LightMix for Influenza A H5N1 detection

TIB MOLBIOL

LightMix® Products – General Information

LightMix® products consist of premixed primers and hybridization probes for use with the LightCycler® instruments series 1.x and 2.0 from Roche Diagnostics.

The product is lyophilized in 6 individual vials, 16 reactions each, providing a total of 96 reactions. Also included, a lyophilized standard row for quantitative analysis ranging from 10 to 10^6 target equivalents per reaction. Products are tested with FastStart /FastStart Plus DNA Master Hybridization Probes and the LightCycler® instruments versions 1.x and 2.0. LightMix® products do not contain polymerase, buffer or dNTPs. [Research Use Only]

Influenza A virus genome detection

The Influenza A virus is a RNA virus. Detection of the genomic RNA requires the preparation of cDNA prior to DNA amplification by PCR, which can optionally be performed in a One-Step RT-PCR reaction (see protocols below). LightMix® M2 targets the matrix protein gene to detect broad range Influenza A including the current Asian H5N1 isolates (2004/2005).
The following products are available for the specific detection of Influenza A virus:

Order no.	Description	Instrument	rxns	Price
40-0219-16	**LightMix® for the detection of *Influenza virus A H5***	1.x, 2.0	96	290.00
	LOD = 10, LOQ = 100	5 kits	480	1,160.00
		10 kits	960	1,890.00
40-0230-16	**LightMix® for the detection of *Influenza virus A N1***	1.x, 2.0	96	290.00
	LOD = 10, LOQ = 10	5 kits	480	1,160.00
		10 kits	960	1,890.00
40-0234-16	**LightMix® for the detection of *Influenza virus A M2***	1.x, 2.0	96	490.00
	Detection of the Matrix protein gene M2 with LC640	5 kits	480	1,960.00
	Internal control (IPC) with LC705 (Duplex PCR)	10 kits	960	3,270.00
40-0242-16	**LightMix® for the detection of *Influenza virus A H5N1***	1.x, 2.0	96	490.00
	Detection of the H5 gene with LC640, N1 gene LC705	5 kits	480	1,960.00
	LOD = <20, LOQ = 100 (H5), LOD = <10, LOQ = 10 (N1)	10 kits	960	3,270.00

LOD = Limit Of Detection, LOQ = Limit Of Quantification (estimated using a plasmid target)

Specificity and Sensitivity

All LightMix products have been tested using cDNA obtained from Asian H5N1 virus isolates. Sensitivity was measured by means of plasmid dilution rows containing the respective targets. The sensitivity of all PCR assays is at least 10 genome equivalents.

Samples

All types of samples are fit for analysis; however Nucleic acid extraction has to be adapted to the sample material. Please refer to the manufacturer's instructions for the isolation of virus RNA from samples like feces, tissue, food, blood, serum, sputum or other samples obtained from birds or other infected species.

Detecting strategy – screening and testing for the H5 and N1 genes

Wild bird populations display 1-5% Influenza A positive animals, which can go up to 15% during bird migration (Virology Erasmus University Rotterdam). Screening with the Matrix assay will detect these individuals and will yield a comfort number of positive samples, which could be subjected to tests on the aggressive H5N1 variant.

To screen samples for Influenza A virus cDNA/RNA (detected with probes labeled with LightCycler® Red 640) use the **LightMix® for the detection of *Influenza virus A M2***. This LightMix also contains an internal PCR control (IPC, detected with probes labeled with LightCycler® Red 705) preventing false negative results due to PCR inhibition.

In case of positive results for Influenza A virus cDNA the sample can be further analyzed with the **LightMix® for the detection of *Influenza virus A H5*** and if further specification is necessary with the **LightMix® for the detection of *Influenza virus A N1***.

Alternatively the detection of the features H5 and N1 genes of Influenza virus A can be accomplished with the **LightMix® for the detection of *Influenza virus A H5N1*** in a single capillary. This LightMix® is based on a duplex PCR reaction for the detection of Influenza virus A H5 cDNA with probes labeled with LightCycler® Red 640 and for Influenza virus A N1 cDNA with probes labeled with LightCycler® Red 705.

Background - Selection of Sequences

The primers and probes used for the detection of the H5 gene have been developed since January 2004 based on virus sequences isolated in Thailand. The amplified gene fragment is a part of the PCR product recently recommended (June 2005) by the WHO, Geneva. Our Blast analysis revealed 179 and 360 matches with 100% identity to the WHO primers; while the LightMix H5 primers match 337 and 375 entries and 366 and 356/375 matches for the LightMix H5N1 duplex PCR primers. The first version of the LightMix H5 product has been tested on more than 50,000 samples in Asian countries and can be considered field-tested.

The German Reference Laboratory for Human Influenza certified a satisfactory sensitivity for the LightMix H5 product; other validation studies are under-way.

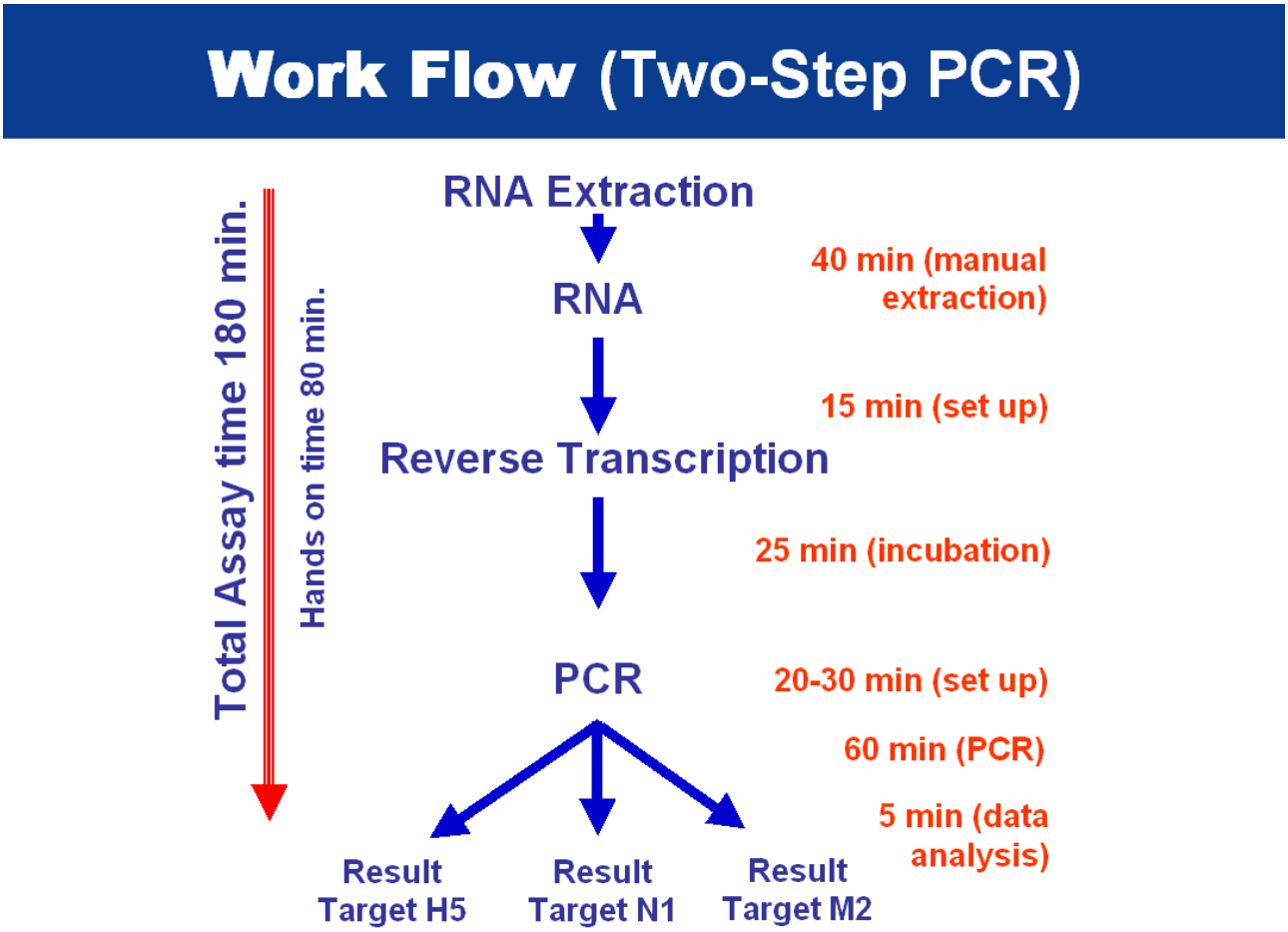
The primers and probes used for the detection of the N1 gene were selected based on alignments of Asian H5N1 virus sequences. Comparison among the primers, WHO show 114 and 124 matches in contrast to 357 and 258 matches with the LightMix primers.

The German Reference Laboratory for Human Influenza found superior sensitivities.

The LightMix product for the detection of the Matrix gene is based on earlier designs published by Smith et. al., 2003. The detection probes were changed and the primers selected according to the publication from Schweiger et al, 2000 then modified to detect the actual H5N1virus isolates.

Additional validation studies for all products are under-way.

Two-Step protocol



Two-Step protocol: cDNA synthesis

10 µl of RNA can be converted into cDNA with the Transcriptor First Strand cDNA Synthesis Kit (04 379 012 001) using the Random Primers supplied in the kit.
The cDNA can be amplified for the desired LightMixes.

Two-Step protocol : PCR Amplification starting from cDNA

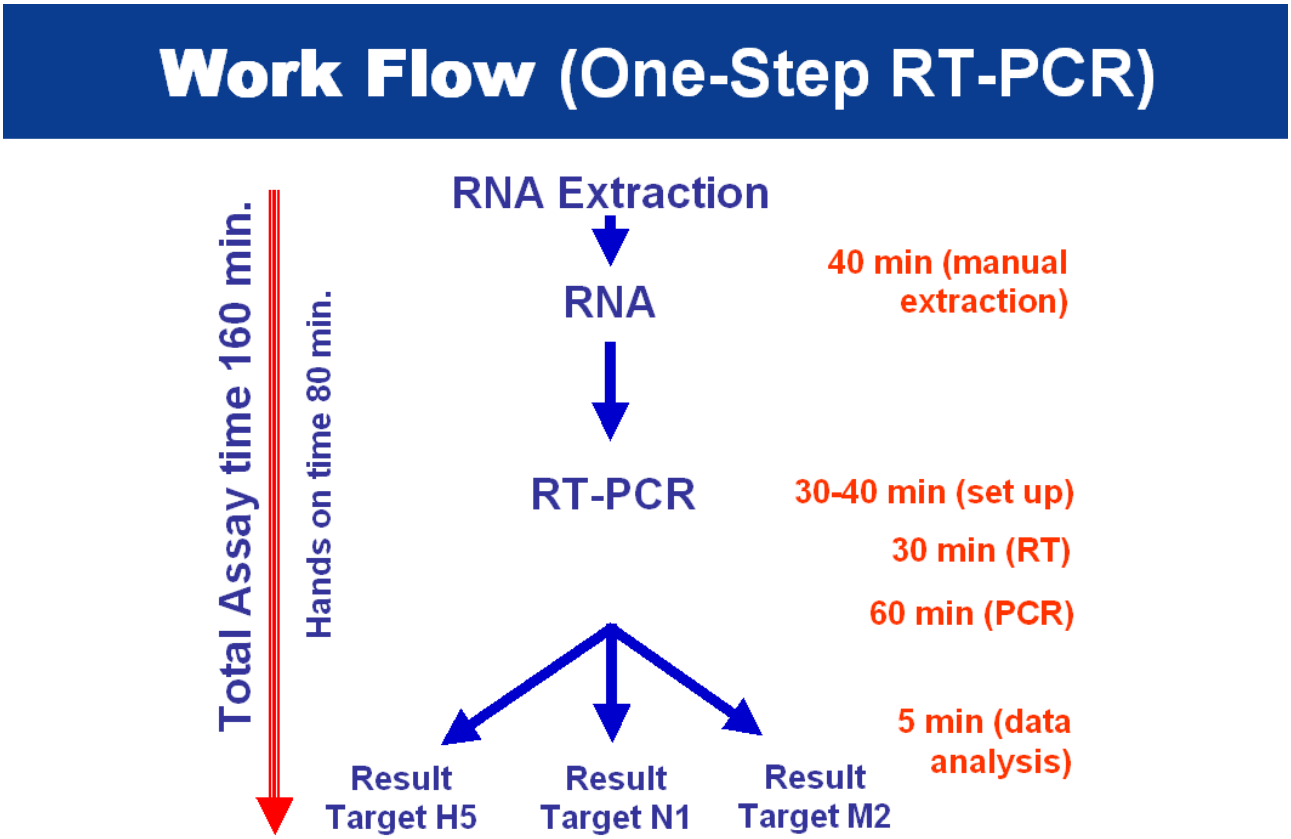
5 µl of cDNA can be amplify following the LightMix manual using :
03 515 575 001 LightCycler® FastStart^{PLUS} DNA Master Hybridization Probes or
03 003 248 001 LightCycler® FastStart DNA Master Hybridization Probes

		Denaturation	*Cycling*			*Melting*			*Cooling*
Parameter									
Analysis Mode		*None*	*Quantification*			*Melting Curves*			*None*
Cycles		*1*	*55*			*1*			*1*
Segment		*1*	*1*	*2*	*3*	*1*	*2*	*3*	*1*
Target [°C]		95	95	55	72	95	40	95	40
Hold [hh:mm:ss]		00:10:00	00:00:05	00:00:15	00:00:08	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]		20	20	20	20	20	20	0.1	20
Acquisition Mode		None	None	Single	None	None	None	Continu.	None

Additional Reagents required (Roche Diagnostics):

LightCycler capillaries (LightCycler instruments 1.x and 2.0)

One-Step RT-PCR (Single Target LightMix H5 or N1 only)



Use a mixture of:

4 µl LightCycler® FastStart DNA Master PLUS HybProbe (03 515 575 001) and
0.2 µl (4 Units) of Transcriptor Reverse Transcriptase (20 U/µl).
Random Hexamer shows significant inhibition.

03 531 295 001 Transcriptor Reverse Transcriptase kit - or
03 531 287 001 Transcriptor Reverse Transcriptase enzyme and Random Hexamer

or use the new One-Step RT-PCR Transcriptor Kit (contact lightmix@tib-molbiol.de)

	RT-step	*Denaturation*	*Cycling*			*Melting*			*Cooling*
Parameter									
Analysis Mode	*None*	*None*	*Quantification*			*Melting Curves*			*None*
Cycles	*1*	*1*	*55*			*1*			*1*
Segment	*1*	*1*	*1*	*2*	*3*	*1*	*2*	*3*	*1*
Target [°C]	60	95	95	55	72	95	40	95	40
Hold [hh:mm:ss]	00:30:00	00:10:00	00:00:05	00:00:15	00:00:08	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	20	0.1	20
Acquisition Mode	None	None	None	Single	None	None	None	Continu.	None

Do not pre-heat the mixture before the reverse transcriptase (RT) reaction.

Additional Reagents required (Roche Diagnostics):

LightCycler capillaries (LightCycler instruments 1.x and 2.0)

RNA Extraction

can be achieved using:

Manual extraction:

- High Pure Viral Nucleic Acid Kit (11 858 874 001) (preferred)
- High Pure Viral RNA Kit (11 858 882 001) or
- High Pure RNA Isolation Kit (11 828 665 001)

Automated Extraction using the MagNa Pure Instrument:

- MagNA Pure LC Total Nucleic Acid Isolation Kit (03 038 505 001) or
- MagNA Pure LC Total Nucleic Acid Isolation Kit -- Large Volume (03 264 793 001)

Automated Extraction using the Magna Pure Compact Instrument:

- MagNA Pure Compact Nucleic Acid Isolation Kit I° (03 730 964 001)
- MagNA Pure Compact Nucleic Acid Isolation Kit I -- Large Volume (03 730 972 001)

Validation

The single target assays for H5 and N1 and the matrix gene have been tested by the German AIV Reference laboratories for human influenza (Robert-Koch-Institut, RKI) and for veterinarian influenza (Friedrich-Löffler-Institut, FLI).

The statement from the RKI from november 2005 was positive. The sensitivity with the N1 assay was better than all other assays available.
The statement from FLI from january 2006 was also positive: 'suitable for the detection of the AIV genome' and 'the H5 assay and the N1 assay detected all current H5N1 isolates'.

References (selection):

Recommended laboratory tests to identify avian influenza A virus in specimens from humans • WHO Geneva • 06-2005

Rapid detection of influenza A and B viruses in clinical specimens by Light Cycler real time RT-PCR. Smith AB, Mock V, Melear R, Colarusso P, Willis DE. J Clin Virol. 2003 Sep 28(1):51-58

Application of a fluorogenic PCR assay for typing and subtyping of influenza viruses in respiratory samples. Schweiger B, Zadow I, Heckler R, Timm H, Pauli G. JCM 38 (2000) 1552-1558

For additional questions and prices please contact TIB MOLBIOL GmbH, Berlin, Germany
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