Deliverables

No.	Deliverable	Due date	Completion/ revised due date
5.4	Development of a library preparation protocol adopted to highly degraded DNA		Sept 2011
5.5	Protocol delivered adopted to the characteristics of hominin aDNA		Sept 2011
5.6	Protocol for the immortalization of aDNA libraries		August 2011
5.7	Development of a showcase capture-NGS protocol		October / November 2012
5.8	Report on the success rate of mtCapture protocols applied to various different museum and archaeological specimens	May 2013	
5.9	Final protocol for mitochondrial and nuclear capture enrichment	August 2013	

Summary of project achievements

5.4 Development of a library preparation protocol adapted to highly degraded DNA

Comparison between a modified library protocol with barcoded adapters based on the Illumina 2009 workflow and a protocol developed by *Meyer et al. 2010*, resulted in a much better performance of the Meyer and Kircher protocol. Nevertheless, we introduced some modifications to it, e.g. we were able to reduce the recommended purification steps, which we think is mandatory when working with highly degraded human material to avoid contaminations and loss of highly fragmented small DNA molecules. We create libraries modified for the specific needs of ancient DNA. This means that:

a) Enzymatic reactions have been adapted to low-input DNA

b) Blunt-end ligation for ligation of adapter sequences to avoid high amounts of adapter dimers

d) Sample specific indices are added during PCR reaction. This allows assigning each library individually with index sequences after preparation.

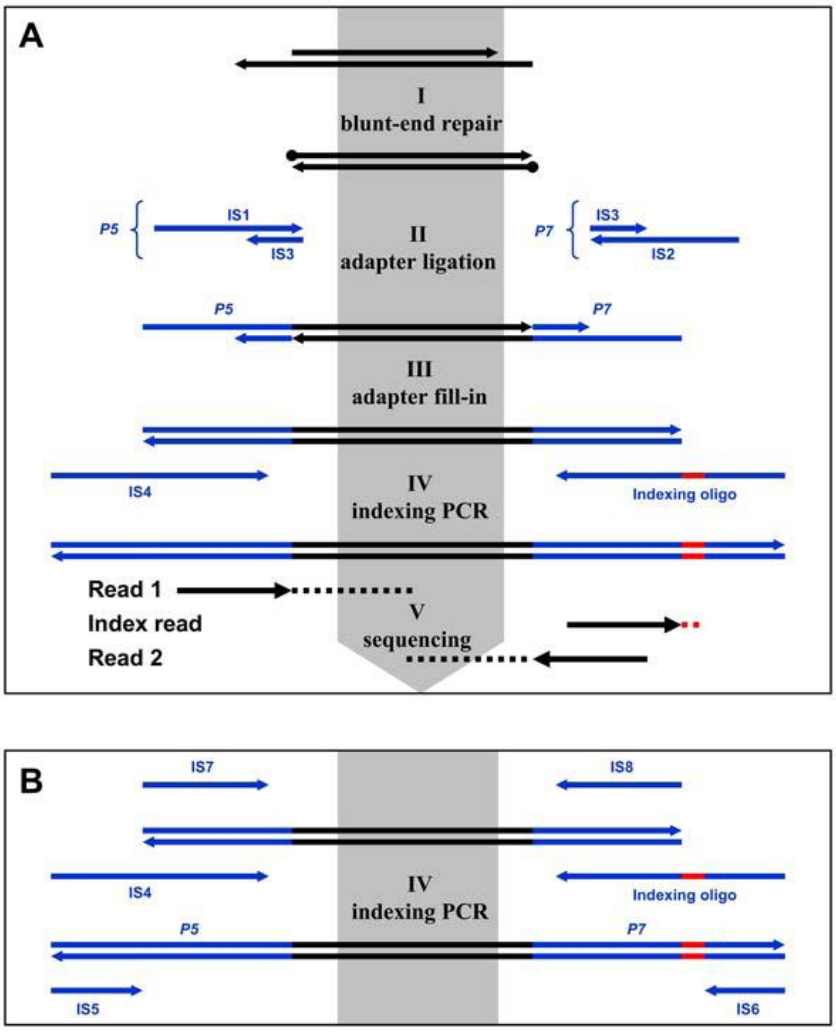

Fig1: Schematic illustration of library preparation (Meyer et al. 2010)

Libraries with our first modified protocol were built in specific ancient DNA laboratory from 50 µl of unshared DNA extract mainly following the protocol developed Meyer et al. 2010 with given modifications. All Index Primers sequences were designed and ordered without the additional base "T" at 3' ends (Biospring GmbH). Extracts are first purified after the adapter ligation step to avoid loss of DNA molecules due to small fragment size in combination with cut-off values of Qiagen MinElute Purification Kit and purification procedure itself. End-repair reaction mix made up as follows: 7,05 µl T4 Ligase Buffer (10x), 0,7 µl dNTPs (10mM each), 0,35 µl BSA (20mg/ml), 3,5 µl T4 PNK (10 U/µl), 1,4 µl T4 DNA Pol (5U/µl) and 7,05 µl UV-HPLC-H2O. After incubation for 15 min at 25°C and 5 min at 12°C, T4 DNA Polymerase has to be inactivated at 75°C for 10 minutes, the reaction should cool down to room temperature. Subsequent reaction volume for Adapter ligation was increased to 100 µl adding 5 µl ATP (10 mM), 10 µl PEG-4000 (50%), 1 µ Adapter-Mix (each 100 µM), 2,5 µl T4 DNA Ligase (5U/µl), and 11,5 µl, UV-HPLC-H2O and incubated at 22 °C for at least 30 min followed by a clean-up step with Qiagen MinElute Purification Kit. Adapter sequence Fill-in reaction was performed

as described by *Meyer et al. 2010*. Libraries were amplified by 20 cycles of PCR comprised 0,5 µl AmpliTaq Gold DNA Polymerase, 5 µl GeneAmp 10 X PCR Gold Buffer, 5 µl MgCl2 Solution (25 mM), 1 µl dNTP Mix (10 mM each), 1 µl BSA (20mg/ml), Is4 Primer (10 µM) and Indexing Primer (10 µM) and 25,5 µl Uv-HPLC-H2O. For amplification of library molecules without adding full adapter-sequence before capture enrichment reaction Primer Is5 (10 µM) and Is6 (10 µM) were used instead of Is4 and Indexing Primer. Indexed or only amplified libraries were purified and eluted in 50 µl elution buffer. Blank controls were carried out for every library preparation and DNA amplification to control the reagents in every step and check for cross-contamination during hands-on time. We created an artificial Library-Control (LC) to guarantee full efficiency of all used enzymes during preparation. LC sequence is assembled from a 40 bp non-matching DNA region and Roche Primer Sequences A and B on both ends of the nucleotide.

(5'CCATCTCATCCCTGCGTGTCAACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAACCTATCCCCTGTGTGCCTTG-3')

Based on the extension of fragments by ligation of adapters a shift in length could act as a positive control. Amplified Libraries, all blank controls and diluted Library-Control (LC) were visualized and screened using an Agilent Bioanalyzer DNA High Sensitivity chip.

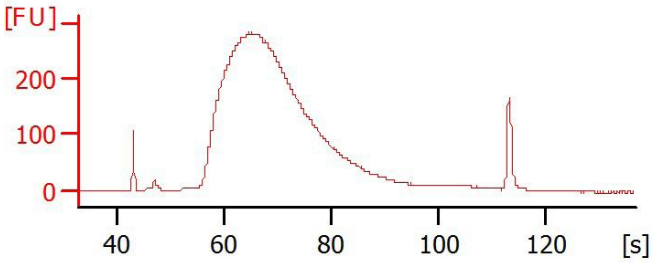

Fig2: Sample with additional Index-Adapter-Sequence after Library preparation and amplification step

5.5 Protocol delivered adopted to the characteristics of hominin aDNA

To provide the best conditions for human mitochondrial and nuclear DNA enrichment we improve the library protocol once more. The aim was to found out at which point the library protocol we could omit one of the cleaning steps without effecting the subsequent enzymatic reaction. For this we compare two different Meyer library protocols (Meyer et al. 2010 and Kircher et al. 2011) and a combination of both with our current library protocol and evaluate the results with a sensitive qPCR measurement.

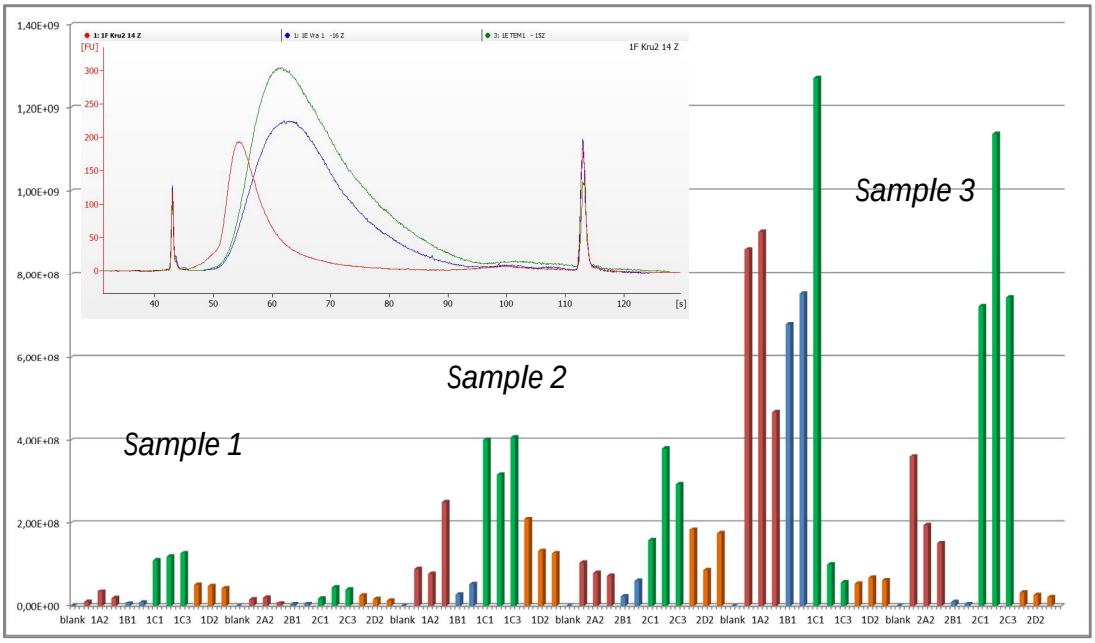

Fig3: Comparison of different library protocols in replicates on three various archeological samples
Blue: Modified Meyer et al. 2010; Red: Meyer et al. 2010; Green: Kircher et al. 2011; Orange: Combination of blue and green

Although the loss of small DNA fragments due the cut-off of a Qiagen MinElute PCR Purification Kit is high it seems to perform best to eliminate the purification step directly before PCR reaction instead of skip it before the ligation step. These results correspond with the library protocol from *Kircher et al. 2011.* As a modification we use our well-established AmpliTaq Gold DNA Polymerase protocol for Library amplification step as described above. Applied Primer and Adapter-Oligonucleotides are the same for both modified protocols.

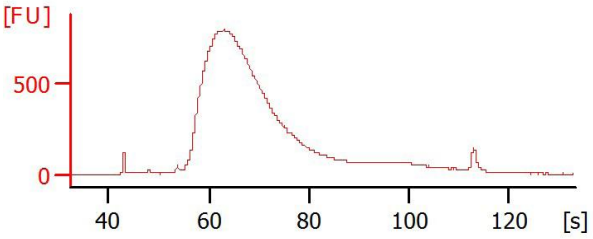

Fig4: Sample with additional Index-Sequence after improved Library preparation and amplification step

5.6 Protocol for the immortalization of aDNA libraries

After library preparation, aDNA molecules can be immortalized by re-amplification using the adapter sequences as priming sites for PCR primers. We could show that we can re-amplify the library from aliquots without losing substantial informational content. We used a multiplex PCR to amplify fragments of varying length and from different chromosomes from a library. After re-amplifying the library four times, we were still able to amplify and sequence the same fragments from it with Sanger sequencing.

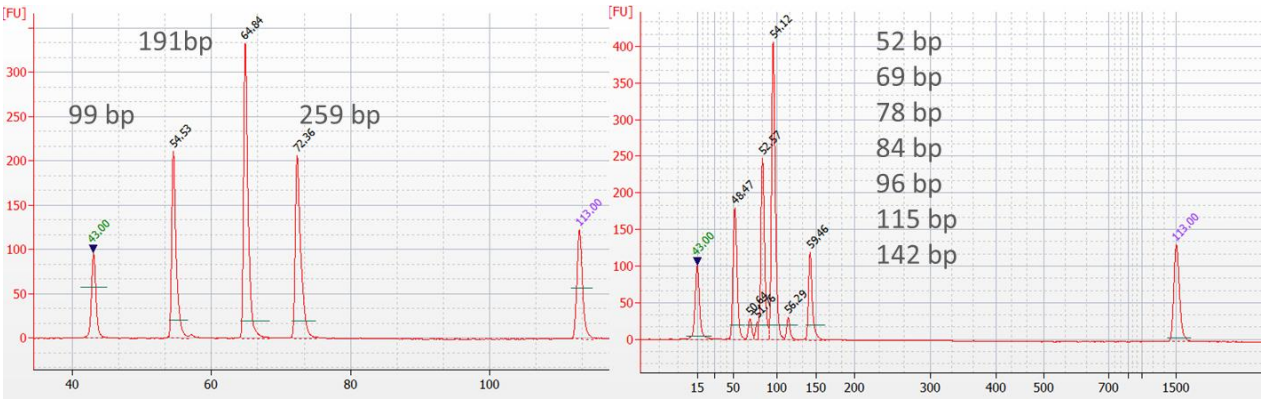

Fig5: Reamplified library after Multiplex-PCR; left: MP-PCR with mitochondrial primer pairs; right: MP-PCR with nuclear primer-pairs

However, we don´t know how much the loss of diversity is on a genomic sequence level and how stable libraries are over longer time periods. Currently, there is no published study on this.

Due to the high data output of Illumina Genome Analyzer a sixth of one lane on a flowcell could reach sufficient data output for further data analyses. In combination with the high sensitivity of Illumina sequencing technology and additional bridge amplification on a cluster station only a small concentration of DNA libraries is needed. On average the recommended concentration lay between 50 to 100 ng and average molarity lay between 4 to 10 nM. This provides the capability to sequence one prepped library up to ten times.

5.7 Development of a showcase capture-NGS protocol

SureSelect In-solution target capture enrichment was performed according to manufacture's protocol except of few modifications. Due to our tiling design of mitochondrial bait library the recommended volume could be diluted by 6-fold to achieve a theoretical coverage of nearly 100 fold.

For libraries which are already indexed the *Agilent SureSelectIndexing Block #3* was replaced by our index-sequence specific self-designed Blocking-Oligo-Mix containing BO1.P5.F, BO2.P5.R, Ind*Block_F, Ind*Block_R (100 µM each). Whereby asterisk symbol acts as a placeholder for individual index number. Capture products were amplified divided in three to four parallels with AmpliTaq Gold DNA Polymerase as described above by using Primer-pair Is5 and Is6. To add individual index sequence different primer system (Is4 and Index-Primer) had to be chosen.

Reamplification of capture products was performed using Herculase DNA Polymerase, 5 x Herculase Buffer, DMSO, Primer Is5 and Is6 (100 µM) with following cycling conditions: after initial denaturation for 30 s at 98 C, 8-10 cycles 98 C 10 s, 60 C 30 s, 72 C 30 s, 72 5 min.

DNA was purified using MSB Spin PCRapace from STRATEC Molecular GmbH and eluted in 40-60 µl Elution buffer.

All post-library-amplification experiments and hybridization reactions were carried out in specific laboratory that is exclusively used for NGS experiments. Working steps were performed in UV-radiated boxes. To avoid cross-contamination between indexed or non-indexed library molecules all suppliers were incubated with bleach and UV-irradiated after usage.

Libraries and all blank controls were visualized and screened using an Agilent Bioanalyzer DNA High Sensitivity chip.

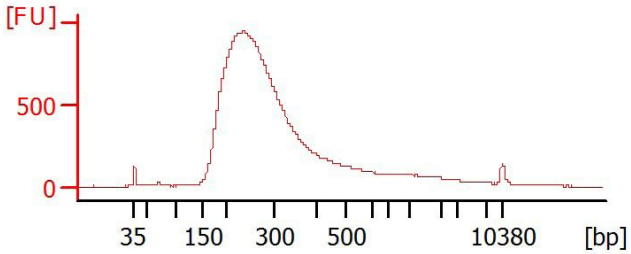

Fig.5: Sample after In-Solution enrichment process of human mitochondrial DNA

Capture efficiency is here measured as the percentage of sequences belonging to one sample (including amplicon duplicates) that can be mapped to the human mtDNA reference sequence (rCRS).

For the modern samples the capture efficiency of 92,76% (Figure 2: modern A) and 70,81% (Figure 2: modern B) was reached. This is coherent as the latter was artificially degraded to simulate the conditions of prehistoric DNA.

On average an efficiency of 25,3% (⍰ 29,5%) can be reported for the 33 archaeological samples, with a maximum at 80,24% in a sample from Kaliningrad (Figure 2: 8700BC) and a minimum at 0,03% in a sample from Greece (Figure 2: 7000BC). The success rate of the human mtDNA capture varies drastically between samples.

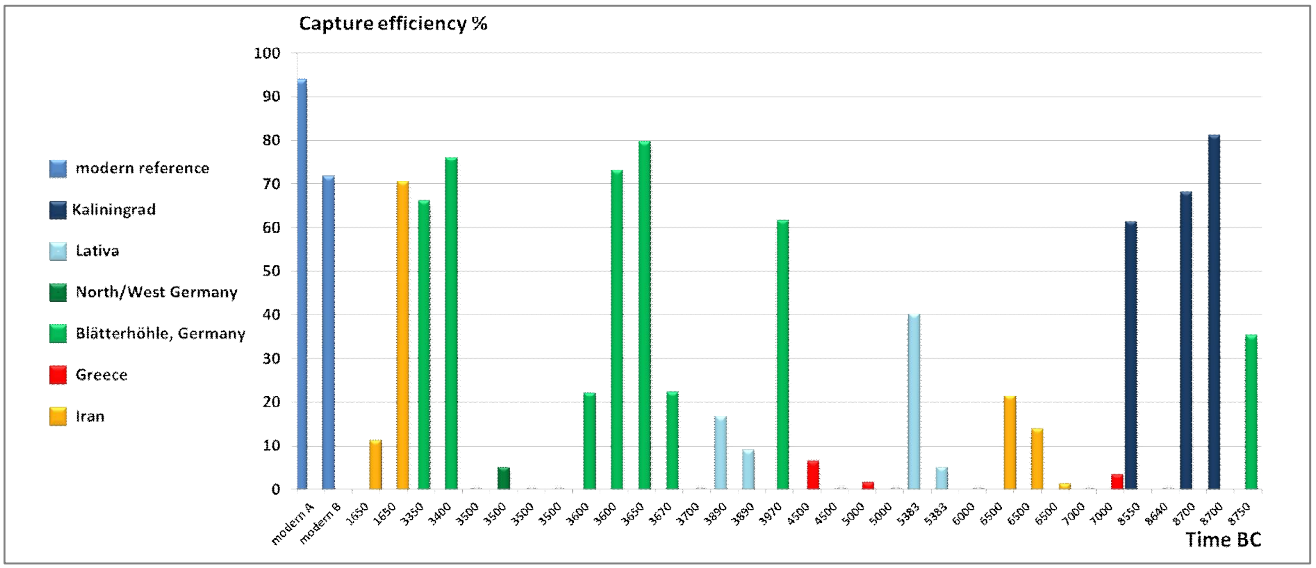

Fig6: x axis shows samples distributed according to their age; y axis shows the percentage of reads that mapped to the human mitochondrial genome (capture efficiency); different colours indicate a different region of sample origin

In-solution full mtDNA genome capture-NGS can successfully be performed on a large number of prehistoric archaeological samples as long as the sample is in a good state of preservation. To determine the sample state, PCR success rate alone is not a sufficient criterion. However, less well-preserved samples from southern latitudes do not produce sufficient sequences reads, probably due to the oversized bait length of 120 bp and low stringency of hybridization reaction.

5.8 Report on the success rate of mtCapture protocols applied to various different museum and archaeological specimens

These deliverables will be appear as a publication in May / June 2013. We will summarize our results of applying different protocols on various archeological samples and present our self-written bioinformatics script for correction of index misidentification between samples on one Illumina lane.

Abstract

Since the advent of second-generation sequencing the potential for studies concerning ancient DNA (aDNA) has grown enormously. If combined with targeted capture-enrichment methods, two major hindrances in aDNA research can theoretically be overcome, the low amount of endogenous DNA and the presence of contaminating DNA. To make economical use of this approach, samples can be provided with specific indices and sequenced in parallel. Here we used Agilent's SureSelect in solution capture with different protocols, on 38 Human archaeological samples of different age and state of preservance. To allow for simultaneous sequencing of all samples on six lanes of the Illumina Hiseq, each sample was provided with one specific Index. Although it is known that this multiplexing approach will lead to miss assignments of indices between simultaneously sequenced Samples, the herby obtained results show that it is possible to identify and remove miss assigned indices and still be able to perform downstream analysis. Further the data gained during these experiments allows us to state the efficiency of several adjustments made to the standard SureSelect protocol.

Protocoll	Nr. of samples	On Target [%]	Genome covered no decon [%]	Genome covered decon [%]
Flow Cell	38	22.81 +/- 26.40	92.37 +/- 14.53	70.98 +/- 31.13
Index	22	13.86 +/- 22.61	88.56 +/- 17.73	62.47 +/- 33.46
No index	16	35.12 +/- 26.89	97.60 +/- 5.46	82.68 +/- 23.89
Reamplified	19	17.01 +/- 22.14	89.93 +/-19.23	70.82 +/- 31.04
Not reamplified	19	28.6 +/- 29.49	94.79 +/- 7.18	71.13 +/- 32.05

Table 1: Showing average values for the data of the whole Flow Cell and groups within all samples according to different lab protocols used; Samples occur in two protocols either in Index or No index and in Reamplified or Not reamplified; +/-Value = standartdeviation ; Index = capture of indexed library; No index = capture with non indexed library; Reamplified =additional PCR of capture products; Not reamplified = only one round of PCR after capture; On Target = Percentage of reads from total aligned to the mtDNA; Genome covered no decon = Percentage of Genome covered before decontamination; Genome covered decon = Percentage of mtDNA covered after performing bioinformatical decontamination.

6.2 Protocol for contamination free DNA extraction from human bone for subsequent 454 sequencing reported (Deliverable JRA1)

Different phenol-chloroform based DNA extraction methods for ancient Neolithic cattle bones were investigated to find one which provides the best solution for samples of different states of preservation.

The test series were dealing with EDTA incubation time and temperature, prior treatment with bleach, additional linkage breaking chemicals and raised amounts of EDTA.

Summarizing the results it has been found that for well-preserved samples, every method gives more or less satisfying results. But DNA in worse preserved samples seems to be destroyed by a prior treatment of bone powder with bleach. In general, a longer incubation time with EDTA (48 hours) seems to lead to better results than the standard of about 18 hours. Warmer incubation temperatures of 37°C perform slightly better than ones of 5°C. The usage of additional linkage breaking chemicals (according to Barnett and Larson 2012) supply a worse performance on DNA yield compared to standard – especially for badly preserved samples.

A combining protocol using a) higher amounts of EDTA over 48 hours or b) higher amounts of EDTA for 48 hours plus additional linkages breaking chemicals revealed that we should contemplate the idea of applying differ-ent protocols to bones of different states of preservation. For good samples treatment a) and b) worked equally well and outperform all other protocols. But on the contrary, for more badly preserved samples these treatments lower the total DNA-yield and only an elongation of incubation time and increasing EDTA amounts can be recom-mended.

The improved extraction protocol is initially independent of the further processing of the sample.

But as described in 5.4 and 5.4, our library preparation protocol including technology specific adapter sequences is designed for further Illumina sequencing. Due to shorter read length and the possibility of an additional index read Illumina sequencing is preferred to work with most archeological samples.

After successful extraction we would therefore recommend a library creation with our established protocol (JRA2 5.5).

5.5 Protocol delivered adopted to the characteristics of hominin aDNA

Blunt-End Repair					
reagent	***volume [µl]***	***final conc.***	***MM + 10%***	***sample count***	
H_2O	6,7			1. prepare MM and add 20µl to 50µl of extract	
Tango Puffer (10x)	7	1x			
dNTPs (jedes 10mM)	0,7	100µM (je dNTP)		2. incubate samples in two steps:	
ATP (100mM)	0,7	1mM		I. 25°C - 15 min	
T4 PNK (10 U/µl)	3,5	0,5U/µl		II. 12°C - 5 min	
T4 DNA Pol (5U/µl)	1,4	0,1U/µl		3. place all samples on ice	
target	50			4. purify samples (silica based) and elute with 22µl elution buffer	
total	**70**				

Adapter Ligation					
reagent	***volume (µl)***	***final conc.***	***MM + 10%***	***sample count***	
H_2O	10			1. add 1µl adapter-mix (100µM) to the purified DNA and mix by pipetting at least 10 times	
T4 DNA Ligase Puffer (10x)	4	1x			
PEG-4000 (50%)	4	5%		2. add 19µl MM and mix by pipetting for at least 10 times	
T4 DNA Ligase (5U/µl)	1	0,125 U/µl		3. incubate the samples at 22°C for 30 min	
Adaptermix (je 100µM)	1	2,5 µM		4. purify the samples: a) silica method b) bead purification a,b - elute with 22µl elution buffer	
target	20				
total	**40**				

Adapter Fill-In					
reagent	***volume (µl)***	***final conc.***	***MM + 10%***	***sample count***	
H_2O	13,5			1. add 20µl of the MM to the purified, ligated product	
ThermoPol Puffer (10x)	4	1x			
dNTPs (je 10mM)	1	250µM		2. incubate at 37°C for 20 min	
Bst Polymerase, großes Fragment (8U/µl)	1,5	0, 3 U/µl		3. chill samples on ice - seal the tubes with parafilm - place the samples on 80°C for 20 min	
target	20			4. make an 1:40 diluted aliquot for qPCR after Fill In (1µl to 39µl water)	
total	**40**				

Inactivated Fill-In product will be amplified directly in 3 parallels - 11µl each

Index-PCR (Ampli Taq)

Reagenz	***Volume (µl)***	***End conc.***
H_2O	24,5	
Ampli Taq Gold Puffer (10x)	5	1x
$MgCl_2$ (25 mM)	5	2,5 mM
BSA (20 mg/ml)	1	0,4 mg/ml
dNTPs (je 10mM)	1	200µM
Primer IS4 (10µM]	1	200nM
Ampli Taq Pol (5 U/µl)	0,5	0,05 U/µl
separately to each sample:		
Index-Primer (10µM)	1	200nM
target	11	
total	50	

PCR without index Primer system IS7/IS8

Reagenz	***Volume (µl)***	***End conc.***
H_2O	24,5	
AmpliGold Puffer (10x)	5	1x
$MgCl_2$ (25 mM)	5	2,5 mM
BSA (20 mg/ml)	1	0,4 mg/ml
dNTPs (je 10mM)	1	200µM
Primer IS7 (10µM]	1	200nM
Primer IS8 [10µM]	1	200nM
AmpliTaq Gold 5U/µl	0,5	0,05 U/µl
target	11	
total	50	

PCR conditions			
step	***temperature***	***time***	
initial denaturation	95 °C	6 min	
denaturation	95 °C	40 sec	repeat 18 cycles
annealing	60 °C	40 sec	
elongation	72 °C	40 sec	
final elongation	72 °C	10 min	

5.6 Protocol for the immortalization of aDNA libraries

Protocol for re-amplification of indexed libraries

PCR Primer system IS5/IS6

Reagenz	***Volumen (µl)***	***End conc.***
H_2O	24,5	
AmpliGold Puffer (10x)	5	1x
$MgCl_2$ (25 mM)	5	2,5 mM
BSA (20 mg/ml)	1	0,4 mg/ml
dNTPs (je 10mM)	1	200µM
Primer IS5 (10µM]	1	200nM
Primer IS6 [10µM]	1	200nM
AmpliTaq Gold 5U/µl	0,5	0,05 U/µl
target	11	
total	50	

PCR conditions			
step	***temperature***	***time***	
initial denaturation	95 °C	6 min	
denaturation	95 °C	40 sec	repeat 18 cycles
annealing	60 °C	40 sec	
elongation	72 °C	40 sec	
final elongation	72 °C	10 min	

5.7 Development of a showcase capture-NGS protocol

Notes:
Use the same blank control as for library preparation
Prepare LowProfile plate with piercable foile

Target Enrichment Protokoll SureSelect
Day 1:
Storage

Table 16 SureSelect Reagent Kit Components

Kit Component	250 RXN Kit	50 RXN Kit	Storage
SureSelect Hyb # 1	bottle	tube with orange cap	Room Temperature
SureSelect Hyb # 2	tube with red cap	tube with red cap	Room Temperature
SureSelect Hyb # 4	bottle	tube with black cap	Room Temperature
3M Sodium Acetate	tube with clear cap	tube with clear cap	Room Temperature
SureSelect Binding Buffer	bottle	bottle	Room Temperature
SureSelect Wash Buffer #1	bottle	bottle	Room Temperature
SureSelect Wash Buffer #2	bottle	bottle	Room Temperature
SureSelect Elution Buffer	bottle	bottle	Room Temperature
SureSelect Neutralization Buffer	bottle	bottle	Room Temperature
SureSelect Hyb # 3	tube with yellow cap	tube with yellow cap	-20°C
SureSelect Block #1	tube with green cap	tube with green cap	-20°C
SureSelect Block #2	tube with blue cap	tube with blue cap	-20°C
SureSelect PE Block #3	tube with brown cap	tube with brown cap	-20°C
SureSelect RNase Block	tube with purple cap	tube with purple cap	-20°C
SureSelect GA PCR Primers	tube with clear cap	tube with clear cap	-20°C
SureSelect Oligo Capture Library	tube with red cap	tube with red cap	-80°C

Step 1. Hybridize the library
For each sample library prepared, do one hybridization and capture. Do not pool samples at this stage. The hybridization reaction requires 500 ng of DNA with a maximum volume of 3.4 µL.

1 If the prepped library concentration is below 147 ng/µL, use a vacuum concentrator to concentrate the sample at ≤ 45°C.
2 Mix the components in Table 17 at room temperature to prepare the hybridization buffer.

Table 1 Hybridization Buffer

Reagent	Volume for 1 capture (µl)
SureSelect Hyb # 1	25
SureSelect Hyb # 2 (red cap)	1
SureSelect Hyb # 3 (yellow cap)	10
SureSelect Hyb # 4	13
Total	49

4 If precipitate forms, warm the hybridization buffer at *65°C for 5 minutes.*

Bait library: (On ice!!!)
5 Prepare the SureSelect capture library mix for target enrichment:

➔ *Later row C*

A Keep tubes on ice until step 10!!!
B For each sample, add the amount of SureSelect capture library as listed in Table 18 (2µl). On ice!!
C Use nuclease-free water to prepare a dilution of the RNase Block (purple cap) as listed in Table 18. Prepare enough RNase Block dilution for all samples, plus excess.
D Add the amount of diluted RNase Block listed in Table 2 for each capture library, and mix by pipetting.

Table 2 SureSelect Capture Library

Capture Size	Volume of SureSelect Library	RNase Block Dilution (Parts RNase block: Parts water)	Volume of RNase Block Dilution to Add
< 3.0 Mb	2 µL	1:9 (10%)	5 µL
≥ 3.0 Mb	5 µL	1:3 (25%)	2 µL

Block Mix

6 Mix the contents to make the correct amount of SureSelect Block mix for the number of samples used.
→ later *row D*

I.) Without Index, Block mix I (Agilent-protocol)

Reagent	1 Reaction
SureSelect Indexing Block #1 (green cap)	2,5
SureSelect Block #2 (blue cap)	2,5
SureSelectIndexing Block #3 (brown cap)	0,6
Total	5,6

II.) With Index, Block mix II

Reagent	1 Reaction
SureSelect Indexing Block #1 (green cap)	2,5
SureSelect Block #2 (blue cap)	2,5
Total	5,0

Distribute Library
7 Prepare the prepped library for target enrichment.
A Add 3.4 µL of 147 ng/µL prepped library to the "D" row in the PCR plate. Put each sample into a separate well.

Without Index:
B Add 5.6 µL of the SureSelect Block Mix I in row D.
With Index:
B Add 5.0 µL of the SureSelect Block Mix II and 1,2 µl Block Mix to each Sample with Index in row D.
C Mix by pipetting up and down.
D Seal the wells of row "B" with PCR-foile and put the PCR plate in the thermal cycler. Do not heat the Hybridization Buffer or capture library yet, only the prepped library with blockers.
E Run the following thermal cycler program in Table 3.

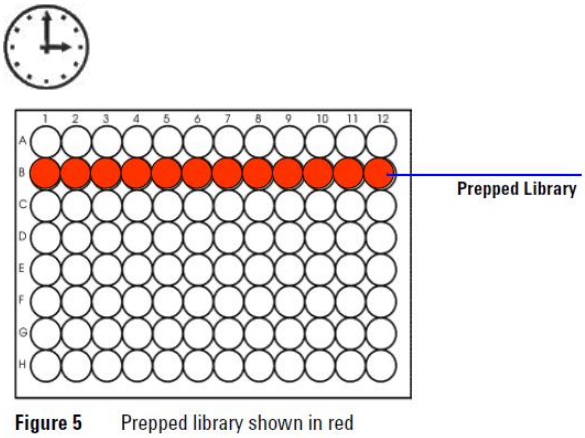

Figure 5 Prepped library shown in red

Table 3

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

8 Use a heated lid on the thermal cycler at 105°C to hold the temperature of the plate on the thermal cycler at 65°C.
9 Maintain the plate at 65°C while you load 40 µL of *hybridization buffer* per well into the "A" row of the PCR plate. The number of wells filled in Row A is the number of libraries prepared.
The example in Figure 6 is for 12 captures.

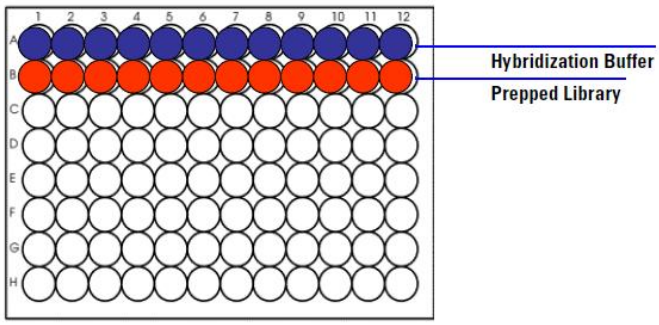

Figure 6 Hybridization buffer shown in blue

!! Make sure that the plate is at 65°C for a minimum *of 5 minutes* before you go to step 10.!!

10 Add the capture library mix from step 5 to the PCR plate:
A Add the capture library mix (7 µL) to the "G" row in the PCR plate.
Keep the plate at 65°C during this time.
B Pierce through the first foil. Seal the wells with new foil. Make sure the fit is tight.

C Incubate the samples at 65°C for 2 minutes.

Transfer all volumes to row "G":
11 Maintain the plate at 65°C while you pipette 13 µL of Hybridization Buffer from the "A" row and add it to the SureSelect capture library mix contained in row "G" of the PCR plate for each sample.
12 Maintain the plate at 65°C while you transfer the entire contents of each prepped library mix in row "D" to the hybridization solution in row "G". Mix well by slowly pipetting up and down *8 to 10 times.* Seal the wells with PCR-foil.
The hybridization mixture is now 27 to 29 µL, depending on degree of evaporation during the pre-incubations.
13 Seal the wells additional with adhesive film. Make sure all wells are completely sealed.
14 Incubate the hybridization mixture for *24 hours at 65°C with a heated lid at 105°C.*
Samples may be hybridized for up to 72 hours, but when you hybridize at longer periods, check that there is no extensive evaporation.

Day 2:

Step 1. Prepare magnetic beads
1 Prewarm SureSelect Wash Buffer #2 at 65°C in a circulating water bath or heat block for use in "Step 3. Select hybrid capture with SureSelect".
2 Vigorously resuspend the Dynal MyOne Streptavidin T1 (Invitrogen) magnetic beads on a vortex mixer. Dynal beads settle during storage.
3 For each hybridization, add 50 µL Dynal magnetic beads to a 1.5-mL microfuge tube.
4 Wash the beads:
A Add 200 µL of SureSelect Binding buffer.
B Mix the beads on a vortex mixer for 5 seconds.
C Put the tubes into a magnetic device, such as the Dynal magnetic separator (Invitrogen).
D Remove and discard the supernatant.
E Repeat step a through step d for a total of 3 washes.
5 Resuspend the beads in 200 µL of SureSelect Binding buffer.

Step 2. Select hybrid capture with SureSelect
1 Estimate and record the volume of hybridization that remained after 24 hour incubation.
2 Add the hybridization mixture *directly* from the thermal cycler to the bead solution, and invert the tube to mix 3 to 5 times.
Excessive evaporation, such as when less than 20 µL remains after hybridization can indicate suboptimal capture performance. See Table 32 on page 65 for tips to minimize evaporation.

3 Incubate the hybrid-capture/bead solution on a Nutator or equivalent for

30 minutes at room temperature.
Make sure the sample is properly mixing in the tube. Seal all tubes with parafilm.

4 Briefly spin in a centrifuge.
5 Separate the beads and buffer on a Dynal magnetic separator and "remove" the supernatant.
6 Resuspend the beads in 500 µL of SureSelect Wash Buffer #1 by mixing on a vortex mixer for 5 seconds.
7 Incubate the samples for 15 minutes at room temperature. Occasionally mix on a vortex mixer.
8 Briefly spin in a centrifuge.
9 Separate the beads and buffer on a Dynal magnetic separator and "remove" the supernatant.
10 Wash the beads:
A Resuspend the beads in 500 µL of 65°C prewarmed SureSelect Wash Buffer #2 and mix on a vortex mixer for 5 seconds to resuspend the beads.
B Incubate the samples for 10 minutes at 65°C in a recirculating water bath, heat block or equivalent. Occasionally mix on a vortex mixer.
Do not use a tissue incubator. It cannot properly maintain temperature.
C Briefly spin in a centrifuge.
D Separate the beads and buffer on a Dynal magnetic separator and remove the supernatant.
E Repeat step a through step d for a total of 3 washes.
Make sure all of the wash buffer has been removed.

11 Mix the beads in 50 µL of SureSelect Elution Buffer on a vortex mixer for 5 seconds to resuspend the beads.
12 Incubate the samples for 10 minutes at room temperature. Occasionally mix on a vortex mixer.
13 Briefly spin in a centrifuge.
14 Separate the beads and buffer on a Dynal magnetic separator.
15 Use a pipette to transfer the supernatant to a new 1.5-mL microfuge tube.
The supernatant contains the captured DNA. The beads can now be discarded.
16 Add 50 µL of SureSelect Neutralization Buffer to the captured DNA.
17 Briefly mix on a vortex mixer.

Step 3. Purify the sample with MSB PCRrapace (Invitek)
Follow the recommended manual
Use 33 µl Elution Buffer

PCR:
3 reactions per sample
Protocols:

a) PCR-Capture Is5+Is5 for all samples with Index already
b) Index-Capture-PCR-Is4 + Index 1.-16.

6.2 Application of the new Phenol-Chloroform protocol

Chemicals

EDTA	0,5 (pH 8,0)	Phenol-chloroform-isoamylalcohol	25:24:1; pH 7,5-8,0
Proteinase K	18 U	Chloroform	≥99 %
N-Laurylsarcosin	0,5%		

Day 1

For 0.5 g bone powder:

- Addition of 6.7ml EDTA,
- 30 µl Proteinase K
- 250 µl N-Laurylsarcosin
- Incubation under permanent rotation for 48 hours at 37°C

Day 2

- Addition of 1 volume phenol-chloroform-isoamylalcohol
- Shake for 1 minute at room temperature
- Centrifugation at 2575 x g for 10 minutes
- Transfer the upper aqueous phase into a new falcon
- Addition of 1 volume chloroform
- Shake for 1 minute at room temperature
- Centrifugation at 2575 x g for 10 minutes
- Transfer the upper aqueous phase onto Amicon® Ultra-15 (50 kDa) filter units
- Centrifugation at 5000 g for 5 minutes
- Washing through stepwise addition of in sum 8 ml HPLC-H_2O and centrifugation at 5000 rpm for 3 minutes
- Concentration to afinal volume of about 250 µl
- Storage of extracts at -20°C

References

Meyer M and Kircher M (2010) Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb. Protoc. doi:10.1101/pdb.prot5448.

Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C et al (2009) Solution hybrid selection with ultralong oligonucleotides for massively parallel targeted sequencing. Nat Biotechnol 27(2):182-189

Kircher M et al. (2011) Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Research, 2011, 1-8. doi:10.1093/nar/gkr771