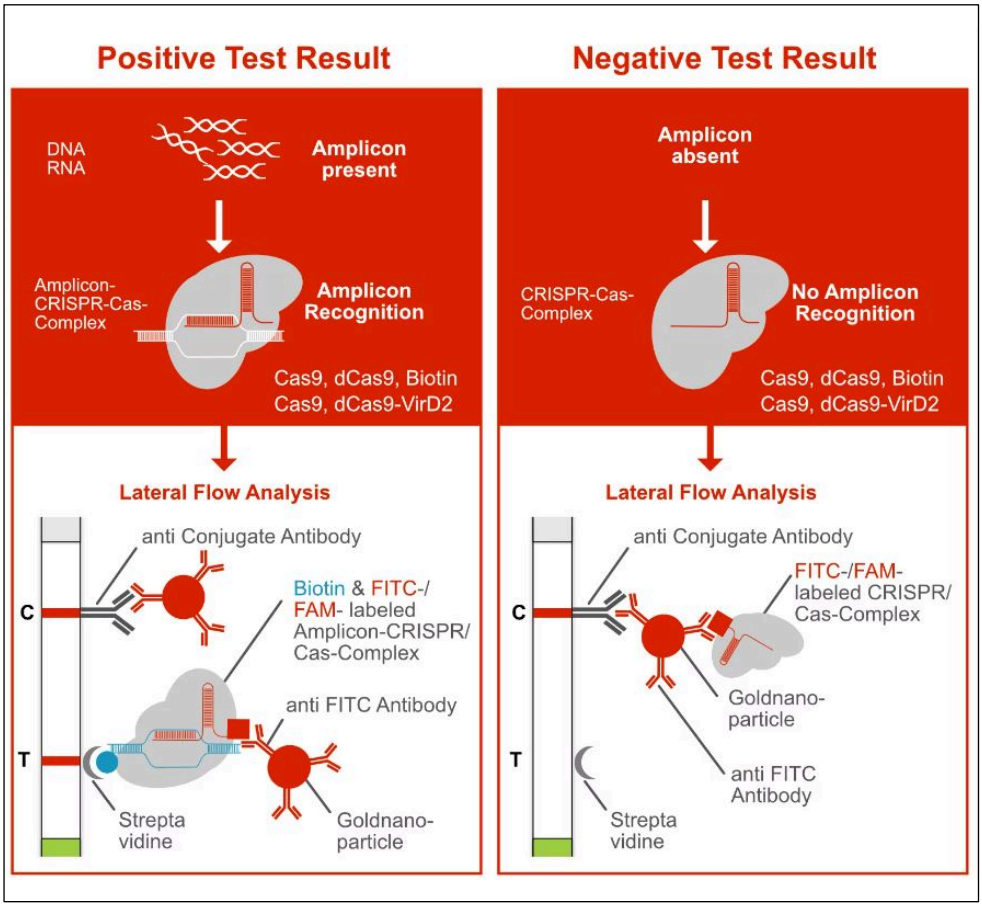

Figure 10: Visualization of the label incorporation method with the Milenia GenLine HybriDetect 2T test strip (MGDS2A).

The most famous method based on the principle of label incorporation is FELUDA (FnCas9 Editor Linked Uniform Detection Assay). For more information regarding this method please see the following link:

- FELUDA

Overview of methods

Besides SHERLOCK, DETECTR and FELUDA there are more methods combining CRISPR/Cas and lateral flow. The following table (Table 3) gives a brief overview of the different methods all relying on the principle of label separation or label incorporation.

Table 3: Selected CRISPR/Cas methods successfully combined with Milenia GenLine HybriDetect (MGHD 1). All methods rely either on the label separation (LS) or label incorporation (LI) strategy.

Method	Detection Strategy	DNA Amplification	Cas Protein	Labeling Strategy	Ref
SHERLOCK	LS	LAMP, RPA (and *in vitro* transcription)	LwaCas13a	collateral cleavage of FAM-Biotin-labelled ssRNA Reporter	(15)
DETECTR	LS	LAMP, RPA	LbCas12a	collateral cleavage of FAM-Biotin-labelled ssDNA Reporter	(16)
CASLFA	LI	PCR, RPA	Cas9 + dCas9	probe-functionalized Nanoparticles, biotinylated amplicon	(17)
FELUDA	LI	PCR	FnCas9	biotinylated amplicon, FAM-labelled guide RNA	(18)
iSCAN	LS	LAMP	LbCas12a	collateral cleavage of FAM-Biotin-labelled ssDNA Reporter	(19)
VIGILANT	LI	RPA	VirD2-SpdCas9 (fusion protein)	FAM-labelled VirD2-dCas9 + non labelled sgRNA + biotinylated amplicon	(20)
"Biotin-dCas9-LFA"	LI	RPA	SpdCas9	biotinylated SpdCas9, FITC-labelled Amplicon	(21)

For more information regarding the combination of CRISPR/Cas and HybriDetect check out the articles Lateral Flow Readout » CRISPR/Cas-based detection strategies on our website.

Do you want to know how other scientists have combined CRISPR/Cas and HybriDetect? Then have a look at our online literature database.

f) Assay Development Guide: Check for primer dimers

The formation of dimers is a major issue for all amplification related assays (PCR, RPA, LAMP). If the sequences of the primers match (partly), they will hybridize and form dimers. Those dimers would have both, a FITC/FAM and a biotin or digoxigenin label which would be shown as positive on the test line by the HybriDetect strip. To avoid dimerization there are several programs to check sequences in silico. We recommend testing the primers for dimerization in lab before your first experiments to exclude false positive signals through primer dimerization. To check for primer dimers, follow this protocol:

1. Add an equal amount of your primers to a reaction tube. We recommend a primer concentration of 1 pmol/µl each.
2. Mix the solution.
3. Incubate for 5 min at room temperature.
4. Add 2 µl of the primer mixture to the Milenia HybriDetect strip and deposit the strip in 80 µl chase buffer (MGCBB) in an upright position for 5-15 min.

The formation of a test line will show you the presence of primer dimers. Sometimes dimers will not show a straight visible line. Instead, they will form dots on the left and right side of the test strip (Figure 11 A) which also indicates the presence of primer dimers. If your primers form dimers, you must redesign them. You can rule out dimer formation if no signal is generated on both test lines (Figure 11 B).

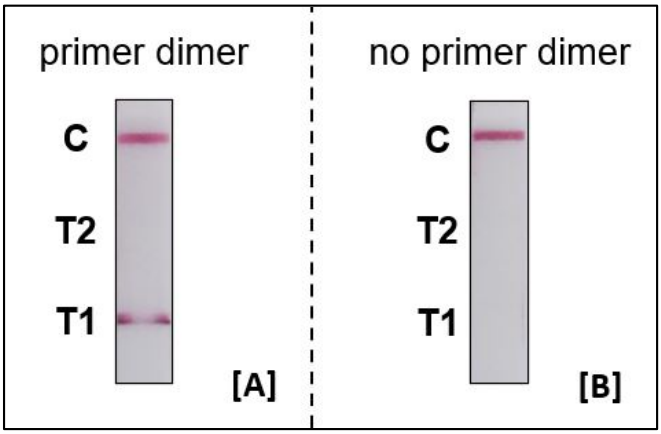

Figure 11: Evaluation of a dual labelled primer pair forming dimers (A) and a dual labelled primer pair without forming dimers (B) with the Milenia GenLine HybriDetect 2T test strip (MGDS2A).

Milenia GenLine HybriDetect Characteristics

a) Limit of Detection

The limit of detection (LOD) of Milenia HybriDetect 2T (MGHD2) was determined by using single-stranded DNA (ssDNA) oligonucleotides labelled with FAM and biotin (Figure 12 A) and oligonucleotides labelled with FAM and digoxigenin (Figure 12 B). After 5 minutes of incubation of the sample loaded test strip in Milenia GenLine Chase Buffer (MGCBB) there is clear signal for a concentration of 5 fmol dual labelled ssDNA on both test lines. Therefore, a concentration of 5 fmol/LFA was determined reproducable as LOD.

Note 1: The signal intensity will get stronger the longer the test strip is placed inside the buffer. Nevertheless 5 fmol/LFA is the minimal concentration needed in order to achieve a positive signal with Milenia HybriDetect 2T (MGHD2).

Note 2: Free labels from unbound antibodies or unbound primers negatively influence the LOD.

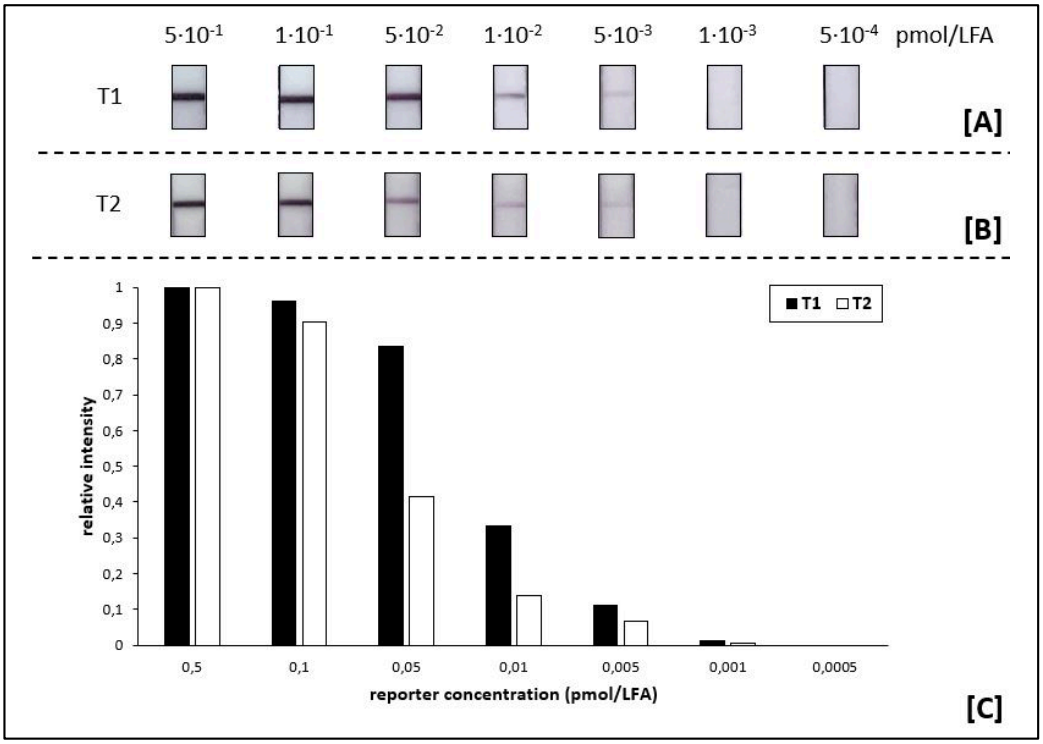

Figure 12: Sensitivity of the Milenia GenLine HybriDetect 2T strip (MGDS2A). Sample material for T1 is a 15 bp ssDNA oligonucleotide labelled with FAM and biotin (A). Sample material for T2 is a 15 bp ssDNA oligonucleotide labelled with FAM and digoxigenin (B). The picture was taken after 5 min incubation of the strip in MGCBB buffer (B). The intensity was measured with the Milenia myilab reader (C).

b) HybriDetect vs Agarose Gel Electrophoresis

The HybriDetect test strip is an alternative for agarose gel electrophoresis (AGE), a standard method when it comes to DNA amplification evaluation. There are several advantages of HybriDetect compared to AGE.

- Customer: While AGE is a molecular method it must be performed by trained personnel with special laboratory equipment. HybriDetect is an easy-to-handle rapid test that can be performed even by untrained end-customers at point-of-care without extra equipment.

- Time: The total time for AGE including gel production, sample application and gel runtime is approximately > 1,5 hours. Evaluation with HybriDetect from sample to answer is possible within 5-10 minutes which reduces the evaluation time significantly compared to AGE.

- Sensitivity: The sensitivity of AGE compared to HybriDetect 2T was determined with a 205 bp dsDNA fragment labelled with FAM and biotin (Figure 13). While HybriDetect 2T can detect 5 fmol dual labelled fragment with a clear visual test line, the sensitivity of AGE is between 50 and 100 fmol. This means a 20 times higher sensitivity of HybriDetect 2T compared to agarose gel electrophoresis.

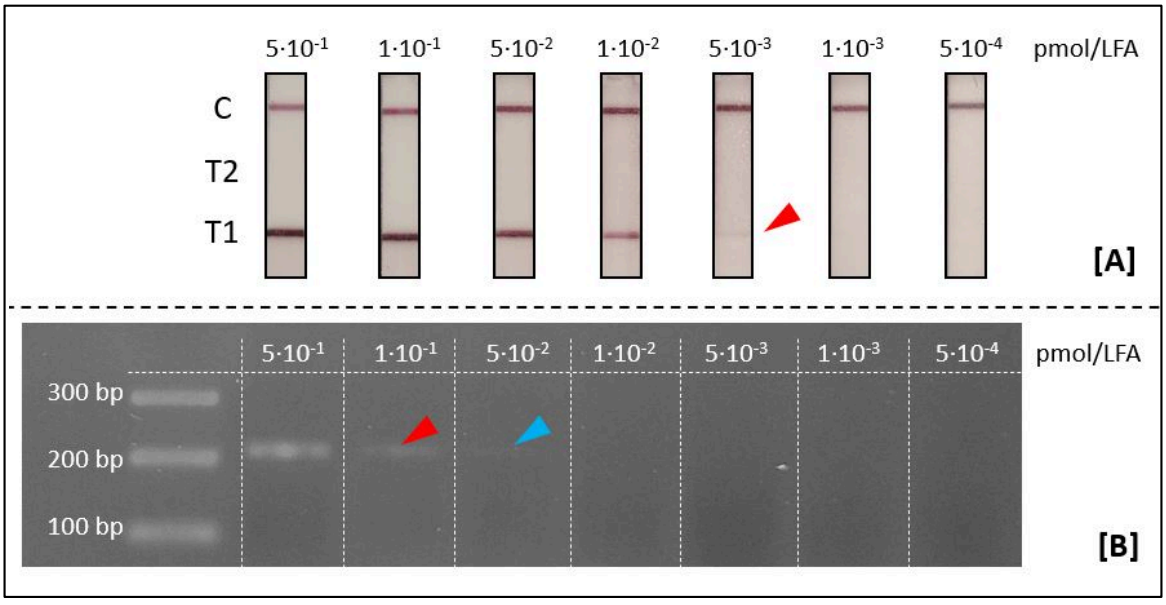

Figure 13: Sensitivity of (A) Milenia GenLine HybriDetect 2T (MGHD2) compared to (B) agarose gel electrophoresis. Sample material is a 205 bp dsDNA oligonucleotide labelled with FAM and biotin. Red arrow: Last good visible sample concentration. Blue arrow: Last faint visible sample concentration. The result for a 205 bp dsDNA oligonucleotide labelled with FAM and digoxigenin is equivalent.

c) High Dose Hook Effect

The high dose hook effect is a typical immunoassay related phenomenon. It occurs if too many LFA-relevant labels (FITC/FAM and biotin or digoxigenin) are introduced into the LFA. The general mechanism is based on a limited number of label-specific binding sites in the lateral flow system. If the number of relevant labels exceeds the number of available binding sites in the lateral flow system, fewer gold nanoparticles can be immobilized on the test line.

A decreasing C-line and increasing T-line intensity is the result of a significant reporter excess. This is an important and characteristic limitation for immunoassay formats like LFAs or ELISAs. The high dose hook effect for test line T1 of MGHD2 begins at approximatly 1 pmol reporter per LFA. The following figure (Figure 14) shows the reporter-induced hook effect in the HybriDetect 2T system for the first test line T1.

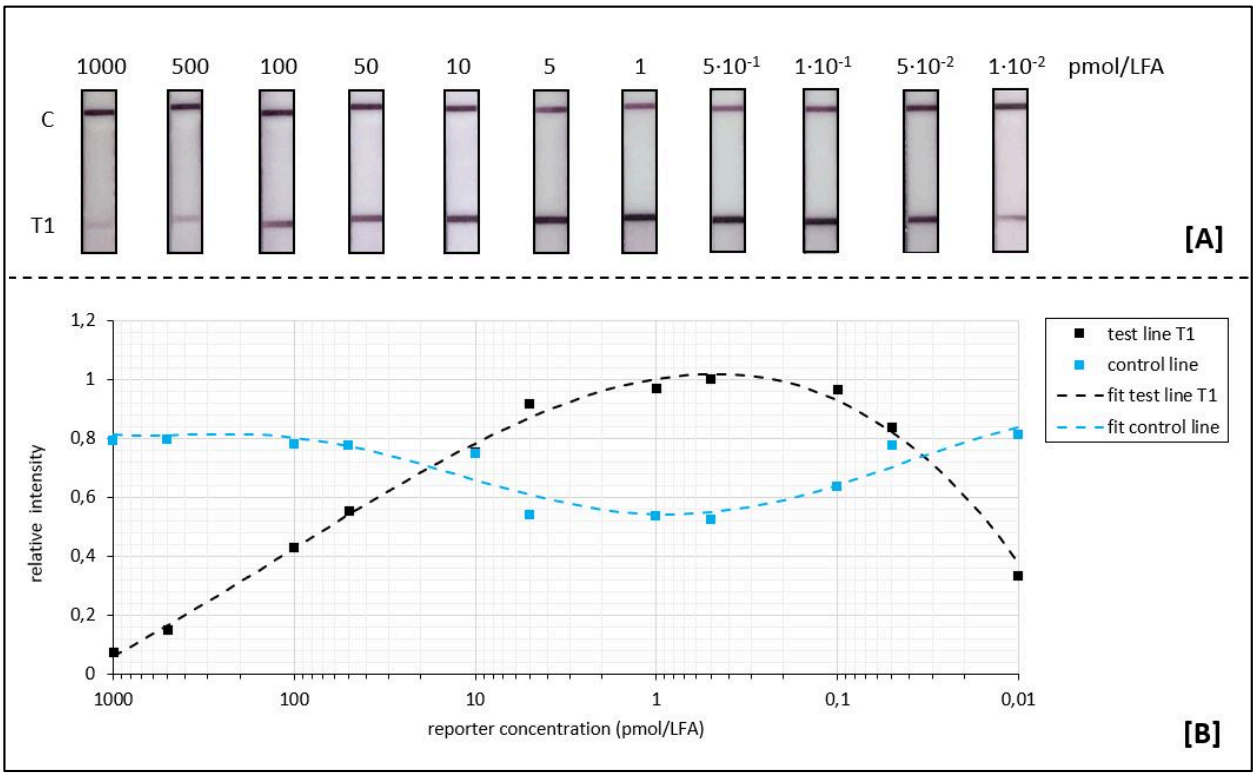

Figure 14: Illustration of the high dose hook effect for Milenia GenLine HybriDetect 2T (MGHD2). Sample material is a 15 bp ssDNA oligonucleotide labelled with FAM and biotin. (A) The picture was done after 5 min incubation of the strip in MGCBB buffer. (B) The intensity was measured with Milenia myilab reader.

For the second test line T2 of MGHD2 the high dose hook effect begins at about 0,5-1 pmol reporter per LFA. The following figure (Figure 15) shows the reporter-induced hook effect in the HybriDetect 2T system for the second test line T2.

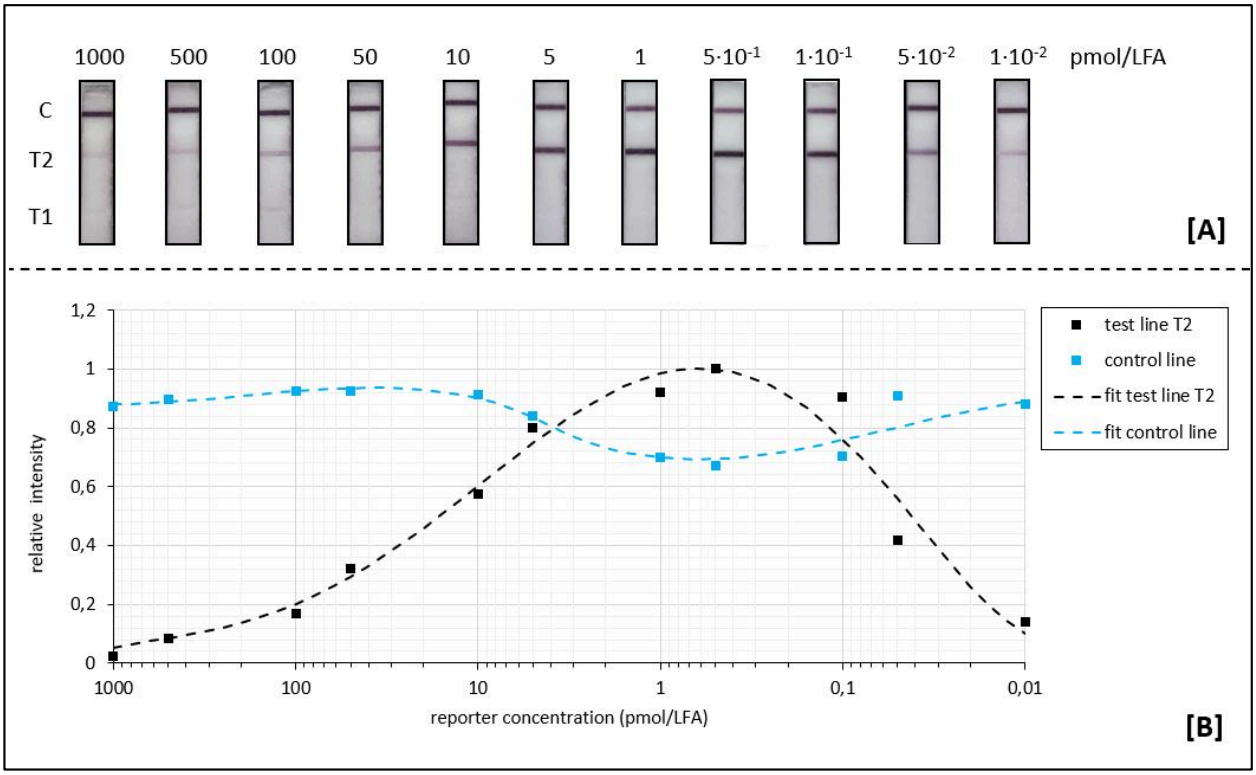

Figure 15: Illustration of the high dose hook effect for Milenia GenLine HybriDetect 2T (MGHD2). Sample material is a 15 bp ssDNA oligonucleotide labelled with FAM and digoxigenin. (A) The picture was done after 5 min incubation of the strip in MGCBB buffer. (B) The intensity was measured with the Milenia myilab reader.

Understanding the effects of the hook effect is important to avoid misinterpretation. According to this effect, different concentrations of double-labeled reporters can produce the same signal intensity. Furthermore, false negative results may be generated especially for assays, which are designed according to the described label incorporation strategy.

The high reporter concentrations in Figure 15 B will not only lower the test line intensity, but also generate false signals that conduct to misleading interpretations. There concentrations are at a rate of 100 pmol FITC/FAM. It is crucial to limit the number of relevant labels in the LFA.

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Additional Products Available

REF	Product Name	Content		Tests	Description
MGHD 1	Milenia GenLine HybriDetect - Universal Lateral Flow Test Strips 	2 x 50 2 x 10 ml	HybriDetect Dipsticks (MGDS) HybriDetect Assay Buffer (MGCB)	100	Dipsticks with **one** test band (biotin) and one control line
MGHC 1	Milenia GenLine HybriDetect Cassette 	4 x 5 1 x 10 ml	HybriDetect Test Units (MGSHC) HybriDetect Assay Buffer (MGCB)	20	Universal lateral flow cassette with **one** test line (biotin) and one control line
MYILABR 1	myilab research - Lateral Flow Reader 	1 x	myilab research device	-	Lateral flow reader for the evaluation of Milenia GenLine HybriDetect test strips and cassettes.

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Change History

Date	Revision	Cause of Revision
2013-01-10	B	Symbols adapted according DIN EN ISO15223
2019-06-06	C	Buffer changed (MGCB2 to MGCBB)
2023-04-28	D	Anti-rabbit antibody exchanged for anti-goat antibody
2024-05-14	E	• German translation removed • Heading changed from *Materials Required (not included)* to *Materials Required but not Supplied* • Heading changed from *Additional available Products* to *Additional Products Available* • The following chapters have been revised: *Explanation of Symbols, Warnings and Precautions, Materials Supplied, Storage and Stability, Method, Additional Products Available, Contact* • The following chapters have been removed: *Necessary Development Work - Development Platform,Testprinciple – Genamplicon Detection, Testprinciple – Protein Detection, Control Band Dipstick, Assay Performance "PCR Products", Assay Performance "RPA Products", Trouble Shooting "PCR", Assay Sensitivity "PCR"* • The following chapters have been added: *Common Test Principle, Sample Application, Assay Development Guide, Milenia GenLine HybriDetect Characteristics* • Chapter *Additional Products Available* updated: New product HybriDetect GenLine Cassette REF MGHC 1 added
2025-07-29	IFU / REF MGHD2 1 / **REV F** / 2025-07-29	• Symbols adapted in chapter *Explanation of Symbols*. • Lateral Flow Reader myilab research included in chapter *Interpretation of Results/ b) Semi-quantitative Evaluation* • Design updated

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