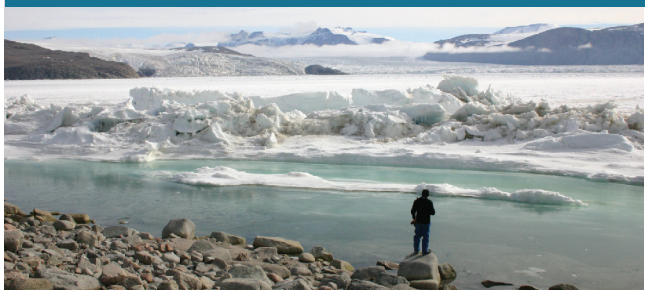
Exymes

Exymes Methods



Antarctica - the source of the Exymes extraction technology (Granite Harbour, Ross Sea)

Exymes Method Optimisation and Troubleshooting

Exymes DNA extraction kits have simple, well-defined protocols that are outlined in our *QuickStart Guides.* All of the procedures have been optimised on a range of samples, but for unusual sample types, the procedures and formulations may need to be fine-tuned.

Exymes kits are ideal for small samples but even when used for low copy material, they provide enough DNA for hundreds or thousands of analyses. It is sometimes tempting to add more sample to an extraction or more extract to a PCR, but often, this is counter-productive. When you first receive your kit, it is advisable to follow the method exactly as laid out in the *QuickStart Guide.* Once you are happy with the results, you will be in a better position to optimise yields and workflows to suit your own needs.

This guide is provided to assist with troubleshooting and method optimisation. It highlights which steps are essential for success and which can be changed.

Top 10 Tips for improving your PCR

1. Add less DNA to your PCR. For most Exymes kits, 5 µl of a 1:5 dilution provides the best results. In your first run, you may want to try a dilution series.
2. Don't overload your extraction by adding more sample. Ask yourself 'how much DNA do I need?'
3. The purpose of the extraction is to release DNA not to make peptide broth. Most of the DNA is released quickly as the Exymes proteinase lyses the cells. Continued digestion may make your tissue disappear, but it contributes nothing to your extract other than loading it with inhibitors. Excessive digestion times should be avoided.
4. If you suspect inhibition, add BSA to your PCR. 1 µl of a 10 mg/ml solution in a 25 µl reaction can dramatically improve results.
5. Use quality reagents. The modern hot-start polymerases far out-perform the older variants.
6. Make sure your primers are fully optimised. Even the best primer design programs have only about a 50% success rate. You may have to design a few variants and try all combinations.
7. Test whether poor results are caused by inhibition or low DNA concentration. Include an inhibition control - a sample of your extract spiked with a known amount of control DNA.
8. Exymes reagents are all DNA-free. This is not the case for many reagents from other manufacturers. If you are using universal mtDNA or rRNA primers, you will need to evaluate different polmerases, water, and reagents for contamination.
9. With high cell densities, your extract may become very viscous because of large amounts of high molecular weight DNA. This can make accurate pipetting difficult. Dilute your samples and if necessary shear the DNA in your sample by vortexing.
10. Use the Exymes support team. Email: support@exymesplc.com for help. Make sure you describe your full method, the results and the problems you faced. We are keen to hear feedback so that we can help you with your problems.

Solving your PCR problems

The table below indicates which sections in the guide are relevant to your sample type.

Sample type	Cause of Problem	Section
Liquid Blood	Inadequate centrifugation	9
	EDTA Vacutainer	4
	Too much blood extracted	1 & 2
	Too much extract in your PCR	1
	Sensitive PCR reagents	5
Buccal swabs	Inhibitory swab types	6
	Too much swab wash extracted	1 & 6
	Inadequate mixing	10 & 6
	Too much extract in your PCR	1
Liquid saliva	Too much saliva extracted	1 & 10
	Too much extract in your PCR	1
	Inadequate mixing	10
Stored extracts	Poor storage conditions	3
Tissue culture	Viscous extracts	Tip 9 above

Sample type	Problem	Section
FTA cards	Pre-washing the punch	7
	Too many punches	1
	Too much extract in your PCR	1
Other storage cards	Is the pre-wash necessary?	7
	Too much extract in your PCR	1
Stored samples	Presence of chelating agents	3
	Too much extract in your PCR	1
Solid tissue/Insects	Dry or tough tissue	8
	Too much tissue added	1 & 8

Workflow optimisation

Buffer compatibility with other applications	12
Quantification	14
Modifying the recommended method	13

For more information visit: www.exymesplc.com
or email: info@exymesplc.com

1. Amount of extract to use in your PCR

Relevant Kits

All *prep*GEM and *forensic*GEM kits

The recommended Exymes methods are optimised to extract DNA while minimising the co-extraction of inhibitors. Adding more sample to your extraction or extracting for longer than is recommended, may do little to improve your DNA yield while being more likely to result in PCR inhibition.

Similarly, adding too much extract to your PCR will generally lower the quality of your results. Most PCR reagents are now robust and capable of amplifying very small amounts of DNA. You will often get better results with smaller samples than when a PCR is overloaded with too much extract.

2. Extraction volume

Relevant Kits

All *prep*GEM and *forensic*GEM kits

The *prep*GEM and *forensic*GEM kits are very effective for processing small samples such as micro-biopsies, laser capture samples and FACS-sorted single cells.

Generally, we recommend using < 5 mg of tissue or 2 mm of a mouse tail, but it is possible to perform the DNA extraction with far smaller samples. This is also true for insect parts and very small whole insects. The minimal volume used in your extraction is not limited by the chemistry but by evaporation in the equipment available to you. However, 5-10 µl extractions can easily be performed in most thermal cyclers with a heated lid.

For smaller volumes, micro tubes, capillaries or specialised equipment should be used. Alternatively, you can cover the liquid surface with a layer of mineral oil.

When scaling, it is important to keep the buffers at 1x and to add sufficient *prep*GEM or *forensic*GEM for efficient digestion to occur. The incubation time can also be shortened to 5 minutes for very small samples.

Recommended amounts of enzyme to add are shown below.

Extraction volume	**Amount of *prep*GEM**
50 µl	1 µl
20 µl	1 µl
10 µl	0.5 µl
5 µl	0.25 µl
1 µl	0.2 µl

3. Storing your DNA extract

Relevant Kits

All *prep*GEM and *forensic*GEM kits

If you do not intend to analyse extracted DNA immediately, then samples should be adjusted to 1 x TE (10 mM Tris [pH7.5], 1 mM EDTA). Stability studies show that samples stored in this way can be kept at 4°C for up to a month. We recommend that for longer storage, the extract should be kept at -20°C.

4. EDTA and other chelating agents

Relevant Kits

All *prep*GEM and *forensic*GEM kits

The proteinase used in Exymes kits is stable at 75°C but like other metalloproteinases, it requires a small amount of Ca^{2+} for stability. This is provided with the enzyme itself, but if chelating agents, such as EDTA or EGTA are present in the sample, it will be necessary to add 0.02 volumes of 10 mM $CaCl_2$ to the extraction. This amount of Ca^{2+} is sufficient to stabilise the enzyme without causing any deleterious effects on the PCR.

A solution of 10 mM $CaCl_2$ is provided with all blood kits (because blood is often collected using EDTA vacutainers).

In some cases, an alternative approach is to remove the liquid from the sample by centrifugation or settling - any residual wetting usually contains insufficient chelating agent to adversely affect the enzyme.

5. PCR inhibition

Relevant Kits

All *prep*GEM and *forensic*GEM kits

Exymes DNA extraction kits do not use a purification step. The proteinase and all the reagents in the buffers were deliberately selected to be fully compatible with PCR.

The Exymes philosophy is to remove the need for purification steps by extracting in mild buffers and selectively releasing DNA into solution using conditions that do not solubilise most inhibitors.

Nonetheless, some substrates can lead to inhibition - especially if large amounts of extract are used in the PCR.

The following suggestions should be tried if you experience any problems with your PCR.

1. Make sure that the problems are caused by inhibition and not low concentrations of DNA template. Inhibition can easily be tested by including a control where one of your extracts is spiked with a known quantity of DNA. Alternatively, if you are using qPCR, the slope and endpoint of the plot can give a good indication that inhibition has occured.
2. Add BSA to your PCR. 1 µl of a 10 mg/ml solution in a 25 µl reaction is sufficient for dramatically reducing the effects of common inhibitors such as blood proteins, tannins and dyes.
3. Try adding less sample to your PCR. Often, inhibition is caused simply by overloading the reaction with too much DNA. Excessive DNA affects Mg^{2+} concentration and PCR efficiency falls. Usually 1 µl or less of the extract is enough for a successful PCR.
4. Try centrifuging the debris out of your extract. This step is not normally needed but sometimes, solid material can reduce PCR efficiency. This is particularly true with some buccal swabs (see section 6).

If problems persist, contact us at info@exymesplc.com

Tip :If you are using adhesive tape lifts, please refer to our Application Note which identifies which tapes are inhibitory.

6. Swabs and PCR inhibition

Relevant Kits

*prep*GEM and *forensic*GEM Universal

Law enforcement agencies and laboratories use a wide variety of swabs for collecting buccal samples from individuals suspected of a crime. Typically, these swabs are either made from cotton or a foam polymer and have either a plastic or wooden handle.
We recommend where possible to use foam swabs with plastic handles for collecting samples. Some cotton swabs release a binding agent or fine particles that can seriously affect PCR and compromise forensic profiling.
We recommend that you test your swabs for inhibition by washing an unused swab in water and adding the eluate to a PCR or profiling reaction containing a known amount of DNA.

In addition, be aware that buccal cells sediment rapidly in the wash solution. Ensure that you mix the suspension immediately before transferring it to the extraction reagents.

7. Storage Cards

Relevant Kits

*prep*GEM and *forensic*GEM Universal

Standard and Elute versions of the Whatman FTA micro-card differ in their properties and type of chemical preservatives impregnated into the paper. When using the standard FTA cards the recommended pre-wash step is necessary to remove these preservatives prior to extraction.

When using Filter paper, Whatman FTA Elute micro-cards or Guthrie cards, the wash step is usually unnecessary - indeed for some card types washing will result in a loss of sample material. It is important to test your cards prior to using them for the release of inhibitors.

8. Animal tissue types

Relevant Kits

*prep*GEM and *forensic*GEM Universal

Whilst the Exymes kits have been validated for a number of tissue types, some material may benefit from minor modifications to the protocol.

Recalcitrant tissues

In the case of tougher tissue, such as lung or kidney, the enzyme concentration may be increased to 2-fold and the 75°C incubation time increased up to 30 minutes.

Dehydrated samples

Hydration can be an issue in the extraction of DNA from a sample. If necessary, leave the sample in buffer overnight at 4°C prior to extraction. We would not recommend adding the enzyme to the overnight soak because some plastics can bind proteins over extended periods. Add sufficient 1x buffer without *prep*GEM or *forensic*GEM to cover the sample, and make up the total volume prior to extraction with the recommended amount of enzyme in an appropriate volume of 1x buffer.

Hair Follicles

1 to 3 hair follicles can be extracted using the standard procedure. Scaling is possible for larger numbers. Generally, a single hair follicle provides ample DNA for typing.

9. Centrifugation - blood

Relevant Kits

All *prep*GEM and *forensic*GEM kits

The centrifugation step used in preparing DNA from blood is critical for optimal PCR performance. The buffers provided in Exymes blood kits are optimised to remove inhibitory substances through sedimentation by centrifugation. If processing single samples in microcentrifuge tubes, then a 5 minute centrifugation at 20,000 x g is sufficient.

When processing samples in 96 well plates, a lower relative centrifugal force (rcf) must be used. We recommend centrifugation at the highest rcf permitted for the rotor and plate. To minimise PCR inhibition, 10 minutes is optimal.

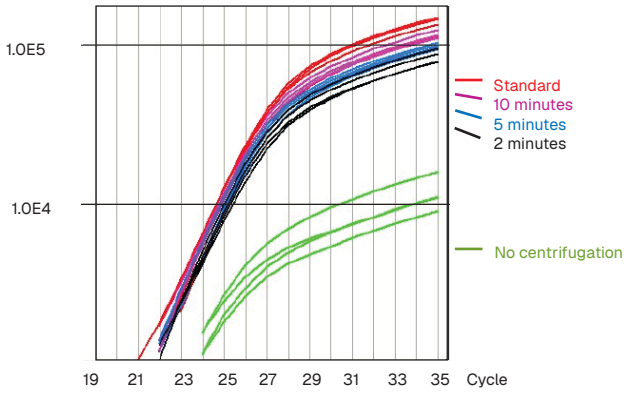


Figure 1 qPCR plots obtained when amplifying a portion of the GAPDH gene from human blood. The extracts were centrifuged in a 96-well plates at 2000 x g for the times shown.

The extracts used for generating the qPCR plots in Figure 1 were processed and centrifuged in a 96-well plate. The green plots (no spin) clearly demonstrate the need for the centrifugation in order to remove inhibition. The black, blue and purple plots demonstrate how the plot profile improves with longer centrifugation. All are acceptable plots but when forced to use a reduced r.c.f., then a longer centrifugation gives a lower C_T value and a higher end point.
We recommend balancing your need for inhibitor-free PCR with the need for fast processing by trying different centrifugation times.

10. Centrifugation - saliva

Relevant Kits

*prep*GEM and *forensic*GEM Universal

If you are using liquid saliva or a suspension of cells from a washed buccal swab, it is important not to allow the cells to sediment and not to add too much of your buccal swab wash. (Also see section 6 for more information about swabs). Extractions from buccal swabs or saliva do not normally need to be centrifuged before adding a sample to a PCR. Be aware, however, that some swabs (most notably those made from cotton wool) can release fine particles that cause PCR inhibition.

If changing the swab brand is not an option, it is sufficient to allow the debris in the extract to settle. A one minute centrifugation at maximum speed may also improve your results.

11. Centrifugation - other sample types

Relevant Kits

*prep*GEM and *forensic*GEM Tissue, *prep*GEM Insect and *livestock*GEM Ear-punch

It is important to understand that the Exymes extraction methods are optimised to release DNA without over-digesting the substrate. In general, over-digestion releases little more DNA but releases many more inhibitory substances. What this means is that when using solid tissue, there will be a substantial portion of the original sample visible in the extract after digestion. This should not cause concern - you will still have enough DNA for many analyses.

A centrifugation step is generally not required to remove this material and simply leaving the sediment to settle is sufficient to clarify the solution. However, it is recommended that you remove solid material if samples are to be stored for long periods and with some sample types, removing debris will make pipetting easier.

In the case of environmental samples (such as sediment and sludge), a centrifugation step will remove the dense material remaining in suspension. Mix the extract by flicking the tubes and centrifuge for one minute at the maximum speed specified for the rotor and tube type.

12. Downstream applications and buffer compatibility

Relevant Kits

All *prep*GEM and *forensic*GEM kits

The buffers supplied in all Exymes DNA extraction kits have been developed to produce DNA suitable for:

- PCR
- qPCR
- Multiplex PCR
- STR (microsatellite) analysis

The kits have also been verified on a number of SNP detection platforms, for whole genome amplification (WGA), and DNA sequencing.

Depending on the sample type and amount of material present, we recommended initially trying more than one dilution of your extract in your PCR. Try 1-5 µl of both an undiluted and a 5-fold dilution of the extract in a 20-25 µl PCR reaction. For low titre samples, more extract can be used - the Exymes extraction reagents will not adversely affect the PCR.

If you experience good results when using standard PCR but the DNA extract fails in more complex procedures, you should contact Exymes Technical Service through www.exymesplc.com.

Validation data from a variety of sources is available on the Exymes website.

Tip
Most DNA-acting enzymes benefit from the presence of protein in the buffers and it is for this reason that BSA is often added.
Residual protein and peptides in your extract are therefore unlikely to affect the efficiency of these reactions - indeed, they can sometimes improve performance!
However, inhibition by blood proteins, haemoglobin, tannins and polysaccharides can be reduced by adding BSA to your PCR reagents. 1 µl of a 10 mg / ml solution of BSA is sufficient for a 25 µl reaction.

13. Omitting the 95°C heat-kill step

Relevant Kits

All *prep*GEM, *forensic*GEM and *vet*GEM kits: excluding Blood kits

Using the recommended procedure provided in the *Quick-Start Guides*, residual proteinase is inactivated by a 5 minute incubation at 95°C. This step denatures the DNA and for complex genomes such as human, subsequent cooling reforms only a limited percentage of double-stranded DNA.

This conformation does not run as a single, clear band on an electrophoresis gel but you can be confident that the extract contains intact, high molecular weight genomic DNA.

In situations where dsDNA is required (particularly when quantifying with fluorescent dyes - see below) the 95°C step can be omitted. The dilution factor when a portion of the extract is used in a PCR, minimises carry-over proteinase. Furthermore, most polymerases are now 'hot-start' and the initial heating cycle of a PCR is sufficient to rapidly remove the small amount of carry-over proteinase. Thermophilic DNA polymerase such as *Taq* are inherently resistant to *prep*GEM and so in the brief time the sample ramps up to 95°C the proteinase has a negligible effect.

With the kits designed to extract DNA from blood or blood stains, the specialised buffer and the 95°C step are necessary to minimise inhibition and so this step should **not** be omitted.

14. DNA Quantification

Relevant Kits

All *prep*GEM, *forensic*GEM and *livestock*GEM kits

The DNA extracted by the recommended Exymes method is largely single stranded (see above) and therefore less suited to fluorescent dye-based quantification. Furthermore, without a solid phase or solvent based purification step, residual protein and peptides in the supernatant can cause inaccuracies in fluorescence and OD quantification. As an example, BSA and IgG both affect the total fluorescence of certain DNA binding dyes.

You should also be aware that many sample types yield DNA from a number of sources. For example as little as 10-20% of the total DNA from a buccal swab may be of human origin. This factor also makes accurate quantification more problematic.

For these reasons, we recommend quantifying extracted DNA using qPCR. This is particularly important when sensitive downstream assays are to be used.

If a rapid estimate of DNA quantity is needed and fluorescent methods are preferred, you can use extracted DNA where the 95°C heat-kill step has been omitted from the extraction cycle. For greater accuracy you can calibrate the amount of interference caused by proteins in the extracts by spiking an extract with pure DNA and noting the percentage change in fluorescence over standards containing no protein.

For more information visit: exymesplc.com
or email: info@exymesplc.com