

IKDT GmbH, Moltkestrasse 31, D – 12203 Berlin

Applied methods in IKDT Laboratory for differential diagnostics of endomyocardial biopsies and molecular diagnostics in cardiology

IKDT is performing diagnostics on endomyocardial biopsies on requests of hospital-affiliated institutions or private doctor offices. For routine diagnostics will be covered three main topics: 1. Histology, 2. Immunohistochemistry and 3. Molecular Virology. 6-8 endomyocardial biopsies at minimum are required for whole routine procedure. Increased number (9-12) will be beneficial for diagnostic accuracy. Sampling error is not negligible for detection of cardiotropic viruses.

Since 2003 IKDT lab is accredited by CAP to perform endomyocardial biopsy diagnostics under certified conditions. CAP accreditation is the only certification process outside of USA which is accepted by US Food and Drug administration (FDA).

IKDT lab offers the most comprehensive approach to analyze myocardial tissue (EMBs) on morphological abnormalities, viral infections and inflammatory processes as causive reasons for heart failure problems.

1. Pre-analytical treatment of patient material

Myocardial biopsies in native or fixed form are the preferred sample materials for these tests. For suitable assays and results it is important that the submitted material undergoes a well defined pre-analytic treatment (Tab.1).

For endomyocardial biopsies should be used a novel reagent for conservation by ambient temperature. ***RNAlater*** is an aqueous, non-toxic tissue storage reagent that stabilizes and protects cellular RNA in intact, unfrozen tissue samples. ***RNAlater*** eliminates the need to immediately process tissue samples or to freeze samples in liquid nitrogen for later processing. ***RNAlater*** is also suitable for preparation for genomic DNA, histological examination and immunohistochemistry.

Table 1: Proposed pre-analytical treatment of endomyocardial biopsies for diagnostics in IKDT lab

Submitted material	Pre-analytic treatment	Detection method	Native/ fixed	Shipment
Myocardial biopsies	<i>RNAlater</i>	PCR & Histology & Immunohistochemistry	Fixed in <i>RNAlater</i>	Ambient temp
<i>Alternative pre-treatment</i>				
Myocardial biopsies	frozen in liquid nitrogen	PCR & Histology & Immunohistochemistry	native	on dry ice
Myocardial biopsies	fixed in 4-5% buffered formalin	Only for histology !	fixed in formalin	Ambient temp, Do not freeze!

IKDT lab is providing submitting institutions by screw-cup tubes filled with ***RNAlater*** for immediate use. Taken biopsies fixed in ***RNAlater*** should be transferred to IKDT for diagnostic procedure.

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Additional analysis

Detection of systemic viral infections or cytokine profiles is performed by analysis of peripheral blood fractions. DNA or RNA from peripheral blood cells is examined by nested- and QPCR on presence of viral genomes for exclusion or confirmation of systemic infection. EDTA-blood is requested for detection of systemic viral infection (Tab. 2).

Table 2: Proposed pre-analytical treatment of peripheral blood fractions for diagnostics in IKDT lab

Submitted material	Pre-analytic treatment	Detection method	Native/ fixed	Shipment
Blood	EDTA-tubes	PCR	native	+4°C or ambient
Serum/Plasma	EDTA-tubes	Immunology	native	frozen, on dry ice below -20°C

2. Histology

For histological examinations 4-5µm thick sections are prepared of paraffin-embedded biopsies by cutting with the rotary microtome. For each staining procedure on one slide are placed between 3-8 serial sections. Routine diagnostics includes always HE, PAS and Elastica v. Weigert staining. The staining of specimens, with the exception of special stainings, is always carried out in the staining machines.

Special staining for amyloid (Congo red), calcium (v. Kossa), acid mucosubstances (Alcian Blue) and iron (Prussian Blue reaction) will be added in clinically suspected cases or on request of submitting physician.

Diameters of cardiomyocytes were routinely measured digitally by application of in-house established digital imaging analysis software for characterisation of dominating atrophy or hypertrophy in myocardial tissue

IKDT Service offers Histology:

- Fixation (e.g. formalin, RNAlater) and storage of tissue samples
- Embedding of tissue in paraffin blocks
- Preparation of serial paraffin sections by rotation microtom
- Staining of tissues on request (manual, automatic)
- Fluorescence and light microscopy
- Digital measurement and evaluation of morphological structures (15 parameters)
- Histological assessment of stained sections
- Digital photographs of representative aspects
- Transfer of data, also via internet
- Archiving of reports, electronic data, paraffin blocks, stained slides according to GLP/GCP

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3. Immunohistochemistry

Immunohistological diagnostics are based on application of specific primary antibodies on cryo-fixed tissue section and following detection of coupled primary antibody by secondary antibody. Secondary antibody is conjugated with enzyme complex which could produce a precipitating coloured complex after use of staining solution.

Five sets of immunohistochemical staining are offered for specialized diagnostics of heart muscle tissue, whereas only the sets *IC1-Heart muscle inflammation* is proposed to perform in routine diagnostics procedure. Set *IC2-Activation marker/Viral proteins* is recommended in clinically suspected cases with high viral load of corresponding cardiotropic virus. Set *IC3-ARVD Diagnostics* is recommended in clinically suspected cases of ARVD to detected disrupted gap junctions of cardiomyocytes.

IC1: Heart muscle inflammation (CD3, CD11a, CD11b, Perforin, HLA class 1, CD54)

IC2: Activation marker/Viral proteins (CD45R0, 27E10, CD69, CD106, EV-VP1, PVB19, HHV6)

IC3: ARVD Diagnostics (*Plakoglobin, Connexin 43, N-Cadherin*):

IC4: Collagen Expression / Fibrosis (*Collagen 1 und 3 incl. Sirius-Red staining*):

IC5: Endothel Activation (*CD31, VCAM1*)

Coloured immunospots are counted digitally by application of in-house established digital imaging analysis software for calculating area fractions, numbers of immuno-spots and area of myocardial tissue (routine diagnostics). Values for counting are fixed by inclusion of digitally produced values in electronic database for in the final report. These reports contains about 20 numeric values for morphological characteristics like biopsy size, quality, fibrosis etc.

IKDT Service offers immunohistochemistry:

- Fixation (e.g. TissueTek, RNAlater) and storage of tissue samples
- Embedding of tissue in cryo blocks
- Preparation of serial cryo sections by cryomicrotome
- Antibody stainings will be tested and performed on request (virus)
- Fluorescence and light microscopy
- Digital measurement and evaluation of stained immunospots and tissue sections (20 parameters)
- assessment of stained sections including numeric values
- Digital photographs of representative aspects
- Transfer of data, also via internet
- Archiving of reports, electronic data, cryo blocks, stained slides according to GLP/GCP

4. Molecular diagnostics of cardiotropic viruses

The molecular diagnostic approach of EMBs are based on detection, quantification and sequencing of viral genomes. With permanently increasing number of virus tests IKDT is focused on common cardiotropic viruses which are described as responsible triggers of heart failure problems. Established virus PCR detection methods of IKDT lab are listed in Table 3.

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Test on cardiotropic viruses are based on qualitative detection of virus by nested-PCR and quantification of virus load by quantitative TaqMan PCR. Depending on the 2 types of viral nucleic acids we perform the isolation of DNA or RNA in separate extraction procedures. The transcriptional activity of virus in myocardial tissue or peripheral blood cells will be determined for the two most frequent cardiotropic viruses –Erythrovirus and HHV6 by nested-RT-PCR and QPCR.

Table 3: Established tests for cardiotropic viruses in IKDT lab

Virus	Nucleic Acid	nested-PCR	TaqMan	Subtypes / variants	Sequencing of positive PCR	Determination of virus subtype by
Erythrovirus (Parvovirus B19)	DNA RNA	X	X	G1, G2	yes	sequencing
Adenovirus	DNA	X	X	52	yes	sequencing
Human Herpesvirus 6 (HHV6)	DNA, RNA	X	X	A and B	yes	sequencing
Cytomegalovirus	DNA	X		no	yes	
Epstein-Barr-Virus	DNA	X	X	no	yes	
Herpes simplex virus 1 and 2	DNA		X	1 and 2		TaqMan
Coxsackievirus	RNA	X	X	various	yes	sequencing
Influenza	RNA		X	A and B		TaqMan
Measles	RNA		X	no		

Monitoring of successful treatment or therapy of infected patients has to be accompanied by estimation of viral load in EMBs at different time points. Viral load is the ratio of viral genome copies to associated amount of extracted myocardial tissue. For quality control and subtyping of infecting agent all PCR positive virus fragments were sequenced routinely.

Expanded analysis of endomyocardial biopsies and peripheral blood preparations

Today IKDT is one of the leading laboratories on viral infections of heart muscle tissue. All necessary analytical devices are existing in IKDT lab. IKDT as one of the first service providers in Europe offers the prompt access to two innovative technologies – GENIOM Biochips (febit biomed, Heidelberg, Germany) and Taqman Low-Density-Arrays (Applied Biosystems, Darmstadt, Germany) for gene expression studies or microRNA profiling.

IKDT is able to measure GENIOM biochips by its own GENIOM RT Analyzer and in-house adapted labeling protocols. The miRNA profiling biochip (FEBIT) always contains all published human miRNAs (more than 1000) from the newest version of the Sanger mirbase database. 8 individual samples can be examined in parallel on one biochip and will be pre-analyzed on different gene expression by existing bioinformatic workflow.

Additionally, IKDT is performing gene expression studies and microRNA profiling by validated TaqMan QPCR assays or TaqMan QPCR Low-Density-Arrays on Micro-Fluidic-Cards (Applied Biosystems) for simultaneous detection of up to 384 genes or microRNAs in a single sample, including RNA isolation, preamplification technologies and final evaluation of expression data. Applying optimized TaqMan SNP Genotyping Assays (Applied Biosystems) IKDT will be able to detect specifically individual SNPs in purified genomic DNA for high throughput screening of patient cohorts.

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The 8-Capillary Sequencer (CEQ8000, Fa. BeckmanCoulter) is routinely applied for genetic analysis for direct read out of genomic sequence for mutation and SNP detection. Extending the full power of this technology IKDT is performing fingerprinting analysis (STR analysis) for authentication and quality control of cell lines (8Stem cells) or biological samples and pathogen typing for expanded analysis of endomyocardial biopsies.

IKDT Service offers Molecular Biology:

- Fixation (e.g. RNAlater) and storage of tissue samples
- Isolation of DNA and RNA from various tissues or blood
- Reverse Transcription of mRNA in cDNA
- Quantification of nucleic acids by TaqMan QPCR
- Detection of viral genomes (nested-PCR, QPCR, sequencing)
- Measurement der gene expression of human, murine or rat genes by TaqMan QPCR
- **TaqMan Low-Density-Arrays - 12 to 384 genes in one sample (human, murine, rat)**
- **MicroRNA Profiling on TaqMan Low-Density-Arrays (660 human microRNAs)**
- Estimation of quantitative gene profiles on *TaqMan Micro-Fluidic Cards*
- Gene profiles are determined by customer (e.g. tumors, inflammation, diabetes)
- **Customized gene arrays of 15.000 probes for mRNA and microRNA (GENIOM)**
- Sequencing of PCR products or plasmids
- Detection of polymorphisms or gene variations by fragment length analysis
- Detection of disease relevant single nucleotide polymorphisms (SNPs)
- **Identity testing by STR analysis**
- Set-up and performance of novel PCR systems (virus, bacteria)
- Transfer of data, also via internet
- Archiving of reports, electronic data, tissues and remaining nucleic acids according to GLP/GCP

5. Autoimmunity Testing

Autoimmune cardiomyopathy is an immune-mediated chronic inflammation of the myocardial tissue. Induction of autoantibodies could be performed by infection of heart muscle by viruses or by intramyocardial inflammation. In order to diagnose autoimmunity in patients with cardiac problems IKDT apply indirect immunofluorescence assays based on BIOCHIP technology of EUROIMMUN (Germany).

Service offers Autoimmunity Testing:

- Detection of autoantibodies by fluorescence and light microscopy
- Simultane investigation of antibodies against various organs or infectious agents
- Transfer of data, also via internet
- Archiving of reports, electronic data and patient sera according to GLP/GCP

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6. Cytokine profiling by Multiplex-ELISA

Inflammation is produced by chemical factors; including specialized chemical mediators, called cytokines, and serves as a protective barrier. Cytokine levels in peripheral blood correspond to systemic situation in patients initiated by various, but often global factors like infections or inflammatory processes. Cytokines or chemokine are estimated in blood serum for characterization of present immune response.

Quantitative detection of cytokines is performed by commercially available immunoassays on full-automated ELISA system DSX (Fa. ThermoLabsystems) or by Multiplex-ELISA (Fa. Bio-Rad).

IKDT Service offers Immunology:

- Extraction and storage of patient samples (sera, tissues)
- Performance of ELISA tests (single parameter, Multiplex – upto 50 analytes)
- Estimation of phosphorylation stage of proteins
- Detection of autoantibodies by fluorescence and light microscopy
- Design or performance of individual ELISA tests or bead-based immunoassays
- Transfer of data, also via internet
- Archiving of reports, electronic data and patient sera according to GLP/GCP

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