

TECHNICAL CONSIDERATIONS  
FOR THE PERFORMANCE OF  
NUCLEIC ACID AMPLIFICATION TECHNOLOGY (NAT)

The NAT Task Force Group

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**Abstract**

The complexity of Nucleic acid Amplification Technology (NAT<sup>1</sup>), comprising sample preparation, amplification and detection methods, requires specific design considerations for both the laboratory and the procedures utilized in such testing. The purpose of this paper is to establish technical considerations for the performance of NAT. These include the collection, handling and assay of specimens and the design of laboratories to routinely and reliably detect low levels of nucleic acid sequences. The sensitivity of NAT due to the exponential amplification of nucleic acids makes contamination a major concern from specimen collection to sample detection. Therefore, laboratories need to be designed to prevent and control contamination through adequate equipment and appropriate workflow. These technical considerations should provide a basis for establishing a robust and reproducible NAT system.

Keywords: NAT, plasma, blood, viral testing minimum requirements.

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<sup>†</sup>Also known as Nucleic Acid Amplification Testing or Technology

## **Scope and purpose**

This document addresses the detection of human infectious agents in blood, plasma, serum and other blood components by NAT. If NAT testing is carried out on other human blood or plasma based materials, it should also be carried out in accordance with this document. While general regulatory requirements apply (see Appendix 1), the purpose of this document is to establish laboratory-specific recommendations as well as general procedural terminology and to set minimum requirements to ensure a robust NAT system. In addition, this document contains a list of citations of currently available guidelines and reference documents.

## **Test Specimens**

### *Specimen collection*

The test specimens in this document refer to blood, blood components, plasma and serum. Specimen collection is the first step in the NAT process. NAT sensitivity and stability of the target are the major factors that require very careful consideration by the collection centers at the time of collection.

Careful specimen handling must be ensured at all times during the collection process. In order to prevent cross-contamination at the specimen level appropriate laboratory procedures such as aseptic techniques should be used. Whenever possible, closed sampling systems should be employed to minimize the risk of specimen cross-contamination.

Measures, such as appropriate handling time, temperature and selection of preservatives, should be taken to ensure stability of pathogens and target nucleic acids.

Preservatives and anticoagulants such as heparin and ethylene-diamine-tetra-acetic acid (EDTA) must be evaluated as they may interfere with the NAT process.

### *Specimen transport and storage*

Transport and storage of test specimens should be done according to applicable regulations concerning biological specimens and should be validated to ensure stability of target sequences (e.g., repeated freezing and thawing of test specimens should be avoided; specimens should be frozen for prolonged storage).

*Specimen pooling and traceability*

Specimen pooling is a mechanism by which individual specimens are combined in a defined order such that NAT testing of the resulting pool and application of an algorithm can, as appropriate, identify an individual positive specimen.

Assurance that each specimen has been added to the pool is necessary.

## **Pooling Environment**

### *Facility*

Appropriate measures to prevent cross-contamination should be employed in the specimen pooling area. This area is one of the most critical regions of the pre-NAT laboratory because specimens, some of which may be of high titer, are manipulated in close proximity to each other. In particular, aerosol formation should be minimized.

### *Materials and Equipment*

Equipment that may be utilized for this process can include robotic pipetting systems, which should employ disposable aerosol-resistant tips. If manual pooling is used, comparable recommendations apply.

### *Procedures*

Workflow procedures should detail mechanisms that prevent cross contamination.

In addition, careful consideration should be given to the time and temperature during pooling processes to ensure pool and specimen stability.

## **Sample Preparation/Extraction**

The goal of sample preparation is the isolation of nucleic acids (the target) from the specimens/pools. In cases where pathogens are concentrated prior to isolation of nucleic acids, care should be taken to ensure adequate recovery.

Transport and storage of samples should follow procedures that ensure stability, e.g. repeated freezing and thawing should be avoided.

## **Data Management**

Data management includes the tracking of all relevant information from specimen acquisition through final reporting of results. NAT is often accompanied by complex specimen pooling and resolution algorithms. When NAT is used as a donor screening tool, it is necessary to have a validated procedure that is able to link the NAT result to a specimen in the pool.

## **Nucleic Acid Amplification Technology (NAT)**

### *Laboratory Design*

Generally, two strategies exist for nucleic acid amplification and detection: open and closed systems. There are inherent advantages and disadvantages to both strategies, and appropriate steps need to be taken depending on the strategy selected.

In open systems, at some point manipulation of the amplification vessel takes place that may result in the physical transfer of amplicons, e.g. the addition of reagents or the removal of amplicons for subsequent processing. The potential for sample contamination with amplicons is significantly increased, and strict separation of reagent preparation, sample preparation, amplification, and detection is crucial to minimize carry-over contamination by amplicons.

In closed systems, the vessels are not manipulated in any way that could result in the physical transfer of amplicons at any stage. This significantly reduces the contamination risk with post-amplified material, and there is no need for a separated detection area.

When using a closed system, strict separation of reagent preparation, sample preparation and amplification should be implemented to minimize contamination.

With respect to the physical structure of the laboratory, applicable regulations should be consulted prior to construction. In addition, facility design should enable uni-directional workflow to minimize contamination. Each distinct area should have dedicated equipment.

### *Assay Controls*

Assay controls are materials that are processed with test specimens to establish the validity of an assay result.

In order to detect contamination and to monitor NAT assay performance (sensitivity and specificity), at least three types of controls should be included in each NAT assay run:

**Negative assay control** - resembles as closely as possible the specimen matrix to be tested (e.g., plasma), but does not contain target sequences (e.g., kit control).

**Positive assay control** - resembles as closely as possible the specimen matrix to be tested and should contain an appropriate and defined amount of target sequences (e.g., kit control).

**Additional assay controls** may be included, e.g., **reagent control**, sometimes referred to as the “blank,” which contains all necessary reagents for amplification, but no target sequence and provides additional information in case of test problems.

**Internal control** - is added to each specimen to ensure the overall validity of the individual test result and should be added as early as possible in the process to control for sample preparation, reverse transcription, amplification and detection. In addition, internal controls may be used to monitor the assay.

### *Interpretation of Results*

If all controls meet acceptance criteria by manufacturers' instructions and/or SOPs, the results are considered valid.

The assay is considered invalid if one or more of the assay controls is not in compliance with acceptance criteria. An exception is the internal control which is added to each specimen. This may be non-reactive when a sample is reactive. In this case, the result for the particular sample is considered valid. If both the internal control and the specimen are non-reactive, the result for the particular sample is considered invalid.

*Standards*

Assay controls, calibrated against WHO International Standards where possible, should be used. A list of currently available standards is given in Appendix 2.

## **Personnel Training**

All personnel involved in the NAT assay and in the operation of the NAT facility need training in good laboratory procedures, appropriate regulatory requirements, and the unique requirements inherent to NAT. Therefore, in the case of personnel directly involved in NAT testing, training should address mechanisms to prevent cross-contamination, workflow, and careful specimen and test sample handling. Such training should include participation in periodic competency assessment, which can either be performed by evaluation of the run control detection performance of a given technician or by participating in internal or external proficiency programs.

*Acknowledgements*

The NAT Task Force Group would like to acknowledge Albrecht Groener, Ilka von Hoegen, Douglas Lee, Lorraine Peddada, Charles Watson, and Dottie Tripp for their valuable contributions to this paper. Their continued and substantial efforts throughout the preparation of this paper contributed to its present state.

## APPENDIX 1

This appendix contains a list of regulations and published guidelines addressing a variety of NAT applications, including transfusion medicine, diagnostics and plasma fractionation.

Guidance for Industry: In the Manufacture and Clinical Evaluation of *In Vitro* Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2. FDA CBER, December 1999.

The Introduction of Nucleic Acid Amplification Technology (NAT) for the Detection of Hepatitis C Virus RNA in Plasma Pools (CPMP/BWP/390/97). Addendum to Note for Guidance on Plasma Derived Medicinal Products (CPMP/BWP/269/95).

Monograph on "Human Plasma for Fractionation 0853," European Pharmacopoeia, 4th Edition, Council of Europe.

General method "2.6.21 Nucleic Acid Amplification Techniques," European Pharmacopoeia, 4th Edition, Council of Europe.

Annex to general method, "2.6.21 Validation of Nucleic Acid Amplification Techniques (NAT) for the detection of Hepatitis C (HCV) RNA in plasma pools," European Pharmacopoeia, 4th Edition, Council of Europe.

## APPENDIX 1

Anforderungen an Validierung bzw. Routinebetrieb der HCV-NAT im Blutspendewesen (1998).

German regulation for blood cellular components (implementation of HCV NAT of blood donation, 1 April; 1999).

## APPENDIX 1

### **Additional web sites and links to Guidelines concerning NAT and to National MOH and International Organizations**

#### **European Guidelines**

Bekanntmachung des Paul-Ehrlich-Instituts vom 27. Oktober 2000 über die Anforderungen an die diagnostische Erprobung von Nukleinsäure-Amplifikationstechniken zum Nachweis, zur Bestätigung und zur quantitativen Bestimmung von Markern von HIV-Infektionen (HIV1 und HIV2) sowie von Hepatitis B und C in Proben menschlichen Ursprungs

Bundesanzeiger Nr. 213 vom 14. November 2000, S. 21805

<http://www.pei.de/banz/2000/banz213.htm>

#### **U.S. Guidelines**

Guidance for Industry: Use of Nucleic Acid Tests on Pooled Samples from Source Plasma

Donors to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV.

Draft Guidance 12/2001

<http://www.fda.gov/cber/gdlns/hivhevnat.pdf>

Previous FDA draft guideline

<http://www.fda.gov/cber/gdlns/hivnas.pdf>

## APPENDIX 1

### Useful Information and Websites

DIN Norm 58967-60: Polymerase-Kettenreaktion (PCR).

DIN Norm 58969-61: Polymerase-Kettenreaktion (PCR); HIV1, HIV2

MiQ - Qualitätsstandards in der mikrobiologisch-infektiologischen Diagnostik; Heft 1/97:

Nukleinsäure-Amplifikationstechniken; im Auftrag der DGHM; Gustav Fischer Verlag, ISBN 3-437-41571-9.

ICH Topic Q2A: Validation of <http://www.eudra.org/humandocs/humans/ICH.htm>  
analytical methods: Definitions and  
terminology. CPMP/381/95

ICH Topic Q2B: Validation of <http://www.eudra.org/humandocs/humans/ICH.htm>  
analytical methods: Methodology.  
CPMP/281/95

**APPENDIX 1**

Austria - Federal Ministry of Labour, <http://www.bmags.gv.at>

Health and Social Affairs

Belgium - Ministry for Social <http://www.afigp.fgov.be>

Affairs, Health and Environment

Council of the European Union <http://ue.eu.int>

Denmark - Danish Medicines <http://www.dkma.dk>

Agency

Drug Information Association <http://www.diahome.org>

European Agency for the Evaluation <http://www.eudra.org>

of Medicinal Products (EMA)

European Commission [http://www.europa.eu.int/comm/index\\_en.htm](http://www.europa.eu.int/comm/index_en.htm)

European Department for the Quality <http://www.pheur.org>

of Medicines (EDQM)

European Union <http://europa.eu.int>

Finland - National Agency for <http://www.nam.fi>

Medicines

France - Agence du Medicament <http://agmed.sante.gouv.fr>

**APPENDIX 1**

Germany - Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM)	<a href="http://www.bfarm.de">http://www.bfarm.de</a>
Germany - Paul Ehrlich Institut	<a href="http://www.pei.de">http://www.pei.de</a>
Ireland, Republic of - Irish Medicines Board	<a href="http://www.imb.ie">http://www.imb.ie</a>
Italy - Ministry of Health	<a href="http://www.sanita.it/farmaci">http://www.sanita.it/farmaci</a>
Japanese Ministry of Health, Labor and Welfare	<a href="http://www.mhlw.go.jp">http://www.mhlw.go.jp</a>
Netherlands - Medicines Evaluation Board	<a href="http://www.cbg-meb.nl">http://www.cbg-meb.nl</a>
Portugal - INFARMED, Instituto Nacional da Farmaci e do Medicamento	<a href="http://www.infarmed.pt/home.html">http://www.infarmed.pt/home.html</a>
Spain - Ministerio de Sanidad y Consumo	<a href="http://www.msc.es/agemed">http://www.msc.es/agemed</a>
Sweden - Medical Products Agency	<a href="http://www3.mpa.se">http://www3.mpa.se</a>
UK - National Institute for Biological Standards & Control (NIBSC)	<a href="http://www.nibsc.ac.uk">http://www.nibsc.ac.uk</a>

## APPENDIX 1

US Centers for Disease Control and <http://www.cdc.gov>  
Prevention (CDC)

US Department of Health and <http://www.dhhs.gov>  
Human Services

US DHHS Advisory Committee on <http://www.os.dhhs.gov/bloodsafety>  
Blood Safety and Availability

US Food and Drug Administration, <http://www.fda.gov>  
FDA,

US National Institutes of Health <http://www.nih.gov>

WHO <http://www.who.int/home-page/>

## HCV

PREPARATION	PROVIDED BY	REMARKS	Reference
WHO international HCV RNA STANDARD	NIBSC	Genotype 1; established 1997; calibrated in International Units, $1 \times 10^5$ IU/ml NIBSC code 96/790	Saldanha J, Lelie N, Heath A; Establishment of the first international standard for nucleic acid amplification technology (NAT) assays for HCV RNA. WHO Collaborative Study Group; Vox Sang 1999; 76(3):149-58
Pelispay	CLB	Genotype 1, $1 \times 10^3$ IU/ml	Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu; Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group; Vox Sang 2000;78(4):217-224
Hepatitis C Virus Biological Reference Preparation H0215000	EDQM	Genotype 1a, $1 \times 10^3$ IU/ml	Nübling M.; IV. Collaborative study for the establishment of Hepatitis C Virus biological reference preparation for testing by nucleic acid amplification techniques; PHARMEUROPA Special Issue, BIO 99-1

PREPARATION	PROVIDED BY	REMARKS	Reference
Human Plasma Pools for NAT Validation H1005000	EDQM	Set of 103 negative plasma pools	Annex to general method “2.6.21 Validation of Nucleic Acid Amplification Techniques (NAT for the detection of Hepatitis C (HCV) RNA in plasma pools,” European Pharmacopoeia, 4 <sup>th</sup> Edition, Council of Europe, 67075 Strasbourg, France
HCV RNA Reference Preparation, Italy	ISS HCV RNA 0498	Genotype 1, 1.7x10 <sup>3</sup> IU/ml	Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu; Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group; Vox Sang 2000;78(4):217-224
Japanese HCV Reference Preparation	JMHW		

PREPARATION	PROVIDED BY	REMARKS	Reference
HCV RNA working reagent for NAT assays	NIBSC	Genotype 3, 710 IU/ NIBSC code 96/586	Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu; Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group; Vox Sang 2000;78(4):217-224
HCV Biological Reference Preparation, PEI Reference 75	PEI	Genotype 1, 2.5x10 <sup>4</sup> IU/ml	Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu; Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group; Vox Sang 2000;78(4):217-224
HCV CBER panel member No. 1	CBER (FDA)	Genotype 1, 250 IU/ml	Saldanha J, Heath A, Lelie N, Pisani G, Nubling M, Yu; Calibration of HCV working reagents for NAT assays against the HCV international standard. The Collaborative Study Group; Vox Sang 2000;78(4):217-224

<b>PREPARATION</b>	<b>PROVIDED BY</b>	<b>REMARKS</b>	<b>Reference</b>
HCV Genotypisierung-Panel	Unversität Essen	Samples: genotype 1a, 1b, 1b, 2a, 2b, 2c, 2i, 3a,3a, 4, 5a IU/ml not known	

**HIV**

<b>PREPARATION</b>	<b>PROVIDED BY</b>	<b>REMARKS</b>	<b>Reference</b>
WHO international HIV-1 RNA STANDARD	NIBSC	Genotype B; established 1999; calibrated in International Units, $1 \times 10^5$ IU/ml NIBSC code 97/656	Holmes H., Davis C., Heath a., Hewlett I., Lelie N. An international collaborative study to establish the 1 <sup>st</sup> international standard for HIV-1 RNA for use in nucleic acid-based techniques; J Virol Methods 2001 Apr;92(2):141-150
Pelispay	CLB	$2.9 \times 10^4$ IU/ml	Final report: Davis C., Holmes H., Heath A. HIV-1 RNA working reagents Calibration Study, 2001
HIV-1 panel	FDA		
HIV-1/2 Genotype Panel	FDA		
HIV Working Reagents	NIBSC	Study (in 2000) to calibrate HIV working reagents	Final report: Davis C., Holmes H., Heath A. HIV-1 RNA working reagents Calibration Study, 2001
HIV-1 RNA Subtype Reference Panel	NIBSC	Collaborative study to evaluate the HIV-1 RNA Subtype Reference Panel, May 2001  Contains HIV-1 RNA subtypes A, B, C, D, E, F, G, H, N, and O  Results pending	

<b>PREPARATION</b>	<b>PROVIDED BY</b>	<b>REMARKS</b>	<b>Reference</b>
HIV-1 working reagent for NAT assays PWS-1	NIBSC	PWS-1: NIBSC code 99/634, genotype B	
HIV-2	NIBSC, Centralized facility for AIDS reagents	HIV-2 ROD	
Candidate Japanese HIV- RNA National Reference Standard	JMHW	Calibration against WHO Standard, study September 2001, results pending	

**HBV**

<b>PREPARATION</b>	<b>PROVIDED BY</b>	<b>REMARKS</b>	<b>Reference</b>
WHO international HBV DNA STANDARD	NIBSC	Genotype A, $1 \times 10^6$ IU/ml, HBsAg subtype adw2 Established 1999 NIBSC code 97/746	Saldhana et al. An international collaborative study to establish a WHO international standard for HBV DNA nucleic acid amplification technology assay. (Expert committee on biological standardization, Geneva, 25 to 29 October 1999)
Pelispy	CLB		
HBV reference preparation	Universität Giessen		
Candidate Japanese HBV Reference Preparations	JMHW	3 candidates (#48, #129, #162), all genotype C calibrated against WHO HBV DNA Standard (genotype A), study December 2000, results by e-mail June 2001 Follow up study to calibrate final candidate #129 against WHO in September 2001, results pending	

**HAV**

<b>PREPARATION</b>	<b>PROVIDED BY</b>	<b>REMARKS</b>	<b>Reference</b>
5 Candidate preparations for a WHO international HAV RNA STANDARD	NIBSC	Calibration in IU/ml, collaborative study June 2001	In preparation
HAV working reagent for nucleic acid amplification techniques	NIBSC	NIBSC code 97/540	Saldanha J; Sensitivity of PCR assays for the determination of hepatitis A virus RNA in plasma pools. A collaborative study. Vox Sang 1999;76(3):163-5

## Parvovirus B19

PREPARATION	PROVIDED BY	REMARKS	Reference
WHO international B19 DNA STANDARD	NIBSC	Established 2000,  1x10 <sup>6</sup> IU/ml  NIBSC code 99/800	Saldanha J., Lelie N., Yu M-Y, Heath A. and the B19 Collaborative Study Group.  Establishment of the first World Health Organization International Standard for human parvovirus B19 DNA nucleic acid amplification techniques. Vox Sang 2002; 82: 24-31
Parvovirus B19 working reagent for NAT assays	NIBSC	NIBSC code 97/542	Saldanha J, Minor P. Collaborative study to assess the suitability of a proposed working reagent  for human parvovirus B19 DNA detection in plasma pools by gene  amplification techniques. B19 Collaborative Study Group. Vox Sang 1997; 73(4): 207-11
Multiplex Working Reagent for NAT	NIBSC code 99/732	Contains HCV  (genotype 3), HBV  (subtype adw), HIV  (genotype B), HAV ,  Parvovirus B19	