Milenia GenLine
HybriDetect Cassette

Universal lateral flow test cassette for the detection of biotin- and FITC/FAM-labelled analytes (proteins, genomic amplicons)

Milenia GenLine **HybriDetect Cassette**	REF **MGHC 1**	Σ **20**

IFU / REF MGHC 1 / B / 2024-11-05

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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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

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).
- Do not use Test Unit if foil pouch is damaged.
- Protect Test Units from humidity.
- The expiration date of all components must be observed. Do not use components past the expiration date!
- Do not interchange components of packages of different batch codes.
- 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
MGHC 1	4 x 5 1 x 10 ml	HybriDetect Test Units (MGSHC) HybriDetect Assay Buffer (MGCB)	20

Storage and Stability

Components	Description	Preparation	Storage	Shelf Life
HybriDetect Cassette (MGSHC)	Membrane coated with biotin-ligand, polyclonal anti-rabbit antibody (goat) and polyclonal (rabbit) anti-FITC/FAM antibody in gold conjugate	Ready to use	2-8°C Use immediately after opening the foil pouch!	Until expiry date
HybriDetect Assay Buffer (MGCB)	Tris-buffered saline	Ready to use	2-8°C	Until expiry date

Materials Required but not Supplied

- Pipets
- Pipet tips (containing protective filters for PCR)

Intended Use

HybriDetect Cassette is a universal lateral flow cassette for the detection of analytes (proteins, genomic amplicons) labelled with fluorescein (FITC or FAM) and biotin. It is a development platform. The test is for research use only and not for diagnostic purposes.

Method

Milenia HybriDetect Cassette is a ready-to-use, universal test unit, which is based on the lateral flow technology using gold nanoparticles. The test unit is designed to develop qualitative or semi-quantitative rapid test systems for several analytes such as proteins, antibodies, or gene amplicons. The user needs to develop an analyte-specific solution, which contains a first detector (e.g. antibody, antigen, specific probe) labelled with FITC or FAM and a second one (e.g. antibody, primer) labelled with biotin (see Common Test Principle on page 5).

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

The complexed analyte, labelled with FITC/FAM and biotin, binds first to the gold-labelled FITC/FAM-specific antibodies in the conjugate release pad of the lateral flow strip. The gold complexes travel through the membrane, driven by capillary forces. Only the analyte captured gold particles will bind when they pass the line with the immobilized biotin-ligand molecules and generate a red-blue band over time. Unbound gold particles migrate up to the control band and will be captured by species-specific antibodies. With prolonged incubation time, an intensely coloured band appears.

Common Test Principle

The lateral flow test strip inside of the cassette consists of four major zones: the sample application pad, the conjugate release pad, the nitrocellulose membrane, and the wicking pad (see Figure 1).

The **sample application pad** (= SAP) is the zone where the sample is applied to the test strip.

The **conjugate release pad** (= CRP) contains the gold nanoparticles which are responsible for the visualization of the test and control line. The gold nanoparticles on the CRP have a diameter of 40 nm and are coupled with polyclonal rabbit anti-FITC/FAM antibodies.

The nitrocellulose **membrane** contains the test line (T) and the control line (C). The test line consists of immobilized streptavidin, the control line consists of immobilized polyclonal goat anti-rabbit 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.

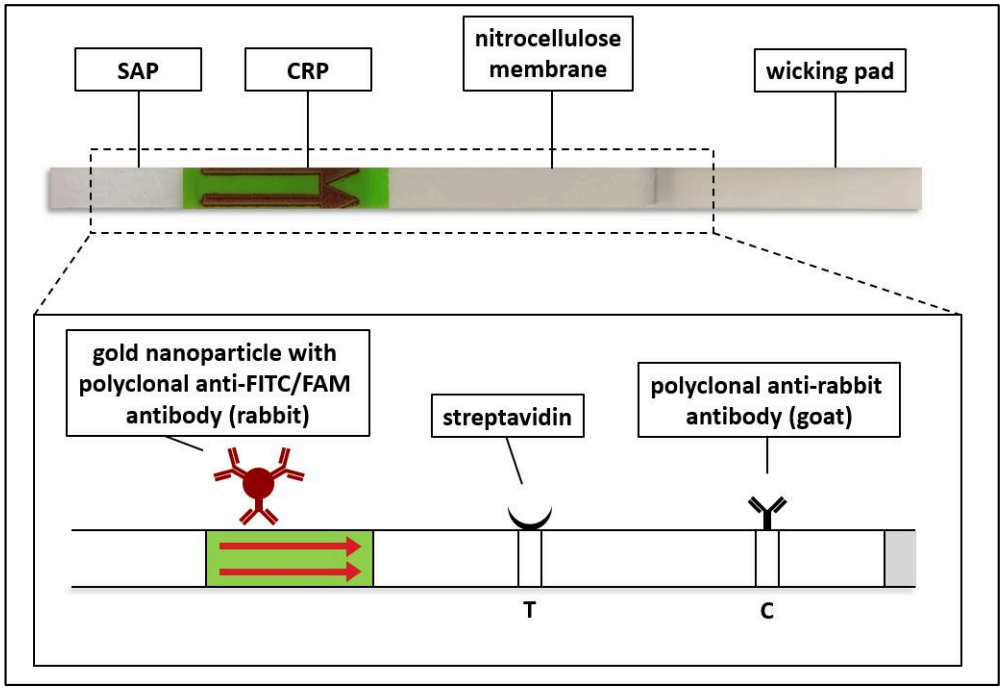

Figure 1: Design of the test strip contained by the Milenia GenLine HybriDetect Cassette (MGHC)

In absence of a dual labelled analyte (i.e., the sample is negative) the test line will stay invisible (see Figure 2). Those unlabelled analytes cannot bind to the streptavidin and will rush through the test line. The gold nanoparticles coupled with rabbit antibodies are caught by the anti-rabbit antibodies immobilized on the control line. This leads to a colouration of the control line.

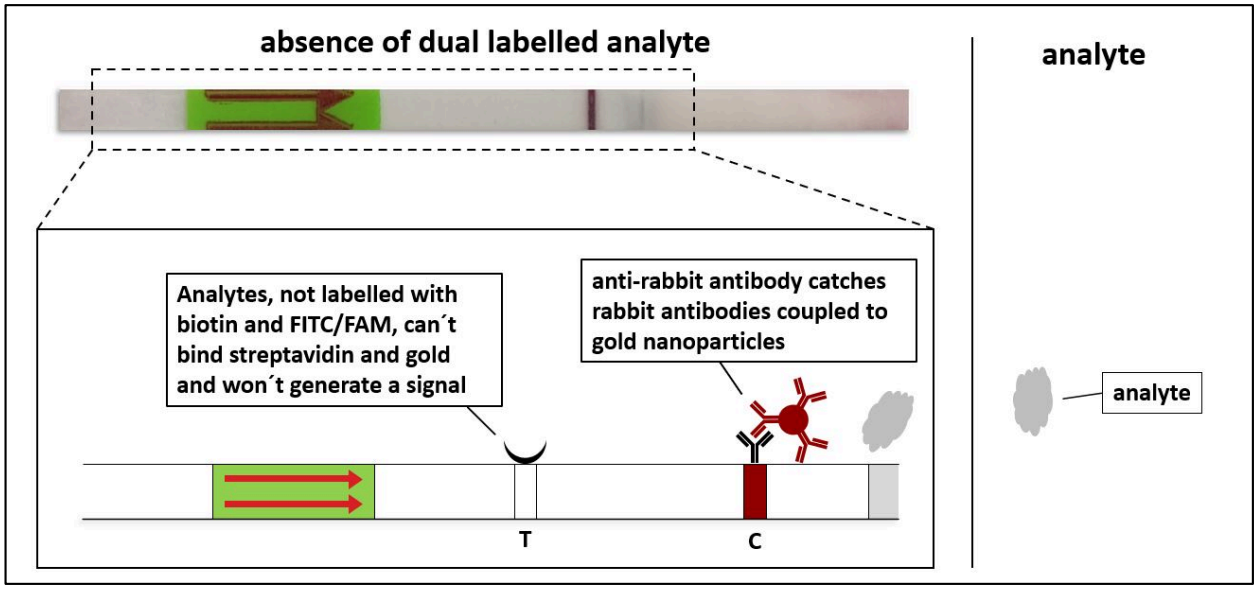

Figure 2: Result of a negative sample applied to the test strip contained by the Milenia GenLine HybriDetect Cassette (MGHC)

In the presence of a dual labelled analyte (i.e., the sample is positive) the test line will turn red (see Figure 3). The biotin bound to the analyte is captured by the streptavidin immobilized on the test line. The FITC/FAM of the analyte interacts with the anti-FITC/FAM antibodies of the gold nanoparticles and will hold them back which leads to an accumulation of the gold. This finally colours the test line 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.

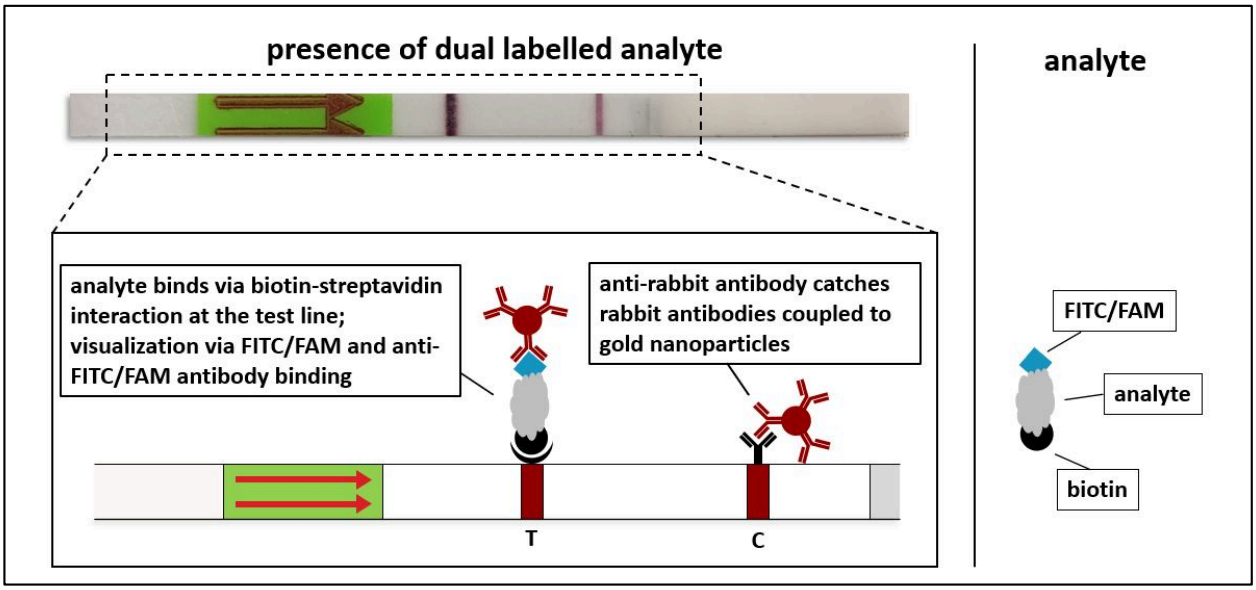

Figure 3: Result of a positive sample applied to the test strip contained by the Milenia GenLine HybriDetect Cassette (MGHC)

Note: This test principle is valid for most applications like PCR, RPA, LAMP or protein detection. However, the test unit can be used in combination with CRISPR/Cas methods. In this case, the test principle is reversed (for more information see Assay development guide: CRISPR/Cas on page 17).

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 Cassette (MGHC).

1. The sample is applied directly to the SAP (sample application pad) through the sample application area of the cassette (see Figure 4). Typical sample volumes are 10 µl. Subsequently the chase buffer (MGCB) is applied to the SAP. The cassette is incubated for several minutes in a horizontal position. Use a buffer volume of 60-80 µl.

2. The sample can be mixed with the chase buffer (MGCB). Subsequently the solution containing sample and MGCB is applied to the SAP and the cassette is incubated for several minutes in a horizontal position. Use about 80 µL fluid (sample material and analyte-specific solution/chase buffer) for the assay procedure.

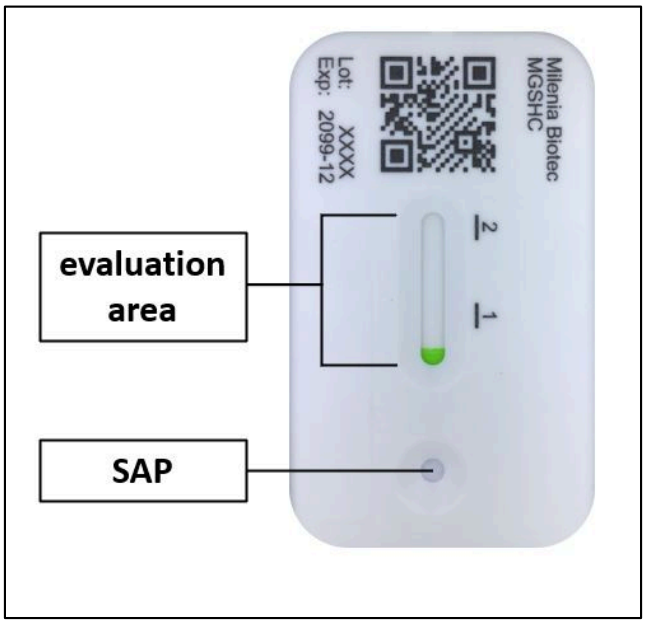

Figure 4: Evaluation Area and sample application area of the Milenia GenLine HybriDetect Cassette (MGHC)

There is no general recommendation for one sample application method. Which method works best for your specific assay is to be chosen by your own personal preference. The provided assay buffer (MGCB) 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 Cassette (MGHC) 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 line 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 Cassette: a qualitative evaluation and a semi-quantitative evaluation.

a) Qualitative Evaluation

With our Milenia GenLine HybriDetect Cassette 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 5 and Table 1).

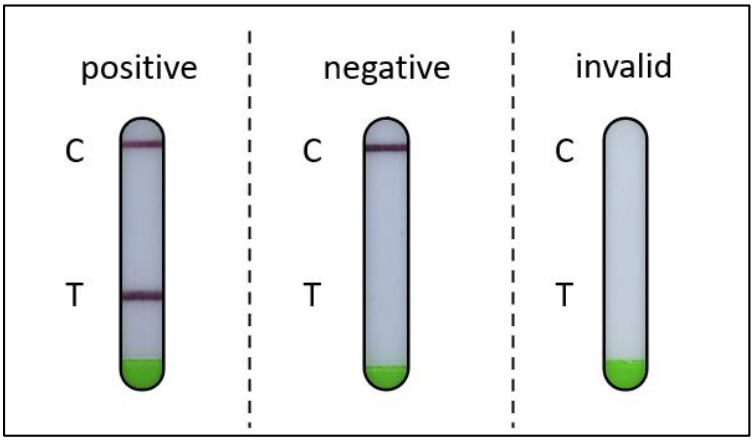

Figure 5: Visual evaluation of the Milenia GenLine HybriDetect Cassette (MGHC). C indicates the position of the control line. T indicates the position of the test line.

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 cassette!

Table 1: Correct interpretation of the Milenia GenLine HybriDetect Cassette (MGHC)

Test Band	Control Band	Interpretation
positive	positive	• Control band is clearly visible, test run is valid • **Amplicon is detected (positive)**
negative	positive	• Control band is clearly visible, test run is valid • **Amplicon is not detected (negative)**
negative	negative	• Control band is not visible, **test run is invalid** • No reliable information regarding amplicon detection • Repeat the test with a new cassette

Note: This interpretation of results is valid for most applications like PCR, RPA, LAMP or protein detection. However, the test unit can be used in combination with CRISPR/Cas methods. In this case, the test principle is reversed (for more information see Assay development guide: CRISPR/Cas on page 17).

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. The easiest way for a semi-quantitative evaluation is via evaluation cards (see Figure 6). Other common ways are the use of extra devices and/or evaluation apps. For more information regarding semi-quantitative evaluation please contact us.

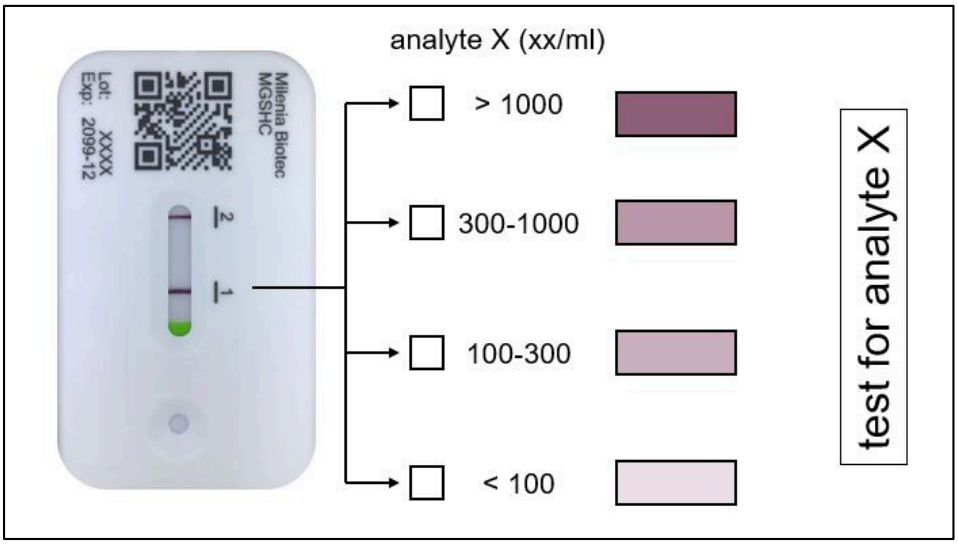

Figure 6: Example for a semi-quantitative evaluation of the Milenia GenLine HybriDetect Cassette (MGHC) via evaluation card for the analyte X

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 Cassette 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 FITC/FAM. For the detection of proteins and antibodies this can be achieved due to two 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 with labelled oligos. 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 should target different locations of the protein to prevent competition for the same binding location. One antibody must be labelled with biotin, 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. One fraction gets labelled with biotin, 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 also shown below (Figure 7).

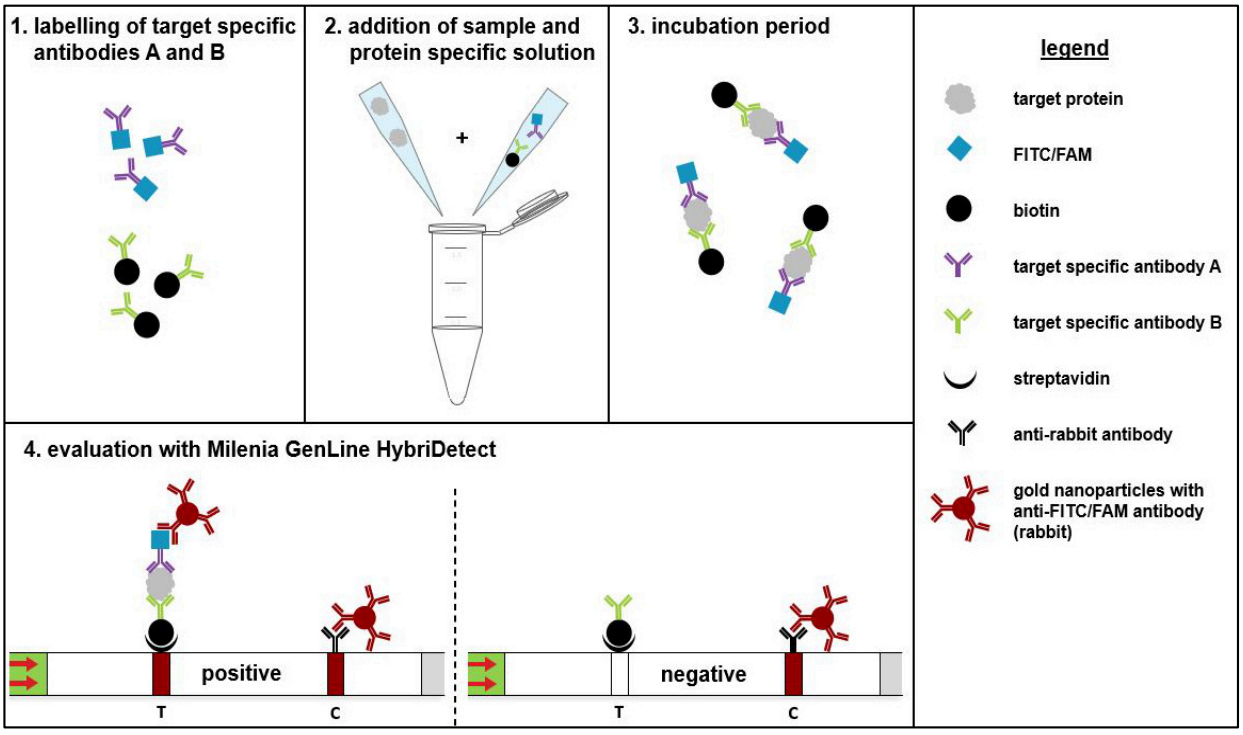

Figure 7: Test procedure for proteins and antibodies with Milenia GenLine HybriDetect Cassette (MGHC)

b) Assay Development Guide: PCR

The polymerase chain reaction (PCR, RT-PCR) is the easiest way to detect DNA/RNA. The HybriDetect Cassette can visualize dual labelled oligo sequences. The label incorporation is achieved during the PCR due to one FITC/FAM and another biotin labelled primer.

Primer design

One (forward or reverse) primer must be labelled with FITC/FAM. The other (forward or reverse) primer must be labelled with biotin. 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 would have both a FITC/FAM and biotin label which would be shown as positive by the HybriDetect Cassette. 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 cassette. 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 label. The label incorporation is achieved during the RPA reaction due to one labelled primer, one labelled probe and a non-labelled primer.

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 but primers up to 45 bp can work too.
- 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. Too short primers will reduce the reaction speed and will 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 itsself by adding endonuclease IV to the TwistAmp® Basic-Kit.

For the use of Endonuclease IV, a special nfo probe is needed (see 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´ends

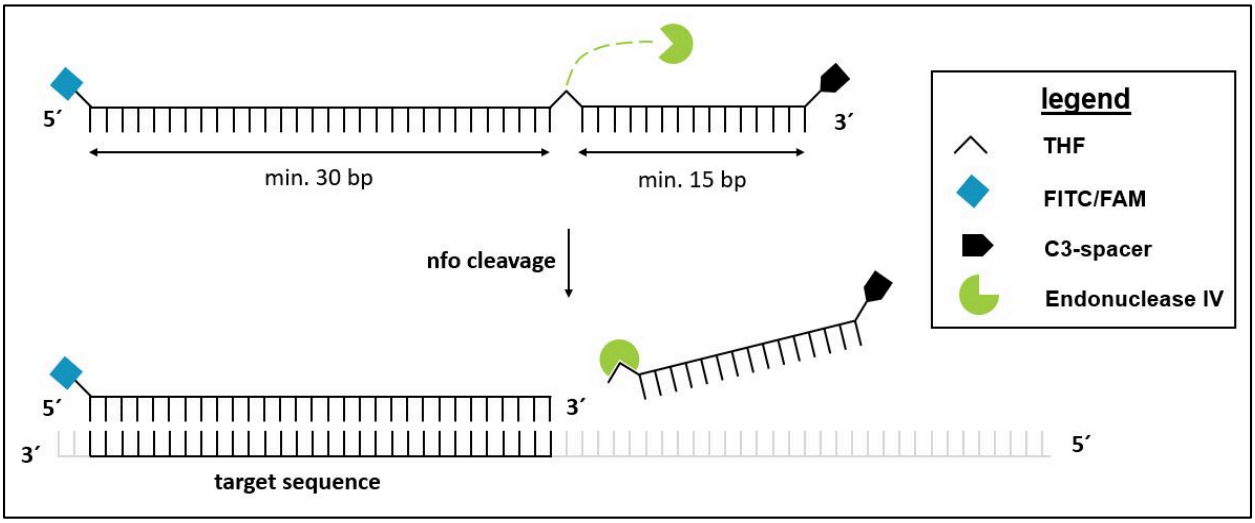

Figure 8: Structure of a nfo probe for the combination of RPA and lateral flow. As label FITC/FAM is shown. It can also be labelled with biotin instead of FITC/FAM. Endonuclease IV (nfo) cleaves nfo probe after completely binding to the target sequence.

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 label which would be shown as positive by the HybriDetect Cassette. 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 Cassette 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 Cassette. LAMP amplicons can be visualized due to FITC/FAM and biotin label. The label incorporation is achieved during the LAMP reaction due to two labelled primers and at least four non-labelled primers.

Primer design

In contrast to PCR and RPA, the LAMP reaction is more complex due to the presence of more primers. 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.

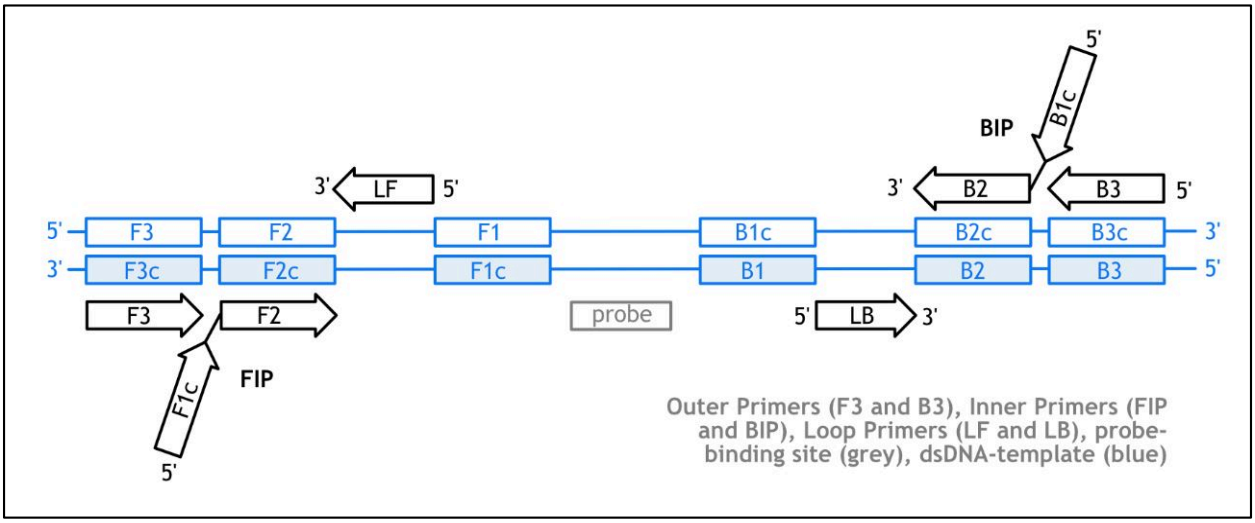

Figure 9: Primers (and probe) needed for LAMP reaction

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 Cassette (MGHC)

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. CRISPR/Cas is one of the most used methods in combination with our HybriDetect products. In general, there are two different mechanisms of CRISPR/Cas based methods: label separation and the label incorporation (Figure 10).

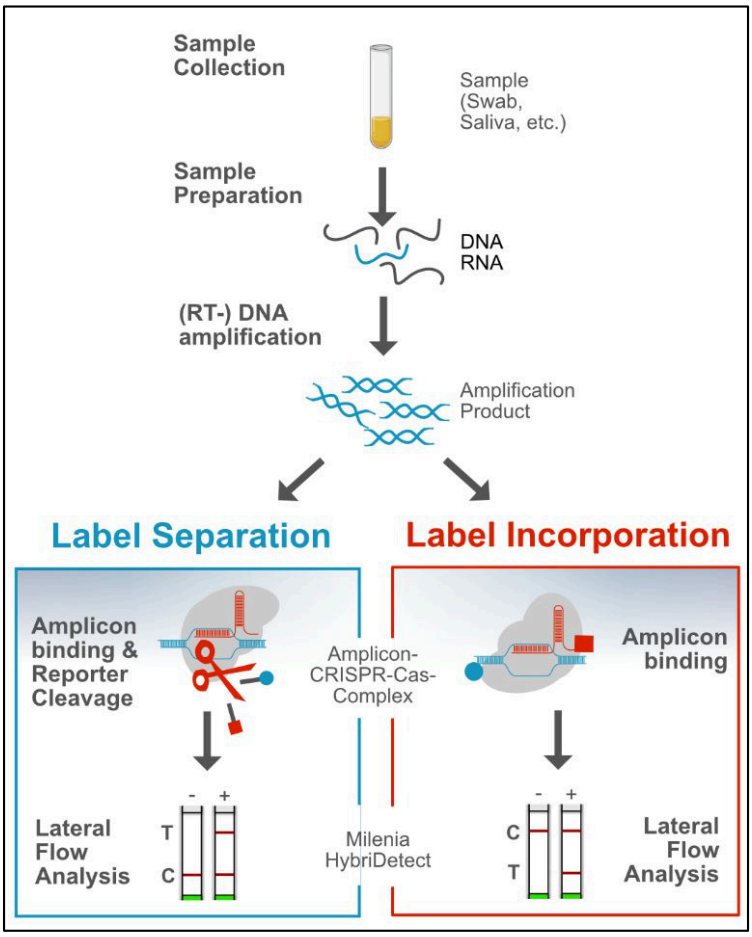

Figure 10: General mechanism of CRISPR/Cas based methods. Label separation is based on the cleavage of reporter oligonucleotides while label incorporation is based on the recognition and binding of target DNA. Both methods can be visualized by Milenia GenLine HybriDetect.

Label separation

The CRISPR-Cas-complexes recognize DNA or RNA with excellent specificity. This leads to the activation of the collateral nuclease activity of the Cas-protein. As a result, reporter molecules are efficiently cleaved by the Cas-protein. Depending on the Cas-enzyme used, ssDNA (Cas12a) or ssRNA (Cas13a) reporter can be cleaved. Those reporters are dual labelled with FITC/FAM and biotin. The separation of terminal labels is detectable with the Milenia HybriDetect (Figure 11).

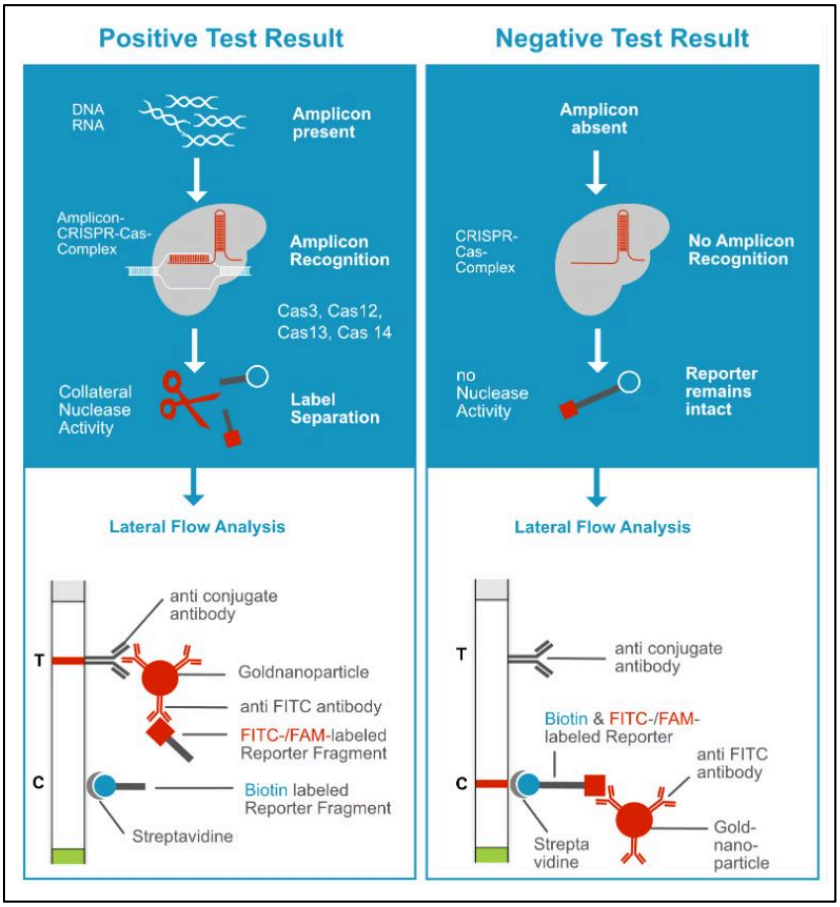

Figure 11: Visualization of the label separation method with the Milenia GenLine HybriDetect Cassette (MGHC).

Note: For the label separation method, the test principle of the HybriDetect Cassette is reversed. The former upper control line is now the test line (T). The former lower test line is now the control line (C).

With the CRISPR method, a fragment marked by FAM and biotin is cleaved in the presence of the target DNA (i.e., the sample is positive). This prevents or attenuates the creation of a control line, and the test line turns red. If the target DNA is not present (i.e., the sample is negative), the FAM/biotin fragment is not cut and binds via the biotin to the control line. The gold is caught by the FAM and colours the control line red. There is not enough gold left to bind on the T line and it stays invisible or is less visible.

The most famous methods based on the principle of label separation are SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) and DETECTR (DNA endonuclease-targeted CRISPR trans reporter). For more information regarding those methods please see the following links:

- SHERLOCK
- DETECTR

Reporter design for label separation

The choice of the optimal reporter sequence depends on the CRISPR-Cas system used. Ultimately, it is important to determine the exact reporter concentration which works for the nuclease reaction and for the lateral flow analysis. The following table (Table 3) gives a brief overview of the most used reporters for Cas13a and Cas12a dependent test formats.

Table 3: Most used reporter oligonucleotides for the combination of label separation method and HybriDetect

Cas protein	Reporter Type	5` Label	3´ Label	Sequence (5' > 3')
LwaCas13a	ssRNA	6-FAM	Biotin	poly-U (≥ 10)
		6-FAM	Biotin	$(RU)_{14}$
		6-FAM	Biotin	$(MARARUGRGRCMA)_2$
LbCas12a	ssDNA	6-FAM	Biotin	TTATT
		6-FAM	Biotin	TTATTATT
		6-FAM	Biotin	$(TTATT)_3$

Test line extinction for label separation

The almost complete extinction of the T-line is the basis for an easy and intuitive interpretation of the test strips. In order to eliminate T-line intensity as much as possible, here are some guidelines on how to achieve this:

- Reporter quantity: You must find the perfect reporter quantity, so that the majority of the gold conjugate is retained at the C-Line. Only few gold nanoparticles will be able to travel to the T-line, leading to a missing or very weak T-line. For an almost complete elimination of the T-Line a specific amount of reporter is needed. The amount of reporter depends on the specific reporter and the sample application. In general, a range of 1 – 5 pmol reporter per LFA is good for an almost complete T-line elimination. Higher reporter concentrations will not decrease the T-line intensity further due to the high dose hook effect (see High dose hook effect on page 25).

- Reporter sequence: Avoid long reporter sequences. Longer reporter sequences are more likely to form secondary structures and are more likely to interact with other sequences which would influence the detectability.

- Reporter quality: Just use HPLC purified reporters to guaranty the concentration and quality of dual labelled reporters.

- Assay buffer modification: The Milenia GenLine HybriDetect Buffer (MGCB) is a universal buffer which works for the most applications but is not optimized for CRISPR/Cas readout. It might be helpful to adjust the buffer. For example, Polyethylenglycole (PEG) might help to improve T-line elimination. Increased viscosity of the buffer slows down lateral flow speed. Moreover, interaction time of mobile and stationary phase of LFD is increased. This can be beneficial for better accumulation of reporter at C-line and thus result in improved T-Line elimination in negative controls.

For more detailed information about test line elimination please take a look at our Short Guide for Improved CRISPR/Cas Readout-Performance.

Note: All data and experiences regarding the combination of CRISPR/Cas label separation method and our lateral flow tests was determined with the Milenia GenLine HybriDetect dipstick!

Label incorporation

Focus of the label incorporation method is the best known Cas9 protein. For this method the Cas9 protein is labelled with FITC/FAM. The amplicon is labelled with biotin 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 label on the other. This amplicon-CRISPR-Cas9 complex is detectable via Lateral Flow using the Milenia HybriDetect Cassette (Figure 12).

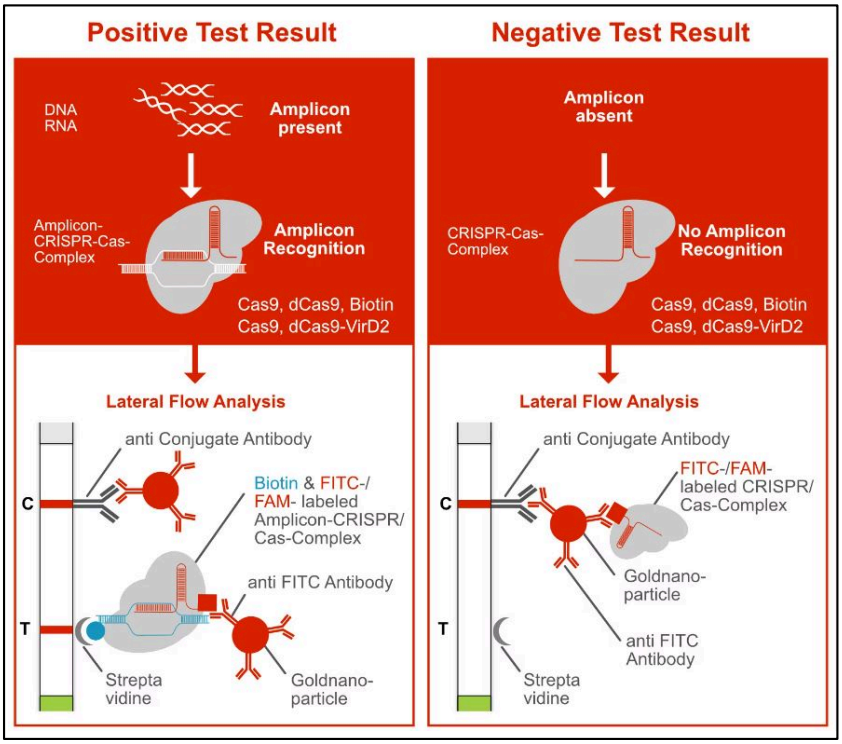

Figure 12: Visualization of the label incorporation method with the Milenia GenLine HybriDetect Cassette (MGHC).

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 4) gives a brief overview of the different methods all relying on the principle of label separation or label incorporation.

Table 4: Selected CRISPR/Cas methods successfully combined with Milenia GenLine HybriDetect. 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 label which would be shown as positive on the test line by the HybriDetect Cassette. To avoid dimerization there are several programs to check sequences in silico. We recommend testing the primers for dimerization in lab too 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 (MGCB) 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 13 A). This 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 the test line (Figure 13 B).

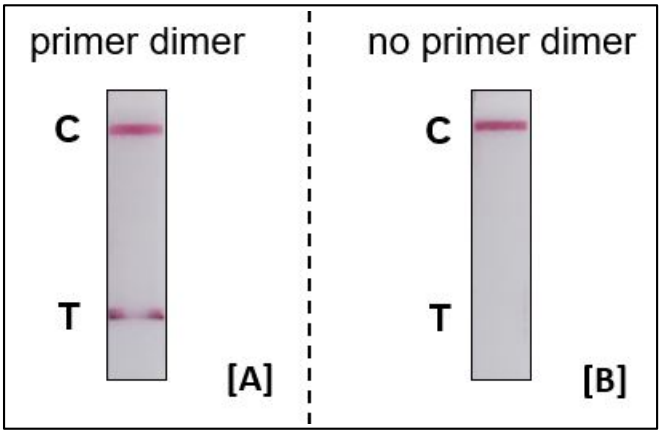

Figure 13: Evaluation of a dual labelled primer pair forming dimers (A) and a dual labelled primer pair without forming dimers (B) with the Milenia GenLine HybriDetect Cassette (MGHC).

Milenia GenLine HybriDetect Characteristics

a) Limit of Detection

The limit of detection (LOD) of Milenia HybriDetect Cassette (MGHC) was determined by using single-stranded DNA (ssDNA) oligonucleotides labelled with FAM and biotin (Figure 14). After 5 minutes of incubation of the sample loaded test strip with the Milenia GenLine Chase Buffer (MGCB) there is clear signal for a concentration of 1 fmol dual labelled ssDNA on the test line. Additionally, there is a faint signal for a concentration of 0,5 fmol/LFA. A concentration of 1 fmol/LFA was therefore determined as LOD.

Note 1: The signal intensity will get stronger the longer you incubate the cassette. Nevertheless 0,5 fmol/LFA is the minimal concentration needed in order to achieve a positive signal with Milenia HybriDetect Cassette (MGHC).

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

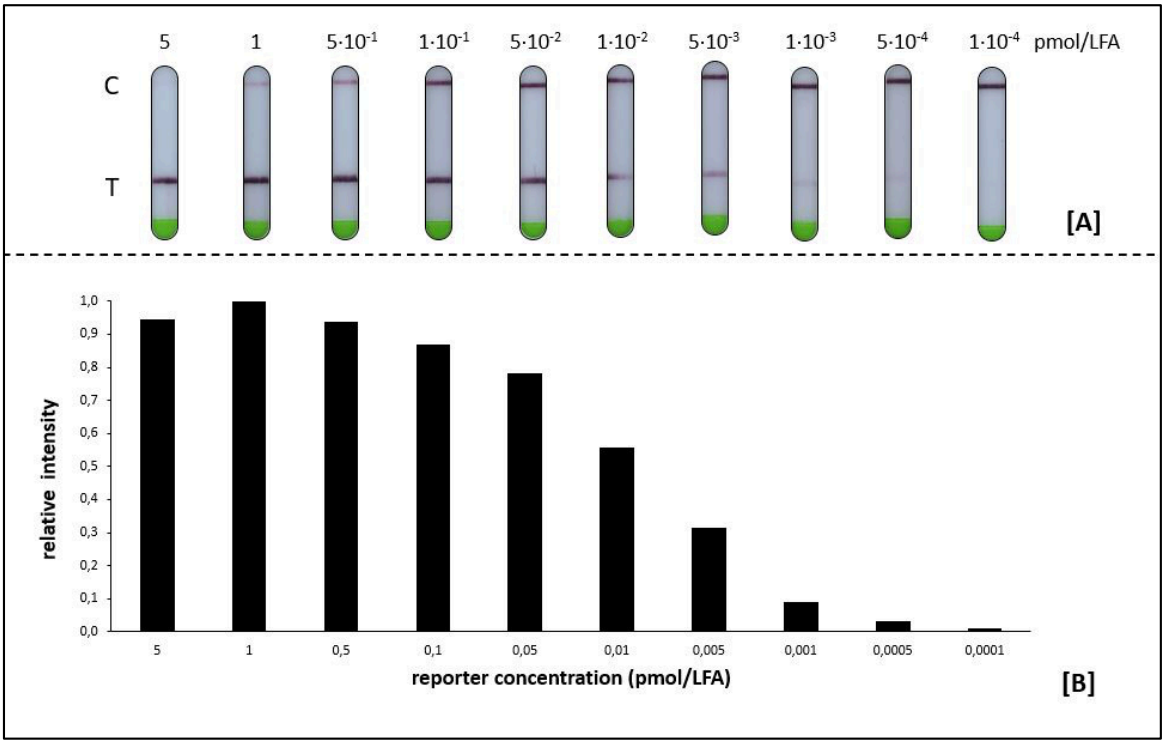

Figure 14: Sensitivity of the Milenia GenLine HybriDetect Cassette (MGHC). Sample material is a 15 bp ssDNA oligonucleotide labelled with FAM and biotin. (A) The picture was taken after 5 min incubation with MGCB buffer. (B) The intensity was measured with the Milenia myilab reader.

b) HybriDetect vs Agarose Gel Electrophoresis

The HybriDetect Cassette 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 was determined with a 205 bp dsDNA fragment labelled with FAM and biotin (Figure 15). While HybriDetect Cassette can detect 1 fmol dual labelled fragment with a clear visual test line within 5 minutes, the sensitivity of AGE is between 50 and 100 fmol. This means a 50 to 100 times higher sensitivity of HybriDetect compared to agarose gel electrophoresis.

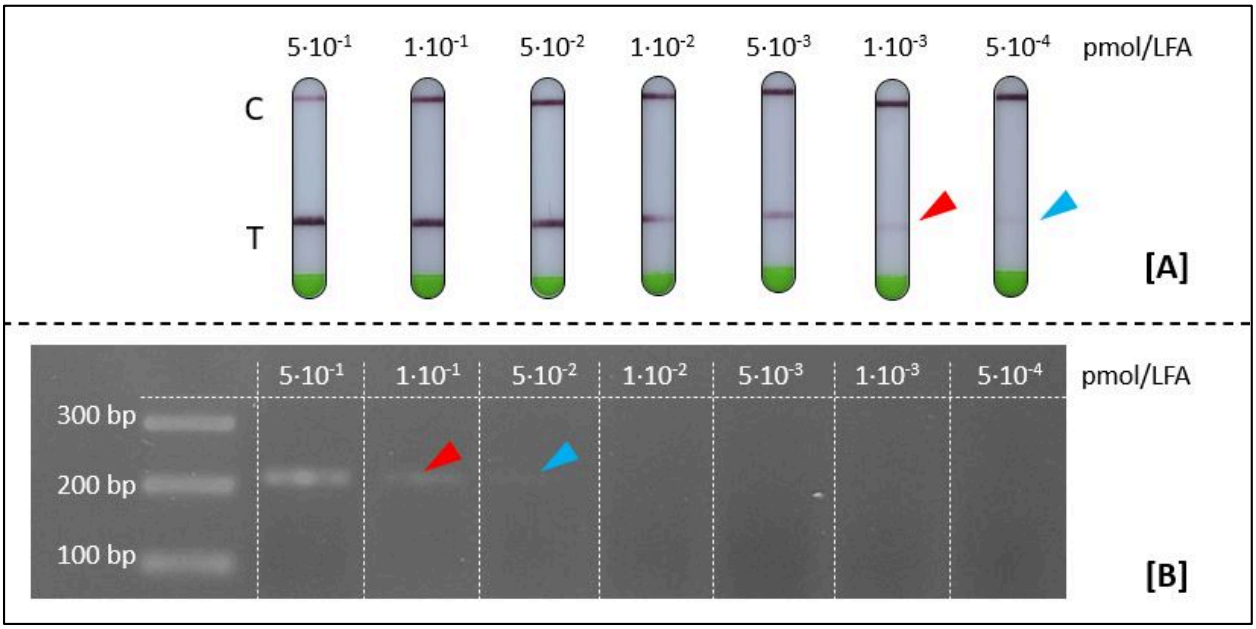

Figure 15: Sensitivity of (A) Milenia GenLine HybriDetect Cassette (MGHC) compared to (B) agarose gel electrophoresis. Sample material is a 205 bp dsDNA oligonucleotide labelled with FAM and biotin. Red arrow: Last good visible sample concentration. Blue arrow: Last faint

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) 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. For MGHC the high dose hook effect begins at about 10 pmol reporter per LFA. This is an important and characteristic limitation for immunoassay formats like LFAs or ELISAs. The following figure (Figure 16) shows the reporter-induced hook effect in the HybriDetect system.

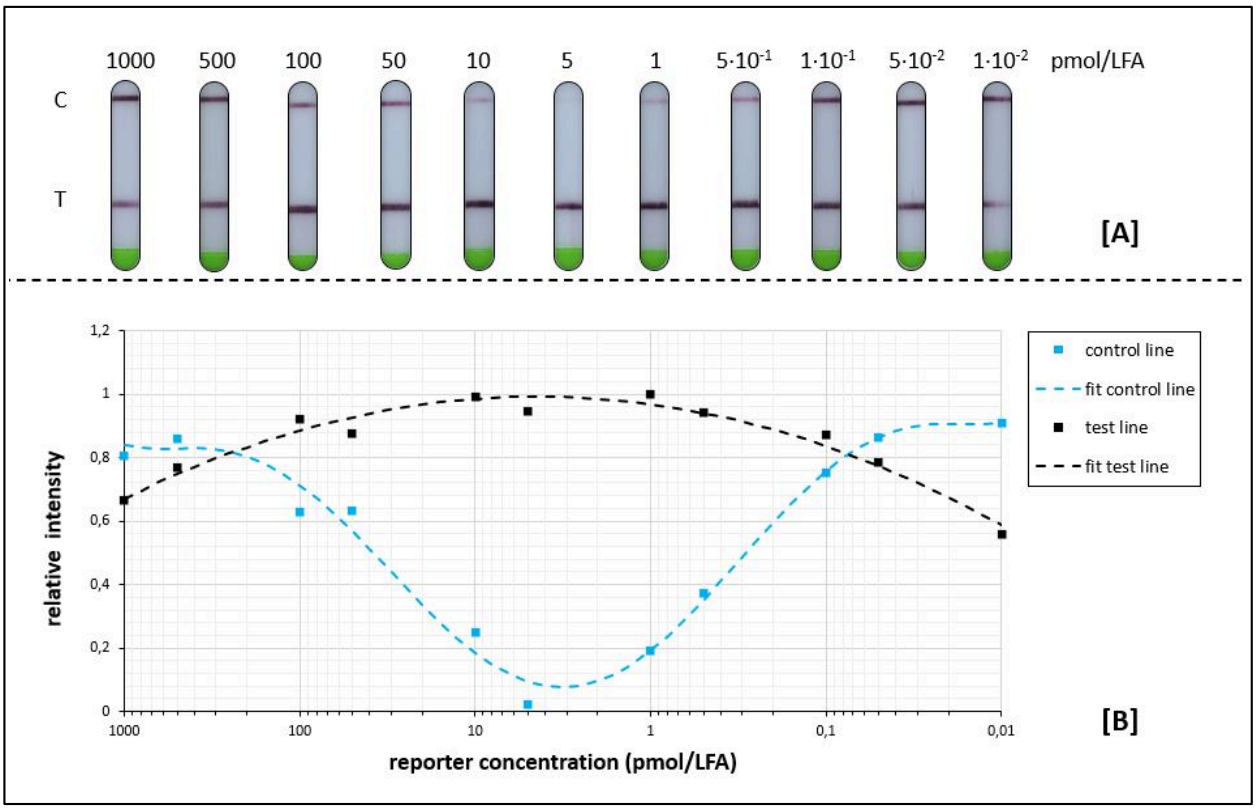

Figure 16: Illustration of the high dose hook effect for Milenia GenLine HybriDetect Cassette (MGHC). Sample material is a 15 bp ssDNA oligonucleotide labelled with FAM and biotin. (A) The picture was done after 5 min incubation with the MGCB buffer. (B) The intensity was measured with the Milenia myilab reader.

It is necessary to understand the hook effect to avoid misinterpretation and handling errors. According to this effect it is possible to create identical signal intensities with different concentrations of the dual labelled reporter. Furthermore, it is possible to create false negative results especially for assays, which are designed according to the described label incorporation strategy. 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 	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
MGHD2 1	Milenia GenLine HybriDetect 2T 	2 x 50 2 x 10 ml	HybriDetect 2T Dipsticks (MGDS2A) HybriDetect 2T Assay Buffer (MGCBB)	100	Dipsticks with **two** different test bands (biotin and digoxigenin) and one control line

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Change History

Date	Revision	Cause of Revision
2024-05-02	A	Creation of the HybriDetect Cassette instructions for use
2024-11-05	B	Sample and buffer volume adjusted

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