

Evaporation-Controlled Automated Embedding and Polymerization

**Electron
Microscopy
Sciences**

Evaporation-Controlled Automated Embedding and Polymerization

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Resin embedding is traditionally performed via sequential applications of a graded series of solvent/resin mixtures [1]. The procedures are time consuming (see also [2]) and the chemicals used can pose health hazards. In addition there is an amount of waste, usually containing both solvent and resin and often larger in volume than the polymerized specimens. When processing large numbers of samples there is also hands-on time to consider. Finally there is the risk of losing specimen material especially in the exchange steps with high viscosity resin.

We have looked into alternatives for the classic embedding procedures, aiming to improve on the drawbacks described above. We describe an approach and an instrument that

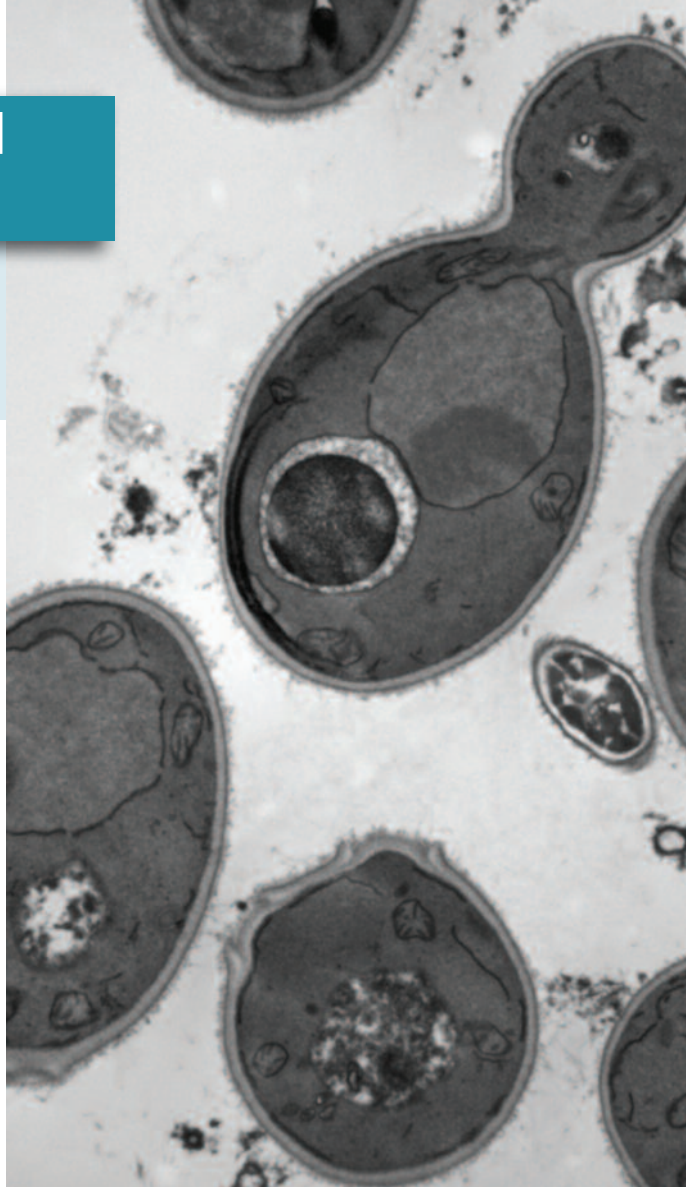
- (i) automates embedding from the first solvent/resin step up to pure resin,
- (ii) reduces solvent and resin use with no waste, except what is left in the mixing vials,
- (iii) minimises exposure to hazardous and irritating chemicals,
- (iv) reduces hands-on time to the bare minimum,
- (v) facilitates the processing of up to 52 samples in one instrument run and
- (vi) prevents any specimen loss.

These goals were achieved by eliminating the step-procedure and removing solvent by making use of its volatility. Common solvents for dehydration and embedding are highly volatile whereas most resin components do not evaporate readily. Evaporation depends on the kinetic energy of solvent molecules, the degree to which solvent is held back by the resin and how easily molecules can stay in the gas phase (saturation pressure). Temperature plays the most prominent role, next to vacuum pressure and the concentration of 'solvent' gas molecules in the gas phase over the samples. Evaporated solvent needs to be removed or diluted by flushing air over the liquid surface at set intervals.

To be able to have full control over temperature, pressure and flushing with fresh air, an instrument was developed that achieves these goals, the EMS Poly III. At the heart of the instrument is a specimen chamber that is temperature controlled over a range of 10°C - 70°C. The pressure in the chamber can be reduced from ambient pressure to a controlled level as low as 10kPa with a built-in vacuum pump. The instrument chamber accepts up to 52 BEEM specimen vials. Preset and user-adjustable programs coordinate temperature, pressure and pressure-increase-induced flushing. This results in an efficient and reproducible removal of solvent from the specimens. Initial steps aiming at bulk removal of solvent are followed by steps for the thorough removal of trace amounts.

In practice: the instrument can be loaded with specimens in e.g. acetone/resin by the end of a workday. The next morning the vials are ready for polymerization once they have been topped up with pure resin to compensate for the removed solvent.

The efficacy of the approach was evaluated by embedding yeast cells (see micrograph at right) in epoxy resin, using acetone as the solvent according to protocol. This leaves the specimens covered in a thin layer of resin by the end of the embedding run.



Yeast cells were fixed with glutaraldehyde in cacodylate buffer, washed in distilled water and postfixed with 1% KMnO₄ in distilled water. After washing in distilled water the cells were dehydrated in a graded series of acetone/water mixtures: 50% - 70% - 80% - 90% - 100% - 2x100% absolute. Each step lasted 10 min. Cells were then resuspended in resin/solvent 1:3 (v/v) in BEEM capsules.

Once the BEEM vials had been transferred into the instrument chamber, the embedding program was started. Upon completion it was followed by the polymerization program, once the BEEM vials had been topped up with pure resin.

References:

[1] J. J. Bozzola, L.D. Russell "Electron Microscopy 2nd edition", ed. Jones & Bartlett Publishers, Boston.

[2] K. L. McDonald, *Microsc. Microanal.* 20 (2014) 152–163.

Acknowledgement: TEM image courtesy of OCEM, University of Otago

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Background

Vacuum conditions in a transmission electron microscope require that liquid water is removed from specimens before introducing them into the column. This is usually achieved by dehydration, i.e. replacing the water by a solvent such as acetone which in turn is replaced by a resin suited for ultrathin sectioning after polymerization. Resin embedding is traditionally performed using a graded series of dehydration solvent and resin mixtures. In the first step, the solvent / resin ratio is in favor of the solvent. This warrants low viscosity and thereby easy penetration of resin components into the specimen. Sequential steps aim at a stepwise increase in the resin concentration until finally the specimens are in pure resin, ready for polymerization.

A typical sequence can be as follows:

3:1 Solvent: Resin

1:1 Solvent: Resin

1:3 Solvent: Resin Pure Resin (2x)

A protocol based on sequential steps can take up to 48 or even 72 hrs for specimens which are dense and hard to infiltrate.

Resin embedding

When during the embedding procedure a solvent-resin mixture is exchanged for a higher resin concentration mix, only the bulk of the volume is replaced; the specimen will slowly follow suit when the solvent diffuses from the specimen into the bulk mixture. This is a slow process as it is predominantly driven by diffusion and thus depends on the temperature and the viscosity of the resin. The exchange is furthermore driven by the difference in concentration of the solvent (gradient) and therefore depends on continuous refreshing of the bulk mix close to the specimen surface by liquid movement. Hence, the traditional way of embedding depends heavily on mechanical movement.

Removing solvent in an alternative way

Instead of removing solvent using sequential steps with increasing resin concentration, solvent can also be removed by making use of the volatility of the solvent. Common solvents for dehydration and embedding are highly volatile whereas most resin components do not evaporate readily. Evaporation depends on the kinetic energy of solvent molecules. If the kinetic energy is high enough, a molecule can escape from a liquid into the gas phase. From the liquid point of view the evaporation rate is therefore related to the temperature of the liquid but also to the interaction the solvent molecules may have with other molecules such as resin components. From the gas phase point of view the evaporation rate depends on how easily molecules can stay in the gas phase. This is temperature

Table 1: Antoine Constants for common solvents

Solvent	A	B	C	T-range	Vp 25°C
Acetone	7.1327	1219.97	230.653	-64 – +70	31 kPa
Acetonitrile	7.33986	1482.29	250.523	-27 – +82	12 kPa
Chloroform	6.95465	1170.97	226.232	-10 – +60	26 kPa
Ethanol	8.20417	1642.89	230.3	-57 – +80	8 kPa
Methanol	8.08097	1582.27	239.7	+15 – +100	17 kPa
Water	8.07131	1730.63	233.426	+1 – +100	3 kPa

Data from DDSST GmbH, Germany.

An on-line calculator and source for the Antoine equation constants can be found at: <http://ddbonline.ddbst.de/AntoineCalculation/AntoineCalculationCGI.exe>

Data for propylene oxide were obtained from:

<http://www.lyondellbasell.com/techlit/techlit/2710.pdf>

related as well, but also depending on the concentration of 'solvent' gas molecules and the presence of other molecules in the gas phase. A saturation condition is reached when as many molecules evaporate from the liquid surface as there are molecules from the gas phase returning to the liquid phase. This saturation condition is characteristic for each solvent and is reflected in the saturation vapor pressure. Saturation conditions and even near-saturation conditions are to be avoided for effective solvent removal. This is realized when the evaporated gas is continuously removed or diluted by forced movement of air over the liquid surface: an open container holding a volatile solvent such as acetone 'dries' more quickly at higher temperatures and under forced air flow. Air flow and on-going removal of the gas under vacuum prevent saturation.

Solvent vapor pressure

Vapor pressure, or more accurately saturation vapor pressure, is the pressure resulting from evaporation when the number of molecules leaving a liquid equals the number that return to it from the vapor/gas phase. The vapor pressure increases with temperature. By definition, at the boiling point of a solvent the vapor pressure equals 1 atmosphere (101.3kPa). Under lower pressure conditions the boiling point is reached at lower temperatures. The vapor pressure graph presented in figure 1 shows the relation of vapor pressure and temperature. The data were calculated using the so-called Antoine equation, which in its turn derives from the Clausius-Clapeyron relation that describes the tangent at any point of the coexistence curve of a phase diagram.

Antoine equation: $\text{Log}(p) = A - C/(B+T)$

where p = pressure (mmHg) and T temperature in °C. A, B and C are substance specific constants which are valid for a limited temperature range. Table 1 shows a list of constants for common solvents. Please note that these values apply to pure substances only. Mixtures incorporating the solvent will show lower evaporation rates and vapor pressures will take longer to establish, especially with gradually decreasing solvent concentrations.

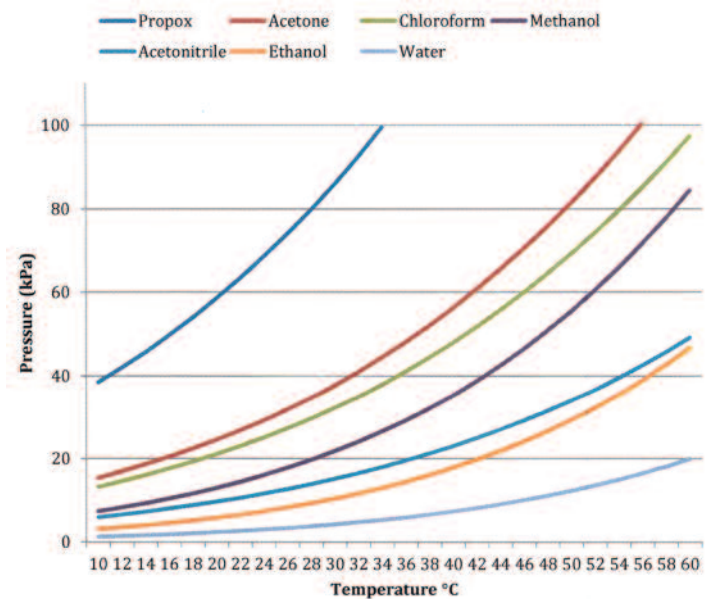


Figure 1: Saturated vapor pressure (kPa) vs temperature (°C) of common solvents.

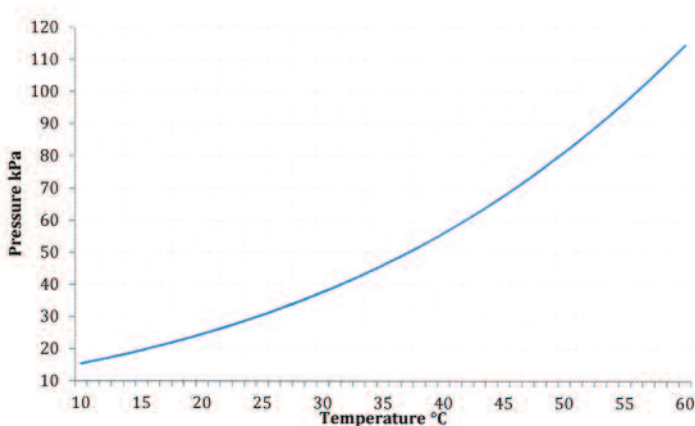
At pressures slightly higher than the values on the vapor pressure line the solvent will not boil but still evaporate. At pressures below the vapor pressure line the solvent will quickly evaporate by forced boiling.

Evaporation-Controlled Automated Embedding and Polymerization (cont.)

An example applying to acetone dehydration and epoxy embedding

Many ultra structural studies involve the use of epoxy resins and acetone. Acetone is highly volatile. The vapor pressure curve for acetone is singled out and presented in figure 2. From the graph it is clear that at 25°C the vapor pressure is ~30 kPa, in other words acetone boils at 25°C if the pressure is lowered to 30 kPa or below. In the case of boiling evaporation occurs not only at the surface of a solution, but throughout the whole volume. This is unfavorable for embedding mixtures as it leads to splashing and potential loss of specimens. At pressures slightly above the saturated vapor pressure, evaporation is still proceeding progressively but only from the surface of the mixture. This is a safe condition for a mixture of resin and acetone to gradually evaporate acetone from the surface. When a preset raise in pressure is exceeded, the pressure is released by air admission into the recipient up to a preset level. The evaporated acetone is diluted with air and removed by the vacuum pump. The dilution prevents that saturation will ever occur. The remaining mixture will become more concentrated in resin. Thus by carefully controlling temperature and vacuum pressure, air admission and flushing the acetone can be removed quantitatively and within hours; upon completion the specimens are left in for all practical purposes pure resin ready for polymerization after topping up with pure resin to compensate for the removed solvent.

Figure 2: Saturated Vapor Pressure of Acetone



Instrumentation

From the graphs it is obvious that fluctuations in temperature as well as in pressure may seriously influence the evaporation process. Without proper control of the vacuum pressure and the temperature the solvent may boil off explosively when the saturated vapor pressure is undercut. Furthermore, extraction of solvent forces the release of the latent heat of evaporation which leads to a significant drop in temperature in the mixture and concomitantly a drop in the evaporation rate. It is imperative that the conditions of pressure and temperature are thoroughly controlled.

Introducing the EMS

poly III



The EMS POLY III is an instrument for the embedding of specimens by the proper combination of pressure and temperature. Central to the instrument is a specimen chamber that is temperature controlled and which can be heated up from room temperature to 70°C. The pressure in the chamber can be reduced from ambient pressure to a controlled level with an inbuilt vacuum-pump. The instrument chamber accepts up to 52 BEEM specimen vials. The vacuum pressure the specimens are exposed to is controlled within narrow limits and warrants solvents will evaporate in a controlled way with the risk of explosive boiling. The exhaust of the vacuum pump is connected to a chemical vapor resistant hose, which can vent into a fume hood in compliance with laboratory practices and general health and safety regulations when working with volatile substances. The instrument features preset programs which can be modified according to the user's preference. In the presets pressure and temperature settings have been coordinated and optimized for an efficient removal of solvent from the specimens eliminating the risk of boiling. Bulk removal of solvent is followed by steps for the thorough removal of trace amounts. As a practical approach the instrument can be loaded by the end of a workday and (when using acetone or propylene oxide) by the next morning the vials are ready for polymerization after the vials have been topped up with pure resin. A lengthy and sometimes tedious manual procedure now reduced to a few simple steps.

Alternatively, to free up the instrument for a next run, polymerization can take place in a stove. The instrument is (after cooling) immediately available for a next run.

Which solvents can be evacuated with the EMS POLY III?

The instrument handles any of the solvents currently used in embedding protocols. In principle any solvent can be safely and gradually evaporated as long as the proper settings for pressure vs temperature are observed. The one important rule is that the pressure should initially NEVER be lower than the saturated vapor pressure at that temperature. Values can be calculated with the Antoine Equation or derived from the graph (Figure 1). For the bulk removal of solvent it is advised to work at a pressure that is approximately 10% higher than the saturated vapor pressure. Failing to do so may lead to sudden boiling of the solvent and spilling of mixture and specimens from the vials. The risk of sudden spilling is higher with more volatile solvents such as propylene oxide and instrument settings need to be accurate.

Important: at no stage should solutions be allowed to bubble. Gas bubble formation needs to be avoided to prevent splashing and damage to the specimens when bubbles should form inside the specimen.

Although extensive testing has been carried out, specific combinations of solvent and resin need to be tested for their suitability for embedding and polymerization in the Poly III on an individual basis.

The EMS poly^{III} in practice

The EMS POLY III instrument is capable of effectively removing any of the solvents commonly used in embedding as long as the following rules are observed:

- use small tissue pieces, smaller volume of cell pellets
- use separate programs for embedding and polymerization
- use the smallest amount of resin in the embedding step
- during polymerization do NOT lower the pressure to a level equal to or lower than the saturated vapor pressure as any trace amount of solvent will result in the formation of holes in the specimen

Small specimens are more easily embedded because of their favorable surface/volume ratio. Likewise, solvents are more easily extracted.

Along those lines it is obvious that solvent is more easily removed if the specimens are covered (or even just merely contained) in a minimum amount of resin. For instance, when using BEEM capsules it is typically sufficient to end up with 30-50 µl of resin at the end of the embedding run.

Using a dilute resin/solvent mixture, or a small volume of mixture in the embedding program leaves the specimens covered in a thin layer of resin by the end of the embedding run, just enough to cover or contain the specimen.

A procedure that has been tested for embedding yeast cells is described below and may serve as a starting point.

Yeast cells were fixed with glutaraldehyde in cacodylate buffer, washed in distilled water and postfixed with 1% KMnO₄ in distilled water.

After washing in distilled water the cells were dehydrated in a graded series of solvent/water mixtures: 50% - 70% - 80% - 90% - 100% - 2x100% absolute. Each step lasted 10min.

Cells are then resuspended in resin/solvent 1:10 (v/v) and after 10 min. pelleted. The pellet is resuspended in fresh mix (appr 10x the volume of the pellet) and then transferred into one or more BEEM capsules.

It is strongly recommended to transfer the suspension into BEEM vials OUTSIDE the POLY III vacuum recipient to avoid spilling of resin inside the recipient. Any spilled resin on the vials should also be removed before inserting the BEEM vials into the recipient recessions.

Once all the BEEM vials have been transferred, the embedding program can start. The embedding program is followed by the polymerization program, after the BEEM vials have been topped up with pure resin.

Alternately, silicone molds can be used in the polymerization program.



front panel



back panel



sample block holds up to 52 samples

Specifications

5 programs, up to 5 steps each

Temperature of the Sample Block 10 – 70 deg C.

Temperature set point in increments of 1 deg C.

Temperature Control Accuracy at the Sensor: +/- 2.0 deg. C in heat mode, +/- 3 deg. C in cooling mode.

Sample Block: Up to 52 samples

Sample Block for BEEM Capsules, silicone molds

Vacuum Level: 10kPa (or 3" of Hg at sea level) – 100kPa

Introduction of fresh air during vacuum cycle and flush cycle.

Lid for the Processing Block: Anodized aluminum with plastic cover
Heated Lid

2 line x 16 character LCD Display with 6 keys membrane keypad

Thermoformed Enclosure

Dimensions: 9" wide (23 cm) wide x 20" (51 cm) deep x 11" (28 cm) tall (with lid)

Weight: 21 lbs (10 Kg)

Power Requirements: 200 watts max, 100 – 240 volts AC, 50 or 60 Hz

Fuse: 2.5 Amp x 250 VAC, Time Delay/Slo Blo fuse

Ordering Information

Cat No.	Description	Qty.
4444	EMS Poly III	each
70020-B	BEEM® Embedding Capsules Size 00 Polyethylene embedding capsules with 1x1mm face at the tip of a truncated pyramid, each with a hinged cap.	1000/pkg
70900	Standard flat embedding mold, 21 numbered cavities measures: 14mm(L) x 5mm(W) x 4mm(D). (clear silicone)	each
70901	Same as #70900, but each of the 21 numbered cavities measures: 14mm(L) x 5mm(W) x 6mm(D). (white silicone)	each
70902	Same as #70900, but each of the 21 numbered cavities measures: 14mm(L) x 5mm(W) x 3mm(D). (white silicone)	each

poly^{III} Embedding Programs

There is a choice of 3 embedding programs in the EMS POLY III catering for 3 different solvents. They have been pre-programmed for general use but the user can change the programs to fit specific specimens.

Program 1: for removal of ethanol

Program 2: for removal of acetone

Program 3: for removal of propylene oxide

The programs each have up to 5 steps that bring the specimens from the resin/solvent mix through removal of the solvent into pure resin.

The individual steps, using Program 1 as an example:

The Program starts at a temperature of 25°C.

The vapor pressure of ethanol at that temperature is ~8kPa.

Note: Steps 4 and 5 are mandatory steps when working with low vapor pressure solvents such as ethanol. More volatile solvents can usually be removed in a shorter procedure only using Step 3, or using Step 3 and 4.

Step 1 serves the gentle removal of air bubbles that may have been introduced.

Step 2 aims at the bulk removal of ethanol, using a pressure of 15kPa at 25°, which is slightly above the max vapor pressure. Ethanol will evaporate from the surface of the resin/solvent mixture. The pressure will increase as a result.

Evaporated ethanol is diluted with air that is admitted into the recipient and then pumped away. These events are controlled by the setting of global variables:

$\Delta P1$, which relates to the pressure increase resulting from evaporation and

$\Delta P2$, which determines how much air is admitted to dilute the evaporated solvent.

The technical manual provides information on how to set the parameter values for these variables.

When, as a result of evaporation, the pressure has increased by preset threshold level $\Delta P1$, the air admission valve opens. The time that passes before this happens is in the order of minutes and depends on the number of specimens, more accurately on the total evaporating area in the recipient, the pressure in the recipient and on the volatility of the solvent.

Example: Let us assume $\Delta P1$ was set to 1kPa and $\Delta P2$ to 25kPa, which are recommended settings.

When the pressure in the recipient has gone up by 1kPa to 16-17kPa (error margins \pm 1kPa) the air admission valve opens. The pressure in the recipient increases as a result and when it has gone up by 25kPa the air admission valve is closed again. The pump is activated and diluted ethanol vapor is removed until the set pressure of 15kPa has been reached again and the pump switches off. As long as ethanol keeps evaporating this process will be

repeating itself, initially with short time intervals, later with gradually increasing intervals. When the interval time has increased to >30 min, indicating that the bulk of the ethanol has been removed, the instrument automatically switches to Step 3, regardless of whether the set time (in the default set up this is 5 hrs) has not yet been completed.

Step 3 starts with raising the temperature and lowering the pressure. Residual ethanol is more easily removed under these conditions and the process described for Step 2 continues until the interval time between pump cycles has increased to >30 min. Then a flush/pump cycle is started: the air valve is opened and the pump switched on so the pump works under gas ballast conditions, improving evaporated solvent removal. This flush/pump cycle is repeated twice within a few minutes, after which the system is pumped down to the set vacuum level. After 30 min the process is repeated. After a total number of twelve of these cycles the system goes to Step 4.

Step 4 starts with raising the temperature after which the same process as described for Step 3 is activated.

Step 5 as Step 4.

The program ends after Step 5 has been completed.

The Technical Manual describes programming steps as well as deleting steps.

Polymerization programs

There is a choice of 2 programs for polymerization in the EMS POLY III. They have been pre-programmed for general use but the user can change the programs to fit specific specimens.

Program 4: for 24 hr polymerization (temperature curing)

Program 5: for 48 hr polymerization (temperature curing)

The programs each have up to 5 steps.

The individual steps are explained using Program 4 as an example. Note: each step switches to the next one based on the programmed time, unlike the steps in Programs 1, 2 and 3.

When embedding has been completed, specimens need to be polymerized. The programs use a minimum of 2 steps.

Step 1 raises the temperature over a period of approximately 30-40 minutes to the 60°C whereas the vacuum is set at 10kPa. Under these conditions the viscosity of epoxy resin is much reduced and the specimens yield the last traces of solvent.

Step 2 starts with releasing the vacuum and slightly raising the temperature. The start of step 2 is the preferred moment for the BEEM vials to be topped up with pure resin. After the polymerization time has passed the polymerized specimens are removed from the recipient.

Table 2: Factory preset programs 1-3 and steps

Program	1	2	3
Solvent			propylene oxide
Resin	ethanol	acetone	
Step 1			
Duration	00:05:00	00:05:00	00:30:00
Temperature	25°C	25°C	15°C
Pressure	65kPa	65kPa	100kPa
Step 2			
Duration	05:00:00	05:00:00	00:05:00
Temperature	25°C	25°C	15°C
Pressure	15kPa	30kPa	65kPa
Step 3			
Duration	02:00:00	02:00:00	05:00:00
Temperature	30°C	30°C	20°C
Pressure	10kPa	20kPa	60kPa
	6 hr. flush	6 hr. flush	6 hr. flush
Step 4			
Duration	02:00:00	02:00:00	02:00:00
Temperature	35°C	30°C	25°C
Pressure	10kPa	10kPa	40kPa
	6 hr. flush	6 hr. flush	6 hr. flush
Step 5			
Duration	02:00:00	02:00:00	05:00:00
Temperature	45°C	45°C	45°C
Pressure	10kPa	10kPa	20kPa
	6 hr. flush	6 hr. flush	6 hr. flush

Table 3: Factory preset programs 4-5 and steps

Program	4	5
Polymerization	short	long
Resin	epoxy methacrylate	epoxy methacrylate
Step 1		
Duration	2:00:00	2:00:00
Temperature	60°C	60°C
Pressure	10kPa	10kPa
Step 2		
Duration	22:00:00	46:00:00
Temperature	65°C	60°C
Pressure	100kPa	100kPa
Step 3, 4, 5		
Duration	unused	unused
Temperature		
Pressure		

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