101194735 - VIROMARKERS

Virus Related Biomarkers to Improve Management of Chronic Conditions

WP2 – HDV-related biomarkers of treatment response

D2.2 HDV Biomarkers Study Protocol

Lead contributor	11 - UCL - Alesandro Cozzi-Lepri

Other contributors	6 - UTOV - Valentina Svicher 12 - Policlinico MI - Pietro Lampertico 9 - ELPA - Veronika Vsetickova 1.1 - I-PRO - Dario Corsini

Reviewers	3 - UniSiena - Maurizio Zazzi 7 - ASST - Giulia Marchetti

Document History

Version	Date	Description
V1.0	30 Apr 2025	First version

List of Acronyms

BLV	Bulevirtide
CHD	Chronic Hepatitis D
CRF	Case Report Form
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation
IEC	Institutional Ethics Committee
HBsAg	Hepatitis B Surface Antigen
HDV	Hepatitis D Virus
IRB	Institutional Review Board
mL	Milliliter
mg	Milligram
EC	European Commission
PID	Participant Identification Number
VIROMARKERS	Virus related biomarkers to improve management of chronic conditions
WBC	White Blood Cell Count
WHO	World Health Organization

1 Executive Summary

This document reports the study protocol for the HDV Biormarkers study, a multicentre, observational, retrospective study aimed at evaluating whether, in HDV patients who start a treatment with bulevirtide, HDV-RNA levels at baseline or their kinetics during the first 12 weeks of the treatment can predict the efficacy of the bulevirtide treatment (achievement of undetectable serum HDV-RNA).

The protocol defines the sponsor, the coordinator of the study, the main investigators and the involved centres. A synopsis section provides a summary of the protocol. The protocol then details the research background, the premises supporting the project, materials, methods and the primary and secondary objectives, including all the variables that represent the measure of the outcome (mainly HDV-RNA and ALT). The number of objectives was kept to a number which is realistic given the nature of the available data and the duration of the grant. A description is given of the general characteristics of the population that will be enrolled in the study. The sample size was planned and justified on the basis of a statistical calculation considering the incidence and prevalence of the disease and expected effect of the drug. The type of statistical analysis is described too. The modality and frequency of participants' examinations are detailed: basal evaluation, follow-up schedule, treatment strategy plan, frequency and dosage of treatment with bulevirtide (this part was enhanced by means of study design flowcharts and tables). Timing and duration of the study are indicated.

The informed consent form for the participants to the study is attached. It has been prepared under the supervision of the Community Advisory Group (CAG) and member of the consortium ELPA. Here premises, methods, and aims of the research together with advantages, modality to request withdrawal and possible risks derived from the participation to the study are clearly stated.

Also, a detailed Laboratory Manual is attached as Appendix D.

A final paragraph is dedicated to selected references.

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2 Study sponsor and participating centres

Sponsor of the study is the University of Rome Tor Vergata (UTOV).

CENTRE	PRINCIPAL INVESTIGATOR	ROLE
Policlinic of Milan	Pietro Lampertico	Coordinating Center
University of Rome Tor Vergata	Valentina Svicher	Co-Coordinating Center
Heinrich-Heine-Universitaet Duesseldorf	Björn-Erik Ole Jensen	Participant

3 Synopsis

3.1 Purpose

Chronic hepatitis Delta (CHD) constitutes the most severe form of viral hepatitis, leading to the development of cirrhosis in 50%-60% of cases. Interferon-alpha (IFNα) has been for several years the only therapeutic option against HDV, although associated with a very poor rate of sustained virological response. In 2020, bulevirtide (BLV) was approved by EMA for the treatment of patients with CHD at a dosage of 2mg/day. A recent European multicenter study, enrolling 244 patients with HDV-related compensated cirrhosis, showed that the percentage of patients achieving virological response (defined as HDV RNA undetectable or >−2 log10 IU/ml decline vs. baseline) at week 48 and 96 of BLV treatment was 65% and 79%, respectively [Degasperi et al., J Hepatol 2025]. Similarly, the percentage of patients achieving undetectable HDV-RNA was 28% and 48% at week 48 and 96, respectively [Degasperi et al., J Hepatol 2025]. Notably, ALT normalization was achieved in 61% and 64% of patients after 48 and 96 weeks of treatment. Although the optimal duration of BLV therapy is still under debate, the current guidelines recommend BLV treatment be continued for as long as it is associated with a clinical benefit [EASL CPG 2024].

To date, serum HDV-RNA is considered the hallmark for monitoring virological response to BLV. Although several in-house and commercial Real-Time PCR assays are available for the quantitative detection of HDV RNA, a relevant degree of variability in their diagnostic performance has emerged in preliminary inter-laboratory studies [Salpini et al., EASL 2024]. Furthermore, each step of HDV-RNA quantification from nucleic acid extraction up to reverse-transcription and real-time PCR amplification lacked robustness, raising the need to share laboratory procedures and protocols to ensure an accurate and precise quantification of HDV RNA across different laboratories [Steltz, 2021; Wedemeyer, 2023].

The harmonization of HDV-RNA assays by utilizing standardized and validated flowcharts still represents a relevant diagnostic unmet clinical need for the appropriate monitoring of patients with CHD receiving BLV treatment. Furthermore, information on long-term virological response and factors predictive of virological outcome is scarce.

Specific primary objectives to characterise the response to bulevirtide in CHD include:

1) To estimate the percentage of participants who will achieve virological response (defined as a decline in serum HDV RNA of >2 log or to undetectable HDV-RNA) and the proportion achieving undetectable HDV-RNA at week 48, 96 and 144 weeks of BLV treatment.

2) To estimate the percentage of participants who will achieve biochemical response and combined response (defined as achievement of virological response and ALT normalization) at week 48, 96 and 144 weeks of BLV treatment.

3) To evaluate whether HDV-RNA levels at baseline or their kinetics during the first 12 weeks of BLV treatment can predict the achievement of undetectable serum HDV-RNA during BLV treatment.

Specific secondary objectives include comparing the diagnostic performances of available assays for HDV-RNA quantification; comparing the rates of undetectable serum HDV-RNA at week 48, 96 and 144 by different HDV-RNA assays; comparing the kinetics of HDV-RNA during BLV treatment by different assays for HDV-RNA quantification; evaluating if the levels of HBsAg isoforms, alone or in combination with serum HDV-RNA at baseline, can predict the achievement of virological response to BLV at at week 48, 96 and 144 and comparing the rate of virological response to BLV according to HDV genotypes/subgenotypes.

The information used from this study on participants with CHD will be rapidly analyzed and shared broadly to guide policymakers for the use and monitoring of response to BLV and to design future studies.

3.2 Participant Selection

The study will include 220 patients who initiated BLV from January 2023 to February 2025 for whom retrospective data and samples are available. Follow-up and sample collection will be extended for this population over the period of the study grant.

To be eligible for inclusion in both the retrospective and prospective part of the study, participants have to be ≥18 years of age, have signed an informed consent and satisfy the following additional criteria:

- A diagnosis of chronic HDV infection with or without compensated cirrhosis
- Having started BLV monotherapy 2mg/day according to the criteria reported in the recent clinical practice guidelines on HDV infection promoted by EASL [EASL CPG 2024]
- Be under effective treatment with nucleoside analogues with HBV-DNA <20 IU/ml at the time of starting BLV

3.3 Study Plan

This is an observational study, in which clinical data, samples, and any other procedures possibly already performed on participants, were performed within the scope of clinical practice or within the scope of otherwise authorized projects, and that therefore there are no procedures performed specifically for this study, which instead uses data collected previously or prospectively during an extended follow-up, but in any case within the scope of current clinical practice. Most participants

have been enrolled and followed up at the project partners' clinical sites over 2023-2025. Their follow-up and sample collection will continue over the duration of the project. At the time of enrollment, demographics, medical history, medications and treatments prescribed were recorded and will be used in this study if the participant consent to be included also in VIROMARKERS. HDV genotypes/subgenotypes will be determined ex-novo on existing baseline stored samples.

For each participant, a blood sample was obtained at baseline and every 3 months during BLV treatment up to week 96. For a subset of participants, blood samples collected up to 144 weeks of treatment will be also available for analysis. At each time point, serum HDV-RNA was quantified with the reference RoboGene 2.0 assay used in clinical studies aimed at evaluating the efficacy of BLV [Wedemeyer et al., 2023; Degasperi et al., J Hepatol 2025].

For a subset of 80 participants followed-up at the Infectious Disease and Hepatology Unit of the University of Rome Tor Vergata and at the Policlinico of Milano, HDV RNA quantification using Altona (AltoStar assays), Qiagen and RoboGene 2.0 will be performed retrospectively in samples stored in 2023-2025 as well as in newly collected samples in 2025-2028. This will allow a comparison of the diagnostic performances of these assays and their capability to predict the achievement of virological response or undetectable HDV-RNA at week 48 and 96 and at week 144 in a subset. As such for the association analysis we will conduct separate analyses according to the method of quantification used. Particular attention will be dedicated at comparing the sensitivity of the above-mentioned assays by evaluating their ability to detect residual HDV viremia during treatment.

Also, on this subset of approximately 80 individuals, the levels of the three different isoforms of HBsAg (L-, M- and S-HBs) will be quantified by using three different ad-hoc designed ELISA assays [Brancaccio et al., 2021, D'Anna et al., 2025] at the following time points: baseline, 48, and 96 weeks and 144 weeks for selected patients. These assays have been demonstrated to have high sensitivity (detection limit for each protein is 0.1 ng/ml) and a high specificity, taking advantage of a sandwich system based on anti-PreS1, anti-PreS2 and anti-S antibodies, respectively. For each patient and for each time point, total HBsAg, L-HBs, M-HBs and S-HBs levels will be quantified in duplicate.

In the analysis, the virological status of each participant enrolled will be assessed every 3 months according to the guidelines promoted by European Association for the Study of the Liver [EASL CPG 2024] and up to 144 weeks.

The use of stored clinical specimens within this protocol will be reviewed by the Protocol Team, the VIROMARKERS Scientific Steering Committee, and EC Horizon Europe.

4 Background and Rationale

Hepatitis D virus (HDV) is one of the smallest human RNA viruses that can replicate only with the help of hepatitis B virus (HBV). Indeed, HDV hijacks HBV surface glycoproteins for its proper assembly and propagation in the hepatocytes. Chronic hepatitis Delta (CHD) constitutes the most severe form of viral hepatitis, leading to HCC and the development of cirrhosis in 50%-60% of cases. Interferon-alpha (IFNα) has been for several years the only therapeutic option against HDV, although associated with very poor rates of sustained virological response. In 2020, BLV was approved by EMA for the treatment of patients with CHD. This drug binds to the sodium

taurocholate cotransporting polypeptide, thus blocking the entry of HDV (and HBV) into hepatocytes.

A recent European multicenter study, enrolling 244 patients with HDV-related compensated cirrhosis, showed that the percentage of patients achieving virological response ((defined as HDV RNA undetectable or >−2 log10 IU/ml decline vs. baseline) at week 48 and 96 of BLV was 65% and 79%, respectively [Degasperi et al., J Hepatol 2025]. Similarly, the percentage of patients achieving undetectable HDV-RNA was 28% and 48% at week 48 and 96, respectively [Degasperi et al., J Hepatol 2025]. Notably, ALT normalization was achieved in 61% and 64% of patients after 48 and 96 weeks of BLV. Furthermore, the 96-week cumulative risks of de-novo HCC and decompensation were 3.0% (95% CI 2-6%) and 2.8% (95% CI 1-5%), respectively, suggesting a low incidence of liver-related complications [Degasperi et al., J Hepatol 2025].

Although the optimal duration of BLV treatment is still debated, current guidelines recommend BLV treatment be continued for as long as it is associated with a clinical benefit (EASL CPG 2024).

BLV has revolutionized the management of patients with CHD, even if there are gaps to be filled for an optimized use of this drug. Firstly, serum HDV-RNA is so far considered the hallmark for monitoring virological response to BLV. Although several in-house and commercial Real-Time PCR assays are available for the quantitative detection of HDV RNA, a relevant degree of variability in their diagnostic performance has emerged in preliminary inter-laboratory studies. Furthermore, each step of HDV-RNA quantification from nucleic acid extraction up to reverse-transcription and real-time PCR amplification is subject to variations, raising the urgent need to share laboratory procedures and protocols to ensure accurate and precise quantification of HDV RNA across different laboratories [Steltz, 2021; Wedemeyer, 2023]. The harmonization of HDV-RNA assays by utilizing standardized and validated flowcharts still represents a relevant diagnostic unmet clinical need for the appropriate monitoring of patients with CHD receiving BLV treatment. Furthermore, information on long-term virological response and factors predictive of virological response is scarce. Preliminary data, derived from small studies, have shown that an HDV-RNA serum level >5log IU/ml correlates with a lower rate of undetectable HDV-RNA after 48 weeks of BLV treatment [Degasperi et al., J of Hepatol 2025]. Similarly, preliminary evidence highlights the role of early decline of serum HDV-RNA in predicting virological response to BLV at week 24 [De Lédinghen et al., 2022]. Nevertheless, these results need to be corroborated in a larger cohort of patients and with a longer follow up. So far, BLV monotherapy is considered a long-term therapeutic strategy aimed at maximally suppressing HDV replication. However, recent studies are evaluating if the combination of BLV with immunomodulant agents (such as interferon alpha) could ensure a finite course of treatment [Asselah et al., 2023]. Thus, there is the need to identify patients who can safely suspend treatment without incurring the risk of virological relapse. In this setting, the availability of highly sensitive assays for serum HDV-RNA quantification can provide an added value. Furthermore, recent studies have raised the interest in evaluating the differential composition of HBsAg isoforms as novel biomarkers for HBV and HDV infection staging and for predicting treatment outcome [Pfefferkorn 2018; Charre, 2019; Rodgers, 2023]. It is known that HBsAg consists of three different proteins encoded within the same S open reading frame (ORF S): (i) large HBsAg (L-HBs), consisting of preS-1, preS-2 and S regions; (ii) middle HBsAg (M-HBs), consisting of pre-2 and S regions, and small HBsAg (S-HBs), containing only S region [Heermann at al., 1984; Glebe et al., 2013]. All the three HBs proteins share the same C-terminal S region, representing the targets of the commercially available serological assays routinely used for HBsAg quantification. Consequently, these commercial assays permit the quantification of only the

overall amount of the three HBs isoforms, while they cannot distinguish the different contribution of the three HBs isoforms [Charre et al., 2019]. Recently, novel assays capable of allowing the quantification of the different HBsAg isoforms have been developed. By using these assays, it has been demonstrated that HBsAg composition significantly changes across the different stages of HBV infection, showing lower proportions of L- and M-HBs in the HBeAg-negative infection compared to HBeAg-negative chronic hepatitis [Pfefferkorn et al, 2018; Roade et al., 2023], and during nucleos(t)ide analogues (NUC) or pegylated interferon alpha (Peg-IFNα) treatment [Pfefferkorn, 2021; Lazarevic, 2024]. Notably, a recent study has highlighted the role of HBsAg isoforms in modulating virological response to BLV [Salpini et al., 2023]. However, their clinical validation in larger sample sizes is still missing.

Finally, HDV is also endowed with an extraordinary degree of viral genetic variability that has enabled viral differentiation into 8 genotypes with genotype 1 being the most prevalent and geographically widespread. Within a single infected patient, this high degree of genetic variability gives origin to different genotypes and sub genotypes whose role in modulating virological response to BLV has not been fully clarified.

5 Methodology

5.1 Study Design

This is a mainly retrospective and partly prospective observational study which will enrol patients with CHD who started BLV over 2023- March 2025. Participants were enrolled in other projects and most have already been followed for some time as part of these studies and will be enrolled in this new study at the time of signing the consent to participate in VIROMARKERS.

Participants have been followed for a minimum of 96 weeks for achievement of virological response. Similar inclusion criteria and follow-up with additional sample collection and testing (levels of HBsAg isoforms, HDV genotypes/sub genotypes) are planned for the prospective part which encompasses an extension of follow-up for those already recruited in the retrospective phase. No new enrolments are planned for this protocol.

5.2 Study Objectives

Primary and secondary objectives are listed below.

Primary

1. Characterize individuals with CHD in terms of demographics, co-morbidities and HDV disease status
2. Describe the virological and clinical course under treatment with BLV for all participants
3. Estimate the proportion of participants achieving a virological response (defined as a decline in serum HDV RNA of >2 log or undetectable HDV-RNA) and undetectable HDV-RNA by 48, 96, 144 weeks from starting BLV

4. Estimate the percentage of patients who achieve biochemical response and combined response (defined as achievement of virological response and ALT normalization) at 48, 96, 144 weeks from starting BLV

5. Identify potential predictors of response: HDV-RNA levels at baseline, HDV-RNA kinetics during the first 12 weeks

6. In a subset of the study population, to measure levels of HBsAg isoforms, along or in combination with serum HDV-RNA at baseline and over follow-up and HDV genotypes/subgenotypes at baseline.

Secondary

1. In a subset of the study population with multiple measures, to compare the diagnostic performances of the assays for HDV-RNA quantification by Robogene, Altona (AltoStar assays) and Qiagen, against the WHO reference standard and clinical samples with known serum HDV RNA.

2. To trace and compare the kinetics of HDV-RNA during BLV treatment by using different assays for HDV-RNA quantification.

3. To compare the rate of undetectable serum HDV-RNA up to 144 weeks by different assays for HDV-RNA quantification.

5.3 Sample Size

Sample size for the retrospective part was closed at 220 individuals. We expect further recruitments over the 3 years of the grant. It is uncertain how many participants will start treatment each year. With the infrastructure that is in place, recruitment of patents in need for treatment will be rapid although it is a rare disease, so we predict to further enroll a maximum of 30 additional individuals.

Based on the population of individuals previously studied and who can be potentially enrolled and follow-ed up in VIROMARKERS, we expect to have a study population with a median age of 50 years (range 30-70) with a comparable gender distribution, 95% of European origin. Sites in diverse geographic locations in several countries have participated. By contrast, the prospective samples will be collected only in Italy at the project sites in Policlinico University of Milan and Rome (University of Tor Vergata).

For CHD participants on BLV, data from a single trial exist to estimate the percentage of participants who will achieve virological response by week 48 and to determine power for studying risk factors related to response.

Statistical considerations are summarized below. We considered binary as well continuous predictors, e.g., HDV RNA, HDV RNA slope, etc.). For the latter, power was estimated from comparing the upper versus lowest quartile of the factor.

In a recent analysis of 244 persons with CHD (the SAVE-D study), 48% achieved undetectable HDV-RNA and 79% virological response. Using these data, relative risks of 2.21 and 2.50 can be detected with 80% and 90% power, respectively, assuming 100 participants will achieve undetectable serum HDV-RNA). For virological response and ALT normalization which have higher response rates, the power will be greater. We expect to have HBsAg isoforms measured in a

subset of 80 individuals, so the power will be limited for this analysis for ORs in the range of 1.8-2.8, depending on the rate of enrolment of new initiators.

Table 1 – Odds ratios of response that can be detected with 80% and 90% power assuming 100 achieving undetectable HDV-RNA		
	Power	
Prevalence of high level of biomarker/high risk genotype	**80%**	**90%**
5%	2.78	3.26
10%	2.25	2.55
25%	1.86	2.06
50%	1.80	1.99

5.4 Participant Selection

5.4.1 Inclusion Criteria

To be eligible for enrollment participants must:

- Be > 18 years of age
- Sign an informed consent
- Have a diagnosis of HDV infection with or without compensated cirrhosis
- Having started BLV monotherapy 2mg/day according to the criteria reported in the recent clinical practice guidelines on HDV infection promoted by EASL (EASL CPG 2023)
- Be under effective treatment with nucleoside analogues with HBV-DNA <20 IU/ml at the time of starting BLV

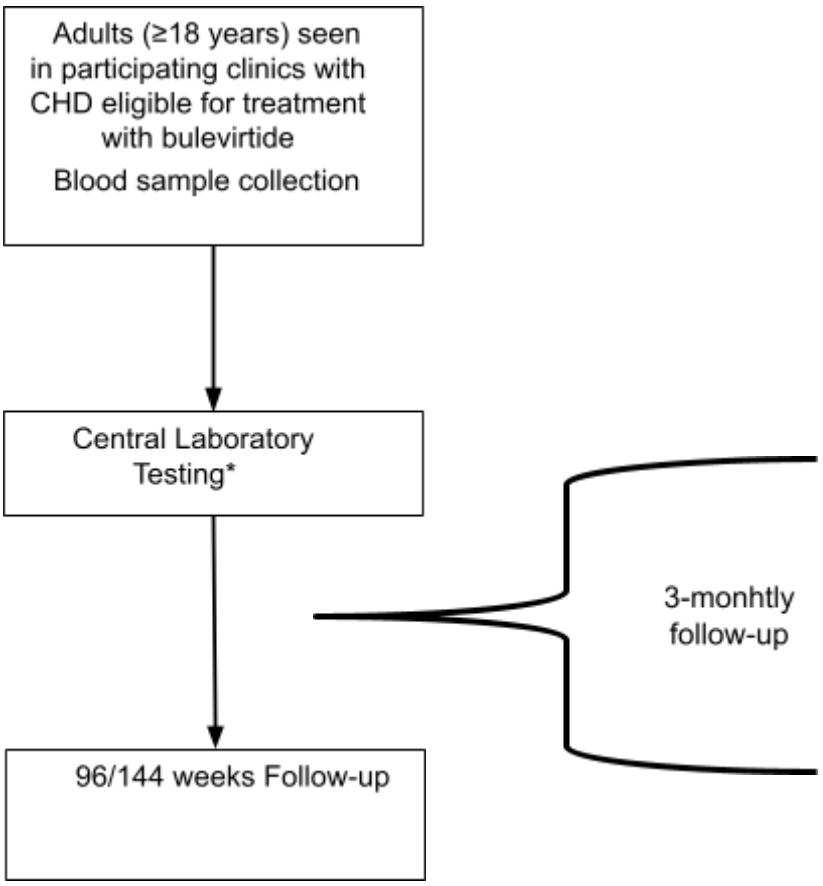
5.4.2 Exclusion Criteria

- Decompensated liver disease
- BLV combined with pegIFN
- Past treatment with BLV

5.5 Study Plan

The study plan is depicted in Figure 1.

Figure 1. *Flow Diagram of the Study.*



At the time of starting BLV (enrolment), information regarding demographics and medical history was obtained, including onset date of illness and treatments prescribed. A blood sample was obtained for all participants at enrollment and new samples will be collected in the newly enrolled to be shipped to a central repository for testing of levels of HBsAg isoforms, HDV genotypes/subgenotypes and Altona/Qiagen HDV RNA quantification.

The virological status of all participants enrolled will be assessed every 3 months according to the guidelines promoted by European Association for the Study of the Liver (EASL CPG 2023) at week 48, 96 and 144 after enrollment. The 48-, 96- and 144-week follow-up status assessment will include:

- HDV RNA levels
- ALT levels
- Adverse events
- Liver-related events
- Hospitalization, including length of stay

The use of stored specimens will be reviewed and approved by the Protocol Team, the VIROMARKERS Scientific Steering Committee, and EC Horizon Europe.

5.6 Study Withdrawal

Participants may withdraw from this observational study at any time at their request.

6 Evaluation

6.1 Data Analysis

Simple descriptive statistics will be used to describe all participants enrolled. Characteristics of the study population overall and according to genotypes/subgenotypes, geographic region, and month of enrollment will be summarized.

The proportion of participants achieving virological response and undetectable HDV RNA level will be computed with 95% confidence limits, overall and by identified potential predictors (HDV-RNA levels at baseline, HDV RNA kinetics during the first 12 weeks, levels of HBsAg isoforms, along or in combination with serum HDV-RNA at baseline, HDV genotypes/subgenotypes). Linear mixed models with random intercept and slope will be used to describe the trajectories of HDV-RNA decline over the course of treatment overall and, in the subset of samples with multiple measurements, after stratification by exact assay used (Robogene vs. Altona (AltoStar assays) vs. Qiagen).

Predictors of achievement of virological response and undetectable HDV-RNA by 48, 96, 144 weeks after starting BLV will be examined using logistic regression analysis. Predictors for major outcomes will include HDV-RNA levels at baseline, HDV RNA kinetics during the first 12 weeks, levels of HBsAg isoforms, along or in combination with serum HDV-RNA at baseline, HDV genotypes/subgenotypes. To account for variability due to year of starting bulevirtide and site of enrollment, mixed models that consider calendar year and site as random effects, will also be considered. All analyses will be repeated separately for the Robogene, Altona (AltoStar assays) and Qiagen assays quantifications.

In the subset of participants with multiple measures, to compare the diagnostic performance of the Robogene, Altona (AltoStar assays) and Qiagen assays against the WHO reference standard, several key metrics will be employed: sensitivity (true positive rate), specificity (true negative rate), accuracy (defined as the proportion of true positive plus true negative over the total sample). A logistic regression model will be used also to derive Receiver Operating Curves (ROC) and calculate the area under the ROC (AUC).

6.2 Data Monitoring

The prospective part of this observational study (extension of follow-up after February 2025) will be conducted under the direction of the Protocol Team, the VIROMARKERS Scientific Steering Committee, and VIROMARKERS leadership. Members of the protocol team are given in Appendix C.

There are minimal risks associated with this study. The risks of having blood taken include transient pain, bleeding, bruising, lightheadedness, fainting and rarely infection or a blood clot where the needle enters the body. If any adverse events occur that result from the blood draw, sites will follow local IRB/IEC procedures for reporting. For a small proportion of participants recruited, the study requires extended follow-up and long-term participation of up to 144 weeks, frequent blood draws, and multiple clinic visits, and this may not fit with all participants' daily lives. Study staff will offer accommodations for those with work, caregiving, or health-related constraints.

Unfortunately, because physical presence is needed to allow treatment infusion and samples collection, remote follow-up is only viable for clinical assessment. Access to the study will be universal but restricted to patients in care at sites participating in the study, as such we do not envisage non-equity concerns regarding the access to the drug.

7 Protection of Human Subjects & Other Ethical Considerations

7.1 Local Review of Protocol and Informed Consent

Participants already enrolled in previous projects for whom we will use already recorded clinical and laboratory results and in whom follow-up will be extended and new data collected will have to sign an informed consent form to be included in VIROMARKERS. Both the informed consent form and participant's information materials will be submitted to and approved by the site's IRB or EC. Likewise, any future amendments to the study protocol will be submitted and approved by each site's IRB or EC. After IRB/EC approval, sites must register for the protocol before enrolling potential participants and must register for any protocol amendments.

7.2 Ethical Conduct of the Study

The study will be conducted according to the Declaration of Helsinki; the requirements of Good Clinical Practice (GCP) as defined in Guidelines, EU Clinical Trials Directive, and EU GCP Directive; Human Subject Protection and Data Protection Acts or with the local law and regulation, whichever affords greater protection of human subjects.

7.3 Participants and Public Involvement

During the retroprospective part of this study, engagement and feedback was provided by community representatives. For the prospective part of this study (which involves only the extension of follow-up of those retrospectively recruited), we envisage to formally establish a Community Advisory Group (CAG) which will provide ongoing engagement and feedback at various stages of the new recruitment. Besides contributing to the writing of the protocol, we will encourage the advisory board to provide input to check the existing, and in case modify, the informed consent form and study procedures to ensure that participation is practical, accessible and aligned with participants' needs.

7.4 Informed Consent of Study Participants

All study participants must sign the IRB/EC approved informed consent form prior to any study-related procedures. See sample informed consent, Appendix A. Patient advisory board will contribute to the development of the form.

7.5 Confidentiality of Study Participants

The confidentiality of all study participants will be protected in accordance with GDPR Guidelines and national regulations. Participants' data and biological samples will be stored only for the length of the study period and will not be used for future research without additional consent.

8 Other Important Documents and Policies

8.1 Data Collection and Monitoring

Study data will be collected on standardized case report forms. Data will be collected during participant visits to health-care providers and possibly via chart abstraction.

In some instances, it may be necessary to obtain and abstract records from another clinic, hospital or healthcare facility; written permission for this is generally required and should be obtained at the time of the signing of the study consent. Study data and case report forms will be made available to site monitoring personnel. Monitoring may be performed by staff from the VIROMARKERS Centers. The retrospective part of the study will foresee data collection from 2 previous studies under the Coordination of the Policlinico of Milan. In compliance with the GDPR and applicable European Regulations, data will be transferred to the Viromarkers Study Database anonymized.

As part of routine clinical care, participants will receive information on key health indicators, such as their HDV RNA levels or treatment response, as well as a clear process so they will be able to share concerns or suggestions for improving the research process.

8.2 Publications and Presentations

Publications and presentations related to data obtained from this study will adhere to the VIROMARKERS Publications and Presentations Policy found on the VIROMARKERS website (www.viromarkers.eu).

9 Appendix A: Sample Informed Consent Form

Protocol Title: HDV-related biomarkers of treatment response

In English (below, the version in Italian):

WRITTEN PATIENT INFORMATION

Title of the study:

Dear Madam / Dear Sir,
You have been asked to participate in an experimental clinical study and this document is intended to inform you about the nature of the study, its intended purpose, what such participation will mean for you, and your rights and responsibilities.
Please read this written information carefully before making a decision about whether to participate in the study. You will have all the time necessary to decide whether to participate or not.
Furthermore, you will be able to freely ask any questions for clarification and ask any questions that have not received a clear and exhaustive answer.
In the event that, after having read and understood all the information provided herein, you decide that you wish to participate in the clinical study, I will ask you to sign and personally date the Informed Consent form attached to this document.

WHAT THE STUDY AIMS FOR

The aim of the study is to collect data relating to patients with chronic HDV infection and to their response to bulevirtide treatment.

WHAT DOES YOUR PARTICIPATION IN THE STUDY INVOLVE

In the event that you decide to participate in the study, we inform you that, after having evaluated the possibility of including you in the research and after having carried out all the medical/instrumental treatments envisaged for you regardless of whether or not you participate in this research, the study involves the collection of data relating to your health, your ethnicity, your sex, your age, your sexual life and risk factors.

There is no end date for data collection, as the network will continue its data updating and collection activity as long as the operational conditions for exercise by the centers and the Scientific Board exist.

We also inform you that participation in the research does not entail any increase in expenses for you, which will all be borne by the hospital.

SURVEYS TO WHICH YOU WILL BE SUBJECTED DURING THE STUDY

No additional investigations.

WHAT ARE THE BENEFITS YOU CAN RECEIVE BY PARTICIPATING IN THE STUDY

Although this cannot be guaranteed a priori, the study is expected to acquire scientific information useful for the clinical management and treatment of the pathologies under study.

WHAT ARE THE RISKS ARISING FROM PARTICIPATION IN THE STUDY

None, as it is a simple collection of data.

WHAT HAPPENS IF YOU DECIDE NOT TO PARTICIPATE IN THE STUDY

Authorization to process data is an essential condition for participating in the study.
You are free not to participate in the study or, if you decide to participate, you will have the right to withdraw from the study at any time and without the obligation to provide explanations. The doctor who performs the Research may also decide to interrupt participation in the study at any time he deems appropriate, without this being detrimental to your health.

CONFIDENTIALITY OF PERSONAL DATA

Pursuant to the legal provisions regarding the protection of personal data (Legislative Decree 30/6/2003 n. 196, Guidelines for the processing of personal data in the context of clinical trials of medicinal products - 24 July 2008 - Official Gazette n. 190 of 14 August 2008, General Data Protection Regulation [GDPR, in particular as specified in Article 9 par. 2(i), par. 2(j) and Art. 89 par.1, par. 2], the following is specified:

Data controllers and related purposes

The centers participating in the data collection and the Sponsor University of Rome*,* each for the areas of their own competence and in accordance with the responsibilities established by the rules of good clinical practice (Legislative Decree 211/2003) and the legislation on the protection of personal data (GDPR 679/2016 and Legislative Decree 196/2003 as amended by Legislative Decree 101/2018), will process your personal data, in particular health data and, only to the extent that they are indispensable in relation to the objective of the study, other data relating to your origin, your lifestyle and your sexual life, information relating to infections from human immunodeficiency virus infection, hepatitis B virus, hepatitis C virus (HIV, HBV and HCV) or other viruses, exclusively for the purpose of carrying out the study.

To this end, the data indicated will be collected by the testing center and transmitted to the Sponsor (University of Rome Tor Vergata - data controller) to external people or companies acting on their behalf, or statistician in charge for the statistical analysis, based in Europe.

The personal data you provide for the purposes described above will be processed on the basis of your express consent, which therefore constitutes the legal basis for the processing.

The processing of personal data relating to your health, your origin, your age, your lifestyle, your sexual life and viral infections is essential for carrying out the study: refusal to provide them will not allow you to participate.

The processing of data is essential for carrying out the study: refusal to provide it will not allow you to participate.

Nature of the data

The doctor who will follow you in the study will identify you with a code: the data concerning you collected during the study, with the exception of your name, will be transmitted to the Sponsor, recorded, processed and stored together with this code, your date of birth, your sex, your weight and your height. Only your doctor and authorized parties will be able to link this code to your name.

Treatment methods

The data, processed using electronic tools, will only be disseminated in a strictly anonymous form, for example through scientific publications, statistics and scientific conferences. All analyzes derived from the study will be conducted and presented in an aggregate manner, in no case making reference to a specific subject. Your participation in the study implies that, in compliance with the legislation on clinical trials of medicinal products, the staff of the Sponsor or of the external companies that carry out the monitoring and verification of the study on behalf of the Sponsor, the Ethics Committee and the Italian and foreign health authorities will be able to know the data concerning you, also contained in your original clinical documentation, in ways that guarantee the confidentiality of your identity.

The data may also be used for the Sponsor's membership in international registries, where they are stored and shared by researchers, in accordance with the principle of open science and open access to research data

Duration of treatment

In accordance with EU Regulation 2016/679 (GDPR), the study data, including anonymized data, will be stored and managed exclusively for the period of time necessary for the purposes of the study, and, in the particular case, as long as they are useful for producing scientific publications aimed at improving clinical and laboratory practice for the treatment of patients with viral infections.

The data will also be kept for the time prescribed by European and National regulations for observational clinical studies, currently equal to seven years after the end of the study. The end of the study will be defined as the moment in which the Sponsor will communicate to the centers the closure of the project, based on an indication to this effect from the Scientific Committee of the study or, in any case, once four years have passed since the last scientific publication produced on the study data and for the pursuit of the objectives of the study.

All the data of subjects who withdraw consent to the processing of data or for whom such consent is withdrawn by those entitled will be eliminated from the study database starting from the date of withdrawal of consent and can therefore no longer be included in any analysis produced by the study, without prejudice to the responsibility of the centers where the subjects are or were being cared for and treated, to conserve the data according to the rules and procedures of the public health facility to which they belong.

Exercise of rights

You may exercise the rights referred to in the art. 15 and Sections 3 and 4 of EU Regulation 2016/679 (e.g. access your personal data, integrate them, update them, rectify them, request their cancellation, limitation and oppose the processing in the cases provided for by law or by the Regulation) by contacting the trial center directly, in the person who, as local Principal Investigator, is delegated to process the data.

We remind you that, in the event that you notice a violation of your rights regarding the protection of personal data, you can lodge a complaint with the Guarantor for the Protection of Personal Data.

Below is the information for contacting the Guarantor Authority for the Protection of Personal Data:

headquarters in Piazza Venezia n. 11 - 00187 Rome

telephone switchboard: 06.696771
email: protocollo@gpdp.it
certified email: protocollo@pec.gpdp.it

You can contact the Personal Data Protection Officer (*Data Protection Officer*, DPO) of the Sponsor to the e-mail address privacy@uniroma2.it

You can interrupt your participation in the study at any time and without providing any justification. In this case, no further data concerning you will be collected, without prejudice to the use of any data already collected to determine the results of the research, without altering them.

Finally, we remind you that the essential documents relating to the study will be kept at the Sponsor and the participating centers for seven years after the completion of the study.

INFORMATION ABOUT THE RESULTS OF THE STUDY

If you request it, at the end of the study the results of the study in general and those that concern you in particular may be communicated to you.

FURTHER INFORMATION

For further information and communications during the study the following staff will be available:

Dr./Prof. …… ……
Surname Name

Telephone ……..……..……

The study protocol proposed to you was approved by the Ethics Committee which, among other things, verified compliance with the Standards of Good Clinical Practice of the European Union and in accordance with the ethical principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee of this structure.

You may report any fact you deem appropriate to highlight, in relation to the research that concerns you, to the Ethics Committee and/or the Health Management of this hospital facility.

INFORMED CONSENT FORM

Title	
Version	Version

I signed up ……..……
……..……
Surname name
age (years) …… date of birth ……. / ……. /

……. Address

……..……

……

Square / Street / V.le House number

City ……..……..……. telephone
……..

Date ……. / ……. / …….

……..……..

Patient's signature (adult, mature minor)……

Signature of both parents in the case of a minor…….

Signature of the legal representative (in the case of an incapacitated, interdicted patient or with a support administrator)
Signature of the doctor who provided the information……

Secondary use of data.

If the patient authorizes the secondary use of data - in anonymized form - in future scientific research projects that researchers may initiate on their own or in which they may participate - a separate signature is required for this specific authorization. This consent may be denied and its absence does not in any way compromise participation in this study.

I the undersigned: NAME AND SURNAME OF THE PATIENT

authorize the secondary use of my data - in anonymized form - in future scientific studies for research purposes.

Date ……. / ……. / …….

Patient's signature (adult, mature minor)……

Signature of both parents in the case of a minor……..

Signature of the legal representative (in the case of an incapacitated, interdicted patient or with a support administrator)

Signature of the doctor who provided the information……

I the undersigned:

NAME AND SURNAME OF THE DOCTOR: _____

I declare that the patient has spontaneously signed his participation in the study
I also declare that:

- having provided the patient with comprehensive explanations regarding the aims of the study, the procedures, the possible risks and benefits and the possible alternatives;
- having verified that the patient has sufficiently understood the information provided to him
- giving the patient the necessary time and the opportunity to ask questions about the study
- not have exercised any coercion or undue influence in the request for Consent

DATE _____

DOCTOR'S SIGNATURE _____

PLEASE NOTE
a copy of this form, signed and dated, attached to the "Written Information for the Patient" must be delivered to the Patient himself

In Italian (above, the version in English):

INFORMAZIONI SCRITTE PER IL PAZIENTE

Titolo dello studio:

Gentile Signora / Egregio Signore,
Le è stato chiesto di partecipare ad uno studio clinico sperimentale e questo documento ha lo scopo di informarLa sulla natura dello studio, sul fine che esso si propone, su ciò che comporterà per Lei una tale partecipazione, sui suoi diritti e le sue responsabilità.
La prego di leggere attentamente queste informazioni scritte prima di prendere una decisione in merito ad una eventuale Sua partecipazione allo studio. Lei avrà a disposizione tutto il tempo necessario per decidere se partecipare o meno.
Potrà, inoltre, porre liberamente qualsiasi domanda di chiarimento e riproporre ogni quesito che non abbia ricevuto una risposta chiara ed esauriente.
Nel caso in cui, dopo aver letto e compreso tutte le informazioni ivi fornite, decidesse di voler partecipare allo studio clinico, Le chiederò di voler firmare e personalmente datare il modulo di Consenso Informato allegato a questo documento.

CHE COSA SI PROPONE LO STUDIO

Lo studio ha come obiettivo quello di raccogliere dati relativi ai pazienti con infezione cronica da HDV e sulla loro risposta al trattamento con bulevirtide

COSA COMPORTA LA SUA PARTECIPAZIONE ALLO STUDIO

Nel caso in cui Lei decidesse di partecipare allo studio, La informiamo che, dopo aver valutato la possibilità di poterLa includere nella ricerca e dopo aver effettuato tutti i trattamenti medici/strumentali per Lei previsti indipendentemente dalla Sua partecipazione o meno a questa ricerca, lo studio prevede la raccolta di dati relativi alla Sua salute, alla Sua etnia, al Suo sesso, alla Sua età, alla Sua vita sessuale e ai fattori di rischio.

Non è prevista una data di fine della raccolta dati, in quanto il network proseguirà la propria attività di aggiornamento e raccolta di dati finché sussisteranno le condizioni operative di esercizio da parte dei centri e del Board Scientifico.

La informiamo, inoltre, che la partecipazione alla ricerca non comporta per Lei alcun aggravio di spese che saranno tutte a carico della struttura ospedaliera.

INDAGINI A CUI SARÀ SOTTOPOSTO/A DURANTE LO STUDIO

Nessuna indagine aggiuntiva.

QUALI SONO I BENEFICI CHE POTRÀ RICEVERE PARTECIPANDO ALLO STUDIO

Benché ciò non possa essere a priori garantito, dallo studio ci si aspetta di acquisire informazioni scientifiche utili alla gestione clinica ed alla cura delle patologie in studio.

QUALI SONO I RISCHI DERIVANTI DALLA PARTECIPAZIONE ALLO STUDIO

Nessuno, trattandosi di una semplice raccolta di dati.

COSA SUCCEDE SE DECIDE DI NON PARTECIPARE ALLO STUDIO

L'autorizzazione al trattamento dei dati è condizione indispensabile per partecipare allo studio. Lei è libero/a di non partecipare allo studio oppure, se decide di partecipare, avrà il diritto di ritirarsi dallo studio in qualsiasi momento e senza l'obbligo di fornire spiegazioni. Anche il Medico Ricercatore potrà decidere di interrompere la partecipazione allo studio in qualsiasi momento lo ritenga opportuno, senza che questo sia pregiudizievole per la Sua salute.

RISERVATEZZA DEI DATI PERSONALI

Ai sensi delle norme di legge in materia di protezione dei dati personali (Decreto Legislativo 30/6/2003 n. 196, Linee guida per i trattamenti di dati personali nell'ambito delle sperimentazioni cliniche di medicinali - 24 luglio 2008 - G.U. n. 190 del 14 agosto 2008, Regolamento Generale sulla Protezione dei Dati [GDPR, in particolare quanto specificato negli Art. 9 par. 2(i), par. 2(j) e Art. 89 par.1, par. 2], regolamento UE n. 2016/679) viene precisato quanto segue:

Titolari del trattamento e relative finalità

I centri partecipanti alla raccolta dei dati (circa 25 centri di cura delle infezioni croniche in Italia) ed il promotore Università degli Studi di Roma Tor Vergata, ciascuno per gli ambiti di propria competenza e in accordo alle responsabilità previste dalle norme della buona pratica clinica (d.l. 211/2003) e alla normativa in materia di protezione dei dati personali (GDPR 679/2016 e D.Lgs. 196/2003 come modificato dal D.Lgs. 101/2018), tratteranno i Suoi dati personali, in particolare quelli sulla salute e, soltanto nella misura in cui sono indispensabili in relazione all'obiettivo dello studio, altri dati relativi alla Sua origine, ai Suoi stili di vita e alla Sua vita sessuale, alle informazioni relative alle infezioni da infezione da virus dell'immunodeficienza umana, virus della epatite B, virus della epatite C (HIV, HBV ed HCV) o altri virus esclusivamente in funzione della realizzazione dello studio.

A tal fine i dati indicati saranno raccolti dal Centro di sperimentazione e trasmessi al Promotore (Università degli Studi di Roma Tor Vergata - titolare del trattamento) alle persone o società esterne che agiscono per loro conto, tra le quali Società InformaPro (Informapro srl - responsabile del trattamento - https://www.informapro.it/), con sede in Roma.

I dati personali che fornirà per le finalità che Le sono state su descritte verranno trattati sulla base del Suo espresso consenso, che costituisce, quindi, la base giuridica per il trattamento.

Il trattamento dei dati personali relativi alla Sua sulla salute, alla Sua origine, alla Sua età, ai Suoi stili di vita, alla Sua vita sessuale e alle infezioni virali è indispensabile allo svolgimento dello studio: il rifiuto di conferirli non Le consentirà di parteciparvi.

Il trattamento dei dati è indispensabile allo svolgimento dello studio: il rifiuto di conferirli non Le consentirà di parteciparvi.

Natura dei dati

Il medico che La seguirà nello studio La identificherà con un codice: i dati che La riguardano raccolti nel corso dello studio, ad eccezione del Suo nominativo, saranno trasmessi al promotore, registrati, elaborati e conservati unitamente a tale codice, alla Sua data di nascita, al sesso, al Suo peso e alla Sua statura. Soltanto il medico e i soggetti autorizzati potranno

collegare questo codice al Suo nominativo.

Modalità del trattamento

I dati, trattati mediante strumenti anche elettronici, saranno diffusi solo in forma rigorosamente anonima, ad esempio attraverso pubblicazioni scientifiche, statistiche e convegni scientifici. Tutte le analisi derivate dallo studio saranno condotte e presentate in modo aggregato, non facendo in alcun caso riferimento ad uno specifico soggetto. La Sua partecipazione allo studio implica che, in conformità alla normativa sulle sperimentazioni cliniche dei medicinali, il personale del Promotore o delle società esterne che eseguono per conto del primo il monitoraggio e la verifica dello studio, il Comitato etico e le autorità sanitarie italiane e straniere potranno conoscere i dati che La riguardano, contenuti anche nella Sua documentazione clinica originale, con modalità tali da garantire la riservatezza della Sua identità.

I dati potranno essere utilizzati anche per l'iscrizione del Promotore a registri internazionali, dove saranno archiviati e condivisi dai ricercatori, nel rispetto del principio di scienza aperta e di libero accesso ai dati della ricerca.

Durata del trattamento

In accordo con il Regolamento UE 2016/679 (GDPR), i dati dello studio, inclusi i dati anonimizzati, saranno conservati e gestiti esclusivamente per il periodo di tempo necessario per le finalità dello studio, e, nel caso particolare, finché essi saranno utili a produrre pubblicazioni scientifiche volte a migliorare la pratica clinica e di laboratorio per il trattamento di pazienti con infezioni virali croniche da HIV, HCV, HBV.

I dati saranno inoltre conservati per il tempo prescritto dalle norme Europee e Nazionali per gli studi clinici osservazionali, attualmente pari a sette anni dopo il termine dello studio. Il termine dello studio sarà definito come il momento in cui il promotore comunicherà ai centri la chiusura del progetto, in base a un'indicazione in tal senso da parte del Comitato scientifico dello studio o, comunque, una volta che siano trascorsi quattro anni dall'ultima pubblicazione scientifica prodotta sui dati dello studio e per il perseguimento degli obiettivi dello studio.

Tutti il dati di soggetti che ritireranno il consenso al trattamento dei dati o per i quali tale consenso sarà ritirato dagli aventi diritto saranno eliminati dal database dello studio a partire dalla data del ritiro del consenso e non potranno quindi essere più inclusi in alcuna analisi prodotta dallo studio, ferma restando la responsabilità, per i centri presso i quali i soggetti sono o erano in cura e trattamento, di conservare i dati secondo le norme e le procedure della struttura sanitaria pubblica di appartenenza.

Esercizio dei diritti

Potrà esercitare i diritti di cui all'art. 15 e Sezioni 3 e 4 del Regolamento EU 2016/679 (es. accedere ai Suoi dati personali, integrarli, aggiornarli, rettificarli, richiederne la cancellazione, la limitazione e opporsi al trattamento nei casi previsti dalla legge o dal Regolamento) rivolgendosi direttamente al centro di sperimentazione, nella persona che, in qualità di Sperimentatore Principale locale, è delegata al trattamento dei dati.

Le ricordiamo che, nel caso in cui ravvisi una violazione dei Suoi diritti in materia di protezione dei dati personali, potrà presentare un reclamo al Garante per la Protezione dei dati personali.

Di seguito riportiamo le informazioni per contattare l'Autorità Garante per la Protezione dei dati personali:

sede in Piazza Venezia n. 11 - 00187 Roma
centralino telefonico: 06.696771
posta elettronica: protocollo@gpdp.it
posta elettronica certificata: protocollo@pec.gpdp.it

Potrà contattare Responsabile della Protezione dei dati personali (Data Protection Officer, DPO) del Promotore all'indirizzo e-mail privacy@uniroma2.it

Potrà interrompere in ogni momento e senza fornire alcuna giustificazione la Sua partecipazione allo studio. In tal caso, non saranno raccolti ulteriori dati che La riguardano, ferma restando l'utilizzazione di quelli eventualmente già raccolti per determinare, senza alterarli, i risultati della ricerca.

Da ultimo, le ricordiamo che i documenti essenziali relativi allo studio saranno conservati presso il Promotore e i centri partecipanti per sette anni dopo il completamento dello studio.

INFORMAZIONI CIRCA I RISULTATI DELLO STUDIO

Se Lei lo richiederà, alla fine dello studio potranno esserLe comunicati i risultati dello studio in generale ed in particolare quelli che La riguardano.

ULTERIORI INFORMAZIONI

Per ulteriori informazioni e comunicazioni durante lo studio sarà a disposizione il seguente personale:

Dott./Prof. …… ……
Cognome Nome

Telefono ……..……..……

Il protocollo dello studio che Le è stato proposto è stato approvato dal Comitato Etico che ha tra l'altro verificato la conformità alle Norme di Buona Pratica Clinica della Unione Europea ed in accordo ai principi etici espressi nelle Dichiarazione di Helsinki ed è stato approvato dal Comitato Etico di questa struttura.

Lei potrà segnalare qualsiasi fatto ritenga opportuno evidenziare, relativamente alla ricerca che La riguarda, al Comitato Etico e/o alla Direzione Sanitaria di questa struttura ospedaliera.

MODULO DI CONSENSO INFORMATO

Titolo	
Versione	Versione

Io sottoscritto ……..……
……..……
Cognome nome
età (anni) ……. data di nascita ……. / ……. /

……. Indirizzo

……..……

……

P.zza / Via / V.le Numero Civico

Città ……..……..……. telefono
……..

Data ……. / ……. / …….

……..……..

Firma del paziente (adulto, minore maturo)……

Firma di entrambi genitori in caso di soggetto minore…….

Firma del rappresentante legale (in caso di paziente inabilitato, interdetto o con amministratore di sostegno)
Firma del medico che ha fornito le informazioni……

Utilizzo secondario dei dati.

Se il paziente autorizza scientifica che i ricercatori potrebbero avviare autonomamente o a cui potrebbe partecipare, è richiesta una firma separata per tale specifica autorizzazione. Tale consenso può essere negato e la sua assenza non compromette in alcun modo la partecipazione al presente studio.

Io sottoscritto ……NOME E COGNOME DEL PAZIENTE…….
autorizzo l'utilizzo secondario dei miei dati - in forma anonima - in futuri progetti scientifici per scopi di ricerca.

Data ……. / ……. / ……. ……..……..

Firma del paziente (adulto, minore maturo)……

Firma di entrambi genitori in caso di soggetto minore…….

Io sottoscritto:

NOME E COGNOME DEL MEDICO:

Dichiaro che il paziente ha firmato spontaneamente la sua partecipazione allo studio

Dichiaro inoltre di:

- aver fornito al paziente esaurienti spiegazioni in merito alle finalità dello studio, alle procedure, ai possibili rischi e benefici e alle possibili alternative;
- aver verificato che il paziente abbia sufficientemente compreso le informazioni fornitegli
- aver lasciato al paziente il tempo necessario e la possibilità di fare domande in merito allo studio
- non aver esercitato alcuna coercizione od influenza indebita nella richiesta del Consenso

DATA

FIRMA DEL MEDICO

NOTA BENE
una copia del presente modulo, firmato e datato, allegato alle "Informazioni Scritte per il Paziente" dovrà essere consegnata al Paziente stesso

In German (above, the version in English):

SCHRIFTLICHE INFORMATIONEN FÜR DEN PATIENTEN

Studientitel:
Sehr geehrte Damen und Herren,
Sie wurden gebeten, an einer experimentellen klinischen Studie teilzunehmen. Dieses Dokument soll Sie über die Art der Studie, ihren Zweck, die Bedeutung dieser Teilnahme für Sie sowie Ihre Rechte und Pflichten informieren.
Bitte lesen Sie diese schriftlichen Informationen sorgfältig durch, bevor Sie eine Entscheidung über die Teilnahme an der Studie treffen. Sie haben ausreichend Zeit, um zu entscheiden, ob Sie teilnehmen möchten oder nicht.
Darüber hinaus können Sie Fragen zur Klärung frei stellen und Fragen, die keine klare und umfassende Antwort erhalten haben, erneut stellen.
Wenn Sie sich nach dem Lesen und Verstehen aller hierin enthaltenen Informationen dazu entschließen, an der klinischen Studie teilzunehmen, werde ich Sie bitten, das diesem Dokument beigefügte Einverständnisformular zu unterschreiben und persönlich zu datieren.

WAS DIE STUDIE VORSCHLÄGT

Ziel der Studie ist es, Daten über Patienten mit chronischer HDV-Infektion und ihr Ansprechen auf die Behandlung mit Bulevirtid zu sammeln.

WAS BEINHALTET IHRE TEILNAHME AN DER STUDIE

Sollten Sie sich für die Teilnahme an der Studie entscheiden, informieren wir Sie darüber, dass wir nach der Prüfung Ihrer Einbeziehung in die Studie und nach Durchführung aller für Sie vorgesehenen medizinischen/instrumentellen Behandlungen – unabhängig davon, ob Sie an dieser Studie teilnehmen oder nicht – im Rahmen der Studie Daten zu Ihrem Gesundheitszustand, Ihrer ethnischen Zugehörigkeit, Ihrem Geschlecht, Ihrem Alter, Ihrem Sexualleben und Risikofaktoren erheben.

Es gibt kein Enddatum für die Datenerfassung, da das Netzwerk seine Datenerfassungs- und Aktualisierungsaktivitäten fortsetzen wird, solange die Betriebsbedingungen für die Zentren und den Wissenschaftlichen Beirat weiterhin bestehen.

Wir weisen Sie außerdem darauf hin, dass für Sie durch die Teilnahme an der Studie keine zusätzlichen Kosten entstehen, die vollständig vom Krankenhaus getragen werden.

UNTERSUCHUNGEN DENEN SIE WÄHREND DER STUDIE UNTERZOGEN WERDEN

Keine weiteren Untersuchungen.

WELCHE VORTEILE ERHALTEN SIE DURCH DIE TEILNAHME AN DER STUDIE

Obwohl dies nicht im Voraus garantiert werden kann, ist davon auszugehen, dass die Studie wissenschaftliche Informationen liefert, die für die klinische Behandlung und Behandlung der

untersuchten Pathologien von Nutzen sind.

WELCHE RISIKEN BESTEHEN BEI DER TEILNAHME AN DER STUDIE

Niemand, da es sich lediglich um eine Datensammlung handelt.

WAS PASSIERT, WENN SIE SICH ENTSCHEIDEN, NICHT AN DER STUDIE TEILZUNEHMEN

Die Einwilligung zur Datenverarbeitung ist eine wesentliche Voraussetzung für die Teilnahme an der Studie. Es steht Ihnen frei, nicht an der Studie teilzunehmen. Sollten Sie sich dennoch für die Teilnahme entscheiden, haben Sie das Recht, jederzeit und ohne Angabe von Gründen von der Studie zurückzutreten. Der Forschungsarzt kann außerdem jederzeit entscheiden, die Teilnahme an der Studie zu unterbrechen, wenn er dies für angemessen hält, ohne dass dies zu gesundheitlichen Schäden führt.

DATENSCHUTZ PERSONENBEZOGENER DATEN

In Übereinstimmung mit den Bestimmungen des Gesetzes zum Schutz personenbezogener Daten (Gesetzesverordnung Nr. 196 vom 30.06.2003, Leitlinien für die Verarbeitung personenbezogener Daten im Rahmen klinischer Prüfungen mit Arzneimitteln – 24. Juli 2008 – Amtsblatt Nr. 190 vom 14. August 2008, Datenschutz-Grundverordnung [DSGVO, insbesondere wie in Art. 9 Abs. 2(i), Abs. 2(j) und Art. 89 Abs. 1, Abs. 2], EU-Verordnung Nr. 2016/679) wird Folgendes festgelegt:

Verantwortliche und damit verbundene Zwecke

Die an der Datenerhebung beteiligten Zentren (etwa 25 Behandlungszentren für chronische Infektionen in Italien) und die Universität Tor Vergata als Veranstalter, Rom, verarbeiten Ihre personenbezogenen Daten, insbesondere Ihre Gesundheitsdaten und – soweit dies für das Ziel der Studie unerlässlich ist – sonstige Daten zu Ihrer Herkunft, Ihrem Lebensstil und Ihrem Sexualleben, Informationen zu Infektionen mit dem humanen Immundefizienzvirus, dem Hepatitis-B-Virus, dem Hepatitis-C-Virus (HIV, HBV und HCV) oder anderen Viren ausschließlich zum Zweck der Durchführung der Studie. Dabei gelten jeweils die eigenen Fachgebiete und die in den Regeln der guten klinischen Praxis (Gesetzesverordnung 211/2003) und den Gesetzen zum Schutz personenbezogener Daten (DSGVO 679/2016 und Gesetzesverordnung 196/2003 in der Fassung der Gesetzesverordnung 101/2018) vorgesehenen Verantwortlichkeiten.
Zu diesem Zweck werden die angegebenen Daten vom Experimentalzentrum erhoben und an den Veranstalter (Universität Tor Vergata Rom – Verantwortlicher) sowie an in seinem Namen handelnde externe Personen oder Unternehmen übermittelt, darunter Società InformaPro (Informapro srl – Verantwortlicher – https://www.informapro.it/) mit Sitz in Rom.
Die Verarbeitung der von Ihnen für die oben beschriebenen Zwecke bereitgestellten personenbezogenen Daten erfolgt auf Grundlage Ihrer ausdrücklichen Einwilligung, die somit die Rechtsgrundlage für die Verarbeitung darstellt.
Die Verarbeitung personenbezogener Daten zu Ihrem Gesundheitszustand, Ihrer Herkunft, Ihrem Alter, Ihrem Lebensstil, Ihrem Sexualleben und Virusinfektionen ist für die Studie unerlässlich; die Verweigerung dieser Angaben führt zur Nichtteilnahme.
Die Verarbeitung der Daten ist für die Durchführung der Studie zwingend erforderlich. Eine Verweigerung der Bereitstellung dieser Daten führt zur Nichtteilnahme.

Art der Daten

Der Arzt, der Sie im Rahmen der Studie betreut, identifiziert Sie mit einem Code: Die im Rahmen der Studie über Sie erhobenen Daten werden, mit Ausnahme Ihres Namens, zusammen mit diesem Code, Ihrem Geburtsdatum, Ihrem Geschlecht, Ihrem Gewicht und Ihrer Größe an den Veranstalter übermittelt, erfasst, verarbeitet und gespeichert. Nur Ihr Arzt und autorisierte Personen können diesen Code mit Ihrem Namen verknüpfen.

Behandlungsmethoden

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Dauer der Behandlung

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Hauptsitz an der Piazza Venezia n. 11 - 00187 Rom

Telefonzentrale: 06.696771

E-Mail: protocollo@gpdp.it

zertifizierte E-Mail: protocollo@pec.gpdp.it

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INFORMATIONEN ZU DEN STUDIENERGEBNISSEN

Auf Ihren Wunsch können Ihnen am Ende der Studie die Ergebnisse der Studie im Allgemeinen und die Sie betreffenden Ergebnisse im Besonderen mitgeteilt werden.

WEITERE INFORMATIONEN

Für weitere Informationen und die Kommunikation während der Studie stehen Ihnen folgende Mitarbeiter zur Verfügung:

Dr./Prof. …… ……

Nachname Name

Telefon ……..……..……

Das Ihnen vorgeschlagene Studienprotokoll wurde von der Ethikkommission genehmigt, die auch die Einhaltung der Standards der Guten Klinischen Praxis der Europäischen Union und die Übereinstimmung mit den in der Deklaration von Helsinki zum Ausdruck gebrachten ethischen Grundsätzen überprüft hat, und wurde von der Ethikkommission dieser Einrichtung genehmigt.

Sie können alle Tatsachen, die Sie im Zusammenhang mit der Sie betreffenden Forschung für besonders wichtig erachten, dem Ethikkomitee und/oder dem Gesundheitsmanagement dieser Krankenhauseinrichtung melden.

Titel	
Version	Version

Ich, der/die Unterzeichnete …… ……

Nachname Name

Alter (Jahre) …… Geburtsdatum……. / ……. / …….

Adresse ……..…… ……

Platz / Straße / Allee Hausnummer

Stadt ……..……..……. telefon ……..

Datum ……. / ……. / …….

……..……..

Unterschrift des Patienten (Erwachsener, Minderjähriger, Reifer) ……

Unterschrift beider Elternteile bei Minderjährigen ……..…….

Unterschrift des gesetzlichen Vertreters (bei geschäftsunfähigen oder entmündigten Patienten bzw. bei einem Betreuungsverwalter)

Unterschrift des Arztes, der die Auskunft erteilt hat

……..……

Sekundärnutzung von Daten.

Wenn der Patient einer Zweitverwendung der Daten – in anonymisierter Form – in zukünftigen wissenschaftlichen Forschungsprojekten zustimmt, die Forscher möglicherweise selbstständig initiieren oder an denen sie teilnehmen, ist für diese spezifische Zustimmung eine separate Unterschrift erforderlich. Diese Einwilligung kann verweigert werden und ihr Fehlen beeinträchtigt in keiner Weise die Teilnahme an dieser Studie.

Datum ……. / ……. / ……. ……..……..

Unterschrift des Patienten (Erwachsener, Minderjähriger, Reifer)……

Unterschrift beider Elternteile bei Minderjährigen…….

Ich, der Unterzeichnete:

VOR- UND NACHNAME DES ARZTES:

Ich erkläre, dass der Patient seine Teilnahme an der Studie spontan unterzeichnet hat

Ich erkläre außerdem Folgendes:

● dem Patienten umfassende Aufklärung über die Ziele der Studie, die Verfahren, die möglichen Risiken und Vorteile sowie die möglichen Alternativen gegeben zu haben;

● nachdem er überprüft hat, dass der Patient die ihm gegebenen Informationen ausreichend verstanden hat

● dem Patienten die nötige Zeit und Gelegenheit gegeben zu haben, Fragen zur Studie zu stellen

● bei der Einholung der Einwilligung keinen Zwang oder unangemessenen Einfluss ausgeübt zu haben

DATUM

UNTERSCHRIFT DES ARZTES

BEACHTEN SIE GUT

Eine Kopie dieses Formulars, unterschrieben und datiert, muss dem Patienten selbst ausgehändigt werden, zusammen mit der „Schriftlichen Patienteninformation“.

10 Appendix B: Time and Events Schedule

Study Requirement	**Enrolment**	**Every 3 months**	**Week 48**	**Week 96**	**Week 144**
Targeted health history, clinical evaluation, interferon use	X				
Stored blood sample	X	X	X	X	X
HBsAg isoforms	X		X	X	X
HDV genotype/sub-genotype	X				
Targeted health history, visit outcomes, hospitalization, use of other drugs, HDV RNA, adverse events	X	X	X	X	X
Informed consent form	X				

11 Appendix C: Protocol Team

The following protocol team will oversee the implementation of this observational study:

- Co-Chairs: Pietro Lampertico and Valentina Svicher
- Statisticians: Alessandro Cozzi-Lepri
- Laboratory Representative: Valentina Svicher
- Community Representatives: Veronika Vsetickova (ELPA)
- VIROMARKERS infrastructure team: Francesca Incardona, Sara Serrao, Chiara Mommo

The protocol team will be supported by others on an implementation team that includes additional representatives from the centers and the Laboratory Group.

12 Appendix D: VIROMARKERS WP2 Laboratory Manual

HDV-RNA quantification

HDV-RNA quantification will be carried out by using the commercial kits by RoboGene HDV RNA Quantification Kit 3.0 – RUO and by the AltoStar® HDV RT-PCR Kit 1.5 RUO following the manufacturer's instructions. Furthermore, HDV-RNA will be quantified also by applying a protocol based on digital PCR. This assay will be optimized using as the basis the protocol reported below for the quantification of HDV-RNA by droplet digital PCR.

HDV-RNA quantification by by ddPCR protocol:

1. Assay characteristics

Forward primer: 5'-*CGTCTGTGCCTTCTCATCTGC-3'*

Reverse primer: 5'-*GCACAGCTTGGAGGCTTGAA-3'*

Probe: 5'-*CTGTAGGCATAAATTGGTCTGCGAA-3'*

Fluorophore: *FAM*

Primers/Probe ratio: *3.6*

2. ddPCR Mastermix preparation

Kit: Bio-Rad One-Step RT-ddPCR advanced kit for probes 200rxn, #1864021

1. The mastermix is prepared according to the table shown below. Per well:

Volume	Component
5 µl	**ddPCR supermix**
1 µl	**Assay**
1 µl	**DTT**
2 µl	**RT**
6 µl	**H_2O DNase/RNase-free**
15 µl	**Total volume per well (before add sample)**

2. 5 µl of sample is then added to each well. Total reaction volume: 20 µl**.**

3. Amplification

A ramp rate of 2°C/ sec is set for each thermal cycling protocol step.

#	Step	Temperature	Time	Cycles
1	**Reverse transcription**	50°C	60min	1
2	**Enzyme activation**	95°C	10min	1
3	**Denaturation**	95°C	30sec	40

	Annealing/Extension	61°C	1min	
4	**Enzyme deactivation**	98°C	10min	1
5	**Hold**	4°C	oo	

Same protocol can be adapted to the QIAcuity OneStep Advanced Probe Kit on the QIAcuity Digital PCR System.

HDV Full Genome Sequencing

RNA Extraction

The HDV whole genome amplification and sequencing protocol was optimized using the commercial QIAmp Viral RNA Mini kit (Qiagen, #52904) for manual extraction of viral RNA from the plasma of infected individuals. Total RNA is extracted from 140µl of plasma and eluted in 50µl according to manufacturer's instructions. For samples with HDV-RNA <10,000 IU/ml, a preliminary ultracentrifuge is recommended (1ml of serum at 47,000 xg for 1h 30min at +4°C). After centrifugation, carefully remove 860µl of sample from the top of the vial and thoroughly resuspend the viral pellet in the residual 140µl of sample and proceed to extraction according to manufacturer's instructions.

Please, note that it's important to keep the extracted RNA on ice (+4°C) if immediately used for PCR. Alternatively, store the extracted RNA at -80°C if PCR is not immediately performed.

HDV Whole Genome Amplification

- Inoculate **8 µL** of viral RNA obtained from the manual extraction into PCR tubes and start the thermocycler for an initial denaturation at 94°C for 3 minutes.
- Quickly transfer the samples to -80°C and leave them for about 5 minutes. Thermal shock facilitates the opening of the HDV genome, which is crucial for primer annealing in the next step.
- In the meanwhile, proceed with the preparation of the first part of the RT-PCR mix, considering for each sample:
    - **3 µL** of nuclease-free H_2O
    - **1 µL** of HDV full reverse primer (2 µM)
      5'-TCCTCGCGGTCCGACCTGGGCAT-3' (856-878)
    - **1 µL** of dNTPs (10 mM)
- Vortex and spin the mix.
- Take the RNA samples out of the -80°C freezer.

- Dispense 5µl of mix into each PCR tube containing the denatured RNA.
- Start the thermocycler with two steps: 65°C for 5 minutes and 4°C for 1 minute.
    - At this point, complete the mix by adding preparation of the second part of the RT-PCR mix, considering for each sample:
        1. **4 µL** of SuperScript IV RT buffer
        2. **1 µL** of DTT (100 mM)
        3. **1 µL** of RNaseOUT
        4. **1 µL** of SuperScript IV enzyme (Invitrogen by Life Technologies)
- Vortex and spin the mix.
- Dispense 7µl of mix into each PCR tube containing the RNA and the first part of RT-Mix. We thus obtain a final volume of 20 µL for each PCR tube.
- Start the thermocycler with two steps: 60°C for 20 minutes and 80°C for 10 minutes. This is the reverse transcription (RT) phase.
- A few minutes before the end of RT, proceed with the PCR mix preparation by using KAPA HiFi ReadyMix (Roche, #07958935001) for the amplification of cDNA considering for each sample:
        1. **25 µL** of KAPA HiFi 2X
        2. **12 µL** of nuclease-free H_2O
        3. **1.5 µL** of HDV full forward primer (10 µM)
           5’-TGGAGATGCCATGCCGACCCGAA-3’ (881-903)
        4. **1.5 µL** of HDV full reverse primer (10 µM)
           5’-TCCTCGCGGTCCGACCTGGGCAT-3’ (856-878)
- Dispense 40 µL of the mix into new PCR tubes.
- Add 10 µL of the cDNA from the first RT phase.
- Start the thermocycler with the following thermal profile:

Phase		**Cycles**	**T °C**	**Time**
DNA denaturation/ Enzyme activation		1	95	5 min
Amplification	Denaturation	40	98	20 sec
	Annealing		70	15 sec
	Elongation		72	1:40 min
Termination	Elongation	1	72	5 min
Cooling down		1	4	30 min
Maintenance		1	10	∞

The successful amplification is verified by electrophoresis on a 2% agarose gel.

NGS with Nextera XT kit (FC-131-1024) by Illumina

1. PCR products purification

The PCR amplicons obtained will be purified using Agencourt® AMPure® XP magnetic beads (Beckman Coulter Inc., A63881), capable of selectively binding DNA fragments.

1. Let the AMPure Beads at room temperature for at least 30 minutes before use.
2. Transfer the PCR amplicons into strips and add the resuspended beads; the beads/amplicon ratio should be 0.8 (e.g., for 25 µL of PCR product, add 20 µL of beads).
3. Pipette up and down 10 times or shake for 2 minutes.
4. Incubate at room temperature for 5 minutes without shaking.
5. Place the strips on the magnet for 2 minutes. The beads, bound with DNA fragments, will separate from the supernatant containing the waste products.
6. Remove the supernatant.
7. Add 200 µL of 80% ethanol (prepared fresh).
8. Incubate for 30 seconds and gently move the beads by sliding the strip on the magnet.
9. Remove the supernatant.
10. Repeat steps 7–9.
11. Let the beads dry for 5-10 minutes while keeping the tubes on the magnet. Avoid over-drying the beads, as this can lead to lower yield.
12. Remove the tubes from the magnet and add 10 µL of H_2O nuclease-free.
13. Pipette up and down 10 times or shake for 2 minutes to resuspend the DNA bound to the beads.
14. Incubate at room temperature for 2 minutes.
15. Place on the magnet for 2 minutes.
16. Recover the purified DNA and transfer it into new pre-labeled strips.
17. The purified products can be stored for up to a week at -20°C.

2. Qubit Quantification (Qubit dsDNA HS Assay Kit, Thermo Fisher, Q32851)

1. Prepare the working solution for the number of samples to be quantified and for two standards. Combine 199 µL of buffer with 1 µL of fluorophore per sample.
2. For the standard curve, dispense 190 µL of working solution and 10 µL of standard 1 in the Std1 tube and 190 µL of working solution and 10 µL of standard 2 in the Std2 tube.
3. For sample quantification, dispense 198 µL of working solution and 2 µL of the sample in Qubit tubes.
4. Incubate for 2 minutes at room temperature.
5. Proceed with Qubit quantification.

According to the quantifications, calculate dilutions to adjust sample concentration to obtain 1 ng of DNA in 5 µl (adjust with nuclease free water).

3. Tagmentation and indexing

The following reagents are provided by Illumina for the tagmentation and indexing process:

- Tagment DNA Buffer (TD): buffer that facilitates the tagmentation reaction.
- Amplicon Tagment Mix (ATM): contains the transposase, which fragments the DNA and adds adapters.
- Neutralize Tagment Buffer (NT): stops the tagmentation reaction.
- Nextera PCR Master Mix (NPM): contains DNA polymerase, buffer, and dNTPs.

Proceed as follows:

1. In each 0.2 mL tube (one per sample), add:
    a. 10 µL of TD
    b. 5 µL of ATM
    c. 1 ng of DNA in 5 µL of H_2O nuclease-free.
2. Run the thermocycler with the following thermal profile: 55°C for 5 minutes, and 10°C hold.
3. Once 10°C is reached, quickly add 5 µL of NT to each sample. This stops the tagmentation reaction, stabilizing the obtained DNA fragments.
4. Incubate at room temperature for 5 minutes.
5. After incubation, add 5 µL of index primer 1 and 5 µL of index primer 2 to each sample. Follow the precise order of primer addition. The different combinations of index 1 and index 2 ensure the uniqueness of the samples during sequencing.
6. Add 15 µL of Nextera PCR Master Mix (NPM) per sample.
7. Run the thermocycler with the following thermal profile:

Cycles	T °C	Time
1	72	3 min
1	95	30 sec
12	95	10 sec
	55	30 sec
	72	30 sec
1	72	5 min
1	10	∞

8. Samples can be stored at +4°C for up to 2 days.

4. Indexed samples purification

1. Let the Ampure Beads sit at room temperature for at least 30 minutes before use.

2. Transfer the entire sample volume (50 µL) into strips and add resuspended beads; the beads/amplicon ratio should be 0.6 (e.g., for 25 µL of PCR product, add 15 µL of beads).
3. Pipette up and down 10 times or shake for 2 minutes.
4. Incubate at room temperature for 5 minutes without shaking.
5. Place the strips on the magnet for 2 minutes. The beads, bound with DNA fragments, will separate from the supernatant containing the waste products.
6. Remove the supernatant.
7. Add 200 µL of 80% ethanol (prepared in advance).
8. Incubate for 30 seconds, gently moving the support.
9. Remove the supernatant.
10. Repeat steps 7–9.
11. Let the beads air-dry for 5-10 minutes while keeping the tubes on the magnet. Avoid overdrying the beads, as this can reduce yield.
12. Remove the tubes from the magnet and add 10 µL of nuclease-free H_2O.
13. Pipette up and down 10 times or shake for 2 minutes to resuspend the DNA bound to the beads.
14. Incubate at room temperature for 2 minutes.
15. Place on the magnet for 2 minutes.
16. Recover the purified DNA and transfer it into new pre-labeled strips.
17. The purified products can be stored for up to a week at -20°C.

5. Qubit Quantification (Qubit dsDNA HS Assay Kit, Thermo Fisher, Q32851)

1. See point 2.

According to the quantification, apply the following formula and calculate the molar concentration of your samples:

$$\left[DNA_{nM}\right] = \frac{[DNA_{\mu l}]}{(400*660)} * 1,000,000$$

Dilute each sample to a final concentration of 20 nM.

Prepare the library for sequencing by transferring 5 µL of each 20 nM sample into a single 1.5 mL tube. After pooling, perform another DNA quantification to ensure the pool has a molarity of 20 nM.

6. Pool Libraries

Before proceeding to the final phase of the protocol, thaw the cartridge in H_2O 1 hour in advance.

Dilute the 20 nM pool by adding 16 µl of nuclease-free H_2O to 4 µl of 20 nM pool, obtaining 20 µl of 4 nM pool.

Denatured as follows:

1. Prepare a fresh 0.2 N NaOH solution in a 1.5 mL tube:
    a. 800 µL of H_2O nuclease-free

b. 200 µL of NaOH (1N)

2. In a new 1.5 mL tube, dispense 5 µL of the 4 nM pool and 5 µL of 0.2 N NaOH, incubating at room temperature for 5 minutes.
3. To adjust the pool concentration to 20 pM, add 990 µL of pre-chilled HT1 Buffer.

Note: HT1 Buffer is specifically formulated to stabilize denatured DNA and promote hybridization to the flow cells. Pre-chilling helps maintain DNA stability and reduces degradation risk.

1. To load the pool at 12 pM, dilute 360 µL of 20 pM denatured DNA with 240 µL of pre-chilled HT1, yielding a total pool volume of 600 µL.

Simultaneously, PhiX is also prepared, a small genome derived from the bacteriophage PhiX174. PhiX is used as a positive control during sequencing, as its sequence is well-characterized and known. Its primary function is to monitor the quality of the sequencing and provide a stable reference point.

The preparation of PhiX is done as follows:

1. Dilute PhiX from 10 nM to 4 nM by combining 2 µL of 10 nM PhiX and 3 µL of H_2O nuclease-free in a 1.5 mL tube.
2. To denature the 4 nM PhiX solution, combine 5 µL of 4 nM PhiX and 5 µL of 0.2 N NaOH in a 1.5 mL tube.
3. Vortex and quickly centrifuge the tube, then incubate at room temperature for 5 minutes.
4. Stop the reaction by adding 990 µL of HT1 buffer, resulting in a 20 pM denatured PhiX solution.
5. Finally, discard 30 µL of the 12 pM pool, and add 30 µL of PhiX, bringing it to 5% concentration.

At this point, the library is ready and can be loaded into the cartridge.

4.7. Loading the Genotypic Library

Proceed with instrument loading after setting up the sample sheet with the list of anonymized samples and the respective index pairs used for each sample in the run.

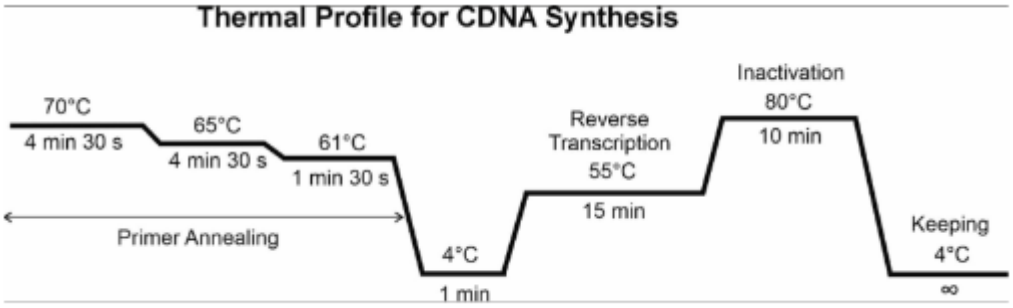
For the final pool loading:

1. Dry the cartridge, which was previously thawed in H_2O for an hour and shake it by inversion 10 times.
2. Clean the flow cell thoroughly with MilliQ water and insert it into the designated slot.
3. Load 600 µL of the pool into the cartridge: 570 µL of the 12 pM denatured pool + 30 µL of 20 pM PhiX. The cartridge is then placed into the machine.
4. Insert the incorporation buffer.
5. Start the sequencing run.

Alternative sequencing protocol using nanopore

cDNA synthesis

For reverse transcription, a master mix of 1 µl dNTP (10mM) and 1 µl reverse primer (5'-TCCTCGCGGTCCGACCTGGCAT, 10 µM) was first prepared for each sample. This mix was then transferred to a multi-ultra tube (0.2 ml). The RNA extract was then heated at 94°C for 3 min to linearize the circular genome. After the three minutes had elapsed, the extract was quickly placed in a cooling block (-18 °C). After snap-freezing, 10 µl of the extract was pipetted to the 2 µl master mix and placed in a Bioer Gene Touch thermocycler with the temperature profile. After the primer annealing step and completed cooling, the master mix consisting of 4 µl 5x buffer, 1 µl DTT and 1 µl of Superscript IV reverse transcriptase was pipetted. This was followed by reverse transcription for 15 minutes and inactivation of the reverse transcriptase for 10 minutes.



Whole genome PCR of HDV (for samples with at least 7000 IU/ml)

Pipetting scheme for the PCR:

- For one sample or positive control:

Q5 HighFielity Mastermix 10 µl

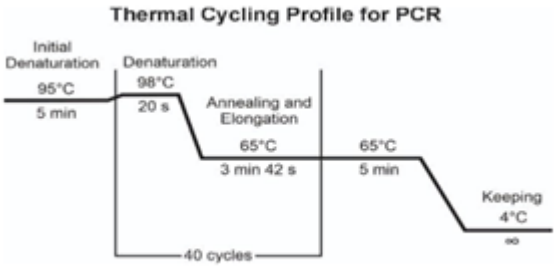
forward Primer 1 µl

reverse Primer 1 µl

cDNA 8 µl

- For higher sample volumes, the volume of the components is multiplied accordingly

- After the master mix has been mixed with the cDNA, the PCR is carried out according to the following thermal profile:



Rapid Barcoding

The Rapid Barcode Plate must first be thawed at room temperature and then centrifuged. Add 2.5 µl of nuclease-free water to a Sentosa™ SX Barcoded PCR Plate according to the number of samples. Next, 5 µl of the PCR products are added using a multichannel pipette. Then transfer 2.5 µl from the Rapid Barcode Plate to this plate and mix thoroughly by pipetting up and down. The plate is then sealed. On the r Thermal Cycler, a program with the following set up is created.:

Temperature (°C) Duration (min)

30 00:02:00

80 00:02:00

After completion, the plate is centrifuged down again. A 1.5 ml reaction tube is labeled with the run name, date and experimenter name and all samples (volume: 10 µl) from the plate are pooled together. Half of this pool (for 48 samples: 480 µl/2=240 µl) is transferred to another reaction tube. One equivalent (same volume as the pool volume) of AMPure XP beads, which were previously well resuspended by vortexing, are added. The reaction vessel is attached to the HulaMixer™ sample mixer (setting: 90 rpm, 10 s rotation time, 90° tiling, 10 s tiling time, 5° vortex, 5 s vortex time) and incubated for a total of 10 min. The reaction tube is then briefly centrifuged and placed on a magnetic rack. After 2 min, when the solution has become clear, the liquid is discarded. The beads are washed with 1 ml 70% ethanol and after 3 min incubation time on the magnetic rack the solution is removed again.

This step is repeated one more time. The beads are then briefly centrifuged and placed back on the magnetic rack. The residual ethanol is removed using a small pipette and the reaction tube is then left open for 30 s to allow the remaining ethanol to evaporate. The beads are then eluted with 15 µl Elution Buffer. Resuspend with the buffer and incubate at room temperature for 15 min. The reaction vessel is again placed on the magnetic rack for 3 min and the supernatant is transferred to another reaction vessel, labeled with the run name, date and experimenter name. The concentration of the now final pooled library is measured on the Qubit Flex using the Qubit 1X dsDNA BR assay kit. The manufacturer recommends an absolute DNA mass of 800 ng for loading the flow cell. The pooled library is diluted accordingly with Elution Buffer to a total of 800 ng in 11 µl. Subsequently, 1 µl Rapid Adapter F (RAP F) is added to the diluted pooled library and incubated for at least 5 min at room temperature.

Preparation and loading of the flow cell:

An R9.4.1 flow cell (FLO-MIN106) will last for 3 months if stored unopened at 4°C. The flow cell is first equilibrated to room temperature for approx. 15 minutes. It is then carefully inserted into the sensor area of the MinION Mk1C. A flow cell check is then carried out. Next, mix 30 µl Flush Tether with 1170 µl Flush Buffer. After completing the flow cell check, the flow cell should have at least 800 pores before the first application, otherwise it can be returned under the manufacturer's warranty and a new one requested.

First open the priming port by turning the lid 45° to the left and set a pipette to 200 µl. Attach this to the priming port and draw up to a maximum of 230 µl. A small amount of Storage Buffer should be visible in the pipette tip. To avoid the introduction of air, pipette 800 µl of the Flush Theter-Flush Buffer mixture into the priming port up to the first stop. This is followed by a 5-minute incubation.

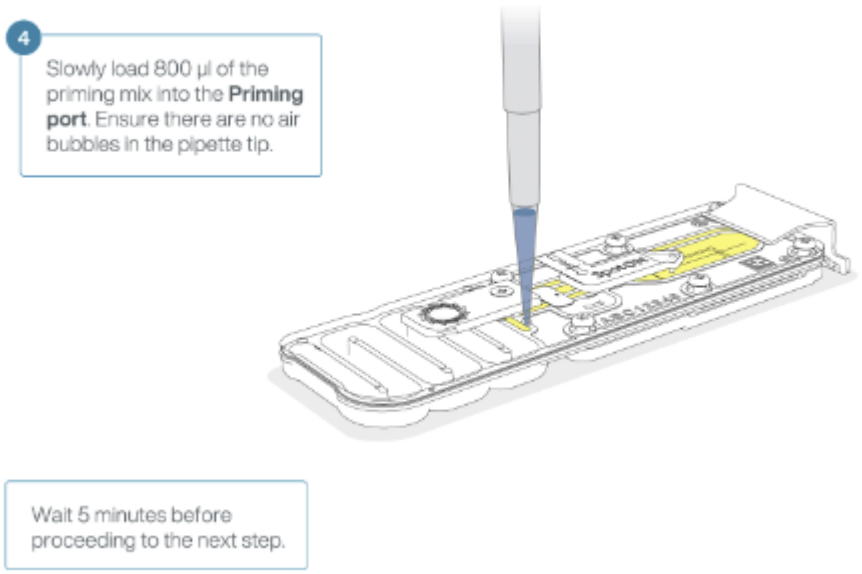


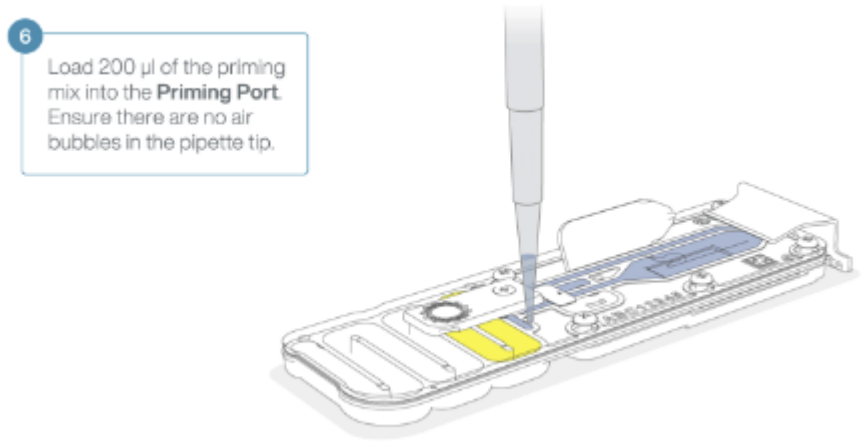
Figure 3 Priming a flow cell-part 1

During this incubation, the master mix is prepared for sequencing. The following pipetting scheme is used:

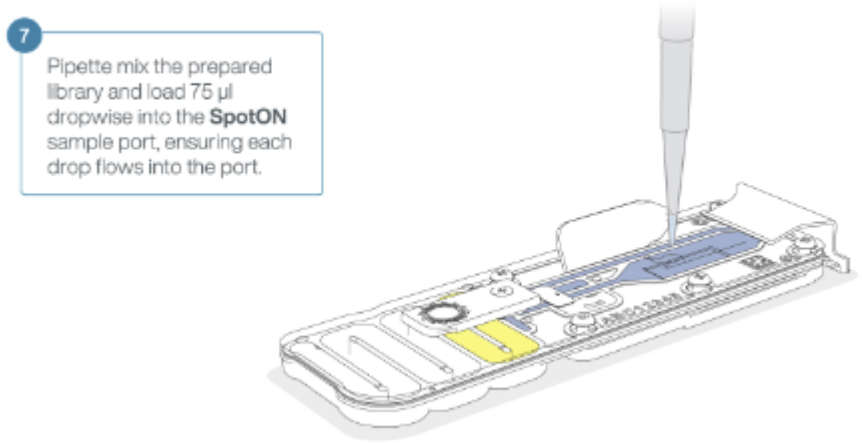
Reagent	volume (µl)
Sequencing Buffer II (SBII)	37.5
Loading Beads II (LBII)	25.5
Pooled library +RAP F	12
	∑ 75

It should be noted that the Loading Beads II (LBII) settle very quickly. Good mixing by vortexing is therefore essential.

Next, open the lid of the spot-on port. Then carefully pipette 200 µl of the Flush Theter-Flush Buffer mixture through the priming port so that any trapped air can escape through the spot on port.



Finally, the master mix is pipetted up and down 10 times before it is dripped onto the Spot On Port with a pipette set to 75 µl. The spot-on port and the priming port are then closed.



Start sequencing on the Minion Mk1C/Mk1D by performing the following steps in sequence:

1. select Start (“Play sign”) and press “start sequencing”

2. define the run name (Nanopore+run number)

3. select the barcode kit (Rapid Barcoding Kit: SQK-RBK110.96) and then the Midnight RT PCR Expansion (EXP-MRT001)

4. select Start Sequencing

5. note: the base pair length to be detected must be 200 bp

Quantification of HBsAg isoforms

The levels of the three different isoforms of HBsAg (L-, M- and S-HBs) will be quantified by using three different *ad hoc* designed ELISA assays, developed in collaboration with Beacle Incorporation (Kyoto, Japan) [24]. These assays have been demonstrated to have high sensitivity (detection limit for each protein is 0.1 ng/ml) and high specificity, with the aid of a sandwich system based on anti-PreS1, anti-PreS2 and anti-S antibodies, respectively.
First, total serum HBsAg will be quantified by using the kit HBs S Antigen Quantitative ELISA Kit, Rapid-II (defined in the formula below as S assay) (Beacle Inc., Kyoto, Japan), by targeting the S region, common to all HBsAg isoforms. Previous results showed high concordance (Rho=0.94, P<0.001) with results from total HBsAg quantified by the commercial assay. Subsequently, the kit HBs Pre-S2 Antigen Quantitative ELISA Kit, Rapid (Beacle Inc., Japan) will be used to quantify the L- and M-HBs, targeting the Pre-S2 region (defined as Pre-S2 assay). Lastly, the L-HBs will be quantified by the kit HBs Pre-S1 Antigen Quantitative ELISA Kit, Rapid-II (Beacle inc., Japan), targeting the Pre-S1 region (defined as Pre-S1 assay). For all assays, the experimental procedure will be carried out according to manufacturer’s instructions (reported attached to this lab manual).

The following formulas were applied in order to obtain the levels of:
S-HBs = (Quantification by S assay) − (Quantification by Pre-S2 assay)
M-HBs = (Quantification by Pre-S2 assay) − (Quantification by Pre-S1 assay)

Total HBsAg and HBsAg isoforms were quantified in two or three samples, collected at the previously mentioned timepoints (baseline, week 48 and week 96 of BLV).

In all our experiments, the coefficient of determination (R^2) of the standard curve was always more than 0.99. As certified by the company, when measuring a specific concentration of antigen using standard antigen, % of coefficient of variation (%CV) of intra-run is less than 10% and %CV of inter-run is less than 15%.

HBs S Antigen Quantitative ELISA Kit, Rapid-II (2-step)

PREPARATION OF REAGENTS AND SAMPLES

1. Warm **Buffer A** (20x) in T_{room} for complete dissolution. Dilute 1:20 with distilled water.
2. Warm **Reaction Buffer** (3x) at T_{room} for complete dissolution. Dilute 1:3 with distilled water.
3. **Standard Antigen** preparation. Pour **1 mL** of **Buffer A** into the standard to obtain 100nUnit/mL (100ng/mL) of solution.
4. Dilute samples with **Buffer A** so that the measuring range is between 0.05 and 10 nUnit/mL.

PREPARATION OF THE STANDARD CURVE

1. Prepare 7 eppendorf tubes and follow the table for the preparation

STD ID	**Buffer A** (2-step)	Antigen solution for dilution	Final concentration
1	900 µl	100 µL (100 nUnits /mL)	10 nUnits /ml
2	500 µl	500 µL (10 nUnits /mL)	5 nUnits /ml
3	600 µl	400 µL (5 nUnits /mL)	2 nUnits /ml
4	500 µl	500 µL (2 nUnits /mL)	1 nUnit /ml
5	500 µl	500 µL (1 nUnit /mL)	0.5 nUnit /ml
6	800 µl	200 µL (0.5 nUnits /mL)	0.1 nUnit /ml
7	500 µl	500 µL (0.1 nUnit /mL)	0.05 nUnit /ml

PROTOCOL

1. Dispense 100 uL of sample (including curve wells) + the **blank** into the wells to be used;
2. Cover with an adhesive film and incubate for 1h (±5min) at Troom;
3. Prepare **Anti S Detection IgG** (HRP- labeled) (1000x) + Reaction Buffer. Prepare 1 mL per strip;
4. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;
5. Wash 3 times (300 uL) with **Buffer A** solution (1x). Make sure that the residual volume does not exceed 10uL , otherwise invert the plate on absorbent paper;
6. Dispense 100 uL of **Anti S Detection IgG** (HRP- labeled) previously prepared in each well;
7. Cover with an adhesive film and incubate for 1h at Troom;
8. About 10 min before the end of the previous reaction, prepare the **chromogenic solution** (1:2 dilution) by mixing **Substrate Solution A** and **Substrate solution B**. Prepare 1 mL per strip.
9. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;

10. Wash 5 times (300 uL) with **Buffer A** solution (1x);
11. Add 100 uL of previously prepared **chromogenic solution** to each well and incubate for 30 min in the dark;
12. Finally, add 50 uL of **Stop solution**;
13. Measure the absorbance at 450 nm and calculate the specific absorbance by subtracting the blank value;
14. Calculate the curve using the *4-parameter logistic model*.

HBs Pre-S2 Antigen Quantitative ELISA Kit, Rapid-II (2-step)

PREPARATION OF REAGENTS AND SAMPLES

1. Warm **Buffer A** (20x) in T_{room} for complete dissolution. Dilute 1:20 with distilled water.
2. Warm **Reaction Buffer** (3x) at T_{room} for complete dissolution. Dilute 1:3 with distilled water.
3. **Standard Antigen** preparation. Pour **1 mL** of **Buffer A** into the standard to obtain 100nUnit/mL (100ng/mL) of solution.
4. **Detection Antibody Solution**. Use 1.5 uL of antibody in 500 uL of **Red coloured solution** (for 1 strip)

PREPARATION OF THE STANDARD CURVE

1. Prepare 9 eppendorf tubes and follow the table for the preparation

STD ID	**Buffer A** (2-step)	Antigen solution for dilution	Final concentration
	900 µl	100 µl (10 µUnit/mL)	1000 nUnits /ml
	900 µl	100 µl (1000 nUnits / mL)	100 nUnits /ml

1	700 µl	300 µl (100 nUnits / mL)	30 nUnits /ml
2	600 µl	300 µl (30 nUnits / mL)	10 nUnits /ml
3	500 µl	500 µl (10 nUnits / mL)	5 nUnits /ml
4	800 µl	200 µl (5 nUnits / mL)	1 nUnit /ml
5	500 µl	500 µl (1 nUnit / mL)	0.5 nUnit /ml
6	600 µl	400 µl (0.5 nUnits / mL)	0.2 nUnit /ml
7	500 µl	500 µl (0.2 nUnits / mL)	0.1 nUnit /ml

PROTOCOL

1. Dispense 100 uL of sample (including curve wells) + the **blank** into the wells to be used;
2. Cover with an adhesive film and incubate for 1h (±5min) at Troom;
3. Prepare **Anti Pre-S2 Detection IgG** (HRP- labeled) (1000x) + Reaction Buffer. Add 1 mL of Reaction Buffer for each (1.5 ul of antibody + 500 uL red coloured solution) per strip;
4. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;
5. Wash 3 times (300 uL) with **Buffer A** solution (1x). Make sure that the residual volume does not exceed 10uL , otherwise invert the plate on absorbent paper;
6. Dispense 150 uL of previously prepared **Anti Pre-S2 Detection IgG** (HRP- labeled) into each well;
7. Cover with an adhesive film and incubate for 1h at Troom;

8. About 10 min before the end of the previous reaction, prepare the **chromogenic solution** (1:2 dilution) by mixing **Substrate Solution A** and **Substrate solution B**. Prepare 1 mL per strip.
9. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;
10. Wash 5 times (300 uL) with **Buffer A** solution (1x);
11. Add 100 uL of previously prepared **chromogenic solution** to each well and incubate for 30 min in the dark;
12. Finally, add 50 uL of **Stop solution**;
13. Measure the absorbance at 450 nm and calculate the specific absorbance by subtracting the blank value;
14. Calculate the curve using the *4-parameter logistic model*.

HBs Pre-S1 Antigen Quantitative ELISA Kit (Genotype D)

PREPARATION OF REAGENTS

1. Warm **Buffer A** (20x) in T_{room} for complete dissolution. Dilute 1:20 with distilled water.
2. Warm **Reaction Buffer** (3x) at T_{room} for complete dissolution. Dilute 1:3 with distilled water.
3. **Standard Antigen** preparation. Pour **1 mL** of **Buffer A** into the standard to obtain 100nUnit/mL (100ng/mL) of solution.
4. **Detection Antibody Solution**. Use 1 uL of antibody in 1 mL of **Dilution solution** (for 1 strip)

PREPARATION OF THE STANDARD SOLUTION

1. Prepare 7 eppendorf tubes and follow the table for the preparation

STD ID	Buffer A	Antigen solution for dilution	Final concentration
1	800 µl	200 µl (100 nUnits / mL)	20 nUnits /ml
2	600 µl	200 µl (20 nUnits / mL)	5 nUnits /ml
3	800 µl	200 µl (5 nUnits / mL)	1 nUnit /ml
4	600 µl	400 µl (1 nUnit / mL)	0.4 nUnit /ml
5	600 µl	200 µl (0.4 nUnits / mL)	0.1 nUnit /ml
6	600 µl	400 µl (0.1 nUnit / mL)	0.04 nUnits /ml
7	600 µl	200 µl (0.04 nUnits / mL)	0.01 nUnit /ml

PROTOCOL

1. Dispense 100 uL of sample (including curve wells) + the **blank** into the wells to be used;
2. Cover with an adhesive film and incubate for 1h (±5min) at Troom;
3. Prepare **Detection IgG solution** (HRP- labeled). 1 mL per strip.
4. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;
5. Wash 3 times (300 uL) with **Buffer A** solution (1x). Make sure that the residual volume does not exceed 10uL, otherwise invert the plate on absorbent paper;
6. Dispense 100 uL of **Anti S Detection IgG** (HRP- labeled) previously prepared in each well;
7. Cover with an adhesive film and incubate for 1h at Troom;
8. About 10 min before the end of the previous reaction, prepare the **chromogenic solution** (1:2 dilution) by mixing **Substrate Solution A** and **Substrate solution B**. Prepare 1 mL per strip.
9. Remove the adhesive film and remove the solution by a multichannel pipette and invert the plate on a sheet of paper to remove any residual volume;
10. Wash 5 times (300 uL) with **Buffer A** solution (1x);

11. Add 100 uL of previously prepared **chromogenic solution** to each well and incubate for 30 min in the dark;
12. Finally, add 50 uL of **Stop solution**;
13. Measure the absorbance at 450 nm and calculate the specific absorbance by subtracting the blank value;
14. Calculate the curve using the *4-parameter logistic model*.

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