Userguide

Eppendorf Thermomixer, ThermoStat plus | No 001 | November 2010

Applications for Eppendorf Thermomixer® comfort*1, Thermomixer® compact*2 and ThermoStat plus™

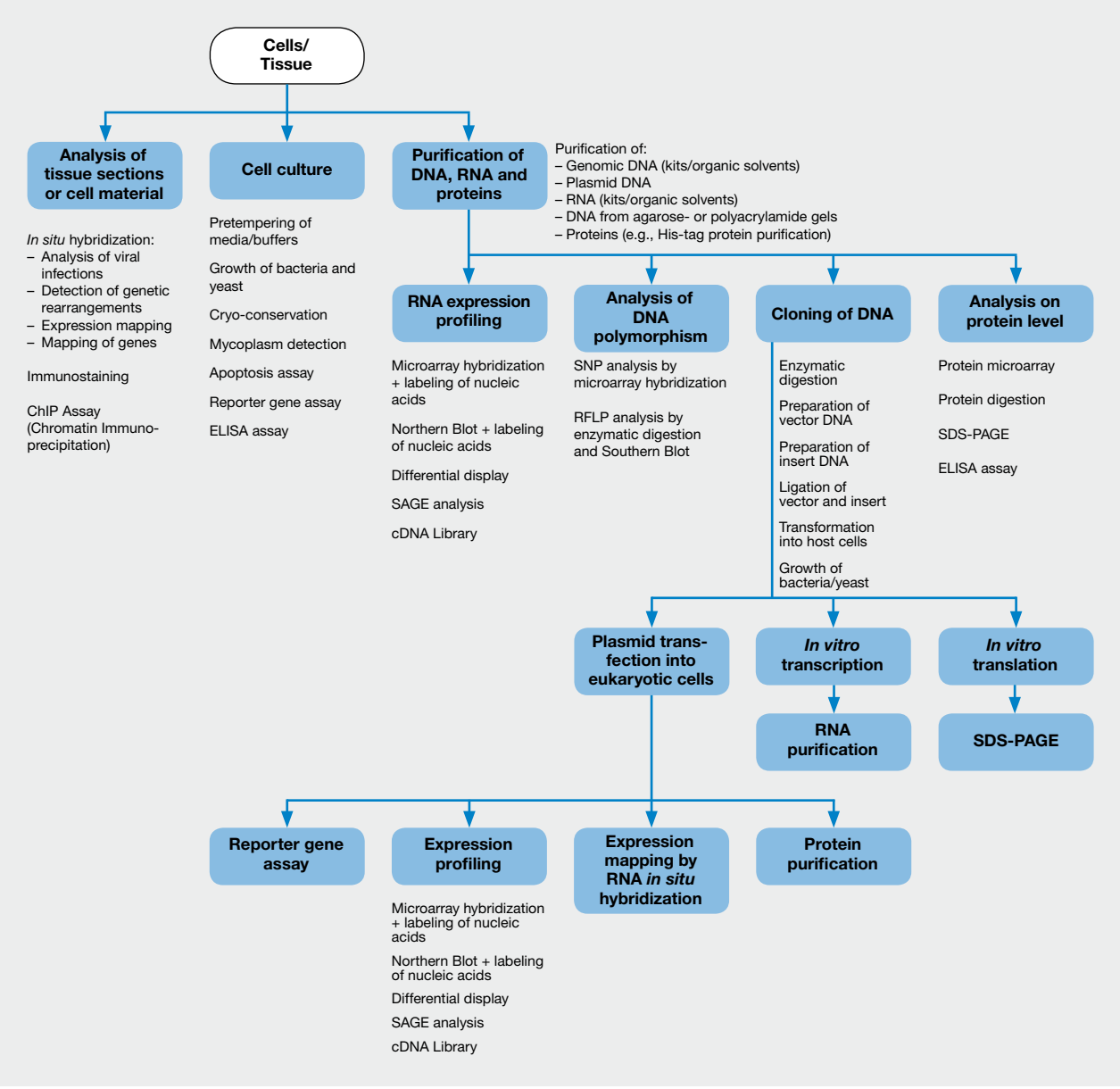

Figure 1: Applications for Thermomixer comfort, Thermomixer compact and/or ThermoStat plus.

*1 Product name in USA: Thermomixer R
*2 Product name in USA: Thermomixer

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As shown in Figure 1, Thermomixer comfort, Thermomixer compact and ThermoStat plus can be used in almost any field of molecular and cell biology. Important methods are outlined below, and detailed descriptions begin on page 6. Determination which device is most appropriate for your application depends on the type of reaction tubes being used, the desired temperature range and whether or not the tubes need to be mixed during incubation. Thermomixer comfort offers the highest degree of flexibility, as it can accommodate 10 different exchangeable thermoblocks (figure 2) as well as heat, actively cool and mix samples. The smaller Thermomixer compact is optimized for heating and mixing samples in 1.5 mL micro test tubes. The ThermoStat plus, which can also be used with different thermoblocks, is perfect for applications where samples require heating or cooling to -5 °C but do not require mixing. Tables 1 (below) and 2 (pages 4–5) outline the key features and technical specifications of these devices and their exchangeable thermoblocks.

Table 1: Product features and technical specifications of Thermomixer comfort, Thermomixer compact and ThermoStat plus

Thermomixer comfort	Thermomixer compact	ThermoStat plus
Features		
heats	heats	heats
cools	–	cools
mixes	mixes	–
Mixing speed		
300 rpm – 1,500 rpm, in steps of 50 rpm	300 rpm – 1,400 rpm, in steps of 50 rpm	–
3 mm mixing stroke (orbital)	3 mm mixing stroke (orbital)	–
Timer		
1 min to 99:59 h, continuous	–	1 min to 99:59 h, continuous
Temperature control range		
Control range: 13 °C below RT to 99 °C Setting range: +1 °C – 99 °C	Control range: 4 °C above RT to 99 °C Setting range: +1 °C – 99 °C	Control range: 30 °C below RT to 99 °C Setting range: -5 °C – 99 °C
Temperature accuracy		
±0.5 °C between 20 °C and 45 °C	±1 °C between 20 °C and 45 °C	±1.0 °C at 0 °C
±2.0 °C below 20 °C and above 45 °C	±2.0 °C below 20 °C and above 45 °C	±0.5 °C at 37°C
		±1.0 °C at 90 °C
Heat-up speed		
approx. 5 °C/min	approx. 5 °C/min	approx. 5 °C/min
Cool-down speed		
2 °C – 3 °C/min between 99 °C and RT	–	6 °C/min between 99 °C and 25 °C
0.5 – 1 °C/min between RT and 13 °C below RT		1.5 °C/min between 25 °C and –5 °C
Additional Features		
● Exchangeable thermoblocks ● Short-mix function ● Intervall mixing ● Pause function ● Programmable: 2 program steps with separate mixing and rest phases ● Selectable “time” and “temp” controls: timer function starts with program activation or when set temperature is reached ● RS 232 interface for remote control	● Fixed thermoblock for 1.5 mL tubes ● Short-mix function ● RS 232 interface for remote control	● Exchangeable thermoblocks ● Pause function ● Programmable: 4 program steps with separate time phases and temp. ramps ● RS 232 interface for remote control

Product name in USA: Thermomixer/Thermomixer R

Exchangeable thermoblocks for	Suitable for		Shape of boreholes			Limits	
	Thermomixer comfort	ThermoStat plus	Ø/LxW	depth	bottom-shape	max. temp.	max. rpm
0.2 mL PCR tubes and PCR plates 96 (adapter plate for MTP thermoblock)	yes	yes	5.1 mm	10 mm	conical	99 °C	1400 rpm
0.5 mL micro test tube (24x)	yes	yes	8 mm	24 mm	conical	99 °C	1500 rpm
1.5 mL micro test tube (24x)	yes	yes	11.1 mm	33 mm	conical	99 °C	1400 rpm
2.0 mL micro test tube (24x)	yes	yes	11.3 mm	33.5 mm	round	99 °C	1400 rpm
Tubes with Ø 11 mm–11.9 mm (24x)	yes	yes	12 mm	34 mm	flat	99 °C	1400 rpm
Cryo tubes 1.5–2.0 mL (24x)	yes	yes	12.7 mm	32.9 mm	flat	99 °C	1400 rpm
15 mL Falcon tubes (8x)	yes	yes	18.5 mm	103 mm	conical	99 °C	750 rpm
50 mL Falcon tubes (4x)	yes	yes	29.5 mm	96 mm	conical	99 °C	750 rpm
MTPs and deepwell plates	yes	yes	130 x 88 mm	24.5 mm	flat	99 °C	1400 rpm
Slides (4x)	yes	yes	76.9 x 26.5 mm	–	flat	99 °C	1400 rpm
CombiBox	no	yes	135 x 94 mm	63.5 mm	flat	99 °C	–

Figure 2: Exchangeable thermoblocks for Thermomixer comfort and ThermoStat plus; list of technical specifications.

Table 2 on the next spread lists typical applications for the Thermomixers and ThermoStat plus along with suitable devices and exchangeable thermoblocks. Pages 6–14 give more detailed information on the most relevant methods used in nucleic acid and protein purification, expression profiling, and DNA cloning.

Product name in USA: Thermomixer/Thermomixer R

Table 2: Typical applications for Thermomixer compact, Thermomixer comfort and ThermoStat plus; the red and gray dots identify those devices (●) and exchangeable thermoblocks (●) that are suitable for a given application.

		Thermomixer compact	Thermomixer comfort	ThermoStat plus	Exchangeable thermoblocks for Thermomixer comfort and ThermoStat plus					
					Micro test tubes 0.5/1.5/2.0 mL or Ø 11–11.9 mm	**Cryo tubes** 1.5 – 2.0 mL	**Falcon tubes** 15/50 mL	**MTP, Deepwell plates, 0. 2 mL PCR tubes, PCR plates (96 wells)**	**Slides**	**CombiBox** (ThermoStat plus only)
Analysis of cells and tissues										
Cell culture	Pretempering of media and buffers			●						●
	Growth of bacteria/yeast in 2.0 mL tubes		●		●					
	Growth of bacteria/yeast in Falcon® tubes **(page 13)**		●				●			
	Growth of bacteria/yeast in deepwell plates **(page 13)**		●					●		
	Cryo-conservation		●	●		●				
	Mycoplasm detection by ELISA (plate coating/Ag-Ab reaction)		●	●				●		
	Mycoplasm detection by PCR (denaturation of culture supernatant)	●	●	●	●			●		
Cell manipulation	Transformation of bacteria by heat-shock method **(page 12)**	●	●	●	●					
	Transfection of eukaryotic cells (pretempering of media and buffers)			●						●
Functional cell analysis	Apoptosis assay (96-well format)		●					●		
	Apoptosis assay by *in situ* detection		●	●					●	
	ELISA test (antigene-antibody reaction)		●	●				●		
	Reporter gene assay (cell lysis)		●					●		
Structural cell analysis	*in situ* hybridization (e.g., FISH, CISH) **(page 9)**		●	●					●	
	Labeling of nucleic acid probes for *in situ* hybridization **(page 8)**	●	●	●	●					
	Immunostaining		●	●					●	
	ChIP Assay (Chromatin Immunoprecipitation)		●		●				●	
Purification of nucleic acids and proteins										
General	Preheating of elution buffer for increased yields with matrix-based purification methods	●	●	●	●					●
Genomic DNA	Proteinase K digestion	●	●	●	●		●			
	Resuspension of genomic DNA **(page 6)**	●	●		●					
Plasmid DNA	Resuspension of bacteria pellets **(page 6)**	●	●		●			●		
RNA	Resuspension of RNA pellets **(page 6)**	●	●		●					
	DNase I treatment	●	●	●	●					
DNA from agarose gel	Dissolving agarose gel in high-salt buffer **(page 6)**	●	●	●	●					
Protein	His- or GST-tag protein purification using magnetic beads **(page 6)**	●	●		●		●			

Product name in USA: Thermomixer/Thermomixer R

		Thermomixer compact	Thermomixer comfort	ThermoStat plus	Exchangeable thermoblocks for Thermomixer comfort and ThermoStat plus: Micro test tubes 0.5/1.5/2.0 mL or Ø 11–11.9 mm	Cryo tubes 1.5 – 2.0 mL	Falcon tubes 15/50 mL	MTP, deepwell plates, 0.2 mL PCR tubes, PCR plates (96 wells)	Slides	CombiBox (ThermoStat plus only)
Expression profiling										
Microarray	RNA isolation **(page 8)**	●	●		○					
	cDNA labeling by reverse transcription	●	●	●	○					
	Hybridization of microarray slide **(page 9)**		●	●					○	
Northern blot	RNA denaturation prior to formaldehyde gel electrophoresis	●	●	●	○					
	Probe labeling and denaturation **(page 8)**	●	●	●	○					
Differential display	RNA isolation **(page 6)**	●	●	●	○					
	cDNA synthesis **(page 7)**	●	●	●	○					
	Sample denaturation prior to polyacrylamide gel electrophoresis	●	●	●	○					
	Isolation of DNA bands from polyacrylamide gel	●	●	●	○					
Serial analysis of gene expression (SAGE)	Generation of sequence tags from isolated RNA by cDNA synthesis, linking of tags to serial molecules, cloning and sequencing		●		○					
cDNA-library construction	RNA isolation **(page 6)**	●	●		○					
	cDNA synthesis with linker primers	●	●	●	○					
	Cloning of cDNA fragments **(page 7)**		●	●	○					
Analyses at DNA level										
Cloning of DNA fragments	Preparation of vector and insert DNA for ligation (enzymatic digestion, modification, DNA purification) **(page 10 ff.)**	●	●	●	○		○			
	Ligation of vector and insert DNA **(page 12)**	●	●	●	○					
	Transformation in bacteria (e.g., heat-shock method) **(page 12)**	●	●	●	○					
	Growth of bacteria/yeast in 2.0 mL tubes, Falcon tubes or deepwell plates **(page 13)**		●		○		○	○		
Further downstream applications	*in vitro* transcription **(page 14)**	●	●	●	○					
	in vitro translation **(page 14)**	●	●	●	○					
	Transfection in eukaryotic cells (pretempering of media and buffers)			●	○					○
SNP analysis using microarrays	Template labeling	●	●	●	○					
	Microarray hybridization **(page 9)**		●	●	○				○	
RFLP analysis	Enzymatic digestion of gDNA	●	●	●	○					
	Labeling of probes for Southern blot hybridization **(page 8)**	●	●	●	○					
Analyses on protein level										
Protein microarray	Protein microarray incubation		●	●					○	
Protein digestion	Incubation of protein digestion	●	●	●	○					
SDS-PAGE	Sample denaturation prior to gel electrophoresis	●	●	●	○					
ELISA assay	Coating of ELISA plate		●	●				○		
	Incubation of antigen-antibody reaction		●	●	○		○	○		

Product name in USA: Thermomixer/Thermomixer R

1. Purification of nucleic acids and proteins

Isolation of genomic DNA and RNA

Application for Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

Isolation of genomic DNA or RNA from different sources such as blood, tissue sections, or cell culture, using organic solvents (e.g., phenol) or kits for nucleic acid purification.

- gDNA: Digestion of cellular proteins with Proteinase K at 37 °C and slight mixing
- RNA: Optional digestion of contaminating gDNA with DNase I at 37 °C
- Resuspension of genomic DNA following alcohol precipitation can be accelerated by careful mixing (300 rpm) at room temperature or at 65 °C

Purification of DNA from agarose gels

Application for Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

For purification of DNA from agarose gels, the gel structure is dissolved at 50 °C by using chaotropic salts. The DNA is subsequently bound to silica particles (e.g., glass-milk) or to a column matrix. After several washing steps the DNA can be eluted with low-salt buffers (e.g., 10 mM Tris-HCl).

- Addition of high-salt buffer to the gel slice and interval mixing at 50 °C for 10 min at 1,400 rpm every 1–2 min for 15 sec each
- Preheating of elution buffer to 50 °C for increased DNA recovery

Purification using magnetic separation techniques

Applications for Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

Principle of biomagnetic separation technology
The principle of biomagnetic separation technology is based on the interaction of special ligands (e.g., antibodies, proteins, oligos) that are coupled onto the surface of paramagnetic beads with specific target molecules (e.g., nucleic acids, proteins, cells). These interactions can be either hybridization or antigen/antibody reactions. After the targets have bound to the ligands (attached to paramagnetic particles) these complexes can be isolated from the sample mixture using a magnet. The bead-bound complexes are drawn to the side of the tube facing the magnet, and the supernatant can be removed with a pipette. Mixing of the sample thereby improves bonding efficiency.

Applications:
mRNA isolation using paramagnetic oligo(dT)$_{25}$ beads
Basic principle:
Isolation of mRNA due to the hybridization of the oligo(dT)$_{25}$-ligands with the poly(A)-tail of mRNA.

- Incubation for 2 min at 65 °C to break down secondary structures, followed by an incubation on ice (e.g., IsoTherm-System)
- Increased binding efficiency of polyA-RNA to the oligo(dT) beads by mixing the sample at RT
- For sample elution, heat sample in elution buffer for 2 min at 80 °C–90 °C

1. Purification of nucleic acids and proteins

Purification using magnetic separation techniques

Purification of biotin-labeled molecules (e.g., nucleic acids, proteins, antibodies) using streptavidin-coated beads
Basic principle: Protein ligand interaction between streptavidin and biotin.
- Mixing of the reaction set-up at RT for approx. 10 min

Isolation of immunoglobulins (IgG) using protein A- or protein G-coated beads
Basic principle: The cell wall protein A or G from *Staphylococcus aureus* or Group G *Streptococcus*, respectively, are able to bind to immunoglobulin.
- Resuspension of beads coupled with either Protein A or G: Mixing for 1 min at RT
- Protein binding: Mixing for 10 min at RT
- Elution of the proteins: Mixing for 2 min at RT

Plasmid DNA purification

Application for ThermoStatplus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes and CombiBox

Applications: Kits for nucleic acid purification
- Bacteria growth in 2.0 mL micro test tubes, in Falcon tubes or in deepwell plates
- Resuspension of the bacteria pellet
- A better plasmid DNA yield can be achieved when the reaction buffer is preheated to 65 °C

2. Expression profiling

Reverse transcription

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

Synthesis of cDNA (copy DNA) from mRNA by reverse transcriptase involves the incubation of reaction preparations at various temperatures.
- Denaturing of RNA and primer, e.g., at 70 °C for 10 min
- First-strand cDNA synthesis: Incubation of the preparation at 37 °C– 42 °C for 1 h (with reverse transcriptase)
- Stopping of the reaction via heat deactivation, e.g., at 70 °C for 30 min

Construction of cDNA libraries

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

Following RNA isolation from the cells or tissue to be examined (e.g., mRNA isolation using paramagnetic beads), the RNA is reverse transcribed into cDNA and then followed by second-strand synthesis using linker primer. The double-stranded DNA generated in this way is suitable for direct cloning in special vector systems (due to the linker).

Application:
- RNA isolation
- Reverse transcription
- Cloning of DNA fragments

2. Expression profiling

Probe-labeling for hybridization experiments

Applications for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

To perform hybridization experiments, such as Southern blot, Northern blot, microarray analysis and *in situ* hybridization – or to visualize its hybridization signals – it is essential to label the probes with suitable dyes. Both radioactive (e.g., ^{32}P, ^{14}C, ^{3}H, ^{35}S) and non-radioactive (e.g., biotin, digoxigenin, Cy3, Cy5 or Alexa Fluor dyes) dyes are available. Incorporation of those dyes (typically covalently bound to nucleotides) into probe molecules can be achieved through different methods:

Applications:
Nick translation
Basic principle: Template DNA is digested with DNase in the presence of Mg^{2+} ions. Under these conditions, the enzyme cuts only one of the two strands, thus maintaining the double-stranded structure. In the second step, the template is processed with DNA polymerase in the presence of nucleotides, one of which is labeled. The polymerase recognizes the cut and extends the free 3' end, while the 5' end is simultaneously broken down. The labeling can be carried out through the incorporation of radioactive – or non radioactive – labeled nucleotides.

- Incubation at 12 °C–15 °C for 15 min–45 min
- Deactivation at 70 °C for 10 min

Random priming
Basic principle: Denaturing of double-stranded template DNA and subsequent hybridization with random primers that serve as primer for a DNA polymerase (e.g., Klenow fragment). The labeling can be carried out through the incorporation of radioactive – or non radioactive – labeled nucleotides.

- Denaturing at 95 °C for 3 min
- Subsequent cooling of the sample on ice/IsoTherm-System for 3 min
- Incubation at 37 °C for 2 h
- Deactivation at 70 °C for 10 min

5'-labeling with polynucleotide kinase (PNK)
Basic principle: The labeling takes place through the exchange of the phosphate residue at the 5' end with a radioactively labeled phosphate. For this purpose, molecules that have to be labeled are first dephosphorylated by phosphatase and then labeled using polynucleotide kinase.

- Dephosporylation of the reaction by incubation with phosphatase for 1 h at 37 °C
- Stopping of the reaction with, for example, heat deactivation at 75 °C for 10 min, or at 65 °C for 1 h
- Labeling with [γ-^{32}P]-ATP using PNK at 37 °C for 10 min–30 min
- Stopping of the reaction with 5 mM EDTA

3'-labeling with terminal transferase
Basic principle: The labeling takes place with terminal deoxynucleotidyl transferase, which adheres nonspecifically desoxynucleotides to the 3' end of single- and double-stranded DNA.

- Incubation at 37 °C for 30 min
- Stopping of the reaction at 70 °C for 10 min

2. Expression profiling

Microarray analysis

Application for Thermomixer comfort/ThermoStat plus
Exchangeable thermoblocks for micro test tubes and slides

Target molecules in the form of short, single-stranded DNA molecules (probes) are immobilized on a particular carrier material (e.g., glass slide). The technology is based on the molecules of interest being labeled with suitable dyes (e.g., fluorescence dyes) and, under optimized reaction conditions, being hybridized to the immobilized target molecules on the carrier material. Resulting hybridization bonds between target molecules and complementary molecules of the sample solution can then be visualized by suitable detection methods (e.g., fluorescence detection).
Mixing of the sample during the hybridization reaction increases the homogeneity and can result in an improved signal/noise ratio, which, in turn, increases the sensitivity and reproducibility of the method.

Applications:
- RNA isolation
- cDNA-labeling by reverse transcription
- Hybridization of the microarrays according to optimized hybridization profiles
- Mixing at 1,400 rpm during microarray hybridization may increase the sensitivity of the method

in situ expression profiling (RNA *in situ* hybridization)

Application for Thermomixer comfort/ThermoStat plus
Exchangeable thermoblock for Slides

Principle of *in situ* hybridization
The method of *in situ* hybridization can be used to detect specific DNA or RNA sequences within histological tissue sections or cytological sample material without losing important morphological details. Target sequences within the sample are detected by using either DNA or RNA probes for hybridization. Stringency washes minimize unspecific probe binding yet maintain the specific interaction between probe and target sequence; thus, depending on the type of probe used, various types of information about the analyzed sample material can be obtained. For example, it is possible to detect viral infections in tissues, identify chromosomal aberrations in the context of tumor diagnostics or analyze gene transcription of single cells. Depending on the type of labeling or detection technique, the following *in situ* hybridization (ISH) methods can be performed:
- FISH (fluorescence ISH) using fluorescent-labeled probes and a fluorescence microscope for evaluation
- CISH (chromogenic ISH) with digoxigenin (DIG)-labeled probes which lead to blue-violet precipitate that can be detected by light microscopy
- RISH: the abbreviation RISH is simultaneously used for different methods in literature:
  - Reflection-ISH for probes being labeled with gold particles. The evaluation is performed with confocal laser scanning microscopes
  - Radioactive-ISH for radioactive-labeled probes
  - RNA-ISH for hybridization with RNA probes, regardless of the type of labeling
- DISH (DNA-ISH), a rarely used term for *in situ* hybridization with DNA probes in accordance with the term RNA-ISH (RISH)

Application: RNA *in situ* hybridization (RISH))
- Incubation of slides for preparation of paraffin sections: 56 °C–60 °C for 2 h–16 h
- Proteolytic treatment of tissue sections: 37 °C for 10 min
- Hybridization with RNA probe: 37 °C for 2 h–16 h
- Staining for detection: 37 °C for up to 30 min

3. Cloning of DNA fragments

Genetic engineering makes it possible to isolate targeted regions from the genome of cells or organisms and amplify them for downstream experiments. The desired DNA fragment (insert) is then linked with the vector DNA (generating recombinant DNA) and multiplied in host cells. The vectors are mostly genetically modified constructs derived from the natural plasmids found in bacteria; they protect the cloned DNA fragment from enzymatic breakdown during multiplication and enable selection of suitable clones through their resistance to antibiotics as well as blue/white screening. In addition to these properties, certain vector variants make transcription of the cloned DNA possible due to the existence of various RNA polymerase binding sites (see also: *in vitro* translation, page 14).
The techniques of DNA cloning are used for cloning entire genomes (i.e., genomic libraries), subcloning genomic fragments, cloning PCR fragments (A/T cloning) or cloning of entire transcriptomes of cells by reverse transcription of cellular RNA into cDNA and subsequent ligation into suitable vector systems (i.e., cDNA library, page 7).

The term DNA cloning is generally used for several methods:
- Methods for preparation of insert and vector DNA for ligation
- Ligation of vector and insert DNA
- Transformation into host cells
- Cultivation of host cells (bacteria, yeast)
- Isolation of plasmid DNA
- Downstream methods to analyze cloned DNA

Restriction digest of insert and vector DNA

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

Restriction enzymes (e.g., restriction endonuclease of type II) recognize specific base sequences of four or more base pairs in the DNA double helix and cut these in 2 different ways: in fragments with smooth ends without base projection (blunt ends); or so-called “sticky ends” with either a 5’ or a 3’ projection, depending upon which DNA strand projects. The cut DNA molecules have a phosphate group at their 5’ ends and a hydroxyl group (-OH) at the 3’ ends.
As a result of the digestion of DNA with restriction endonuclease, the DNA molecules that are created can be easily ligated, cloned and later analyzed due to their size and respective base projections.
- Incubation of the DNA molecule at 37 °C for 1 h
- Stopping of the reaction via heat deactivation of the enzyme (e.g., at 65 °C for 20 min) or with the addition of 5 mM EDTA
- Purification of the desired DNA fragments from agarose gels (page 6)

Excursus: Restriction digestion
As an example, the circular, 5243 bp long DNA molecule of the SV40 virus has 1 recognition sequence for the restriction enzyme *Eco*RI, 4 for H*pal* and 11 interfaces for the *Hind*III enzyme. This means that fragments resulting from digestion with an enzyme can still be split into eversmaller DNA fragments via an additional digest with a second or third enzyme.
To represent size differences between the different restriction fragments, gel electrophoresis can separate the fragments according to size (in either an agarose or polyacrylamide gel) followed by detection with a suitable dye method. Comparison with molecular weight standards (markers) permits a more precise estimate of the length of the respective fragments. Additionally, a particular DNA fragment can be specifically identified via Southern blot and subsequent hybridization with a labelled probe that contains a sequence complementing the desired fragment.
Because the number and the position of recognition sequences for restriction enzymes generally differentiate various DNA molecules, a very specific restriction pattern for each DNA molecule can be created through digestion with a suitable enzyme. This in turn can be used as a “DNA fingerprint” for identification and further analysis of DNA molecules.

3. Cloning of DNA fragments

Transformation of “sticky ends” into “blunt ends”

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

To subsequently ligate the insert DNA with the vector, both DNA molecules (insert and linearized vector) must have compatible ends. Ligation can take place either with sticky ends, where the ends of the insert and vector DNA are compatible with each other, or with fragments via blunt ends. The efficiency of the ligation is usually significantly higher for sticky ends than for blunt ends.
Because vectors have multiple cloning sites (MCS), which have recognition sequences for a number of different restriction enzymes, it is generally not difficult to find suitable restriction enzymes for cloning via sticky ends. Sometimes a vector and insert may not have a compatible interface, in which case the smoothing of projecting fragment ends and the subsequent ligation of the fragments in a vector with smooth, dephosphorylated ends is required. This makes it possible to either remove the projecting nucleotides (e.g., with mung bean endonuclease; T4 DNA polymerase) or to fill the gaps through normal polymerization (e.g., with T4 DNA polymerase or Klenow fragment).

- Incubation of the reaction setup for 20 min at RT or at 37 °C for 5 min
- Stopping of the reaction via heat deactivation of the enzyme, e.g., at 75 °C for 10 min
- Purification of the DNA

A-tailing

Application for ThermoStat plus/Thermomixer comfort
Exchangeable thermoblocks for micro test tubes

Another method for cloning DNA is ligation in special vectors with a T-projection. This method is especially important for the cloning of PCR fragments, as the normal *Taq* DNA polymerase always incorporates an additional A-projection at the end of the fragment during the amplification of DNA. In other words, the PCR product produced in this way can be used directly for ligation without further modification. In the case of blunt-end fragments (e.g., PCR fragments that were amplified with proofreading polymerases), this property of the *Taq* DNA polymerase can be used to prepare the DNA fragments for cloning in vectors with T-projections through “A-tailing”.

- Incubation of the reaction setup for 10 min at 72 °C
- Purification of the DNA

Phosphorylation of linker DNA

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

The transfer of phosphoryl groups is a fundamental reaction in biochemistry. Enzymes that transfer the phosphoryl groups from the ATP to an acceptor are generally referred to as kinases. In the field of cloning, a phosphorylation is required, for example to activate artificially produced, cohesive ends (linkers) with a phosphoryl group for subsequent ligations using, as another example, the T4 polynucleotide kinase. The short, chemically synthesized DNA linkers are then linked with the ends of a DNA fragment or vector.

- Incubation of the reaction setup for 30 min –1 h at 37 °C
- Stopping of the reaction via heat deactivation at 65 °C for 20 min
- Purification of linker DNA

3. Cloning of DNA fragments

Dephosphorylation of linear DNA molecules

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes

When a vector is only cut with one restriction enzyme, this results in two complementary ends that can then be ligated with one another. One problem with cloning DNA fragments is that the intramolecular ligation of the vector competes directly with intermolecular insert-vector ligation, which results in a drastic reduction of cloning efficiency. To prevent this, the phosphate groups at the 5' ends of the linearized vector are removed (CIPing – named for the **C**alf **I**ntestinal Alkaline **P**hosphatase – the enzyme used most frequently for this method) with the help of a phosphatase following the restriction digest of the vector DNA. Dephosphorylating the vector in this manner prevents intramolecular ligation of the vector; however, the DNA fragment to be incorporated into the vector contains both phosphate residues and can therefore still ligate with the vector DNA.

- Incubation of the reaction preparation for 1 h at 37 °C
- Stopping of the reaction by, for example, adding 5 mM of EDTA (pH 8.0) and heat deactivation at 75 °C for 10 min, or at 65 °C for 1 h
- Purification of the DNA

Ligation of vector and insert DNA

Application for ThermoStat plus/Thermomixer comfort
Exchangeable thermoblocks for micro test tubes

For a particular DNA fragment to be ligated in a vector, the ends of the DNA fragment must be compatible with the ends of the linearized vector. That requires both vector and insert to have blunt ends or complementary sticky ends (resulting from the *Taq* polymerase, restriction enzyme or artificial linkers). Through base pairing, DNA fragments and linearized plasmid can form layers on one another and then be linked via a DNA ligase that catalyzes a phosphodiester bond between the two DNA chains. The DNA ligase thereby requires a free OH group at the 3' end of the one DNA chain and a phosphate group at the 5' end of the other. For optimal reaction conditions, vectors and insert DNA are generally mixed at a molar ratio of 1:3.

- Incubation of the reaction preparation at certain temperatures, depending upon the type of fragment ends:
  – e.g., for 1 h at RT with cohesive ends
  – e.g., for 16 h–24 h at 14 °C for blunt end ligation

Transformation into host cells

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes + IsoTherm-System

For the amplification of cloned DNA, the constructs of vector and insert are channeled into host cells, where they are multiplied/replicated. The vector thereby codes the genetic elements required for autonomous replication. Generally, different variants of the *Escherichia coli (E. coli)* safety strain K12 (e.g., JM109, DH5α, TOP10) are used as host cells that are not capable of living outside of lab conditions. In some cases yeast is used as a host organism for the amplification (e.g., for the amplification of yeast artificial chromosomes (YAC)). Methods such as $CaCl_2$/heat shock transformation and electroporation are generally used for the directed transfer of DNA into the host organism.

Application: Heat shock transformation

- Slow thawing of the bacteria at 0 °C
- Incubation of the reaction preparation of ligated DNA and bacteria at various temperatures, e.g., on ice/IsoTherm-System at ~0 °C for 30 min –1 h
- Heat shock incubation for 45 sec at 42 °C
- Bacteria incubation for 2 h at 37 °C
- Spreading of the bacteria on agar plates and overnight incubation at 37 °C

3. Cloning of DNA fragments

Cultivation of host cells

Application for Thermomixer comfort
Exchangeable thermoblocks for micro test tubes, Falcon tubes or deepwell plates

For optimized bacteria cultivation, the growth medium and the Thermomixer comfort, in combination with the desired thermoblock, should be preheated to 37 °C. The type of growth medium and appropriate antibiotic depends on the bacteria strains and the vector system used.

Applications:
Bacteria cultivation in deepwell plates (DWP)
For 2.4 mL deepwell plates 1 mL–1.25 mL growth medium should be used, and the plates should be sealed with an air permeable seal to prevent contamination. The enclosed lid must be used during incubation.
- Preheating of the device to 37 °C with closed lid
- Incubation: at 37 °C for 20 h–24 h, 1,200 rpm

Bacteria cultivation in 15 mL or 50 mL Falcon tubes
- Overnight incubation: at 37 °C, 750 rpm

Isolation of plasmid DNA

Application for ThermoStat plus/Thermomixer comfort/Thermomixer compact
Exchangeable thermoblocks for micro test tubes and CombiBox

Applications: Kits for nucleic acid purification
- Bacteria growth in 2.0 mL micro test tubes, Falcon tubes or deepwell plates
- Resuspension of the bacteria pellet
- A better plasmid DNA yield can be achieved when the reaction buffer is preheated to 65 °C

Downstream applications

***in vitro* transcription & *in vitro* translation**

Application for ThermoStat plus/ Thermomixer comfort
Exchangeable thermoblocks for micro test tubes

in vitro translation is the cell-free synthesis of proteins, based on a cloned open reading frame (ORF) that codes the amino acid sequence of the desired protein. Thus, during the generation of the template DNA, it is important to identify the right ORF and clone it correctly into a vector with an ATG-start codon and a stop-codon. Generally, isolated mRNA that has been reverse transcribed into cDNA and made double-stranded by second-strand synthesis is used for cloning into a plasmid vector with SP6, T3 or T7-RNA polymerase promoters. This DNA template is then used for *in vitro* transcription of mRNA by RNA-polymerase. In the last step, this synthesized mRNA is *in vitro* translated into protein. A eukaryotic translation system (e.g., wheat germ extract or reticulocyte lysate) is thereby used for the protein synthesis.
- *in vitro* transcription at 37 °C–42 °C for 1 h
- Purification of transcribed mRNA
- *in vitro* translation at 30 °C for 1 h
- Purification of translated protein for further analysis

Ordering information

Description		International order no.	North America order no.
Eppendorf Thermomixer® compact, with fixed thermoblock for 1.5 mL tubes, incl. IsoPack 0 °C and IsoRack	100–240 V, 50–60 Hz	5350 000.013	–
	120 V, 50–60 Hz	–	022670000
	220 V, 50–60 Hz	–	022670051
Eppendorf Thermomixer® comfort, without exchangeable thermoblock			
	100–240 V, 50–60 Hz	5355 000.011	–
	120 V, 50–60 Hz	–	022670107
	220 V, 50–60 Hz	–	022670158
ThermoStat plus™, without exchangeable thermoblock	100–240 V, 50–60 Hz	5352 000.010	–
	120 V, 50–60 Hz	–	022670204
Exchangeable thermoblocks and accessories for Eppendorf Thermomixer® comfort and ThermoStat plus™:			
0.2 mL PCR tubes and PCR plates 96 (adapter plate for MTP thermoblock)		5363 007.009	022670573
0.5 mL micro test tubes (24x), with IsoPack 0 °C and IsoRack		5361 000.015	022670506
1.5 mL micro test tubes (24x), with IsoPack 0 °C and IsoRack		5360 000.011	022670522
2.0 mL micro test tubes (24x), with IsoPack 0 °C and IsoRack		5362 000.019	022670549
Tubes with Ø 11–11.9 mm (24x)		5364 000.016	022670581
Cryo tubes 1.5–2.0 mL (24x)		5367 000.017	022670557
15 mL Falcon® tubes (8x)		5366 000.013	022670531
50 mL Falcon® tubes (4x)		5365 000.010	022670514
MTPs and deepwell plates, with lid		5363 000.012	022670565
Slides (4x)		5368 000.010	022670590
CombiBox (ThermoStat™ only)		5352 100.007	022670603

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