CERTIFICATION REPORT

The certification of the copy number concentration of solutions of plasmid DNA containing a BCR-ABL b3a2 transcript fragment

Certified Reference Materials: ERM®-AD623a, ERM®-AD623b, ERM®-AD623c, ERM®-AD623d, ERM®-AD623e, ERM®-AD623f

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Summary

This report describes the production of a set of plasmid solutions, ERM®-AD623a, b, c, d, e, and f, certified for the copy number ratio of specific desoxyribonucleic acid (DNA) fragments per plasmid and for the copy number concentration of the plasmid. The material has been produced following ISO Guide 34:2009 [1].

DNA fragments specific for the transcript of breakpoint cluster region gene (*BCR*), the transcript of the glucuronidase beta gene (*GUSB*) and the fusion transcript from the *BCR* gene and the c-abl oncogene 1 (*BCR-ABL* b3a2) were cloned into a pUC18 vector to construct the pIRMM-0099 plasmid. The sequence identity of the pIRMM-0099 plasmid was determined by dye terminator cycle sequencing of the entire plasmid. The plasmid was diluted to six different concentration levels.

Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2]. The material was characterised by an intercomparison among laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005 [3]. Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible heterogeneity and instability and to characterisation.

The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Material consortium.

The certified values and their uncertainties are listed below:

	Number of specific DNA fragments per plasmid	
	Certified value [b]	Uncertainty [c]
BCR-ABL b3a2 transcript [a]	1	negligible
BCR transcript [a]	1	negligible
GUSB transcript [a]	1	negligible

a) The sequence identity has been confirmed by dideoxy termination sequencing of the entire plasmid. The estimated error probability of the sequence identification of each fragment is lower than 0.0002 %

b) The certified value is traceable to the International System of units (SI).

c) The uncertainty of the certified value refers to a standard uncertainty. It is estimated by a type B evaluation based on information provided in Section 6 of the corresponding certification report.

	Copy number concentration of the plasmid [d]	
	Certified value [e] [cp/µL]	Uncertainty [f] [cp/µL]
ERM®-AD623a	1.08×10^{6}	0.13×10^{6}
ERM®-AD623b	1.08×10^{5}	0.11×10^{5}
ERM®-AD623c	1.03×10^{4}	0.10×10^{4}
ERM®-AD623d	1.02×10^{3}	0.09×10^{3}
ERM®-AD623e	1.04×10^{2}	0.10×10^{2}
ERM®-AD623f	10.0	1.5

d) The copy number concentration of the plasmid has been determined by digital PCR using conditions as described in Annex A of the certification report.

e) Unweighted mean of independent measurements obtained in three different laboratories. The certified value and its uncertainty are traceable to the International System of units (SI).

f) The certified uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2009.

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Table of contents

Glossary

6-FAM	6-carboxylfluorescein dye
ABL	C-ABL oncogene 1
ANOVA	Analysis of variance
bp	Base pair
BCR	Breakpoint cluster region gene
cDNA	Complementary DNA
CML	Chronic myeloid leukaemia
cp	Copy number
CRM	Certified reference material
d	Correction factor
DNA	Deoxyribonucleic acid
dPCR	Digital Polymerase Chain Reaction
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ERM®	Trademark of European Reference Materials
GUM	Guide to the Expression of Uncertainty in Measurements [ISO/IEC Guide 98-3:2008]
GUSB	Glucuronidase beta gene
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LB	Luria-Bertani
MGB	Dihydrocyclopyrroloindole tripeptide minor groove binder
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
MRD	Minimal residual disease
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not accepted
n.c.	Not calculated
PCR	Polymerase chain reaction
qPCR	Quantitative real time polymerase chain reaction
rel	Index denoting relative figures (uncertainties etc.)
RNA	Ribonucleic acid
RSD	Relative standard deviation
r^2	Coefficient of determination of the linear regression
s	Standard deviation

s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
SNP	Single nucleotide polymorphism
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
$T_1E_{0.01}$	Buffer contained 1 mmol/L TRIS and, 0.01 mmol/L EDTA at pH 8.0
T	Temperature
t	Time
t_i	Time point for each replicate
TAMRA	Tetramethyl-6-Carboxyrhodamine
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation during real-time PCR
t_{sl}	Proposed shelf life
tRNA	Transfer RNA
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit heterogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit heterogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_d	Uncertainty of the correction factor d; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability
X-gal	5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside

1 Introduction

1.1 Background: need for the CRM

Philadelphia translocation is a specific chromosomal abnormality caused by the exchange of two fragments from the human chromosomes 9 and 22, also described as t(9;22)(q34;q11). This translocation joins two otherwise separate genes: c-abl oncogene 1 (*ABL*) and breakpoint cluster region (*BCR*) gene together to the fusion gene *BCR-ABL*. Breakpoints can occur at different positions within the *BCR* and *ABL* gene. The two major transcripts are called b2a2 (joining exon 13 of *BCR* with exon 2 of *ABL*) and b3a2 (joining exon 14 of *BCR* with exon 2 of *ABL*).

The *BCR-ABL* gene plays a causal role in the pathogenesis of chronic myeloid leukaemia (CML) [7]. CML is characterised by massive increase of premature myeloid cells in the blood and has an incidence of 1 to 2 per 100000 persons [8]. Treatment of CML is focused on the inhibition of the BCR-ABL protein and usually leads to return of normal blood cell counts [9]. Few leukemic cells however persist in most patients, called minimal residual disease (MRD). Therefore, lifelong treatment is required and the risk of therapy resistance and disease relapse is present [10].

Regularly monitoring of MRD can identify patients in need of therapeutic intervention before the onset of overt relapse. Levels of the BCR-ABL transcript have shown to be a good indicator for the amount for leukemic cells present in the blood of the patients [11]. The measurement procedure consists of several steps. White blood cells are isolated from a blood sample and messenger RNA (mRNA) is extracted. After transfer of the mRNA to complementary DNA (cDNA) by reverse transcription the amount of *BCR-ABL* b3a2 transcript is measured by quantitative real time PCR (qPCR). Together with the *BCR-ABL* transcript levels, the transcript of a control gene is also quantified to control for variations in reverse transcription (RT) PCR and the sample preparation. The results are expressed as a ratio of the number of copies of *BCR-ABL* b3a2 transcript to the number of copies of the control gene transcript.

Results obtained by qPCR are influenced by a larger number of factors including the composition of the mastermix and the instrument used [12]. The copy number ratio of *BCR-ABL* b3a2 transcript to the control gene transcript in the patients' samples is determined using a calibration curve from standards with known copy number concentrations. In addition, the calibration curve is also used to estimate the absolute copy number concentration of the control gene in the patient samples. This value is important to assure the quality of the sample preparation steps before the qPCR including the reverse transcriptase step. Especially for patient samples in which the *BCR-ABL* transcript is not detected the copy number concentration measured for the control gene transcript gives an indication about the sensitivity of the measurement.

1.2 Choice of the material

There are several requirements for a material to be used as a calibrant for the qPCR reactions, in addition to requirements for homogeneity, stability and metrological traceability.

The calibrant is used to determine the ratio of the number of copies of *BCR-ABL* b3a2 transcript and the transcript of the control gene. The calibrant should have a certified value for this copy number ratio and the associated uncertainty should be as small as possible. Therefore it was decided to produce a plasmid that contains the sequences of both the *BCR-ABL* b3a2 transcript and the control gene transcript.

A number of different qPCR methods have been developed and validated. These methods use different control genes and different primer and probe sets. The three following genes have been studied extensively and appear to be the most suitable control genes: the native form of *ABL*, the native form *BCR* and glucuronidase beta gene (*GUSB*) [13]. The sequence of the *BCR-ABL* b3a2 transcript and the native form of *ABL* largely overlap and most primers and probes used to amplify the *ABL* transcript will also amplify the *BCR-ABL* b3a2 transcript. It is therefore not necessary to

add a specific DNA fragment from the native *ABL* transcript. The plasmid should contain DNA fragments specific for the transcripts from *BCR-ABL*, *GUSB* and *BCR*. These fragments should be large enough to allow the use of different primers and probes.

The plasmid calibrant will also be used to estimate the number of copies of the control gene transcript present in the cDNA sample of the patient. The calibrant should therefore have a certified value for the copy number concentration of the plasmid.

Ideally, the calibration curve for a qPCR reaction should contain at least six points and cover a 5-log range [12]. The calibration curve should also comprise the concentration levels of both the *BCR-ABL* b3a2 transcript and the control gene transcript that are usually present in the patient samples. Therefore, it was decided to produce a set of plasmid dilutions with nominal copy number concentrations ranging from 10^6 cp/µL to 10 cp/µL.

1.3 Design of the project

DNA fragments specific for the transcript of *BCR-ABL* b3a2, *BCR* and *GUSB* were obtained by using cDNA from the K562 cell line [14] as a template. These DNA fragments were cloned into a pUC18 vector to construct the pIRMM-0099 plasmid. The different plasmid solutions of ERM-AD623 were processed by diluting the pIRMM-0099 plasmid into a buffer containing 1 mmol/L Tris(hydroxymethyl)aminomethane (TRIS), 0.01 mmol/L Ethylenediamine tetraacetic acid (EDTA) pH 8.0 (called $T_1E_{0.01}$ buffer) and 50 ng/µL transfer RNA (tRNA) from *Escherichia coli* (*E. coli*) as background nucleic acids.

Homogeneity and stability studies were performed with duplex qPCR amplifying the two PCR targets on the DNA fragment specific for the *BCR-ABL* b3a2 transcript. One PCR target is located at the *BCR-ABL* junction site (called *BCR-ABL* target) while the other is located in the DNA sequence originating from the ABL gene (called *ABL* target). The primers and probes and PCR conditions used, have been validated in an intercomparison study [5, 6]. The probe of the *BCR-ABL* b3a2 target was 5' labelled with 6-VIC and the quencher MGB was located at the 3' side. For the probe of the *ABL* target the 5' label was 6-FAM and TAMRA was used as a quencher at the 3' side. The primers and probes and PCR conditions are described in Annex A.

Within the homogeneity and stability studies each vial was measured three times (n = 3) and one measurement result is the mean result from three wells (triplicate). Measurements were performed on 96 well plates and the volume of the qPCR reaction was 25 µL per well. Five µL of DNA solution were added to 20 µL master mix containing the appropriate primers and probe for the duplex qPCR. TaqMan® Universal PCR MasterMix (Applied Biosystems, Lennik, BE) was used and the qPCR reactions were performed on an ABI 7900. Data were analysed with the SDS 2.4 using the automatic base line and threshold settings.

On each qPCR plate a calibration curve was added containing 7 concentration points. This calibration curve was made from a plasmid solution of the pIRMM-0099 which was extracted independently and its copy number concentration was determined by digital PCR (dPCR).

The number of specific DNA fragments per plasmid (the *BCR-ABL* b3a2 transcript fragment, the *BCR* transcript fragment and the *GUSB* transcript fragment) is defined by the DNA sequence of the plasmid. The DNA sequence of the pIRMM-0099 plasmid was determined by dideoxy terminator sequencing by two expert laboratories.

The copy number concentration of the plasmid was determined by dPCR measurements performed in three laboratories. Digital PCR relies on single molecule detection since the PCR solution is distributed across a large number of individual partitions [15]. After amplification, the concentration of target DNA in the solution is estimated using a binominal approximation based on the number of partitions containing amplified product and the total number of partitions analysed.

Digital PCR reactions were performed on the BioMark system (Fluidigm, South San Francisco, US) using the 12.765 Digital arrays (Fluidigm). This nanofluidic digital array comprises 765 individual partitions of approximately 6 nL volume each with total volume per panel of

approximately 4.6 µL. The *BCR-ABL* b3a2 target and the *ABL* target were amplified using the same primers and probes and PCR conditions as for the qPCR (Annex A).

Samples from ERM-AD623a, b, c and were gravimetrically diluted in serial dilution to reach a nominal copy number concentration of 500 cp/µL. Samples from ERM-AD623e and f were analysed undiluted. Afterwards the DNA sample was added to the reaction mix containing Taqman universal mix (Applied Biosystems), sample loading reagent (Fluidigm) and primers and probes and the DNA. This reaction mix was analysed on five panels of one digital array and the mean of these five panels was considered as one measurement result. The obtained raw data were analysed with the BioMark Digital PCR Analysis software.

A limited commutability study was performed by nine expert laboratories using six different qPCR methods to determine the ratio of *BCR-ABL* b3a2 transcript to the control gene transcript. The participating laboratories received sets of the six plasmid solutions of ERM-AD623 and two dilutions of a cDNA sample mimicking real patient samples. From each set of plasmid solutions several calibration curves were measured. The two cDNA solutions were also measured and their copy number ratio of *BCR-ABL* b3a2/control gene was determined using the different calibration curves.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

Salisbury District Hospital, National Genetics Reference Laboratory (Wessex), Salisbury, UK

2.3Homogeneity study and stability study

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO/IEC 17025 for testing and calibration laboratories, BELAC No. 268-TEST)

2.4 Characterisation

2.4.1 Sequence identity of the plasmid

Baseclear BV, Leiden, NL

Eurofins Madigenomix GmBH, Martinsried, DE

2.4.2 Copy number concentration

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO/IEC 17025 for testing and calibration laboratories, BELAC No. 268-TEST)

LGC Limited, Molecular and Cell Biology Team, Teddington, UK

National Measurement Institute (NMI), Department of Innovation, Industry, Science and Research, Bioanalysis Group, West Lindfield, AU

2.5 Commutability study

Academy of Athens, Biomedical Research Foundation, Haematology Research Laboratory, Athens, GR

Catholic University of Korea, Seoul, KR

Erasme Hospital , Medical Genetics Department, Brussels, BE
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IMVS Pathology, Division of molecular pathology, Adelaine, AU
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University of Frankfurt, Department of Haematology, MRD labor, Frankfurt, DE

University medical centre Mannheim, Mannheim, DE

3 Material processing and process control

3.1 Origin of the starting material

3.1.1 Cloning of BCR and GUSB single target plasmids

A 963 bp fragment of the *BCR* transcript was amplified by AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Lennik, BE) using cDNA from the K562 cell line (Hammersmith Hospital, London, UK) as template. The amplicon was ligated in the plasmid vector pCR®2.1 (Invitrogen, Carlsbad, CA, US) and *E. coli* Top10 were transformed with the ligation products. Transformed cells carrying the plasmid with insert were selected on Luria-Bertani (LB) plates supplemented with ampicillin and 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal) to allow colour based selection of the colonies bearing a plasmid with insert. The sequence of the insert was checked by dideoxy terminator sequencing of plasmid DNA isolated from single colonies. One single clone bearing this plasmid (called intermediated plasmid pCR2.1_BCR) was selected.

An 813 bp fragment of the *GUSB* transcript was amplified by AmpliTaq Gold® DNA polymerase using cDNA from the K562 cell line as template. The amplicon was ligated in the plasmid vector pCR2.1 (Invitrogen) and *E. coli* Top10 cells were transformed with the ligation products. Transformed cells carrying the plasmid with insert were selected on LB plates supplemented with ampicillin and X-gal. The sequence of the insert was checked by dideoxy terminator sequencing of plasmid DNA isolated from single colonies. One single clone bearing this plasmid (called intermediated plasmid pCR2.1_GUSB) was selected.

3.1.2 Construction of the multiple target plasmid

The multiple target plasmid was assembled in three consecutive DNA fragment insertions into the plasmid vector pUC18.

Intermediate plasmid pCR2.1_BCR was digested with *EcoR*I and the fragment containing the *BCR* insert was cloned into the pUC18 vector digested with *EcoR*I. The relevant digestion products were ligated and *E. coli* XL1 blue competent cells were transformed with the ligation products. Transformed cells were selected on ampicillin-containing LB plates. Correct insertions were identified by restriction enzyme digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named intermediate plasmid pUC18_BCR.

The fragment of the *GUSB* transcript was inserted by restriction digestion of the intermediate plasmid pCR2.1_GUSB and pUC18_BCR using *Xba*I and *Kpn*I. After ligation *E. coli* XL1 blue competent cells were transformed and plated on LB plates containing ampicillin. Correct insertions were identified by restriction enzyme digestion of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named intermediate pUC18_BCR_GUSB.

A 1397 bp fragment of the *BCR-ABL* b3a2 transcript was amplified by AmpliTaq Gold® DNA polymerase using cDNA extracted from the K562 cell line as template. The PCR primers used had additional *Sal*I restriction sites at the 5' end. The PCR amplicon was then digested with *Sal*I and ligated into intermediate plasmid pUC18_BCR_GUSB that had been digested with *Sal*I. After ligation *E. coli* XL1 blue competent cells were transformed and plated on LB plates containing ampicillin. Correct insertions were identified by restriction enzyme digestion and dideoxy terminator sequencing of plasmid DNA extracts from single bacterial colonies. The corresponding plasmid was named pIRMM-0099. One single clone bearing this final plasmid was plated again on a LB plate supplemented with ampicillin and from this plate one isolated colony was selected.

After a second confirmation of the presence of the correct DNA sequence of the plasmid by restriction digest and sequencing of the inserts this clone was selected as the starting point for the processing of ERM-AD623a, b, c, d, e and f. Figure 1 shows the circular map of the plasmid.

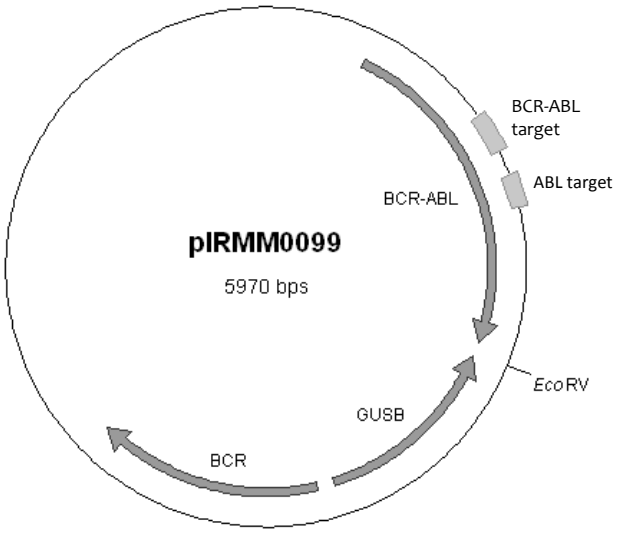

Figure 1: Circular map of the plasmid. The arrows represent the inserts from the transcripts fragments of *BCR*, *GUSB* and *BCR-ABL* b3a2. The rectangles show the location of the PCR targets *BCR-ABL* b3a2 and *ABL* used in this report to quantify the copy number concentration of the plasmid. The restriction site of *EcoR*V is also shown.

3.2 Processing

3.2.1 Preparation of stock solution of plasmid pIRMM-0099

Two hundred mL of LB medium containing 100 µg/mL of ampicillin were inoculated with a single colony of *E. coli* containing plasmid pIRMM-0099 and shaken at 200 rpm overnight at 37 °C. The plasmid was extracted and purified using the QIAfilter Plasmid Maxi Kit (Qiagen Benelux B.V., Venlo, NL). The purified plasmid was dissolved in $T_1E_{0.01}$ buffer. The identity of the plasmid was

checked by DNA restriction analysis and agarose gel electrophoresis as described in Section 3.3.1.

From this solution of the circular plasmid pIRMM-0099 two aliquots were set aside to be sent to two independent sequencing service companies to determine the complete nucleic acid sequence of the plasmid.

The plasmid in the remaining solution was restricted with *EcoR*V-HF™ (New England Biolabs, Ipswich, MA, US). Complete restriction was checked by agarose gel electrophoresis. The linearised plasmid was purified using the Qiaquick® gel extraction kit (Qiagen) and eluted from the Qiaquick silica membrane with $T_1E_{0.01}$ buffer.

The DNA mass concentration of the linearised plasmid solution was determined with fluorometry using the Picogreen dsDNA quantification kit (Molecular Probes Inc, Eugene, OR, US). Measurements were performed according to the manufacturer's instructions using a lambda DNA standard solution. The measured DNA mass concentration and standard deviation were 210 ± 3 µg/mL (*n* = 6). The purity of the nucleic acid preparation was assessed spectrophotometrically by measuring the UV absorbance of the sample at 230 nm, 260 nm and 280 nm. Additional information can be found in Section 3.3.

Based on DNA mass concentration, the copy number concentration of the plasmid solution was estimated, applying the following equation.

$$copy\ number\ concentration = \frac{C_{DNA} \cdot N_A}{M_{plasmid}} \qquad \text{Equation 1}$$

C_{DNA} DNA mass concentration

N_A Avogadro constant

$M_{plasmid}$ molar mass of the pIRMM0099 plasmid

Calculation of the molar mass of the pIRMM0099 plasmid was based on the DNA sequence of the plasmid and the molar mass of each nucleotide monohosphate [16].

The estimated copy number concentration of the linearised plasmid solution with a DNA mass concentration of 210 ng/µL was 3.43 x 10^{10} cp/µL.

The solution containing linear plasmid was diluted to 35 ng/µL (estimated copy number concentration: 5.71 x 10^9 cp/µL) in $T_1E_{0.01}$ buffer to prepare the stock solution for processing the individual CRMs. This stock solution was separated in 8 aliquots which were stored at -20 °C until further use.

3.2.2 Preparation of individual CRMs

Each CRM was processed separately starting from one individual aliquot of the stock solution. Dilutions were made in $T_1E_{0.01}$ buffer contained 50 ng/µL tRNA from *E. coli* (Sigma-Aldrich, Bornem, BE).

Afterwards, the plasmid solution was sterilised by filtration through a polyethersulfone (PES) filter (Merck Millipore, Overijse, BE) and filled manually in pre-labelled high recovery polypropylene vials under sterile conditions. Each vial was filled with 600 µL of plasmid containing solution. Measures were taken to avoid agglomeration and to keep the plasmids evenly distributed in the solution during processing. The plasmid containing solution and the vials were kept cool with cooling elements.

After filling, the vials for the homogeneity study, the stability studies, and the characterisation study were selected according to a random stratified sampling scheme from the entire batch. The vials were frozen either at (-20 ± 5) °C or at (-70 ± 10) °C.

3.3 Process control

3.3.1 Identity confirmation

The identity of the plasmid pIRMM-0099 was confirmed by restriction with the *EcoR*V enzyme. The circular plasmid was first analysed by DNA restriction analysis and agarose gel electrophoresis. The plasmid was restricted with *Hinc*II (Figure 2, lane 1), *Pvu*II (Figure 2, lane 2) and *Xho*I (Figure 2, lane 3) and the resulting DNA bands were made visible on an agarose gel by UV after ethidium bromide staining. No other DNA bands than those expected could be observed.

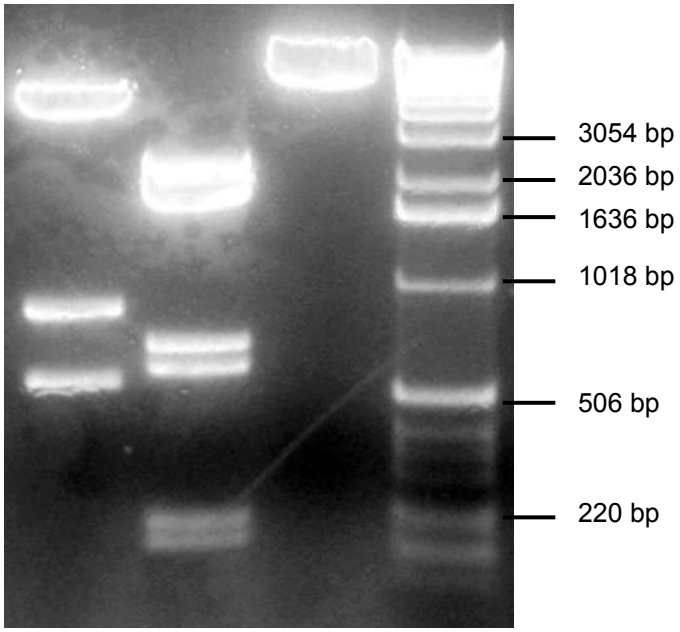

Figure 2: Restriction analysis of pIRMM-0099. Lane 1: pIRMM-0099 restricted with *Hinc*II (expected fragments: 4573 bp, 860 bp and 544 bp); Lane 2: pIRMM-0099 restricted with *Pvu*II (expected fragments: 2372 bp, 1965 bp, 709 bp, 598 bp, 195 bp and 132 bp; Lane 3: pIRMM-0099 restricted with *Xho*I (plasmid linearisation expected); Lane 4: DNA ladder (Invitrogen, Life Technologies Europe, Gent, BE).

3.3.2 Purity of the plasmid extract

In theory three different types of contaminants could be present in the plasmid extract: a) intermediate plasmids used for the assembly of pIRMM-0099, b) nucleic acids originating from host bacterial cells and c) non-nucleic acid contaminants.

During the cloning process the bacterial cells could have been transfected with different populations of plasmids: pIRMM-0099 and one or more of the intermediate plasmids used the assembly of pIRMM-0099. The synthetic plasmids used in our cloning strategy (pUC18 and pCR 2.1) have the same origin of replication (*ori*V from ColE1 plasmid) which allows them to replicate independently of the host chromosome. A bacterial cell however cannot replicate different plasmids with the same mechanism of replication. As a consequence only one plasmid will remain present in a bacterial clone while others are lost during cell division. As the plasmid production was started from a single colony, only one type of plasmid can be present in the plasmid extract [17]. To ensure that the plasmid production started from a single colony, one additional plating step was included. A single colony containing the pIRMM0099 was plated on a LB plate and from this plate one isolated colony was picked.

After restriction with *EcoR*V and purification with the Qiaquick® gel extraction kit; the plasmid solution was analysed by gel electrophoresis. As there was no smear and/or RNA bands observed, it can be reasonably concluded that the plasmid preparation was not contaminated with large amounts of genomic DNA or RNA molecules from host bacterial cells. However, traces of genomic DNA or RNA molecules cannot be detected by gel electrophoresis.

The linearised plasmid was investigated by spectrophotometry to detect the presence of non-nucleic acid contaminants like proteins, or chemical residues from nucleic acid extraction like phenol and guanidine. The UV absorbance of the plasmid in solution was measured at 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). Taking into account the generally accepted mean extinction coefficient for double-stranded DNA at 260 nm and 280 nm, pure nucleic acid samples are expected to have an A260/A280 ratio of approximately 1.8 and an A_{260}/A_{230} ratio of approximately 2.0 or higher [18]. The A_{260}/A_{280} and A_{260}/A_{230} ratios measured were 1.86 and 1.97 respectively, indicating a sufficient DNA purity; however, such values do not exclude traces of contaminating proteins or chemical residues from nucleic acid extraction.

Contamination of the stock solution of plasmid pIRMM-0099 with traces of nucleic acids for the host bacterial cells proteins may affect the DNA mass concentration measured by spectrophotometry and fluorometry and lead to an overestimation the copy number concentration of a plasmid in solution. However, such traces do not affect the dPCR measurements as the used primers and probes are highly specific for the targeted sequences and do not hybridise to other DNA fragments that could be present in the final plasmid preparation. A BLASTN 2.2.24+ analysis of the PCR primers did not reveal more than 70 % nucleic acid sequence identity with the genomic DNA of *E. coli* from the NCBI database (data not shown). Traces of or chemical residuals from nucleic acid extraction like phenol and guanidine might, however, inhibit the PCR reaction and lead to an underestimation of the copy number concentration of a plasmid solution. Inconsistencies between the estimations of the copy number concentration based on spectrophotometry and fluorometry and dPCR can therefore be used as an indication for the presence of contaminants in the plasmid solution. Table 1 shows the results obtained with these three different techniques. The measurement results and their standard deviations obtained for the copy number concentration estimates overlap.

Table 1: The DNA mass concentrations and the estimated copy number concentrations obtained for the stock solution of linearised plasmid pIRMM-0099 using three different methods

Method	Number of replicates	Mean $C_{DNA} \pm s$ [ng/µL]	Copy number concentration $\pm s$ [10^{10}cp/µL]
Fluorometry (picogreen)	6	210 ± 2.8 (measured)	3.43 ± 0.05 (estimated)
Spectrophotometry	3	214 ± 5.6 (measured)	3.49 ± 0.09 (estimated)
dPCR	2	205 ±17.8 (estimated)	3.34 ± 0.29 (measured)

Based on all these observations it can be reasonably concluded that there are no major contaminations present in the plasmid solution that might affect the behaviour of the plasmid in qPCR reactions.

4 Assessment of homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value. In contrast to that it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit heterogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

4.1 Homogeneity assessment of the number of DNA fragments per plasmid

The common certified value for ERM-AD623a, b, c, d, e and f is expressed as number of DNA fragments per plasmid. As each plasmid contains the three fragments, obtaining the certified value is independent of the number of plasmids contained in each vial and even a single plasmid would be sufficient to obtain the certified values. Homogeneity testing therefore needs to ensure that each plasmid contains both fragments once. The studies confirming the purity of the material are described in Section 3.3.

4.2 Homogeneity assessment of the copy number concentration of the plasmid

4.2.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all vials of the material, within the stated uncertainty. The same analyses were performed for each concentration level of ERM-AD623.

For the between-unit homogeneity test, 23 vials were selected using a random stratified sampling scheme covering the whole batch. For this, the batch was divided into 23 groups (with similar number of vials) and one vial was randomly selected from each group. The number of selected vials corresponds to approximately the cubic root of the total number of the produced vials. Each vial was measured three times with duplex qPCR amplifying the *BCR-ABL* b3a2 target and the *ABL* target as described in Section 1.3. The measurements were performed on three 96 wells plates and each plate contained a calibration curve and one measurement of every vial. This study design allows separation of a potential analytical drift (caused by a plate effect) from a trend in the filling sequence. The measurements were done under intermediate precision conditions, while evaluation was done under repeatability conditions as a correction was applied when a significant plate effect was present. Only the qPCR results of the *BCR-ABL* b3a2 target were used to assess the homogeneity of the material as the method repeatability of this target was the best.

Analysis of variance (ANOVA) was performed to evaluate potential significant difference between the measurements performed on different PCR plates. Some significant (95 % confidence level) differences between the plates were visible, pointing at instability of the analytical system. As each vial is measured once on each of the three PCR plates, correction for this bias can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, the bias was corrected as shown below if the difference between the PCR plates was significant on at least a 95 % confidence level.

$$corrected\,result = measured\,result \times d_i \qquad \text{Equation 2}$$

With $d_i = \overline{x_1} / \overline{x_i}$

d_i correction factor for plate i

$\overline{x_1}$ mean value measured for all vials on plate 1

$\overline{x_i}$ mean value measured for all vials on plate i (2 or 3)

Regression analyses were performed on the corrected datasets to evaluate potential trends in the filling sequence. No trends in the filling sequence were observed.

The corrected dataset was tested for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No outlying individual results and outlying unit means have been detected. The results of the measurements are shown in Annex B.

Quantification of between-unit heterogeneity is most easily done by ANOVA, which can separate the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the

method repeatability if the individual samples are representative for the whole unit. Evaluation by ANOVA requires unit means which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Too few data are available for each unit to make a clear statement of the distribution of individual results. Therefore, it was checked whether all individual data follow a unimodal distribution using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not grossly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 2.

Table 2: Results of the statistical evaluation of the homogeneity study

CRM	Significant difference between plates (p < 0.05)	Significant trend in filling sequence (p < 0.05)	Outliers (confidence level 99 %)		Distribution	
			Individual results	Unit means	Individual results	Unit means
ERM-AD623a	no	no	none	none	normal	unimodal
ERM-AD623b	yes (corrected)	no	none	none	normal	unimodal
ERM-AD623c	yes (corrected)	no	none	none	normal	unimodal
ERM-AD623d	yes (corrected)	no	none	none	normal	unimodal
ERM-AD623e	yes (corrected)	no	none	none	unimodal	unimodal
ERM-AD623f	no	no	none	none	normal	unimodal

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum heterogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [19]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum heterogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$ Equation 3

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$ Equation 4

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}}$$ Equation 5

MS_{within} mean square within a unit from an ANOVA

$MS_{between}$: mean squares between-units from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n: mean number of replicates per unit

$v_{MSwithin}$: degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 3. In most cases, the uncertainty contribution for homogeneity was determined by the method repeatability.

Table 3: Results of the homogeneity study

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
ERM-AD623a	13.02	n.c.	3.57	3.57
ERM-AD623b	10.93	n.c.	2.88	2.88
ERM-AD623c	9.84	n.c.	2.59	2.59
ERM-AD623d	9.38	n.c.	2.47	2.47
ERM-AD623e	9.82	2.75	2.59	2.75
ERM-AD623f	14.78	4.37	4.36	4.37

n.c.: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits for the detection power of the study, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential heterogeneity.

4.2.2 Within-unit homogeneity and minimum sample intake

Homogeneity/stability experiments were performed using a 5 µL sample intake. This sample intake gives acceptable repeatability, demonstrating that the within-unit heterogeneity does no longer contribute to analytical variation at this sample intake.

5 Stability

Time, temperature and radiation were regarded as the most relevant influences on stability of the materials. Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability).

The sensitivity of a DNA molecule to degradation is influenced by the GC content, the length of the strand and the buffer composition [20]. The three inserts are located on the same DNA strand and have a very similar GC content (54 % for the *BCR-ABL* b3a2 insert, 57 % for the *BCR* insert and 51 % for the *GUSB* insert). The stability of the three inserts is therefore considered to be identical. During the stability studies the copy number concentrations of the BCR-ABL b3a2 and the ABL PCR targets have been measured.

The stability studies have been carried out using an isochronous design [21]. In that approach, samples are stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

For the short-term stability study, samples from the final batch have been stored at 4 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to -70 °C. Five vials per storage time were selected using a random stratified sampling scheme. From each vial three samples were measured by duplex qPCR. The measurements were performed on three PCR plates and one plate contained one sample of each vial. The measurements were done under intermediate precision conditions, while evaluation was done under repeatability conditions as a correction was applied when a significant plate effect was present. This study design allows distinguishing a potential analytical drift from a trend over storage time. Only the qPCR results of the *BCR-ABL* b3a2 target were used as the method repeatability of this target was the best.

ANOVA was performed to evaluate potentially significant differences between the measurements performed on different PCR plates. Some significant (95 % confidence level) differences between the plates were detected, and results were corrected using Equation 2 as described in Section 4.2.1.

No outlying individual results were found using a Grubbs outlier test at a level of confidence of 99 %.

Furthermore, the data were plotted against storage time and regression lines of copy number concentration versus time were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to shipping conditions). For all six concentrations of ERM-AD623, the slopes of the regression lines were not significantly different from zero (99 % confidence level).

The results of the measurements are shown in Annex C.

No technically unexplained outliers were observed and none of the trends with storage time was statistically significant on a 99 % confidence level. As a consequence of this, no uncertainty contribution has been added to the combined uncertainty budget for u_{sts}.

The material was found stable at 4 °C for the period of 4 weeks. Nevertheless, the material is stored frozen and therefore freeze/thaw cycles should be kept at minimum. With this consideration, the material shall be shipped frozen on dry ice.

5.2 Long-term stability study

Before processing ERM-AD623 a, b, c, d, e, and f a smaller pilot batch was produced for each of the six CRMs. These pilot batches were processed in the same way as the final CRM batches. It was therefore possible to combine the data from three isochronous stability studies to assess the stability of the CRMs. Two of the studies were performed on the pilot batches and one on the final batches of ERM-AD623a, b, c, d, e, and f.

In the first stability study, vials of the pilot batches have been stored at -20 °C for 0, 4, 8 and 12 months. For the second study on the pilot batches the storage times were 0, 12, 18 and 24 months at -20 °C. Vials of the final batches have been stored at -20 °C for 0, 3, 6 and 9 months. The reference temperature of all three studies was set at -70 °C.

Within one study five vials were selected per storage time using a random stratified sampling scheme. From each vial, three samples were measured by duplex qPCR. The measurements were performed on three PCR plates and one plate contains one sample of each vial. This design allows separation of a potential analytical drift (plate effect) from a trend over storage time. The measurements were done under intermediate precision conditions, while evaluation was done under repeatability conditions as a correction was applied when a significant plate effect was present. Only the qPCR results of the *BCR-ABL* b3a2 target were used as the method repeatability of this target was the smallest. ANOVA was performed to evaluate potential significant difference between the measurements performed on different PCR plates. Some significant (95 % confidence level) differences between the plates were detected, and results were corrected using Equation 2 as described in Section 4.2.1.

The obtained data were first evaluated individually for each study. The results were screened for outliers using the single and double Grubbs test (99 % confidence level). Only for the third isochronous study of ERM-AD623c one outlying result was identified. As no technical reason for this outlier could be found all data were retained for statistical analysis.

Furthermore, the data were plotted against storage time and regression lines of copy number concentration versus time were calculated. The slope of the regression lines was then tested for statistical significance (99 % confidence level). A positive trend was observed in the second isochronous study of ERM-AD623e. As the analyte cannot be created in the sample, a positive trend could only be due to degradation of the matrix. This, however, should be seen for all concentration levels, which is not the case. The observed trend was therefore regarded as a statistical artefact.

Afterwards the results of the three isochronous studies have been combined to one long-term stability study as described in [22]. There was a measurement bias between the three studies. As study 2 has the longest storage times the results of the study 1 and 3 have been normalised to the results of study 2 using the correction factor *d* as calculated in Equation 6. The relative uncertainty of these corrections $u_{d,\,rel}$ is calculated with Equation 7.

$$d = \overline{x_2} / \overline{x_i} \qquad \text{Equation 6}$$

$\overline{x_2}$ mean measurement result of study 2

$\overline{x_i}$ mean measurement result of study i (1 or 3)

$$u_{d,i,rel} = \sqrt{\frac{1}{n_i} RSD_i^2 + \frac{1}{n_2} RSD_2^2} \qquad \text{Equation 7}$$

$u_{d,i,rel}$ relative uncertainty of the correction of study i

RSD_i relative standard deviation of all results in study i

RSD_2 relative standard deviation of all results in study 2

n_i number of data points is study i

n_2 number of data points in study 2

The normalised data were plotted against storage time and regression lines of copy number concentration versus time were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to storage conditions). For all six CRMs the slopes of the regression lines were not significantly different from zero (99 % confidence level). The significant trend observed in study 2 of ERM-AD623e is no longer present in the combined stability study. These results confirm that the observed trend for the individual study is a statistical artefact.

The results of the measurements are shown in Annex D.

Based on these measurements, it can be concluded that the plasmid solutions can be stored at -20 °C.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means, even under ideal conditions, the outcome of a stability study can only be "degradation is (0 ± x) % per time".

The uncertainty of stability during dispatch was not considered as the samples will be shipped on dry ice and the storage temperature is -20°C ± 5°C.

The uncertainty of stability during dispatch was estimated as described in [21] and [22]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contribution u_{lts} is then calculated as the product of the chosen shelf life and the uncertainty of the regression lines. As the long-term stability study is a combined study of two isochronous studies the uncertainty of the correction (u_d) should also be taken in account (Equation 8).

$$u_{lts} = \frac{RSD_{x^*y}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \text{Equation 8}$$

RSD_{x^*y} relative standard deviation of the estimate

t_i time point for each replicate

$\bar{t}$ mean of all time points

t_{sl} proposed shelf life (two years at -20 °C)

The standard error on the estimate s_{x^*y} should be calculated with Equation 9

$$RSD_{x^*y} = \sqrt{RSD^2_{pooled} + \frac{n_1}{n_2 + n_1} u_{d,rel,1} + \frac{n_3}{n_2 + n_3} u_{d,rel,3}} \qquad \text{Equation 9}$$

RSD^2_{pooled}: relative standard deviation of the combined stability studies

n_1 number of measurement results in study 1

n_2 number of measurement results in study 2

n_3 number of measurement results in study 3

$u_{d,rel,1}$ uncertainty of the correction of study 1

$u_{d,rel,3}$ uncertainty of the correction of study 3

The uncertainty contribution related to the stability during storage ($u_{lts,rel}$) was estimated for 24 months and describes the possible degradation at -20 °C. The results of these evaluations are summarised in Table 4.

Table 4: Uncertainties of stability during storage. $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 24 months

CRM	$u_{lts,rel}$ [%]
ERM-AD623a	2.75
ERM-AD623b	2.21
ERM-AD623c	2.81
ERM-AD623d	2.11
ERM-AD623e	2.43
ERM-AD623f	3.83

The uncertainty of the long-term stability has been considered in the combined uncertainty (Section 7.2). After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

5.4 Stability after freeze/thaw cycles

Since the minimum sample intake is 5 µL, one experiment measuring *BCR-ABL* b3a2 and one control gene by simplex qPCR requires at least 30 µL. One vial contains approximately 600 µL and can therefore be used for less than 20 experiments. The storage temperature is -20 °C and repeated use of the CRM will require repeated freeze/thaw cycles. In these stability studies the effect of repeated freeze/thaw cycles is investigated.

These stability studies have been carried out using an approach similar to the isochronous design. Vials from the pilot batches from ERM-AD623a, ERM-AD623c and ERM-AD623f have been used to perform these studies. For each of these three concentrations five vials were exposed to 0, 5, 10 and 20 freeze/thaw cycles and moved to the reference condition (-70 °C) afterwards.

Each vial was measured three times with duplex real time PCR as described in Section 1.3. The measurements were performed on three 96 wells plates and each plate contained a calibration curve and one sample of every vial. This study design allows separation of a potential analytical drift (caused by a plate effect) from a trend generated by the freeze/thaw cycles. The measurements were done under intermediate precision conditions, while evaluation was done under repeatability conditions as a correction was applied when a significant plate effect was present. The qPCR results of the *BCR-ABL* b3a2 target were used as the method repeatability of the target was the smallest.

ANOVA was performed to evaluate potential significant differences between the measurements performed on different PCR plates. Some significant (95 % confidence level) differences between the plates were detected, and results were corrected using Equation 2 as described in Section 4.2.1. No outlying individual results were found using Grubbs outlier tests (99 % confidence level). Furthermore, the data were plotted against the number of freeze/thaw cycles and regression lines of copy number concentration versus number of freeze/thaw cycles were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to freezing thawing). For the test batches of ERM-AD623c and ERM-AD623f the slope of the regression lines were not significantly different from zero (99 % confidence level). For the test batch of ERM-AD623a the slope of the regression line was significantly different from zero (99 % confidence level). Closer investigation of the results for the test batch of ERM-AD623a showed that there was no significant degradation after 5 and 10 cycles. However, the results obtained for the vials that were exposed to 20 freeze/thaw cycles were significantly lower. The results are shown in Annex E.

Although this degradation was only observed for one concentration level, precautions should be taken and the CRMs should not be exposed to more than ten freeze/thaw cycles.

6 Characterisation

The material characterisation is the process of determining the property value of a reference material.

6.1 Characterisation study for the number of DNA fragments per plasmid

The number of DNA fragments per plasmid is defined by the structural identity of the plasmid i.e. the sequence. Dideoxy terminator sequencing (or Sanger sequencing) was used to determine the sequence of the plasmid.

6.1.1 Study set up

Two laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field dideoxy terminator sequencing by submitting results for intercomparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

Each laboratory received one vial containing the circular pIRMM-0099 plasmid dissolved in $T_1E_{0.01}$ buffer at a concentration of approximately 150 ng/µL. These vials were set aside during the processing process as described in Section 3.2.1. The laboratories were requested to perform double stranded sequencing of an entire circular plasmid by using primer walking. Sequences were generated using the BigDye® Terminator chemistry version 3.1 (ABI). The sequence reactions were analysed on ABI3730 capillary sequencers.

6.1.2 Results

Each laboratory provided a full sequence of the pIRMM-0099 plasmid. Comparison of both obtained sequences showed that they were identical. The laboratories also provided the sequence quality values of each nucleotide. This quality value QV is a transformed estimate of the probability of correctness (1-probability of error) and defined by the following equation:

$$QV = -10 \log_{10}(P_e) \qquad \text{Equation 10}$$

P_e estimated error probability of a base

The sequence results generated by the first laboratory had the best quality. Each base pair within the sequence generated had a quality score of at least 67 corresponding to an error probability of less than 1/5000000. Due to the fact that most of the base pairs had an even higher quality score and the calculated cumulative calculated cumulative error of the entire plasmid sequence (5970 bp) is 1/718013375. Based on the Poisson distribution and the length of each cloned DNA fragment, the probability of one sequencing error per DNA fragment can be calculated. This probability is less than 0.0002 % for each cloned fragment.

Within the sequence generated by the second laboratory, each base pair had a quality score of at least 38. These results were considered as confirmatory results.

The sequences of the fragments were also compared with the consensus sequences of the transcripts of *GUSB*, *BCR* and *ABL* available in the GenBank database (NCBI, NIH, Bethesda, MD, US). Two differences were observed between the consensus sequences from Genbank and the sequences of the fragments present in pIRMM-0099. These differences correspond to two known single nucleotide polymorphisms (SNPs) present in the SNP database (dbSNP, NCBI, http://www.ncbi.nlm.nih.gov/projects/SNP). The DNA fragment of the *BCR-ABL* b3a2 transcript

contains the G allele of the SNP with reference number rs140504 while the consensus sequence in the NCBI database contains the A allele. Within the DNA fragment of the *BCR* transcript, the T allele of SNP rs11558697 is present while in the consensus sequence the C allele is present. These SNP can also be present in patients' samples and they should be taken in account when using primers or probes located at these positions. The effect of the presence of the SNPs on the PCR efficiency can be tested by performing cross-over experiment as described in [23]. Otherwise a mixture of two oliginucleotides, each contain one allele of the SNP, should used as primer or probe.

The alignment of the sequences (Annex F) shows that each of the three fragments is present once in the plasmid. As a clear consequence, the structurally defined ratio between the number of DNA fragment specific for *BCR-ABL* b3a2 transcript and the DNA fragments specific for *BCR* and *GUSB* is 1.

6.1.3 Confirmatory measurements

Digital PCR measurements were performed to confirm the structurally defined ratio between the numbers of DNA fragment specific for *BCR-ABL* b3a2 transcript and the DNA fragments specific for *BCR* and *GUSB*. Target sequences located on the *BCR-ABL* b3a2 fragment and the *GUSB* fragment were amplified in a duplex dPCR reaction using primers and probes and PCR conditions described in [5, 6]. The primers and probes and PCR conditions described in [11] were used to amplify target sequences located on the *BCR-ABL* b3a2 fragment and the *BCR* fragment in a duplex dPCR reaction. For each plasmid concentration of ERM-AD623 one vial was measured on five panels of a digital array. The value of 1 was confirmed for both ratios. Results are shown in Table 5.

Table 5: The results of duplex dPCR measurements to confirm the structurally defined copy number ratios

CRM	Copy number ratio *BCR-ABL* b3a2/*GUSB* ± *s*	Copy number ratio *BCR-ABL* b3a2 /*BCR* ± *s*
ERM-AD623a	1.015 ± 0.017	1.000 ± 0.013
ERM-AD623b	0.994 ± 0.016	1.019 ± 0.015
ERM-AD623c	0.990 ± 0.012	1.012 ± 0.033
ERM-AD623d	1.008 ± 0.022	0.994 ± 0.021
ERM-AD623e	1.000 ± 0.018	1.005 ± 0.020
ERM-AD623f	0.989 ± 0.074	0.966 ± 0.050

6.2 Characterisation study for the copy number concentration of the plasmid

The material characterisation was based on an intercomparison of expert laboratories. The certified value for the copy number concentration of the plasmid of the six plasmid dilutions was determined by dPCR measurements performed by three different laboratories. All participants used the BioMark system (Fluidigm) and the 12.765 digital arrays to perform the measurements. This approach aims at randomisation of laboratory bias, which reduces the combined uncertainty.

6.2.1 Study set up

Three laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of dPCR measurements by previous publications. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

Each laboratory received six vials of each plasmid concentration of ERM-AD623 and was requested to provide six independent results, one result per vial. Vials for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. Each of the six vials from one CRM had to be diluted and measured on a different day and on different digital array to ensure intermediate precision conditions.

All laboratories used the BioMark system (Fluidigm) and the 12.765 Digital arrays to perform the measurements. The *BCR-ABL* b3a2 target and the *ABL* target (both located on the *BCR-ABL* b3a2 transcript fragment) were amplified in a duplex PCR reaction as described in Section 1.3. Each sample mix was analysed on five panels of one digital array. The results of both the *BCR-ABL* b3a2 and the *ABL* target were used to demonstrate the absence of a bias which might be due to one specific set of primers and probe. The mean value of both PCR targets (n = 10) obtained was considered as one independent measurement result.

6.2.2 Results

The characterisation campaign resulted in 18 independent measurement results per CRM. The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- compliance with the analysis protocol: sample preparations and measurements performed on six different days
- correctness of the dilutions steps: all dilutions steps had to be prepared gravimetrically and masses had to be recorded
- visual check if there was no air bubble present in the panels
- each independent measurement result should be based on the results of 5 panels

Based on the above two datasets were rejected as not technically valid: one for ERM-AD623a and one for ERM-AD623f. In both cases there was an air bubble present in one of the panels.

All accepted results of the participants, grouped per CRM, are displayed in tabular form in Annex G.

Statistical analyses were performed to identify the major source of variation in this characterisation study. One way ANOVA analyses were performed to calculate the standard deviation between independent measurements performed within one laboratory ($s_{within\ labs}$), the standard deviation between laboratories ($s_{between\ labs}$) and the standard deviation of all independent measurements ($s_{all\ measurements}$). Standard deviations between all independent measurements are considerably larger than the standard deviation within laboratories. One way ANOVA analysis also showed that there is no significant difference between the results obtained by the different laboratories (95 % confidence level). The results of these ANOVA analyses are summarised in Table 6. Moreover, the standard deviation between all measurements from this intercomparison is very similar to the standard deviation between measurements obtained during the method validation performed within one laboratory (IRMM). The standard deviation between measurements from the in-house method validation was 14.71 %.

Table 6: Statistical analyses to identify the major source of variation in the characterisation study

CRM	Laboratory number	$s_{within\ lab,\ rel}$ [%]	$s_{between\ labs,\ rel}$ [%]	$s_{all\ measurements,\ rel}$ [%]
ERM-AD623a	1 2 3	12.66 10.11 16.88	n.c.	13.03
ERM-AD623b	1 2 3	17.86 11.64 14.17	5.03	15.12
ERM-AD623c	1 2 3	9.46 12.95 11.73	6.25	12.68
ERM-AD623d	1 2 3	6.40 11.61 10.15	6.70	11.20
ERM-AD623e	1 2 3	11.40 11.43 8.72	8.22	12.81
ERM-AD623f	1 2 3	15.43 18.72 6.68	5.78	15.96

n.c.: cannot be calculated as $MS_{between} < MS_{within}$

This information shows that the variations between independent measurements have a larger impact than the variations between laboratories. Each of the 18 independent measurements was therefore considered as a separated data set.

The datasets accepted on technical grounds were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations (both at 99 % confidence level). The dataset means follow normal distributions. None of the data contains outlying means. The datasets are therefore consistent and the mean of independent measurement results is a good estimate of the true value.

The uncertainty for the characterisation exercise was estimated as the relative standard uncertainty of the independent measurement results, i.e. $\frac{s_{all\ measurements}}{\sqrt{p}}$ with s the relative standard deviation of the independent measurement results and p the number of technically valid independent measurements. The results of these evaluations are shown in Table 7.

Table 7: Statistical evaluation of the technically accepted data sets for ERM-AD623. *p* number of technically valid independent measurements, *s* standard deviation, u_{char} uncertainty related to the characterisation study

Copy number concentration of the plasmid								
CRM	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [cp/µL]	*s* [cp/µL]	u_{char} [cp/µL]	$u_{char, rel}$ [%]
ERM-AD623a	17	none	none	yes	1081264	146173	35452	3.28
ERM-AD623b	18	none	none	yes	107706	16343	3852	3.58
ERM-AD623c	18	none	none	yes	10321	1317	310	3.01
ERM-AD623d	18	none	none	yes	1018	115	27	2.66
ERM-AD623e	18	none	none	yes	103.5	13.5	3.2	3.06
ERM-AD623f	17	none	none	yes	9.96	1.75	0.42	4.28

7 Value Assignment

7.1 Copy number ratio of specific DNA fragments within the plasmid

The plasmid calibrant is characterised for the copy numbers of each of the three specific DNA fragments per plasmid i.e. the *BCR-ABL* b3a2 transcript fragment, the *BCR* transcript fragment and the *GUSB* transcript fragment. Sequence analysis showed that three cloned DNA fragments were present as a single copy in the plasmid and that their sequence was correct.

Based on the sequence identity the copy number ratios *BCR-ABL* b3a2 transcript fragment/plasmid, *GUSB* transcript fragment/plasmid and *BCR* transcript fragment/ plasmid are 1. The sequence identity of the plasmid was determined by two independent laboratory analyses which provided exactly the same results. The DNA sequencing was performed by forward and backward sequencing. The uncertainty on sequencing under those conditions can be considered as negligible.

Table 8: Certified values and their uncertainties for the number of specific DNA fragments per plasmid

Specific DNA fragment	Number of fragment per plasmid	Uncertainty
BCR-ABL b3a2 transcript	1	negligible
BCR transcript	1	negligible
GUSB transcript	1	negligible

Based on the sequence identity and the purity assessment the theoretical copy number ratios *BCR-ABL* b3a2 transcript fragment/ *GUSB* transcript fragment and *BCR-ABL* b3a2 transcript fragment/*BCR* transcript fragment are 1. These copy number ratios are confirmed by dPCR measurements.

7.2 The copy number concentration of the plasmid

The unweighted mean of the accepted datasets as shown in Section 6 was assigned as certified value for the copy number concentration of the plasmid.

The assigned uncertainty consists of uncertainties related to characterisation, u_{char}, potential between-unit heterogeneity, u_{bb} and potential degradation during transport (u_{sts}) and long-term storage, u_{lts}. These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k as

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{lts,rel}^2} \qquad \text{Equation 11}$$

u_{char} was estimated as described in Section 6

u_{bb} was estimated as described in Section 4

u_{lts} was estimated as described in Section 5.

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions, a coverage factor k of 2 was applied to obtain the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 9.

Table 9: Certified values and their uncertainties for the copy number concentration of the plasmid

CRM	Copy number concentration of the plasmid [cp/µL]	$u_{char,\,rel}$ [%]	$u_{bb,\,rel}$ [%]	$u_{lts,\,rel}$ [%]	$U_{CRM,\,rel}$ [%]	U_{CRM} [cp/µL]
ERM-AD623a	1.08×10^{6}	3.28	3.57	2.75	11.15	0.13×10^{6}
ERM-AD623b	1.08×10^{5}	3.58	2.88	2.21	10.19	0.11×10^{5}
ERM-AD623c	1.03×10^{4}	3.01	2.59	2.81	9.73	0.10×10^{4}
ERM-AD623d	1.02×10^{3}	2.66	2.47	2.11	8.40	0.09×10^{3}
ERM-AD623e	1.04×10^{2}	3.06	2.75	2.43	9.56	0.10×10^{2}
ERM-AD623f	10.0	4.28	4.37	3.83	14.42	1.5

8 Metrological traceability

8.1 Metrological traceability

8.1.1. Copy number ratio of specific DNA fragments within the plasmid

The sequence identity of the pIRMM-0099 plasmid is structurally defined and independent of the measurement method. Dideoxy terminator sequencing was used to determine the sequence identity of plasmid pIRMM-0099. Dideoxy terminator sequencing is a method of high metrological order as it does not require a calibrant and the obtained results have a very low uncertainty. Based on this sequence identity the copy number ratio of the specific DNA fragments per plasmid was determined. This copy number ratio was confirmed by an independent method, i.e. dPCR. The value of the copy number ratio of the specific DNA fragment per plasmid is traceable to the SI.

8.1.2 Copy number concentration of the plasmid

The copy number concentration of the plasmid is a method-defined measurand and has been obtained by PCR using the primers and probe set and the PCR conditions as described in Annex A. The value of the copy number concentration of the plasmid is traceable to SI. Traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and calibrated with tools ensuring traceability to the SI. Consistency in the intercomparison demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

9 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups) of analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all the analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CSLI Guideline C-53A [24] recommends the use of the following definition for the term *commutability*:

"The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established in such cases, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrator.

This set of CRM is used to calibrate the qPCR reaction targeting the *BCR-ABL* b3a2 transcript. The plasmid DNA present in these calibrant is different from the cDNA obtained from patient samples. However, based on the traceability chain described above, as well as on additional practical reasons, which include full sequence characterisation, reproduction of additional batches of calibrant and availability, ERM-AD623 is selected as the calibrant of choice to be used for the calibration of the qPCR reactions.

A limited commutability study was performed to test the performance of this set of plasmid solutions using different methods. Nine laboratories were selected on the basis of proven experience and quality management systems in place. The laboratories were asked to perform these measurements with the simplex qPCR method(s) that they routinely use to determine the ratio of *BCR-ABL* b3a2 transcript to the control gen transcript. An overview of the different methods used is given in Annex H.

Per method used, laboratories received two sets of the six plasmid solutions of ERM-AD623 and two dilutions of cDNA sample. This cDNA sample was extracted from K562 cells and diluted to two different concentrations: Based on the results of one dPCR measurement dilution 1 had an estimated *BCR-ABL* b3a2 transcript copy number concentration of $3.6*10^4 \pm 0.3*10^4$ cp/µL and dilution 2 of $1.3 *10^3 \pm 0.04 *10^3$ cp/µL.

From each set of plasmid solution eight calibration curves should be measured: four for the *BCR-ABL* b3a2 transcript and four for the control gene transcript. The two cDNA solutions were also measured and their copy number ratio of *BCR-ABL* b3a2/control gene was determined using the different calibration curves. In total the results of each laboratory for each method consist of the following: eight calibration curves for both the *BCR-ABL* b3a2 transcript and the control gene transcript and eight measured copy number ratio *BCR-ABL* b3a2/ control gene for two cDNA solutions.

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. Reasons for exclusion were: too much variation between triplicates, the control gene and the *BCR-ABL* b3a2 not measured on the same PCR plate, and degradation of the cDNA solution.

The calibration curves were evaluated for their slope and coefficient of determination (r^2). The slope should be between -3.00 and -3.60 and the r^2 should be above 0.99. In total, there were 171 accepted calibration curves: 84 for *BCR-ABL* b3a2, 36 for *ABL*, 23 for *BCR* and 28 for *GUSB*. Only 3 calibration curves were rejected because their slope was outside the range of -3.00 to -3.60. Two of them had a slope of -3.61 and one had a slope of -3.62. All calibration curves had an r^2 above 0.993.

Only the accepted calibration curves were used to determine the copy number ratios *BCR-ABL* b3a2/ control gene of the two cDNA solutions and the results are shown in Annex H. The relative standard deviation between the mean copy number ratio measured by the different laboratories for the two cDNA solutions were 8 % for the ratio *BCR-ABL* b3a2 */BCR*, 14 % for the ratio *BCR-ABL/ABL* and 18 % for the ratio *BCR-ABL* b3a2/*GUSB*. These differences are within the expected variability of qPCR measurements as the RSD of an accurate qPCR method can be up to 30 %.

Based on the results of this commutability study it was concluded that this set of six CRMs with different plasmid concentrations can be used to calibrate different qPCR measurements determining the copy number ratio of the *BCR-ABL b3a2* transcript to three control genes i.e. *BCR*, *ABL* and *GUSB*.

The user should however be aware, that the plasmid calibrant cannot be used to control the sample preparation steps before the qPCR including the reverse transcription step. Therefore additional matrix reference material should be used to control the whole measurement procedure of the patient samples. Secondary reference materials calibrated with the "WHO International Genetic Reference Panel for the quantification of BCR-ABL translocation" can be used for this purpose. The matrix materials should be cell line mixtures which have the same analytical behaviour as patient samples [25].

10 Instructions for use

10.1 Storage conditions

The materials shall be stored at -20 °C ± 5 °C.

Under the condition that contaminations have been excluded, the solutions can be used for several experiments. The material should however not pass more than 10 freeze/thaw cycles. The material can also be stored at 4°C for 4 weeks as it was verified that changes to the certified concentration observed during that period are not significant. It is advisable to close the vial with the original screw cap after use. The plasmid containing solution should not be exposed to direct sun light.
Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

10.2 Safety and protection for the environment

The usual laboratory safety measures apply.

10.3 Preparation and use of the material

To make the plasmid solutions ready for use, the content of the vials has to be thawed completely and mixed gently by inverting the vial several times. The plasmid containing vials should be opened and handled under a laminar flow to reduce the risk of contamination.

10.4 Minimum sample intake

The minimum sample intake representative is 5 µL.

10.5 Use of the certified value

ERM-AD623 is only intended to be used for the calibration of quantitative real time PCR (qPCR) methods for the measurement of the copy number ratio of the *BCR-ABL* b3a2 transcript to one of the following control genes: *BCR*, *ABL* or *GUSB*. A limited commutability study has shown that ERM-AD623 can be used to calibrate different qPCR methods targeting *BCR-ABL* b3a2. A list of the qPCR methods can be found in Annex H. For other targets such as the *BCR-ABL* b2a2 transcript, the suitability of ERM-AD623 has to be verified.
This set contains six concentrations which should be used to construct the calibration curve for both the *BCR-ABL* b3a2 transcript and the control gene. The contribution of this calibrant to the measurement uncertainty on this ratio is negligible.
In addition, the calibration curve is also used to estimate the absolute copy number concentration of the control gene in the patient samples. In this case, the uncertainty of the certified value should be taken into account.

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Annexes

Annex A: Description of the qPCR and dPCR methods used.

The copy number concentrations of the plasmid have been determined by dPCR using the protocol described below. Ten µL of ERM-AD623a, ERM-AD623b, ERM-AD623c and ERM-AD623d were gravimetrically diluted in $T_1E_{0.01}$ buffer to obtain DNA samples at a nominal concentration of 500 cp/µL. ERM-AD623e and ERM-AD623f were used undiluted in the dPCR assay. A volume of 19.70 µL of the diluted or undiluted DNA sample was further mixed with 30.3 µL of pre-sample mix solution. A volume of 9 µL of each of those mixtures was loaded on 5 replicate panels of the 12.765 digital Array ™ IFC's (Fluidigm). The pre-sample mix solution contained the primers and probes for the *BCR-ABL b3a2* transcript and for the *BCR* transcript at final concentrations mentioned in the Table A1 together with 20x GE sample loading reagent and TaqMan® Universal PCR MasterMix as recommended by the manufacturer. The PCR were performed according to the specifications mentioned in Table A2. The PCR runs were then analysed with the Fluidigm Digital PCR software using the following settings (quality threshold: 0.4; linear baseline correction; automatic Ct threshold method; target Ct range between 20 and 49). In case of low background noise, the results were analysed using a linear derivative baseline correction and a manual Ct threshold setting.

For qPCR, the same general PCR conditions listed in Table A1 and A2 have been used.

Table A1: Primers and probes used to amplify the *BCR-ABL* b3a2 and the *ABL* target in a duplex PCR reaction

PCR target	Primer/ probe (EAC code)	Sequence (5'-3')	Concentration in PCR reaction [nM]	Amplicon size
BCR-ABL b3a2	Forward primer (ENF501)	TCCGCTGACCATCAAYAAGGA	300	149
	Reverse primer (ENR561)	CACTCAGACCCTGAGGCTCAA	300	
	Probe (ENP541)	(6-VIC)-CCCTTCAGCGGCCAGTAG CATCTGA-(MGB)	200	
ABL	Forward primer (ENF1003)	TGGAGATAACACTCTAAGCATAAC TAAAGGT	300	122
	Reverse primer (ENPr1043)	GATGTAGTTGCTTGGGACCCA	300	
	Probe (ENPr1043)	(6-FAM)-CCATTTTTGGTTTGGGCTT CACACCATT-(TAMRA)	200	

The sequences of the *BCR-ABL* b3a2 primers and probe are those published by J. Gabert *et al*. 2003 [5].

The sequences of the *ABL* primers and probe are those published by E. Beillard *et al*. 2003 [6].

The European Against Cancer code (EAC) is mentioned for information.

Y (cytidine or thymidine) appears on the BCR primer according to the polymorphism described on the *BCR* gene.

Table A2: Thermal cycling protocol used for the duplex PCR reaction amplifying the *BCR-ABL* b3a2 and *ABL* target

Name	**Phase**	**Time [seconds]**	**Temperature [°C]**	**Repeats**
UNG and Hot start	UNG	120	50	1
	Hot start	600	95	
PCR cycles	Denaturation	15	95	50
	Annealing	60	60	

Annex B: Results of the homogeneity measurements

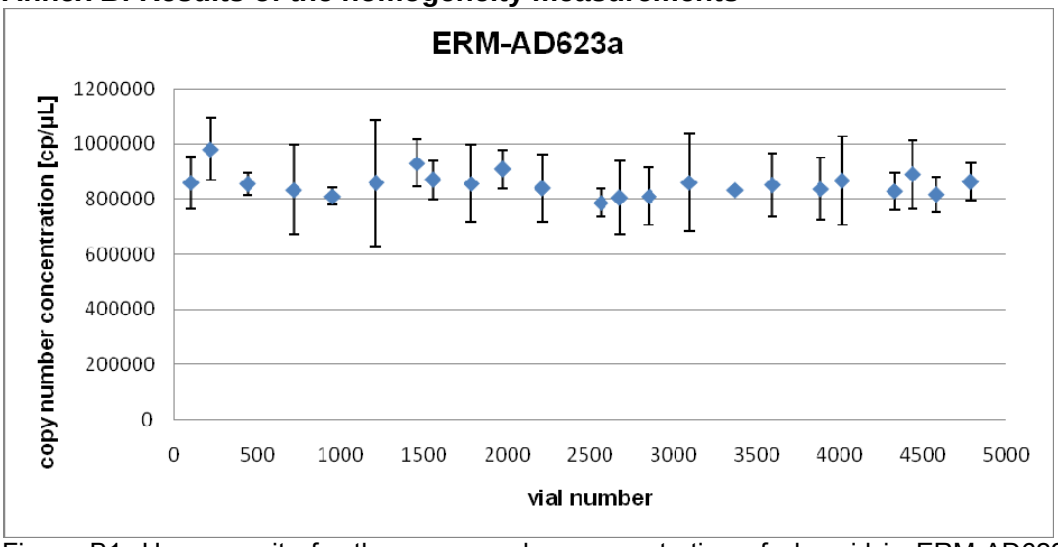

Figure B1: Homogeneity for the copy number concentration of plasmid in ERM-AD623a. The error bars represent the standard deviation from 3 replicates.

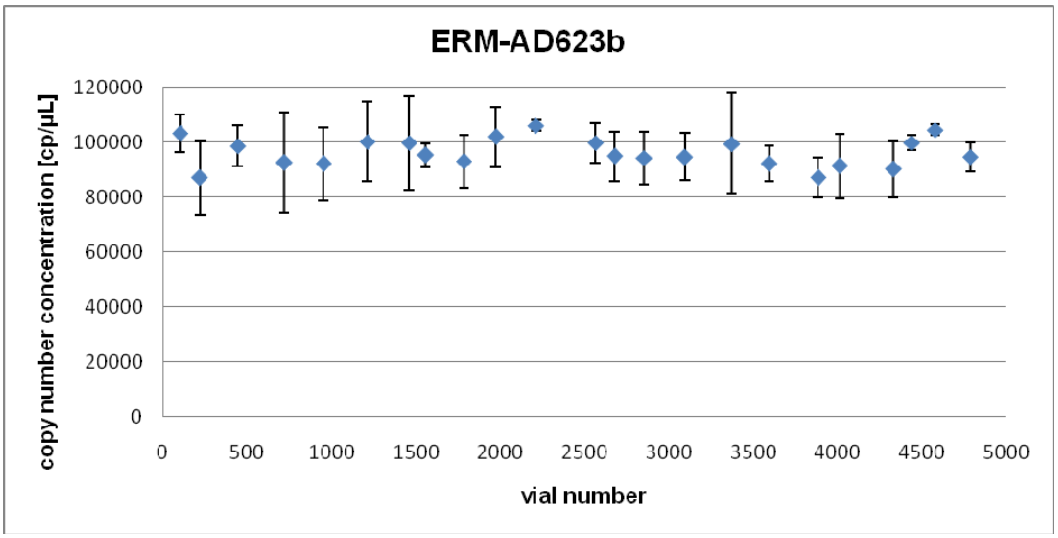

Figure B2: Homogeneity for the copy number concentration of the plasmid in ERM-AD623b. The error bars represent the standard deviation from 3 replicates.

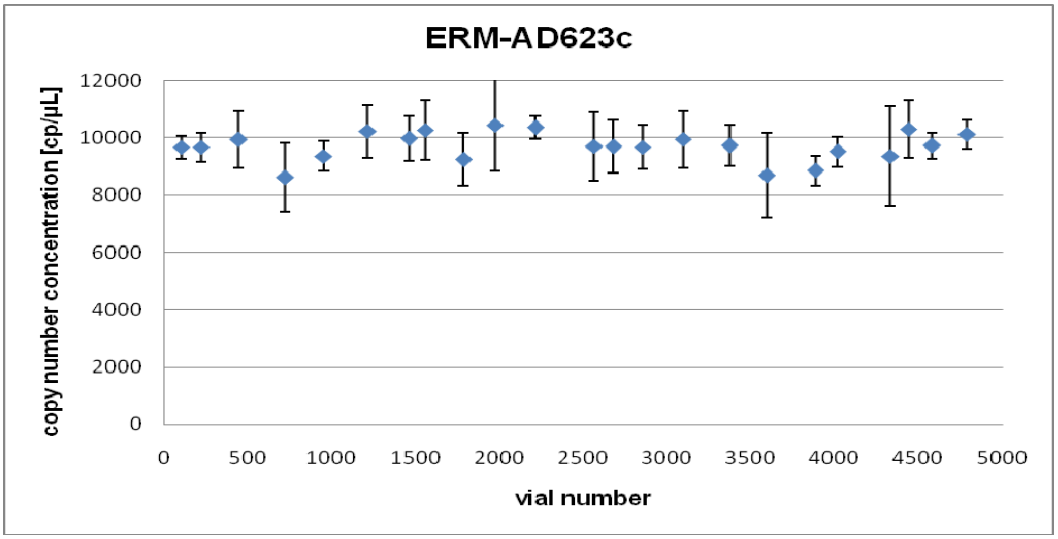

Figure B3: Homogeneity for the copy number concentration of the plasmid in ERM-AD623c. The error bars represent the standard deviation from 3 replicates

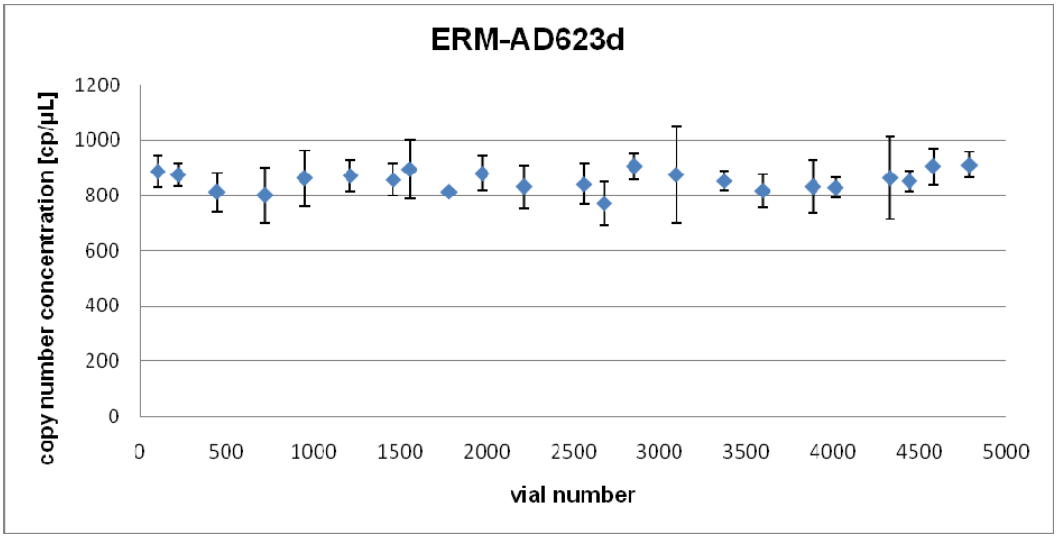

Figure B4: Homogeneity for the copy number concentration of the plasmid in ERM-AD623d. The error bars represent the standard deviation from 3 replicates

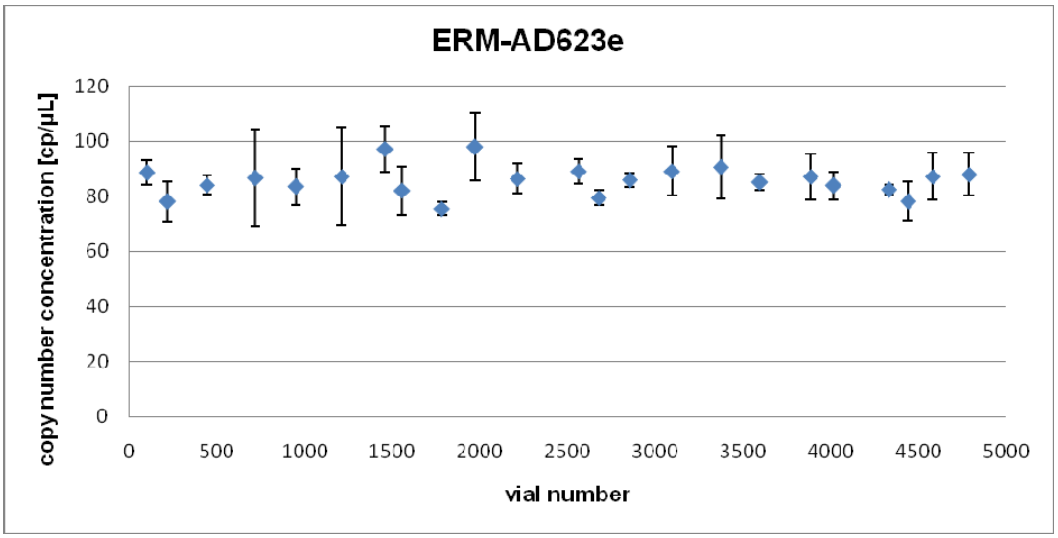

Figure B5: Homogeneity for the copy number concentration of the plasmid in ERM-AD623e. The error bars represent the standard deviation from 3 replicates

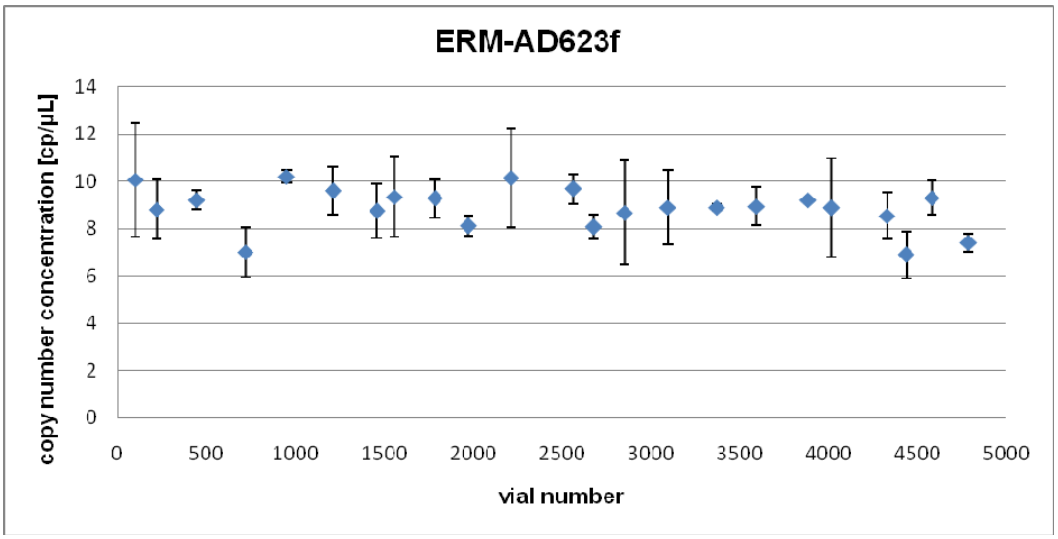

Figure B6: Homogeneity for the copy number concentration of the plasmid in ERM-AD623f. The error bars represent the standard deviation from 3 replicates

Annex C: Results of the short-term stability studies

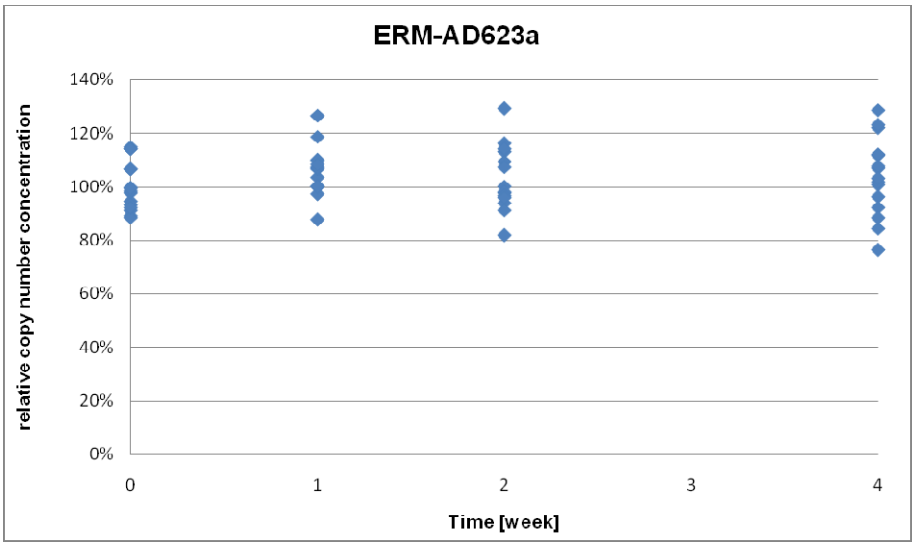

Figure C1: Short-term stability for the copy number concentration of the plasmid in ERM-AD623a.

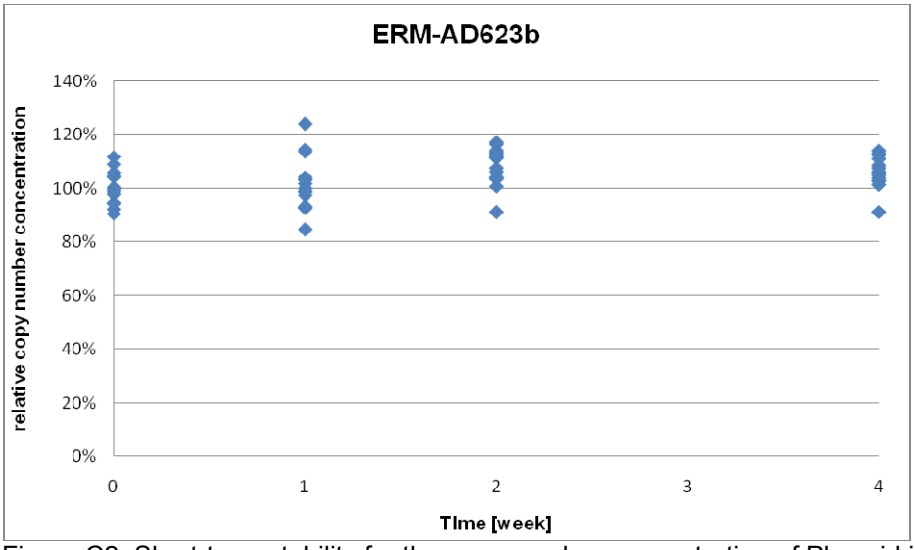

Figure C2: Short-term stability for the copy number concentration of Plasmid in ERM-AD623b.

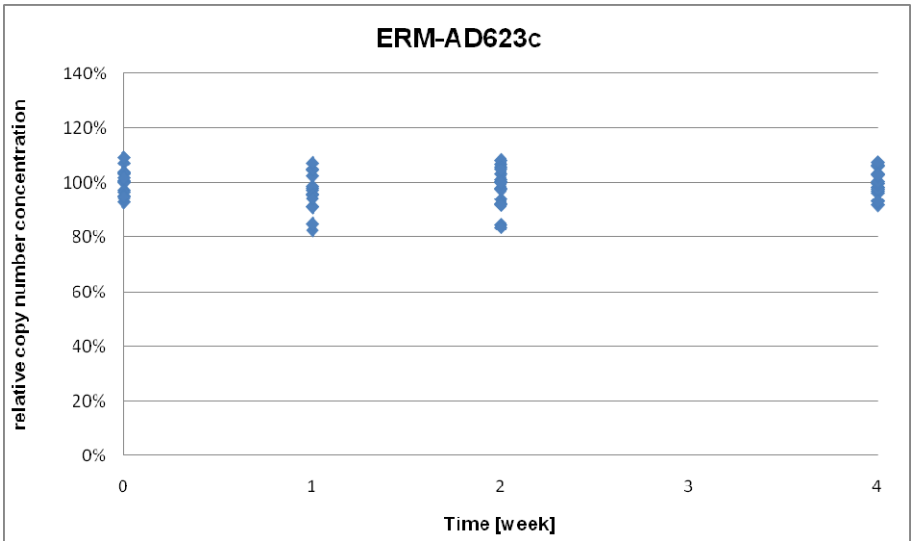

Figure C3: Short-term stability for the copy number concentration of the plasmid in ERM-AD623c.

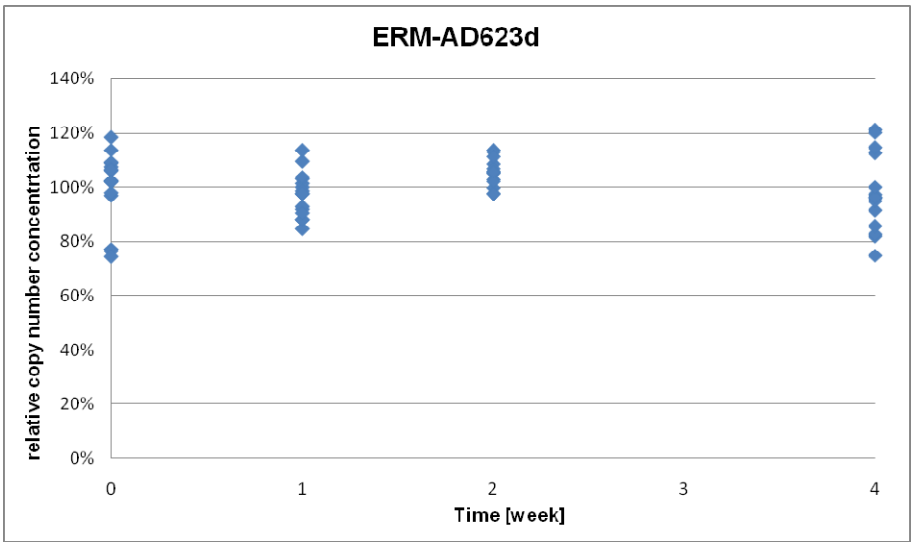

Figure C4: Short-term stability for the copy number concentration of the plasmid in ERM-AD623d.

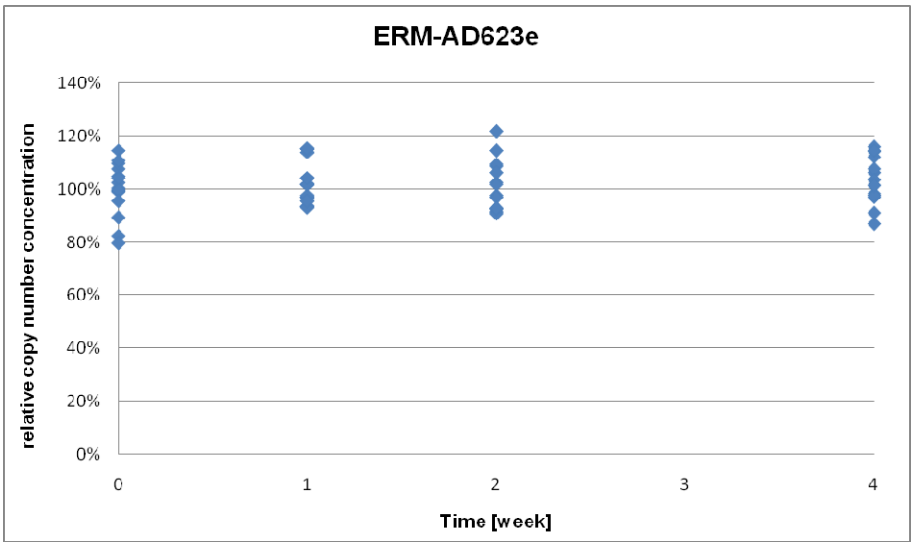

Figure C5: Short-term stability for the copy number concentration of the plasmid in ERM-AD623e.

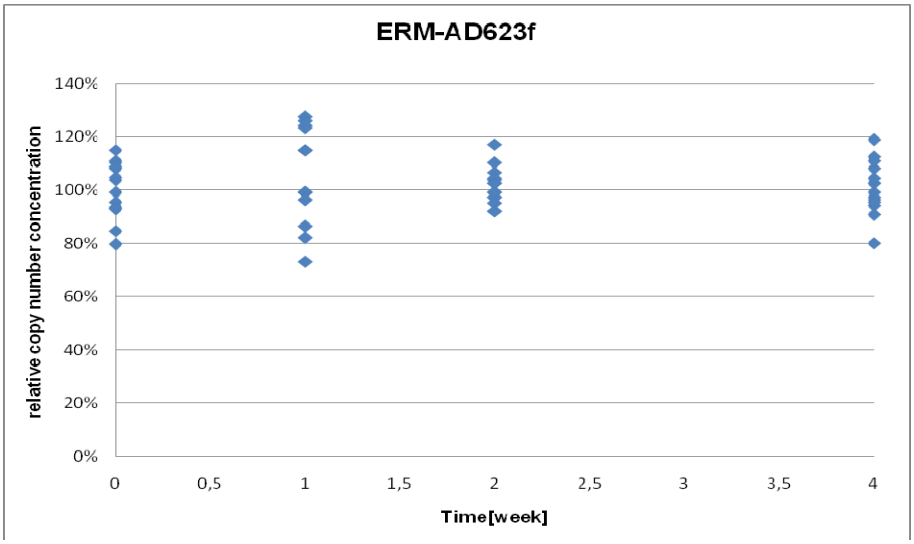

Figure C6: Short-term stability for the copy number concentration of the plasmid in ERM-AD623f.

Annex D: Results of the long-term stability measurements

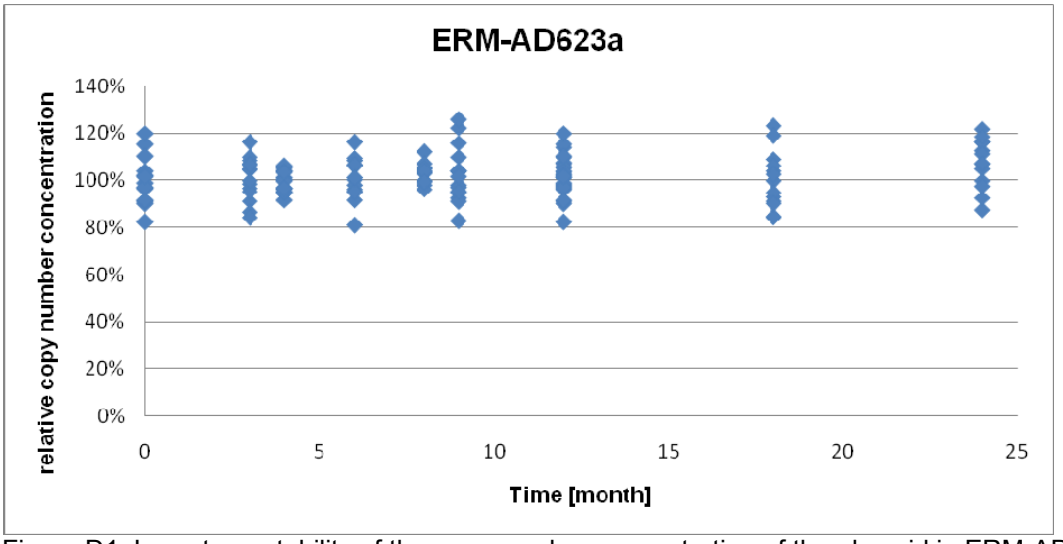

Figure D1: Long-term stability of the copy number concentration of the plasmid in ERM-AD623a.

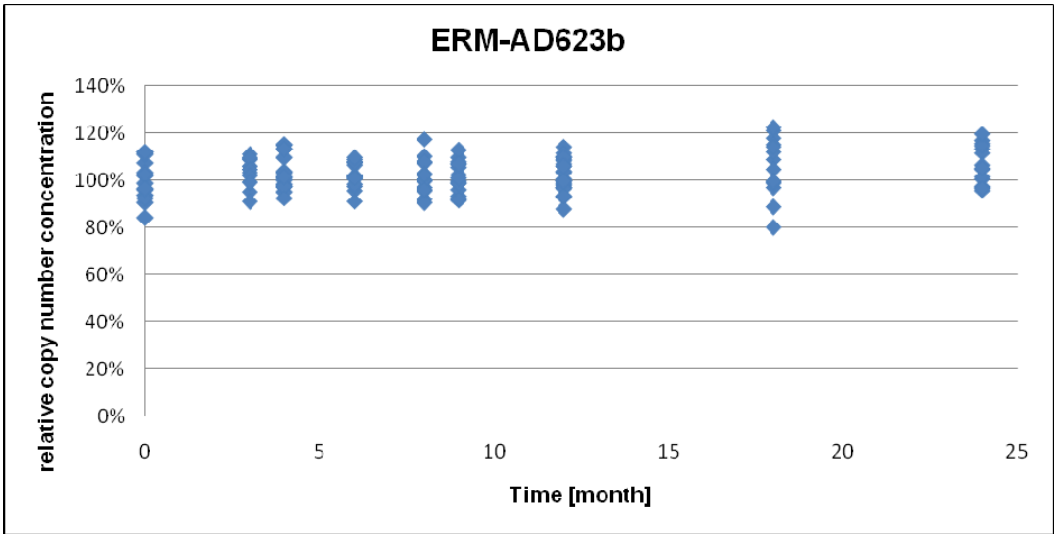

Figure D2: Long-term stability of the copy number concentration of the plasmid in ERM-AD623b.

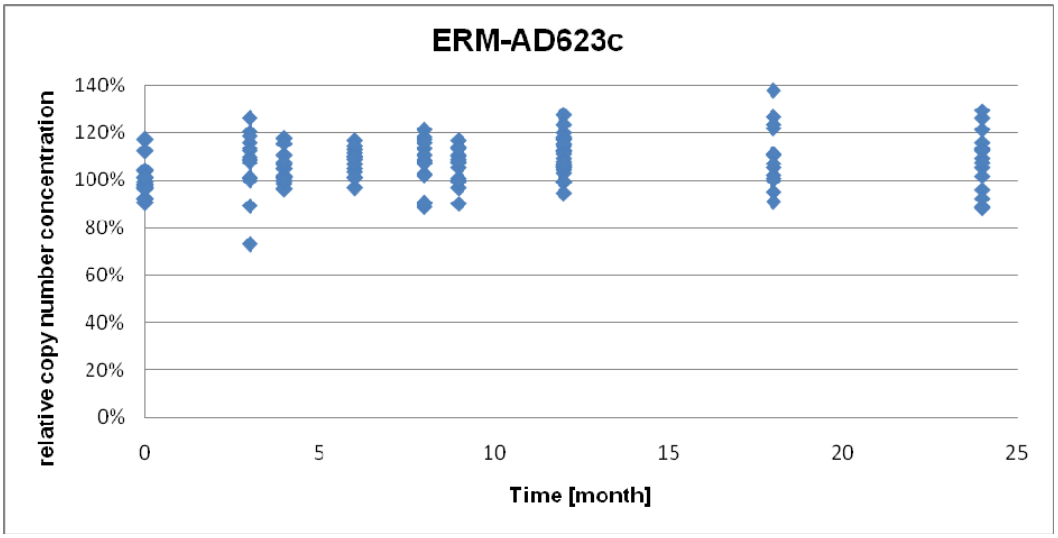

Figure D3: Long-term stability of the copy number concentration of the plasmid in ERM-AD623c.

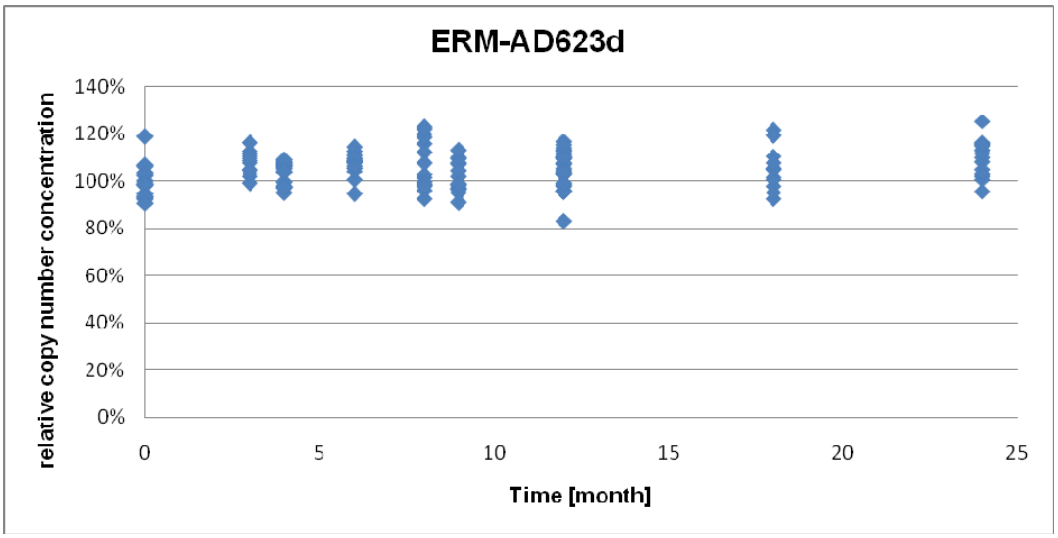

Figure D4: Long-term stability of the copy number concentration of the plasmid in ERM-AD623d.

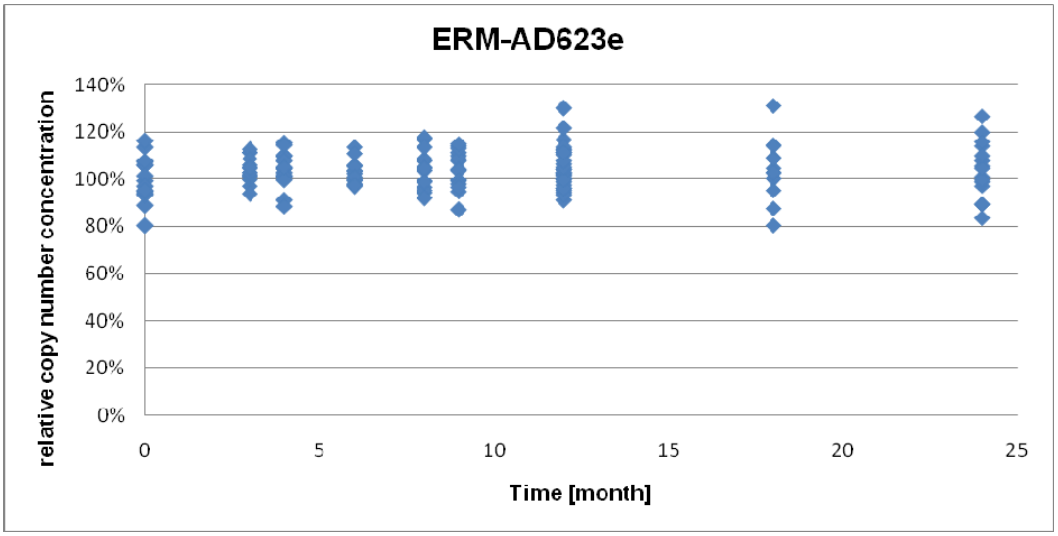

Figure D5: Long-term stability of the copy number concentration of the plasmid in ERM-AD623e.

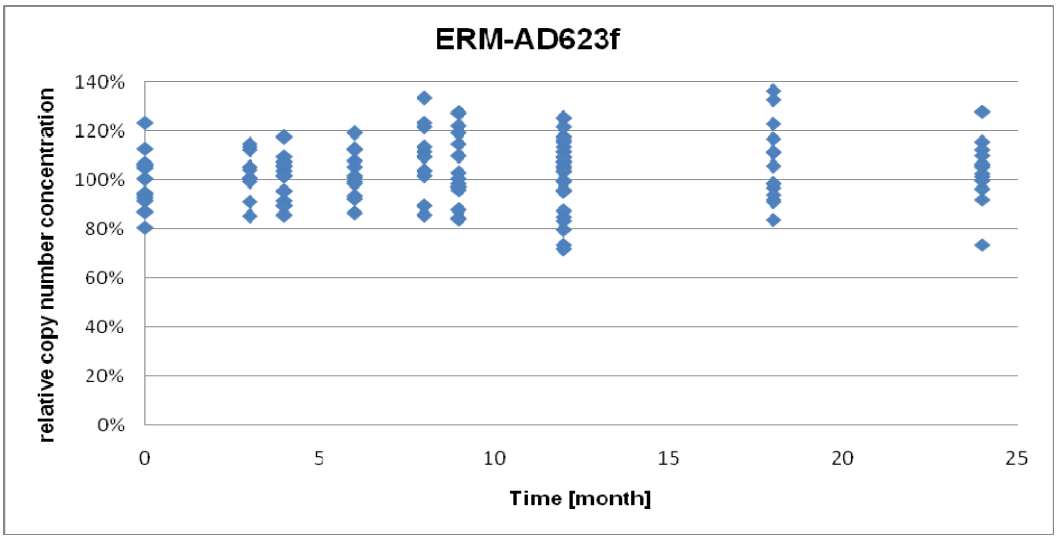

Figure D6: Long-term stability of the copy number concentration of the plasmid in ERM-AD623f.

Annex E: Results of the freeze/thaw stability study

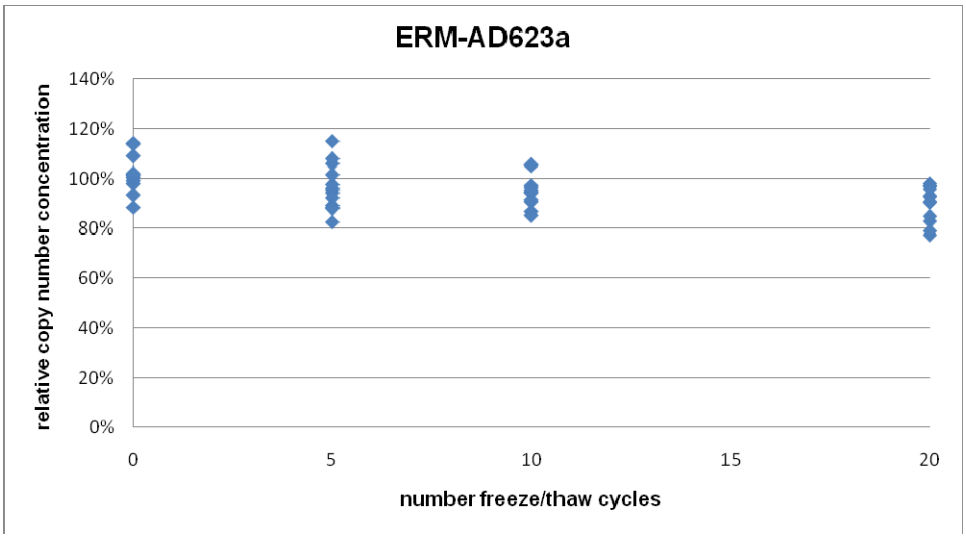

Figure E1: Stability of the copy number concentration of the plasmid in ERM-AD623a after several freeze/thaw cycles.

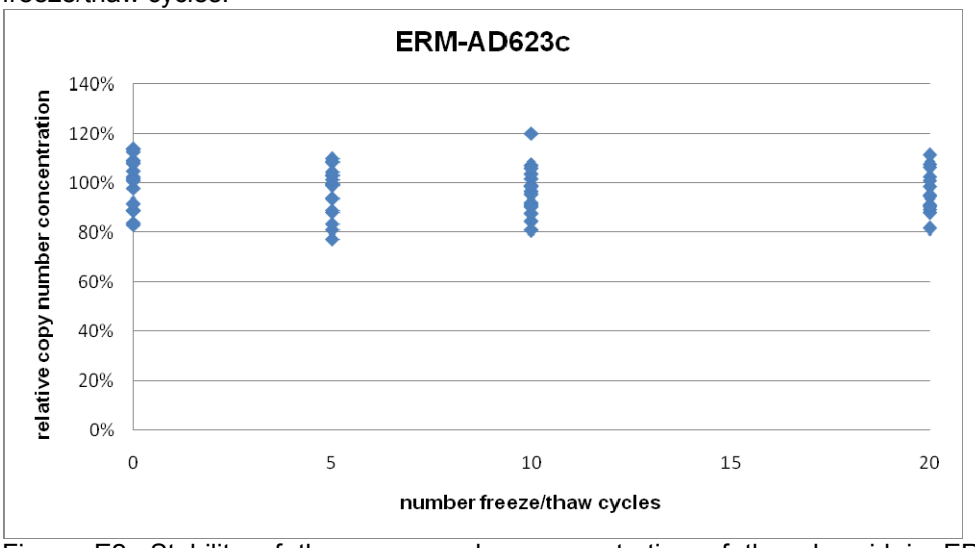

Figure E2: Stability of the copy number concentration of the plasmid in ERM-AD623c after several freeze/thaw cycles.

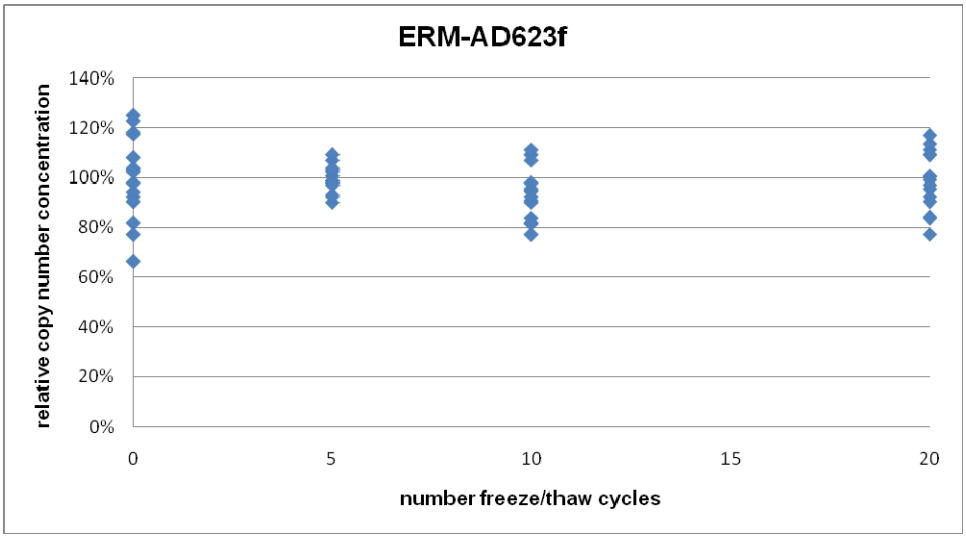

Figure E3: Stability of the copy number concentration of the plasmid in ERM-AD623f after several freeze/thaw cycles.

Annex F: Alignment of the results obtained by sequence analysis and the published consensus sequences

```
Lab1          5970 atctgcagaattcggcttgtaaacgggctgttttccaaacattgtgacttggctactgagtggggatacctggtttcatt
Lab2             1 atctgcagaattcggcttgtaaacgggctgttttccaaacattgtgacttggctactgagtggggatacctggtttcatt
GUSB           813 ------------------gtaaacgggctgttttccaaacattgtgacttggctactgagtggggatacctggtttcatt
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5890 ggcaatcttccagtatctctctcgcaaaaggaacgctgcacttttggttgtctctgccgagtgaagatccccttttttat
Lab2            81 ggcaatcttccagtatctctctcgcaaaaggaacgctgcacttttggttgtctctgccgagtgaagatccccttttttat
GUSB           751 ggcaatcttccagtatctctctcgcaaaaggaacgctgcacttttggttgtctctgccgagtgaagatccccttttttat
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5810 tccccagcactctcgtcggtgactgttcagtcatgaaatcggcaaaattccaaatgagctctccaaccacgtattttctg
Lab2           161 tccccagcactctcgtcggtgactgttcagtcatgaaatcggcaaaattccaaatgagctctccaaccacgtattttctg
GUSB           671 tccccagcactctcgtcggtgactgttcagtcatgaaatcggcaaaattccaaatgagctctccaaccacgtattttctg
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5730 cgtttttgatccagacccagatggtactgctctagcagacttttctggtactcttcagtgaacatcagaggtggatcctg
Lab2           241 cgtttttgatccagacccagatggtactgctctagcagacttttctggtactcttcagtgaacatcagaggtggatcctg
GUSB           591 cgtttttgatccagacccagatggtactgctctagcagacttttctggtactcttcagtgaacatcagaggtggatcctg
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5650 gtgaaaccctgcaatcgtttctgctccatactcgctctgaataatgggcttctgatacttcttataccagttctcaaact
Lab2           321 gtgaaaccctgcaatcgtttctgctccatactcgctctgaataatgggcttctgatacttcttataccagttctcaaact
GUSB           511 gtgaaaccctgcaatcgtttctgctccatactcgctctgaataatgggcttctgatacttcttataccagttctcaaact
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5570 gggtggccagctgcagctgaatcaactccaggtgcccgtagtcgtgataccaagagtagtagctgttcaaacagatcaca
Lab2           401 gggtggccagctgcagctgaatcaactccaggtgcccgtagtcgtgataccaagagtagtagctgttcaaacagatcaca
GUSB           431 gggtggccagctgcagctgaatcaactccaggtgcccgtagtcgtgataccaagagtagtagctgttcaaacagatcaca
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5490 tccacatacggagccccttgtctgctgcatagttagagttgctcacaaaggtcacaggccgggaggggtccaaggattt
Lab2           481 tccacatacggagccccttgtctgctgcatagttagagttgctcacaaaggtcacaggccgggaggggtccaaggattt
GUSB           351 tccacatacggagccccttgtctgctgcatagttagagttgctcacaaaggtcacaggccgggaggggtccaaggattt
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5410 ggtgtgagcgatcaccatcttcaagtagtagccagcagattctaggtgggacgcaggctcgttggccacagaccacatca
Lab2           561 ggtgtgagcgatcaccatcttcaagtagtagccagcagattctaggtgggacgcaggctcgttggccacagaccacatca
GUSB           271 ggtgtgagcgatcaccatcttcaagtagtagccagcagattctaggtgggacgcaggctcgttggccacagaccacatca
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5330 cgaccgcggggtggttcttgtccctacgcaccacttcttccatcacctgcatgtggtgatgcagagaaacgttgttgaag
Lab2           641 cgaccgcggggtggttcttgtccctacgcaccacttcttccatcacctgcatgtggtgatgcagagaaacgttgttgaag
GUSB           191 cgaccgcggggtggttcttgtccctacgcaccacttcttccatcacctgcatgtggtgatgcagagaaacgttgttgaag
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5250 aactgcggcagcgccaggcccacgccgggacactcatcgatgaccacaatcccatagcggtcacacatctgcatcacttc
Lab2           721 aactgcggcagcgccaggcccacgccgggacactcatcgatgaccacaatcccatagcggtcacacatctgcatcacttc
GUSB         1 11 aactgcggcagcgccaggcccacgccgggacactcatcgatgaccacaatcccatagcggtcacacatctgcatcacttc
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5170 ctctgcataggggtagtggctggtacggaaaaagccgaattccagcacactggcggccgttactagtggatccgagctcg
Lab2           801 ctctgcataggggtagtggctggtacggaaaaagccgaattccagcacactggcggccgttactagtggatccgagctcg
GUSB            31 ctctgcataggggtagtggctggtacggaaa----------------------------------------------
BCR                ------------------------------------------------------------------------------
BCR-ABL            ------------------------------------------------------------------------------

Lab1          5090 gtaccgagctcgaattcggcttgtccactcagccactggatttaagcagagttcaaatctgtactgcaccctggaggtgg
Lab2           881 gtaccgagctcgaattcggcttgtccactcagccactggatttaagcagagttcaaatctgtactgcaccctggaggtgg
GUSB               ------------------------------------------------------------------------------
BCR              1 ----------------------gtccactcagccactggatttaagcagagttcaaatctgtactgcaccctggaggtgg
BCR-ABL            ------------------------------------------------------------------------------
```

```
Lab1          5010 attcctttgggtattttgtgaataaagcaaagacgcgcgtctacagggacacagctgagccaaactggaacgaggaattt
Lab2           961 attcctttgggtattttgtgaataaagcaaagacgcgcgtctacagggacacagctgagccaaactggaacgaggaattt
GUSB               ----------------------------------------------------------------------------------
BCR             59 attcctttgggtattttgtgaataaagcaaagacgcgcgtctacagggacacagctgagccaaactggaacgaggaattt
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4930 gagatagagctggagggctcccagaccctgaggatactgtgctatgaaaagtgttacaacaagacgaagatccccaagga
Lab2          1041 gagatagagctggagggctcccagaccctgaggatactgtgctatgaaaagtgttacaacaagacgaagatccccaagga
GUSB               ----------------------------------------------------------------------------------
BCR            139 gagatagagctggagggctcccagaccctgaggatactgtgctatgaaaagtgttacaacaagacgaagatccccaagga
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4850 ggacggcgagagcacggacagactcatggggaagggccaggtccagctggacccgcaggccctgcaggacagagactggc
Lab2          1121 ggacggcgagagcacggacagactcatggggaagggccaggtccagctggacccgcaggccctgcaggacagagactggc
GUSB               ----------------------------------------------------------------------------------
BCR            219 ggacggcgagagcacggacagactcatggggaagggccaggtccagctggacccgcaggccctgcaggacagagactggc
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4770 agcgcaccgtcatcgccatgaatgggatcgaagtaaagctctcggtcaagttcaacagcagggagttcagcttgaagagg
Lab2          1201 agcgcaccgtcatcgccatgaatgggatcgaagtaaagctctcggtcaagttcaacagcagggagttcagcttgaagagg
GUSB               ----------------------------------------------------------------------------------
BCR            299 agcgcaccgtcatcgccatgaatgggatcgaagtaaagctctcggtcaagttcaacagcagggagttcagcttgaagagg
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4690 atgccgtcccgaaaacagacaggggtcttcggagtcaagattgctgtggtcaccaagagagagaggtccaaggtgcccta
Lab2          1281 atgccgtcccgaaaacagacaggggtcttcggagtcaagattgctgtggtcaccaagagagagaggtccaaggtgcccta
GUSB               ----------------------------------------------------------------------------------
BCR            379 atgccgtcccgaaaacagacaggggtcttcggagtcaagattgctgtggtcaccaagagagagaggtccaaggtgcccta
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4610 catcgtgcgccagtgcgtggaggagatcgagcgccgaggcatggaggaggtgggcatctaccgcgtgtccggtgtggcca
Lab2          1361 catcgtgcgccagtgcgtggaggagatcgagcgccgaggcatggaggaggtgggcatctaccgcgtgtccggtgtggcca
GUSB               ----------------------------------------------------------------------------------
BCR            459 catcgtgcgccagtgcgtggaggagatcgagcgccgaggcatggaggaggtgggcatctaccgcgtgtccggtgtggcca
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4530 cggacatccaggcactgaaggcagccttcgacgtcaataacaaggatgtgtcggtgatgatgagcgagatggacgtgaac
Lab2          1441 cggacatccaggcactgaaggcagccttcgacgtcaataacaaggatgtgtcggtgatgatgagcgagatggacgtgaac
GUSB               ----------------------------------------------------------------------------------
BCR            539 cggacatccaggcactgaaggcagccttcgacgtcaataacaaggacgtgtcggtgatgatgagcgagatggacgtgaac
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4450 gccatcgcaggcacgctgaagctgtacttccgtgagctgcccgagcccctcttcactgacgagttctaccccaacttcgc
Lab2          1521 gccatcgcaggcacgctgaagctgtacttccgtgagctgcccgagcccctcttcactgacgagttctaccccaacttcgc
GUSB               ----------------------------------------------------------------------------------
BCR            619 gccatcgcaggcacgctgaagctgtacttccgtgagctgcccgagcccctcttcactgacgagttctaccccaacttcgc
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4370 agagggcatcgctctttcagacccggttgcaaaggagagctgcatgctcaacctgctgctgtccctgccggaggccaacc
Lab2          1601 agagggcatcgctctttcagacccggttgcaaaggagagctgcatgctcaacctgctgctgtccctgccggaggccaacc
GUSB               ----------------------------------------------------------------------------------
BCR            699 agagggcatcgctctttcagacccggttgcaaaggagagctgcatgctcaacctgctgctgtccctgccggaggccaacc
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4290 tgctcaccttcctttccttctggaccacctgaaaagggtggcagagaaggaggcagtcaataagatgtccctgcacaac
Lab2          1681 tgctcaccttcctttccttctggaccacctgaaaagggtggcagagaaggaggcagtcaataagatgtccctgcacaac
GUSB               ----------------------------------------------------------------------------------
BCR            779 tgctcaccttcctttccttctggaccacctgaaaagggtggcagagaaggaggcagtcaataagatgtccctgcacaac
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4210 ctcgccacggtctttggccccacgctgctccggccctccgagaaggagagcaagctccctgccaaccccagccagcctat
Lab2          1761 ctcgccacggtctttggccccacgctgctccggccctccgagaaggagagcaagctccctgccaaccccagccagcctat
GUSB               ----------------------------------------------------------------------------------
BCR            859 ctcgccacggtctttggccccacgctgctccggccctccgagaaggagagcaagctccctgccaaccccagccagcctat
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4130 caccatgactgacagctggtccttgaagccgaattcgtaatcatgtcatagctgtttcctgtgtgaaattgttatccgct
Lab2          1841 caccatgactgacagctggtccttgaagccgaattcgtaatcatgtcatagctgtttcctgtgtgaaattgttatccgct
GUSB               ----------------------------------------------------------------------------------
BCR            939 caccatgactgacagctggtccttg--------------------------------------------------------
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          4050 cacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaa
Lab2          1921 cacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaa
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL            ----------------------------------------------------------------------------------
Lab1          3970 ttgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgggg
Lab2          2001 ttgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcgggg
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          3890 agaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagc
Lab2          2081 agaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL            ----------------------------------------------------------------------------------

Lab1          3810 ggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaag
Lab2          2161 ggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaag
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
```

```
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3730 gccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgccccccctgacgagcatcac
Lab2          2241 gccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgccccccctgacgagcatcac
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3650 aaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccct
Lab2          2321 aaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccctggaagctccct
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3570 cgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctc
Lab2          2401 cgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3490 atagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttcag
Lab2          2481 atagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaacccccgttcag
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3410 cccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagc
Lab2          2561 cccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagcagc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3330 cactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctaca
Lab2          2641 cactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctaca
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3250 ctagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggc
Lab2          2721 ctagaagaacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3170 aaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaaga
Lab2          2801 aaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaaga
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3090 tcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaa
Lab2          2881 tcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaa
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          3010 aaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtct
Lab2          2961 aaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtct
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------
Lab1          2930 gacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccc
Lab2          3041 gacagttaccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          2850 cgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcac
Lab2          3121 cgtcgtgtagataactacgatacgggagggcttaccatctggccccagtgctgcaatgataccgcgagacccacgctcac
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          2770 cggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctcc
Lab2          3201 cggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctgcaactttatccgcctcc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          2690 atccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgc
Lab2          3281 atccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgc
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          2610 tacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacat
Lab2          3361 tacaggcatcgtggtgtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacat
GUSB                ----------------------------------------------------------------------------------
BCR                 ----------------------------------------------------------------------------------
BCR-ABL             ----------------------------------------------------------------------------------

Lab1          2530 gatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgtta
Lab2          3441 gatcccccatgttgtgcaaaaaagcggttagctccttcggtcctccgatcgttgtcagaagtaagttggccgcagtgtta
```

```
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2450 tcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagta
Lab2         3521 tcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagta
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2370 ctcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgc
Lab2         3601 ctcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgc
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2290 cacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttg
Lab2         3681 cacatagcagaactttaaaagtgctcatcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttg
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2210 agatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagc
Lab2         3761 agatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagc
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2130 aaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttccttttc
Lab2         3841 aaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttccttttc
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         2050 aatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaata
Lab2         3921 aatattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaata
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1970 ggggttccgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaa
Lab2         4001 ggggttccgcgcacatttccccgaaaagtgccacctgacgtctaagaaaccattattatcatgacattaacctataaaaa
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------
Lab1         1890 taggcgtatcacgaggccctttcgtctcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggaga
Lab2         4081 taggcgtatcacgaggccctttcgtctcgcgcgtttcggtgatgacggtgaaaacctctgacacatgcagctcccggaga
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1810 cggtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcgg
Lab2         4161 cggtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcgg
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1730 ggctggcttaactatgcggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgt
Lab2         4241 ggctggcttaactatgcggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgt
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1650 aaggagaaaatgccgcatcaggcgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctctt
Lab2         4321 aaggagaaaatgccgcatcaggcgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctctt
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1570 cgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacga
Lab2         4401 cgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacga
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL           ----------------------------------------------------------------------------------

Lab1         1490 cgttgtaaaacgacggccagtgccaagcttgcatgcctgcaggtcgactctctgcaccaagctcaagaagcagagcggag
Lab2         4481 cgttgtaaaacgacggccagtgccaagcttgcatgcctgcaggtcgactctctgcaccaagctcaagaagcagagcggag
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL         1  ------------------------------------------------tctctgcaccaagctcaagaagcagagcggag

Lab1         1410 gcaaaacgcagcagtatgactgcaaatggtacattccgctcacggatctcagcttccagatggtggatgaactggaggca
Lab2         4561 gcaaaacgcagcagtatgactgcaaatggtacattccgctcacggatctcagcttccagatggtggatgaactggaggca
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL          33 gcaaaacgcagcagtatgactgcaaatggtacattccgctcacggatctcagcttccagatggtggatgaactggaggca

Lab1         1330 gtgcccaacatccccctggtgcccgatgaggagctggacgctttgaagatcaagatctcccagatcaagagtgacatcca
Lab2         4641 gtgcccaacatccccctggtgcccgatgaggagctggacgctttgaagatcaagatctcccagatcaagagtgacatcca
GUSB              ----------------------------------------------------------------------------------
BCR               ----------------------------------------------------------------------------------
BCR-ABL         113 gtgcccaacatccccctggtgcccgatgaggagctggacgctttgaagatcaagatctcccagatcaagaatgacatcca
```

```
Lab1          1250 gagagagaagagggcgaacaagggcagcaaggctacggagaggctgaagaagaagctgtcggagcaggagtcactgctgc
Lab2          4721 gagagagaagagggcgaacaagggcagcaaggctacggagaggctgaagaagaagctgtcggagcaggagtcactgctgc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        193 gagagagaagagggcgaacaagggcagcaaggctacggagaggctgaagaagaagctgtcggagcaggagtcactgctgc

Lab1          1170 tgcttatgtctcccagcatggccttcagggtgcacagccgcaacggcaagagttacacgttcctgatctcctctgactat
Lab2          4801 tgcttatgtctcccagcatggccttcagggtgcacagccgcaacggcaagagttacacgttcctgatctcctctgactat
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        273 tgcttatgtctcccagcatggccttcagggtgcacagccgcaacggcaagagttacacgttcctgatctcctctgactat

Lab1          1090 gagcgtgcagagtggagggagaacatccgggagcagcagaagaagtgtttcagaagcttctccctgacatccgtggagct
Lab2          4881 gagcgtgcagagtggagggagaacatccgggagcagcagaagaagtgtttcagaagcttctccctgacatccgtggagct
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        353 gagcgtgcagagtggagggagaacatccgggagcagcagaagaagtgtttcagaagcttctccctgacatccgtggagct

Lab1          1010 gcagatgctgaccaactcgtgtgtgaaactccagactgtccacagcattccgctgaccatcaataaggaagatgatgagt
Lab2          4961 gcagatgctgaccaactcgtgtgtgaaactccagactgtccacagcattccgctgaccatcaataaggaagatgatgagt
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        433 gcagatgctgaccaactcgtgtgtgaaactccagactgtccacagcattccgctgaccatcaataaggaagatgatgagt

Lab1           930 ctccggggctctatgggtttctgaatgtcatcgtccactcagccactggatttaagcagagttcaaaagcccttcagcgg
Lab2          5041 ctccggggctctatgggtttctgaatgtcatcgtccactcagccactggatttaagcagagttcaaaagcccttcagcgg
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        513 ctccggggctctatgggtttctgaatgtcatcgtccactcagccactggatttaagcagagttcaaaagcccttcagcgg
Lab1           850 ccagtagcatctgactttgagcctcagggtctgagtgaagccgctcgttggaactccaaggaaaaccttctcgctggacc
Lab2          5121 ccagtagcatctgactttgagcctcagggtctgagtgaagccgctcgttggaactccaaggaaaaccttctcgctggacc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        593 ccagtagcatctgactttgagcctcagggtctgagtgaagccgctcgttggaactccaaggaaaaccttctcgctggacc

Lab1           770 cagtgaaaatgaccccaacctttcgttgcactgtatgattttgtggccagtggagataacactctaagcataactaaag
Lab2          5201 cagtgaaaatgaccccaacctttcgttgcactgtatgattttgtggccagtggagataacactctaagcataactaaag
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        673 cagtgaaaatgaccccaacctttcgttgcactgtatgattttgtggccagtggagataacactctaagcataactaaag

Lab1           690 gtgaaaagctccgggtcttaggctataatcacaatggggaatggtgtgaagcccaaaccaaaaatggccaaggctgggtc
Lab2          5281 gtgaaaagctccgggtcttaggctataatcacaatggggaatggtgtgaagcccaaaccaaaaatggccaaggctgggtc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        753 gtgaaaagctccgggtcttaggctataatcacaatggggaatggtgtgaagcccaaaccaaaaatggccaaggctgggtc

Lab1           610 ccaagcaactacatcacgccagtcaacagtctggagaaacactcctggtaccatgggcctgtgtcccgcaatgccgctga
Lab2          5361 ccaagcaactacatcacgccagtcaacagtctggagaaacactcctggtaccatgggcctgtgtcccgcaatgccgctga
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        833 ccaagcaactacatcacgccagtcaacagtctggagaaacactcctggtaccatgggcctgtgtcccgcaatgccgctga

Lab1           530 gtatctgctgagcagcgggatcaatggcagcttcttggtgcgtgagagtgagagcagtcctggccagaggtccatctcgc
Lab2          5441 gtatctgctgagcagcgggatcaatggcagcttcttggtgcgtgagagtgagagcagtcctggccagaggtccatctcgc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        913 gtatctgctgagcagcgggatcaatggcagcttcttggtgcgtgagagtgagagcagtcctggccagaggtccatctcgc

Lab1           450 tgagatacgaagggagggtgtaccattacaggatcaacactgcttctgatggcaagctctacgtctcctccgagagccgc
Lab2          5521 tgagatacgaagggagggtgtaccattacaggatcaacactgcttctgatggcaagctctacgtctcctccgagagccgc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL        993 tgagatacgaagggagggtgtaccattacaggatcaacactgcttctgatggcaagctctacgtctcctccgagagccgc

Lab1           370 ttcaacaccctggccgagttggttcatcatcattcaacggtggccgacgggctcatcaccacgctccattatccagcccc
Lab2          5601 ttcaacaccctggccgagttggttcatcatcattcaacggtggccgacgggctcatcaccacgctccattatccagcccc
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL       1073 ttcaacaccctggccgagttggttcatcatcattcaacggtggccgacgggctcatcaccacgctccattatccagcccc

Lab1           290 aaagcgcaacaagcccactgtctatggtgtgtcccccaactacgacaagtgggagatggaacgcacggacatcaccatga
Lab2          5681 aaagcgcaacaagcccactgtctatggtgtgtcccccaactacgacaagtgggagatggaacgcacggacatcaccatga
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL       1153 aaagcgcaacaagcccactgtctatggtgtgtcccccaactacgacaagtgggagatggaacgcacggacatcaccatga

Lab1           210 agcacaagctgggcgggggccagtacggggaggtgtacgagggcgtgtggaagaaatacagcctgacggtggccgtgaag
Lab2          5761 agcacaagctgggcgggggccagtacggggaggtgtacgagggcgtgtggaagaaatacagcctgacggtggccgtgaag
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL       1233 agcacaagctgggcgggggccagtacggggaggtgtacgagggcgtgtggaagaaatacagcctgacggtggccgtgaag

Lab1           130 accttgaaggaggacaccatggaggtggaagagttcttgaaagaagctgcagtcatgaaagagatcaaacaccctaacct
Lab2          5841 accttgaaggaggacaccatggaggtggaagagttcttgaaagaagctgcagtcatgaaagagatcaaacaccctaacct
GUSB               ----------------------------------------------------------------------------------
BCR                ----------------------------------------------------------------------------------
BCR-ABL       1313 accttgaaggaggacaccatggaggtggaagagttcttgaaagaagctgcagtcatgaaagagatcaaacaccctaacct

Lab1             50 ggtgcggtcgactctagatgcatgctcgagcggccgccagtgtgatggat
Lab2           5921 ggtgcggtcgactctagatgcatgctcgagcggccgccagtgtgatggat
GUSB               ---------------------------------------------------
 BCR               ---------------------------------------------------
```

```
BCR-ABL        1393  ggtgc-------------------------------------------
```

Two differences between the consensus sequences from Genbank and the sequences of the fragments present in pIRMM-0099 ware indicated in grey.

Annex G: Results of the Characterisation study for the copy number concentration of the plasmid

Table G1: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623a

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1	973463	12.66	n.c.
	2	884230		
	3	1091851		
	4	1216974		
	5	1085891		
	6	1244606		
2	7	990555	10.11	
	8	1076161		
	9	1075208		
	10	n.a.		
	11	1318816		
	12	1203224		
3	13	850527	16.88	
	14	971317		
	15	888295		
	16	1020358		
	17	1302875		
	18	1187144		
all measurements		1081264	13.03	

n.a.: not accepted, n.c.: cannot be calculated as $MS_{between} < MS_{within}$

Table G2: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623b

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1	89381	17.86	5.03
	2	83629		
	3	101269		
	4	120777		
	5	120027		
	6	131755		
2	7	100105	11.64	
	8	108482		
	9	107063		
	10	131156		
	11	130595		
	12	118884		
3	13	84084	14.17	
	14	93263		
	15	87540		
	16	99239		
	17	120806		
	18	110646		
all measurements		107706	15.12	

Table G3: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623c

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1 2 3 4 5 6	8619.9 10290.2 11565.9 10984.8 10522.5 10582.7	9.46	6.25
2	7 8 9 10 11 12	9067.9 10612.3 10842.0 13205.1 12194.6 10508.6	12.95	
3	13 14 15 16 17 18	9232.2 8475.1 9956.1 8255.1 9512.8 11344.6	11.73	
all measurements		10320.7	12.68	

Table G4: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623d

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1 2 3 4 5 6	939.7 1033.0 1113.7 1074.2 1100.1 984.1	6.40	6.70
2	7 8 9 10 11 12	869.1 1119.0 1077.3 1244.1 1156.7 1039.1	11.61	
3	13 14 15 16 17 18	893.2 838.2 1028.9 818.2 960.8 1041.7	10.15	
all measurements		1018.4	11.20	

Table G5: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623e

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1 2 3 4 5 6	90.1 92.2 104.6 120.4 114.1 99.9	11.40	8.22
2	7 8 9 10 11 12	94.3 110.0 104.3 115.7 127.5 127.7	11.43	
3	13 14 15 16 17 18	92.8 82.7 88.3 95.5 95.8 107.9	8.72	
all measurements		103.5	12.81	

Table G6: Results of the characterisation study of the copy number concentration of the plasmid in ERM-AD623f

Laboratory number	Measurement number	Result	$s_{within\ labs}$ [%]	$s_{between\ labs}$ [%]
1	1 2 3 4 5 6	n.a. 8.72 9.60 12.63 9.66 8.61	15.43	5.78
2	7 8 9 10 11 12	8 11.75 9.6 9.43 13.35 13.3	18.72	
3	13 14 15 16 17 18	9.16 8.88 7.78 9.66 10.54 8.72	6.68	
all measurements		9.96	15.96	

n.a.: not accepted

Annex H: Commutability study

Table H1: qPCR methods used within the commutability study

Laboratory number	Control gene	Reference of qPCR method	qPCR instrument
1	BCR	[11]	ABI 7000 (Applied Biosystems)
2	ABL	[5,6]	ABI 7000 (Applied Biosystems)
3	GUSB	[5,6]	ABI 7500 FAST (Applied Biosystems)
4	ABL	[5,6]	ABI 7900 HT (Applied Biosystems)
5	ABL	[5,6]	ABI 7900 HT (Applied Biosystems)
	GUSB	[5,6]	
6	ABL	Home made	LightCycler480 (Roche)
	GUSB	Home made	
7	ABL	[26]	LightCycler1.5 (Roche)
	GUSB	[23]	
8	BCR	[11]	ABI7500 (Applied Biosystems)
9	ABL	[5,6]	Rotorgene 6000 (Corbett)
	BCR	[11]	
	GUSB	[5,6]	

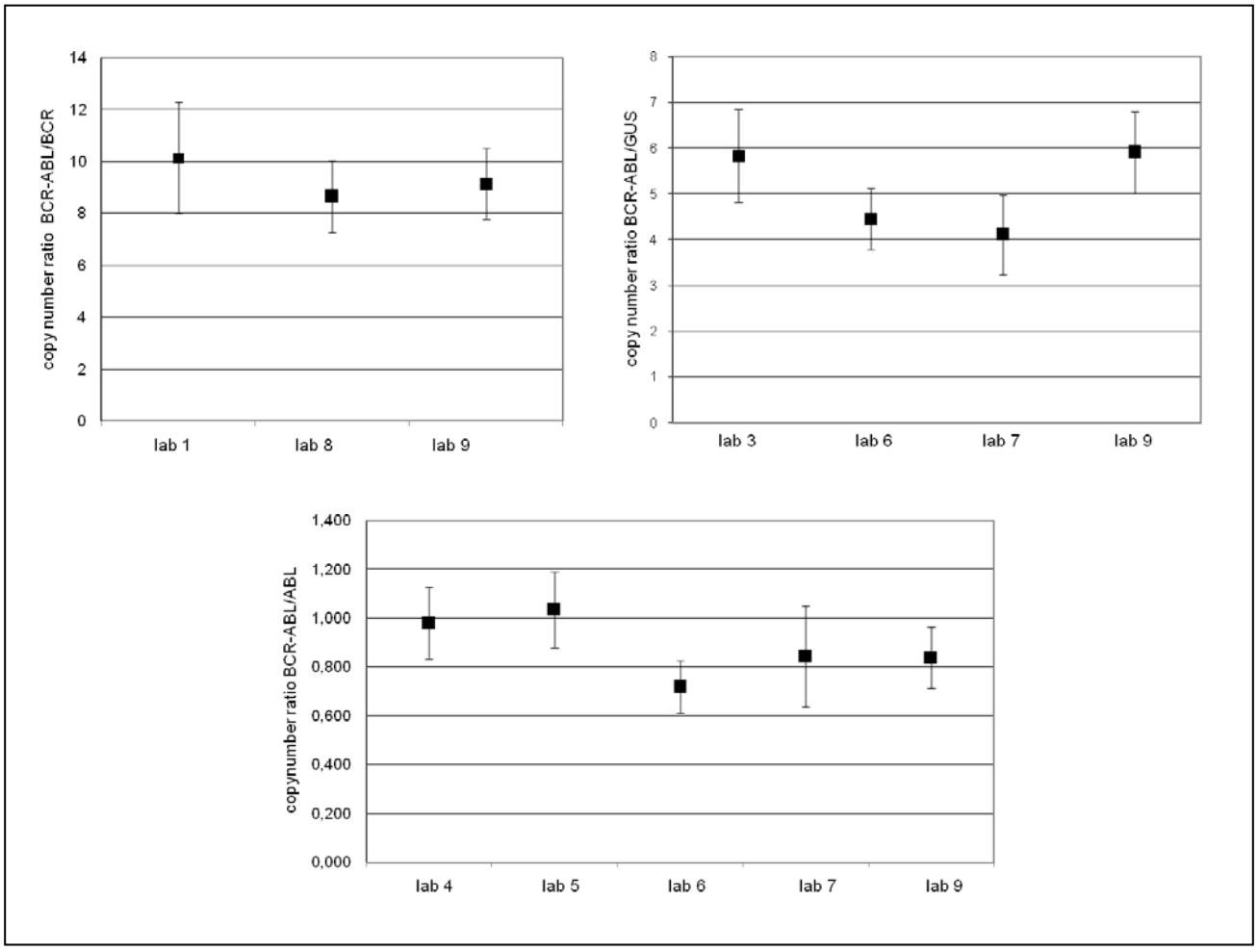

Figure H: Copy number ratios of BCR-ABL b3a2 to the three control genes: BCR, ABL and GUSB measured for two cDNA solutions by different expert laboratories using ERM-AD623 as calibrant. Blackpoint represents the mean of the ratio calculated for the two cDNA solutions. The bars represent an estimation of the measurement uncertainty based on a relative standard deviation of 30 % for qPCR measurements.

European Commission

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Title: The certification of the copy number concentration of solutions of plasmid DNA containing a BCR-ABL b3a2 transcript fragment: Certified Reference Materials: ERM®- AD623a, ERM®-AD623b, ERM®-AD623c, ERM®-AD623d, ERM®-AD623e, ERM®-AD623f

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Abstract
This report describes the production of a set of plasmid solutions, ERM®-AD623a, b, c, d, e, and f, certified for the copy number ratio of specific desoxyribonucleic acid (DNA) fragments per plasmid and for the copy number concentration of the plasmid. The material has been produced following ISO Guide 34:2009 [1].
DNA fragments specific for the transcript of breakpoint cluster region gene (*BCR*), the transcript of the glucuronidase beta gene (*GUSB*) and the fusion transcript from the *BCR* gene and the c-abl oncogene 1 (*BCR-ABL* b3a2) were cloned into a pUC18 vector to construct the pIRMM-0099 plasmid. The sequence identity of the pIRMM-0099 plasmid was determined by dye terminator cycle sequencing of the entire plasmid. The plasmid was diluted to six different concentration levels.
Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2]. The material was characterised by an intercomparison among laboratories of demonstrated competence and adhering to ISO/IEC 17025:2005 [3]. Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible heterogeneity and instability and to characterisation.
The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Material consortium.

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